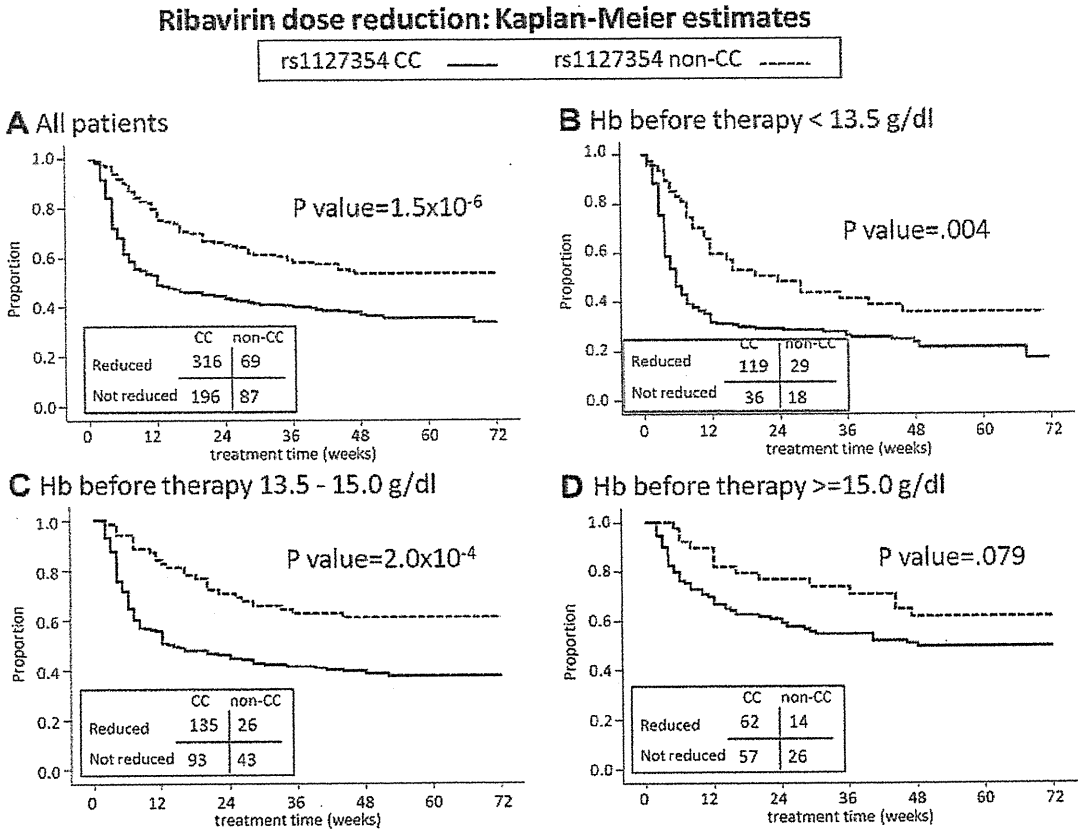

Fig. 2. Dose reduction of ribavirin in patients who were treated with combination therapy. Kaplan–Meier curves for dose reduction grouped by ITPA SNP rs1127354 genotype (solid line: CC, dashed-line: non-CC) among (**A**) all patients, (**B**) patients with low pretreatment hemoglobin levels (<13.5 g/dl), (**C**) patients with intermediate pretreatment hemoglobin levels (13.5–15.0 g/dl), and (**D**) patients with high pretreatment hemoglobin levels (≥15 g/dl).

effect of these factors as well as clinical factors were analyzed for dose reduction of ribavirin. As shown in Table II, univariate analysis identified ITPA SNP rs1127354 genotype, fibrosis stage and inflammatory activity of the liver, white blood cell count, platelet count, hemoglobin, ALT, age, and sex as factors associated with more than 80% ribavirin administration. Multivariate analysis identified age, hemoglobin, and rs1127354 genotype as independent predictive factors.

Effect of ITPA Genotype and Pretreatment Hemoglobin Levels on Outcome of Therapy

As the frequency of patients receiving more than 80% of planned ribavirin administration differed by pretreatment hemoglobin levels and ITPA genotype, treatment outcome might be expected to differ based on these factors. As expected, SVR rate was significantly higher in patients with non-CC genotypes with hemoglobin levels 13.5–15 g/dl, where the frequency of patients receiving 80% ribavirin administration differed most significantly between genotypes CC and non-CC (Fig. 4).

Predictive Factors of the Combination Therapy for SVR and NVR

Predictive factors for SVR and NVR were assessed, including baseline clinical factors, genotype of the recently reported IL28B SNP, and viral factors such as the number of substitutions in the ISDR, and substitutions at core amino acid 70 and 91. By univariate analysis, a number of factors were significantly associated with SVR, including IL28B SNP genotypes (rs8099917 and rs12979860), ITPA SNP rs1127354 genotype, core70 mutation, fibrosis of the liver, white blood cell count, platelet count, hemoglobin, ALT, fasting blood sugar, viral titer, age, sex, body mass index, and duration of the therapy (Table III). Multivariate analysis identified IL28B SNP rs8099917 genotype as the strongest independent predictor for SVR (OR 15.379, $P = 3.48E{-}07$), followed by hemoglobin level, ITPA SNP rs1127354 genotype, fibrosis of the liver, age, and body mass index (Table III). Significant independent predictive factors for NVR included IL28B SNP rs8099917 genotype fibrosis, and age (Table IV).

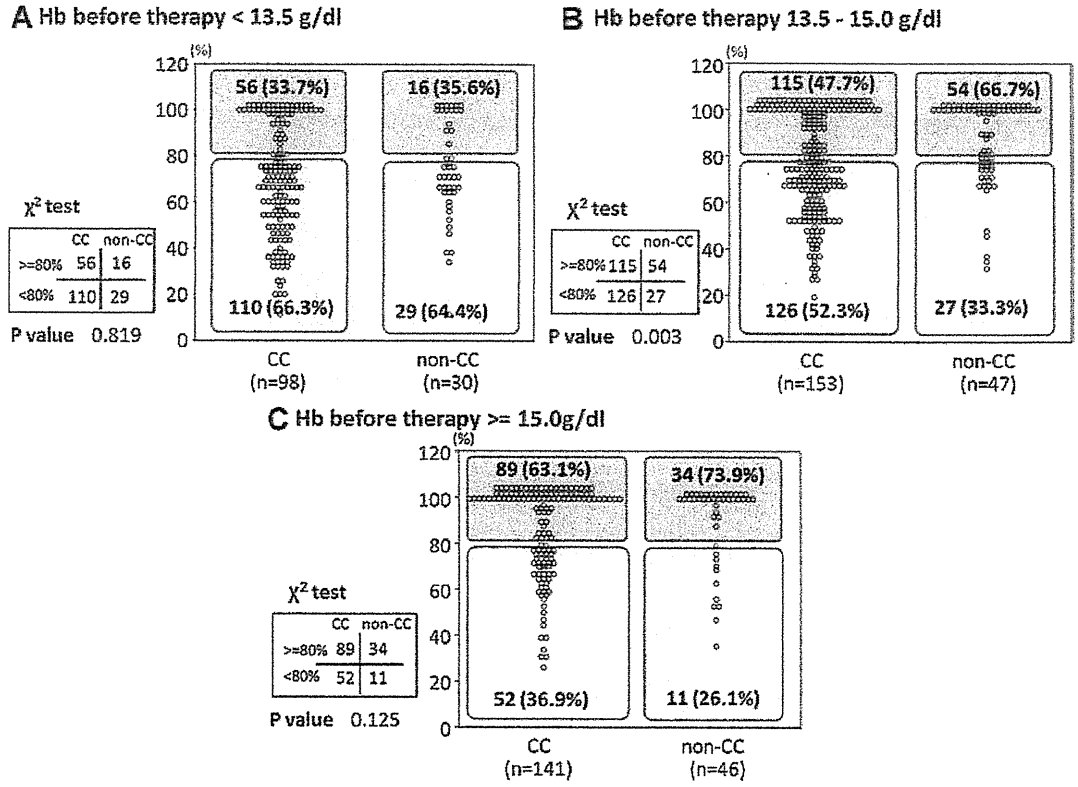

Fig. 3. Dose of ribavirin administered to patients with genotype 1 treated with combination therapy by ITPA rs1127354 genotype and pretreatment hemoglobin levels. Patients with genotype 1b and treated with ribavirin were divided into three groups based on their pretreatment hemoglobin levels: (**A**) <13.5 g/dl, (**B**) between 13.5 and 15.0 g/dl, and (**C**) ≥15 g/dl.

TABLE II. Factors Associated With Ribavirin Dose Reduction (80%) in Hepatitis C Virus Patients Determined by Logistic Regression Analysis

	Simple		Multiple		
Variable	OR	*P*-value	OR	95% CI	*P*-value
rs1127354 CC vs. CA/AA	0.580	0.002**	0.578	0.372–0.897	0.014*
Core70	1.007	0.974			
Core91	0.776	0.244			
ISDR 0/1 vs. >1	1.091	0.743			
BMI (kg/m^2)	1.008	0.740			
Fibrosis 1–2 vs. 3–4	1.676	0.009**	1.409	0.902–2.202	0.132
Activity 0–1 vs. 2–3	1.537	0.013*			
WBC (/mm^3)	1.000	1.2E−05**			
Plt (×10^4/mm^3)	1.070	5.2E−06**	1.000	1.000–1.000	0.178
Hb (g/dl)	1.485	1.7E−10**	1.244	1.066–1.453	0.006**
AST (IU/L)	1.001	0.769			
ALT (IU/L)	1.003	0.035*			
γGTP (IU/L)	1.001	0.362			
Albumin (g/dl)	1.549	0.460			
Total cholesterol (mg/dl)	0.997	0.175			
Triglycerides (mg/dl)	1.000	0.935			
HDL cholesterol (mg/dl)	0.989	0.066			
LDL cholesterol (mg/dl)	0.995	0.503			
Fasting blood sugar (mg/dl)	1.001	0.585			
Virus titer (log IU/ml)	1.047	0.567			
Age	0.936	2.1E−15**	0.934	0.914–0.954	3.5E−10**
Sex	0.586	3.9E−04**			

**$P < 0.01$.
*$P < 0.05$.

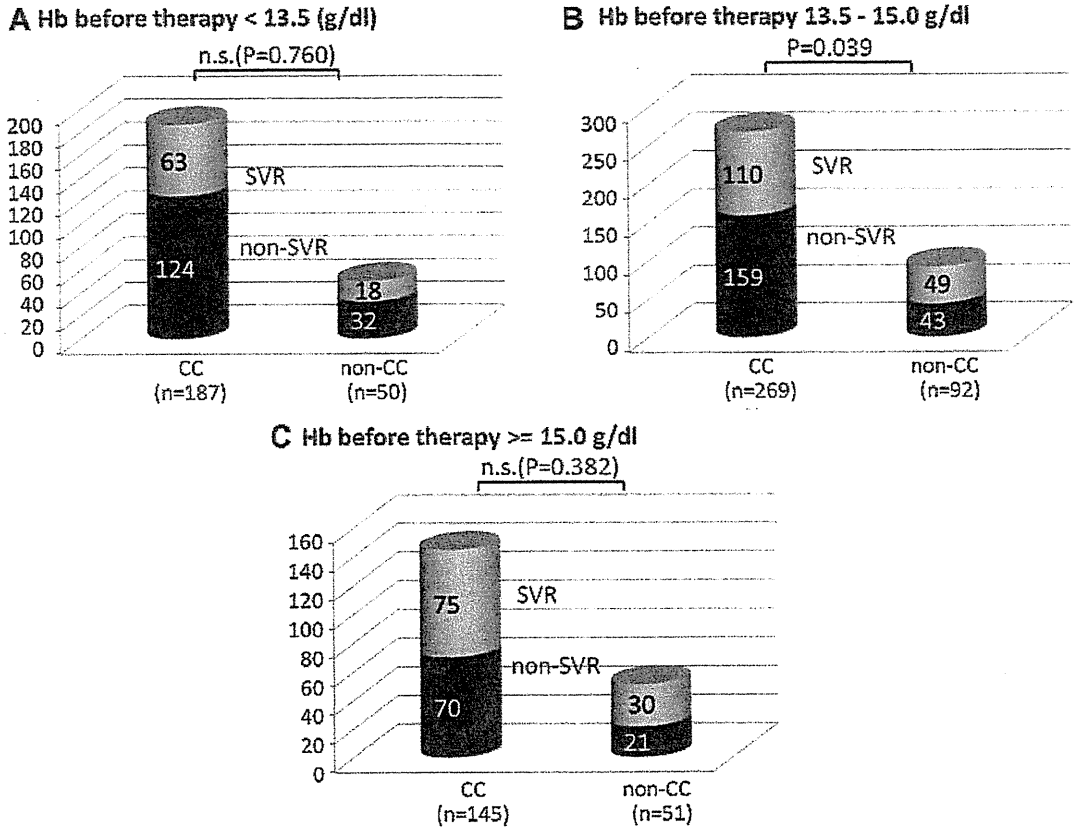

Fig. 4. Effect of combination therapy in patients with genotype 1b by ITPA rs1127354 genotype and pretreatment hemoglobin levels. Patients with genotype 1b and treated with ribavirin were divided into three groups based on their pretreatment hemoglobin levels: (**A**) <13.5 g/dl, (**B**) between 13.5 and 15.0 g/dl, and (**C**) ≥15 g/dl.

DISCUSSION

Ribavirin-induced anemia is one of the most serious side effects resulting from combination therapy [De Franceschi et al., 2000], but a polymorphism within the ITPA gene has recently been shown to affect incidence of this form of anemia [Fellay et al., 2010; Ochi et al., 2010; Thompson et al., 2010]. This study showed that hemoglobin decrease is faster and more severe, especially in the first 12 weeks of treatment, in patients with the anemia-susceptible ITPA rs1127354 CC genotype (Fig. 1). The rapid reduction of hemoglobin observed in genotype CC patients persisted to the end of therapy and was associated with early reduction of ribavirin dosage (Fig. 2), resulting in lower total ribavirin administration. The linear and continuous decrease in hemoglobin seen in non-CC patients also contributed to the reduction of ribavirin administration but not as drastically as in patients with the CC genotype (Fig. 2). The other significant ITPA SNP, rs7270101, is associated with splicing variant formation and reduced activity of the ITPA enzyme in patients of European and African ancestry, but this SNP is absent in the Japanese population [Ochi et al., 2010]. Therefore, only the missense SNP rs1127354, which results in a P32T amino acid change and reduced enzyme activity, was analyzed. Thompson et al. [2010] divided patients into four groups (−, +, ++, +++) based on the genotypes of these two SNPs. According to their classification, CC and non-CC genotypes in this study are almost comparable to "−" and "++" in their study because there are no patients with the rs1127354 AA genotype, and there were only two "+++" patients present in their study. Hemoglobin decrease was slightly milder in the this study compared to Thompson et al. [2010], probably due to early reduction in ribavirin dose in Japanese patients resulting from lower pretreatment hemoglobin levels.

Initial hemoglobin levels indeed had a strong influence on reduction of ribavirin dose. As shown in Figure 3, ITPA genotype did not have a significant influence on patients with <80% ribavirin administration when pretreatment hemoglobin levels were <13.5 or >15 g/dl. Accordingly, because reduction of ribavirin to <80% results in decreased rate of SVR [McHutchison et al., 2002], patients with pretreatment hemoglobin levels below 13.5 g/dl or patients with pretreatment hemoglobin levels between 13.5 and 15 g/dl who have the ITPA anemia-susceptible genotype should receive treatment with drugs such as erythropoietin to prevent reduction of ribavirin.

TABLE III. Predictive Factors Associated With Sustained Viral Response in Hepatitis C Virus Patients Determined by Logistic Regression Analysis

	Simple		Multiple		
Variable	OR	*P*-value	OR	95% CI	*P*-value
rs8099917 TT vs. TG/GG	3.614	1.85E−16**	15.358	5.371–43.919	3.48E−07**
rs12979860 CC vs. CT/TT	4.271	8.87E−16**			
rs1127354 CC vs. CA/AA	0.660	0.006**	0.368	0.161–0.838	0.017*
Core70	1.891	0.005**			
Core91	1.503	.059			
ISDR 0/1 vs. >1	0.660	0.106			
BMI (kg/m^2)	0.944	0.007**	0.865	0.758–0.987	0.032*
Fibrosis 1–2 vs. 3–4	2.290	5.83E−05**	4.540	1.618–12.734	0.004**
Activity 0–1 vs. 2–3	0.869	0.412			
WBC (/mm^3)	1.000	0.008**			
Plt ($\times 10^4$/mm^3)	1.072	4.68E−08**	1.055	0.976–1.141	0.176
Hb (g/dl)	1.172	0.001**	1.505	1.106–2.048	0.009**
AST (IU/L)	1.000	0.824			
ALT (IU/L)	1.003	0.027*			
γGTP (IU/L)	0.998	0.118			
Albumin (g/dl)	2.802	0.089			
Total cholesterol (mg/dl)	1.002	0.345			
Triglyceride (mg/dl)	0.997	0.094			
HDL cholesterol (mg/dl)	1.003	0.670			
LDL cholesterol (mg/dl)	0.999	0.922			
Fasting blood sugar (mg/dl)	0.989	0.001**	0.991	0.977–1.005	0.197
Virus titer (log IU/ml)	0.722	1.83E−04**	0.798	0.567–1.124	0.196
Age	0.960	5.13E−11**	0.957	0.919–0.995	0.028*
Sex	0.713	0.009**			
RBV treatment period (weeks)	1.012	3.86E−04**			

**$P < 0.01$.
*$P < 0.05$.

TABLE IV. Predictive Factors Associated With NVR in Chronic Hepatitis C Virus Patients Treated With Peg-Interferon Plus Ribavirin Combination Therapy

	Simple		Multiple		
Variable	OR	*P*-value	OR	95% CI	*P*-value
rs8099917 TT vs. TG/GG	6.663	6.00E−32**	7.157	3.592–14.262	2.21E−08**
rs12979860 CC vs. CT/TT	7.589	1.07E−30**			
rs1127354 CC vs. CA/AA	0.673	0.027*			
Core70	2.531	5.25E−05**			
Core91	1.951	0.003**	1.604	0.849–3.033	0.146
ISDR 0/1 vs. >1	0.569	0.053			
BMI (kg/m^2)	0.969	0.189**	0.910	0.822–1.008	0.070
Fibrosis 1–2 vs. 3–4	1.826	0.002**	2.941	1.404–6.162	0.004**
Activity 0–1 vs. 2–3	0.866	0.424			
WBC (/mm^3)	1.000	0.052			
Plt ($\times 10^4$/mm^3)	1.048	0.001**			
Hb (g/dl)	1.112	0.046*			
AST (IU/L)	0.999	0.608			
ALT (IU/L)	1.001	0.651			
γGTP (IU/L)	0.996	0.007**			
Albumin (g/dl)	1.534	0.479			
Total cholesterol (mg/dl)	1.005	0.058			
Triglyceride (mg/dl)	0.998	0.100			
HDL cholesterol (mg/dl)	1.003	0.669			
LDL cholesterol (mg/dl)	0.997	0.664			
Fasting blood sugar (mg/dl)	0.998	0.461			
Virus titer (log IU/ml)	0.753	0.006**	0.744	0.534–1.036	0.080
Age	0.977	0.001**	0.958	0.927–0.99	0.010**
Sex	0.830	0.202			
RBV treatment period (weeks)	1.021	1.86E−07**	1.012	0.996–1.027	0.135

Results of simple and multiple logistic regression are shown. The multivariate model was constructed using stepwise selection of significant univariate terms.
**$P < 0.01$.
*$P < 0.05$.

Although the ITPA polymorphism was significantly associated with ribavirin-induced anemia [Fellay et al., 2010; Thompson et al., 2010], no effect on outcome of therapy was found in the two previous studies on ITPA polymorphism from the United States. In contrast, Ochi et al. [2010] reported a possible association between ITPA genotype and outcome of therapy in Japan. Similarly, results of this study suggest an association between ITPA genotype and outcome of combination therapy for HCV genotype 1 in Japanese patients (Table II). There are several potential reasons for the different effects of ITPA genotype among these studies. First, the incidence of anemia-protective (rs1127354 non-CC) genotypes is higher in Japanese patients (20%) compared with patients with European (16.7%) and Sub-Saharan African (6.7%) ancestry [Olivier, 2003], suggesting a lack of power to detect the association in studies based on these populations. Secondly, the age of treated patients is higher in Japan than in the US (50–55 vs. 45) [Kainuma et al., 2010], which may lead to a higher incidence of ribavirin dose reduction during therapy [Hung et al., 2006]. Similarly, lower pretreatment levels of hemoglobin in Japanese patients compared with US patients (13.0 g/dl vs. 14.9 g/dl) [Fellay et al., 2010; Ochi et al., 2010] might result in a greater incidence of ribavirin reduction in Japanese patients and enhance the effects of the ITPA SNP on treatment outcome.

This study showed that a significantly larger number of patients ultimately received <80% of planned ribavirin administration when their hemoglobin levels were either <13.5 g/dl or between 13.5 and 15 g/dl in ribavirin-sensitive patients (ITPA rs1127354 genotype CC) (Fig. 4). As reported previously, administration of <80% of planned ribavirin is associated with poor outcome of therapy, and this study confirmed that reduction of ribavirin is significantly associated with SVR ($P < 0.009$, data not shown). Treatment of these patients with erythropoietin may therefore help prevent ribavirin dose reduction and improve SVR rate. However, in Japan erythropoietin is not available to treat this condition. As erythropoietin has been shown to improve anemia and treatment outcome of combination therapy, administration should be considered, at least for patients matching the criteria in this study, to improve the outcome of therapy.

ACKNOWLEDGMENTS

We thank Mika Tsuzuno, Sakura Akamatsu, Sanae Furuya as well as other clerical and medical staff at Hiroshima University Hospital for their help.

REFERENCES

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380.

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Arase Y, Ikeda K, Kumada H. 2006. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 78:83–90.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 46:403–410.

Alter MJ. 1995. Epidemiology of hepatitis C in the West. Semin Liver Dis 15:5–14.

Chevaliez S, Pawlotsky JM. 2007. Hepatitis C virus: Virology, diagnosis and management of antiviral therapy. World J Gastroenterol 13:2461–2466.

De Franceschi L, Fattovich G, Turrini F, Ayi K, Brugnara C, Manzato F, Noventa F, Stanzial A, Solero P, Corrocher R. 2000. Hemolytic anemia induced by ribavirin therapy in patients with chronic hepatitis C virus infection: Role of membrane oxidative damage. Hepatology 31:997–1004.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis - diagnosis, grading and staging. Hepatology 19:1513–1520.

Dienstag JL, McHutchison JG. 2006. American Gastroenterological Association technical review on the management of hepatitis C. Gastroenterology 130:231–264; quiz 214–237.

Dieterich D, Wasserman R, Bräu N, Hassanein T, Bini E, Bowers P, Sulkowski M. 2003. Once-weekly epoetin alfa improves anemia and facilitates maintenance of ribavirin dosing in hepatitis C virus-infected patients receiving ribavirin plus interferon alfa. Am J Gastroenterol 98:2491–2499.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. 1995a. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis-C virus 1b—Sensitivity to interferon is conferred by amino-acid substitutions in the NS5A region. J Clin Invest 96:224–230.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. 1995b. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 96:224–230.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Fellay J, Thompson A, Ge D, Gumbs C, Urban T, Shianna K, Little L, Qiu P, Bertelsen A, Watson M, Warner A, Muir A, Brass C, Albrecht J, Sulkowski M, McHutchison J, Goldstein D. 2010. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. Nature 464:405–408.

Ge DL, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Jr., Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. 2004. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. Ann Intern Med 140:346–355.

Hung CH, Lee CM, Lu SN, Wang JH, Chen CH, Hu TH, Kee KM, Chang KC, Tseng PL, Yen YH, Changchien CS. 2006. Anemia associated with antiviral therapy in chronic hepatitis C: Incidence, risk factors, and impact on treatment response. Liver Int 26:1079–1086.

Kainuma M, Furusyo N, Kajiwara E, Takahashi K, Nomura H, Tanabe Y, Satoh T, Maruyama T, Nakamuta M, Kotoh K, Azuma K, Shimono J, Shimoda S, Hayashi J. 2010. Pegylated interferon alpha-2b plus ribavirin for older patients with chronic hepatitis C. World J Gastroenterol 16:4400–4409.

McHutchison J, Manns M, Patel K, Poynard T, Lindsay K, Trepo C, Dienstag J, Lee W, Mak C, Garaud J, Albrecht J, IHIT Group. 2002. Adherence to combination therapy enhances sustained

response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology 123:1061–1069.

Ochi H, Maekawa T, Abe H, Hayashida Y, Nakano R, Kubo M, Tsunoda T, Hayes C, Kumada H, Nakamura Y, Chayama K. 2010. ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy-A genome-wide study of Japanese HCV virus patients. Gastroenterology 139:1190–1197.

Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. 2001. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 46:471–477.

Olivier M. 2003. A haplotype map of the human genome. Physiol Genomics 13:3–9.

Romero-Gómez M, Del Mar Viloria M, Andrade R, Salmerón J, Diago M, Fernández-Rodríguez C, Corpas R, Cruz M, Grande L, Vázquez L, Muñoz-De-Rueda P, López-Serrano P, Gila A, Gutiérrez M, Pérez C, Ruiz-Extremera A, Suárez E, Castillo J. 2005. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. Gastroenterology 128:636–641.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J, Hepatitis CS. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1174.

Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. 2003. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 34:395–402.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Thompson A, Fellay J, Patel K, Tillmann H, Naggie S, Ge D, Urban T, Shianna K, Muir A, Fried M, Afdhal N, Goldstein D, McHutchison J. 2010. Variants in the ITPA gene protect against ribavirin-induced hemolytic anemia and decrease the need for ribavirin dose reduction. Gastroenterology 139:1181–1189.

Zeuzem S, Franke A, Lee JH, Herrmann G, Ruster B, Roth WK. 1996. Phylogenetic analysis of hepatitis C virus isolates and their correlation to viremia, liver function tests, and histology. Hepatology 24:1003–1009.

J Gastroenterol (2011) 46:545–555
DOI 10.1007/s00535-010-0358-6

Factors predictive of sustained virological response following 72 weeks of combination therapy for genotype 1b hepatitis C

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Received: 1 November 2010 / Accepted: 25 November 2010 / Published online: 19 January 2011

Abstract
Background Treatment of genotype 1b chronic hepatitis C virus (HCV) infection has been improved by extending peg-interferon plus ribavirin combination therapy to 72 weeks, but predictive factors are needed to identify those patients who are likely to respond to long-term therapy.
Methods We analyzed amino acid (aa) substitutions in the core protein and the interferon sensitivity determining region (ISDR) of nonstructural protein (NS) 5A in 840 genotype 1b chronic hepatitis C patients with high viral load. We used logistic regression and classification and regression tree (CART) analysis to identify predictive factors for sustained virological response (SVR) for patients undergoing 72 weeks of treatment.
Results When patients were separately analyzed by treatment duration using multivariate logistic regression, several factors, including sex, age, viral load, and core aa70 and ISDR substitutions ($P = 0.0003$, $P = 0.02$, $P = 0.01$, $P = 0.0001$, and $P = 0.0004$, respectively) were significant predictive factors for SVR with 48 weeks of treatment, whereas age, previous interferon treatment history, and ISDR substitutions ($P = 0.03$, $P = 0.01$, and $P = 0.02$,

Electronic supplementary material The online version of this article (doi:10.1007/s00535-010-0358-6) contains supplementary material, which is available to authorized users.

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respectively) were the only significant predictive factors with 72 weeks of treatment. Using CART analysis, a decision tree was generated that identified age, cholesterol, sex, treatment length, and aa70 and ISDR substitutions as the most important predictive factors. The CART model had a sensitivity of 69.2% and specificity of 60%, with a positive predictive value of 68.4%.

Conclusions Complementary statistical and data mining approaches were used to identify a subgroup of patients likely to benefit from 72 weeks of therapy.

Keywords CART analysis · Core protein · Decision tree · ISDR · LDL cholesterol

Abbreviations

HCV	Hepatitis C virus
ISDR	Interferon sensitivity determining region
CART	Classification and regression tree analysis
SVR	Sustained virological response
NR	Non-viral response

Introduction

Chronic hepatitis C virus (HCV) infection is a major global cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1–3]. The treatment of chronic hepatitis C has improved with the advent of peg-interferon (IFN) plus ribavirin combination therapy [4–7], but fewer than half of the patients with high viral loads of genotype 1b show a sustained virological response (SVR), defined as testing negative for HCV RNA 24 weeks after cessation of the therapy. To overcome this limitation, recent therapeutic regimens have extended the treatment period to 72 weeks [8–11]. This extension is especially effective in patients whose HCV RNA declines relatively slowly [9–11]. Accordingly, recent treatment protocols have recommended extending the treatment period to 72 weeks in patients who become negative for HCV RNA after 12 weeks of treatment but before 24 weeks [10, 11]. This response-guided decision-making approach to therapy has resulted in improvements of the SVR rate [10, 11]. Following this approach, patients with a non-viral response (NR), i.e., patients who show very poor response to the therapy (defined as less than 2-log decline of HCV RNA during 12 weeks of treatment), should be advised to discontinue therapy because SVR is rare in such patients. While response-guided therapy is useful in determining the appropriate duration of treatment for patients who are likely to respond eventually, predictors that can be assessed before the start of therapy will aid in differentiating which difficult-to-treat patients are likely to achieve an SVR with extended therapy and which may be better served by considering alternative therapy options.

To predict NR, recent studies recommend analysis of amino acid (aa) substitutions in the HCV core protein at positions 70 and 91 [12, 13]. The substitution of arginine with glutamine or other amino acids at core protein aa 70 has been reported to be associated with NR, and this finding was confirmed by several other groups [14–16]. Analysis of core aa 70 has also been shown to be useful to predict the outcome of 72 weeks of combination therapy [17]. While many factors have been reported to be useful predictors of the effect of combination therapy [18–26], many of these factors are mutually interdependent. Furthermore, because almost all of these factors have been reported under conditions in which a majority of patients were receiving 48 weeks of treatment, it is necessary to consider the effect of the treatment period.

In this study, we compiled a database of clinical data from 840 patients from 16 national centers in Japan. We used logistic regression and classification and regression tree analysis (CART) to identify factors predictive of SVR for 48- and 72-week therapy and to assess which patients are most likely to benefit by long-term 72-week therapy.

Methods

Study subjects

In this retrospective study, data from 840 patients with chronic hepatitis C treated at 16 different hospitals in Japan were analyzed for predictive factors for SVR based on

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Table 1 Patient characteristics for 48- and 72-week treatments

	All patients (n = 840)	48-Week therapy (n = 619) 73.69%	72-Week therapy (n = 221) 25.12%
Age (years)	54.4 ± 10.73	53.8 ± 11.21	56.2 ± 9.03
Gender (male/female)	449/391	357/262	92/129
Body weight (kg)	60.9 ± 10.8	61.3 ± 10.6	59.8 ± 11.4
Height (cm)	162.2 ± 9.1	162.7 ± 9.1	160.7 ± 9.0
BMI	23.0 ± 3.05	23.0 ± 2.92	23.0 ± 3.4
HCV core protein aa 70 (wild/mutant)	539/301	396/223	143/78
HCV core protein aa 91 (wild/mutant)	504/336	369/250	135/86
ISDR (0–1/≥2)	714/126	513/106	201/20
Hypertension (present/absent/ND)	538/113/189	395/78/146	143/35/43
Diabetes (present/absent/ND)	634/47/159	457/38/124	177/9/35
Transfusion (present/absent/ND)	505/227/108	379/162/78	126/65/30
Fibrosis stage (0–2/3–4/ND)	604/128/108	448/90/81	156/38/27
Activity stage (0–1/2–3/ND)	382/343/115	287/245/87	95/98/28
Steatosis (present/absent/ND)	158/344/338	119/250/250	39/94/88
AST (IU/l)	65 ± 49	66 ± 47	63 ± 53
ALT (IU/l)	68 ± 56	68 ± 56	66 ± 55
White blood cell count (/mm^3)	4832 ± 1455	4882 ± 1488	4693 ± 1352
Hemoglobin (g/dl)	14.2 ± 1.36	14.3 ± 1.39	14.1 ± 1.29
Platelets ($\times 10^4$/mm^3)	16.9 ± 5.18	17.0 ± 5.11	16.8 ± 5.35
γGTP (IU/l)	56 ± 59	59 ± 64	49 ± 42
Albumin (g/dl)	4.02 ± 0.348	4.01 ± 0.350	4.03 ± 0.343
Uric acid (mg/dl)	5.41 ± 1.29	5.46 ± 1.27	5.25 ± 1.35
Iron (μg/dl)	147.0 ± 69.65	151.0 ± 75.71	136.1 ± 47.45
Ferritin (μg/l)	173.9 ± 167.9	181.7 ± 175.7	153.0 ± 143.7
Fasting blood sugar (mg/dl)	99.8 ± 19.8	99.3 ± 19.1	101.2 ± 21.5
Alpha-fetoprotein (μg/l)	16.3 ± 50.4	14.2 ± 44.8	22.0 ± 62.7
Total cholesterol (mg/dl)	175 ± 32.3	173 ± 31.8	179 ± 33.4
LDL cholesterol (mg/dl)	100.8 ± 29.8	100.2 ± 30.3	102.5 ± 28.4
HDL cholesterol (mg/dl)	52.1 ± 15.5	51.4 ± 15.0	53.9 ± 16.6
Triglycerides (mg/dl)	103.2 ± 48.8	103.8 ± 46.1	101.7 ± 55.1
HCV-RNA (KIU/ml)	3239 ± 4669	3170 ± 4828	3427 ± 4205
Response to treatment (SVR/TR/NR)	465/246/129	341/164/114	124/82/15

BMI body mass index, *HCV* hepatitis C virus, *aa* amino acid, *ISDR* interferon sensitivity determining region, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γGTP* γ-glutamyl transpeptidase, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *SVR* sustained virological response, *TR* transient response/relapsers, *NR* non-viral response, *ND* not determined

treatment duration. Inclusion criteria included testing positive for HCV RNA for longer than 6 months and testing negative for both hepatitis B virus surface antigen and anti-HIV antibody. Patients with confounding conditions such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease were excluded. We excluded patients who were lost for follow up and those who did not show a high level of viremia for genotype 1b, as well as patients for whom we failed to determine both core and IFN sensitivity determining region (ISDR) of nonstructural protein (NS) 5A sequences; 385 patients were treatment-naïve. All subjects gave their written informed consent to participate in the study according to the process approved by the ethics committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Patient profiles are listed in Table 1.

All patients initially received weekly injections of peg-IFN-alpha-2b for 48 weeks (60 μg for body weight (BW) 35–45 kg, 80 μg for BW 46–60 kg, 100 μg for BW 61–75 kg, 120 μg for BW 76–90 kg, and 150 μg for BW 91–120 kg). Ribavirin was administered orally, and the dosage was determined based on the patient's BW (600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg

for >80 kg). Ribavirin dosage was reduced when hemoglobin levels were reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. Successful treatment was ascertained based on SVR, defined as HCV RNA-negative 6 months after cessation of therapy. Using response-guided therapy, slow viral responders, i.e., patients for whom HCV RNA levels became negative after 12 weeks of therapy but before 24 weeks, and some non-responders were recommended for extension of therapy to 72 weeks.

Biochemical tests were performed at the individual hospitals, and pathological diagnosis was made by pathologists in each hospital according to the criteria of Desmet et al. [27]. Fibrosis and activity data were compared among hospitals to ensure that there were no systematic differences.

Analysis of viral titer and amino acid sequences in the core and ISDR region

The HCV RNA level was analyzed using reverse transcription polymerase chain reaction (RT-PCR)-based methods (Amplicor™ high-range test; Roche Diagnostics, Basel, Switzerland, or TaqMan RT-PCR test; Applied Biosystems, CA). The measurement ranges of these assays were 5–5000 KIU/ml and 1.2–7.8 log IU/ml, respectively. For values exceeding the measurable range, the limit value was used as an approximation. The values obtained by the Amplicor test were converted to logarithmic values [28].

Nucleotide and amino acid sequences of the core and the ISDR region were determined by direct sequencing of cDNA fragments amplified by PCR. Arginine and leucine were considered wild-type for core protein aa 70 and aa 91, respectively [12, 13]. The number of aa substitutions in the ISDR was determined by comparison with the reference sequence reported by Kato et al. [29] using the method of Enomoto et al. [30, 31].

Statistical analysis

Statistical analysis was performed using the R software package (http://www.r-project.org). The χ^2 or Fisher's exact and Mann–Whitney *U*-tests were used to detect significant associations. All statistical analyses were two-sided, and $P < 0.05$ was considered significant. Simple and multiple logistic regression analyses were used to examine the association between viral substitutions and clinical factors, using $P < 0.05$ as the criterion for inclusion in the initial multivariate model. Multivariate logistic regression analysis was performed using forward/backward stepwise selection based on the akaike information criterion (AIC) score and validated by bootstrapping, using the rms package in R. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each factor.

CART analysis

CART analysis was used to generate a decision tree by classifying patients by SVR, based on a recursive partitioning algorithm with minimal cost-complexity pruning to identify optimal classification factors. The SimpleCart classifier in the WEKA data mining package [32] was used with a minimal terminal node size of 4 and trained with the variables listed in Table 1. Performance was assessed using tenfold cross-validation, and the sensitivity, specificity, and precision of the model were calculated. Receiver operating characteristic (ROC) curves were generated and results were compared with the logistic regression model.

Results

Patient characteristics

Patients were partitioned into two groups based on whether they received 48 or 72 weeks of therapy (Table 1). In this study 465 patients achieved an SVR, whereas 375 patients were either non-responders or relapsers, yielding an overall SVR rate of 55.4%. The rate of SVR did not differ significantly between the 48- and 72-week treatment groups (55.3 vs. 56.4%, respectively; $P = 0.81$), but the NR rate was significantly lower in patients who were treated for 72 weeks (18.3 vs. 6.4%; $P = 9.3 \times 10^{-6}$).

Predictive factors for SVR

The association between SVR and individual clinical factors was assessed using logistic regression. A number of factors were significant at the $P < 0.05$ level, including age, sex, viral load, aa70/ISDR substitutions, hypertension, fibrosis, steatosis, prior IFN treatment, low-density lipoprotein (LDL) cholesterol, total cholesterol, white blood cell count, platelet count, hemoglobin, γ-glutamyl transpeptidase (γGTP), and albumin (Table 2). On multivariate logistic regression, only age, sex, core aa70, ISDR, LDL, and γGTP were identified as significant independent predictors of SVR. Although length of treatment was not identified as a significant predictor in this analysis, exploratory analysis suggests the presence of potential interactions between treatment length and age and/or sex that are not captured by the first-order terms in the model. When second-order terms were selected a posteriori, however, a significant interaction was found between sex and treatment length ($P = 0.0034$). When analyzed separately, independent predictive factors for SVR for 48 weeks

Table 2 Factors associated with sustained virological response to combination therapy

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age	840	0.393	3.16×10^{-11}***	517	0.386	(0.27–0.56)	5.08×10^{-7}***
Sex (male vs. female)	840	0.521	3.61×10^{-6}***	517	0.52	(0.35–0.78)	0.001459**
BMI (kg/m^2)	834	0.8	0.1094				
Viral load (Log IU/ml)	840	0.761	0.001828**				
Core aa70 substitution	840	0.537	1.98×10^{-5}***	517	0.507	(0.35–0.74)	0.000521***
Core aa91 substitution	840	0.818	0.1568				
ISDR (0–1 vs. ≥2)	840	2.36	5.19×10^{-5}***	517	2.12	(1.19–3.77)	0.01037*
Hypertension	651	0.625	0.02389*				
Diabetes	681	0.794	0.4464				
Blood transfusion	732	1	0.9788				
Fibrosis (F0–1 vs. F2–4)	732	0.674	0.008287**				
Activity (A0–1 vs. A2–4)	725	0.779	0.09567				
Steatosis	502	0.645	0.03413*				
Prior IFN treatment	830	1.37	0.02648*				
HDL cholesterol (mg/dl)	493	0.761	0.1333				
LDL cholesterol (mg/dl)	529	1.46	0.03223*	517	1.61	(1.10–2.38)	0.01521*
Triglyceride (mg/dl)	726	0.913	0.5412				
Total cholesterol (mg/dl)	814	1.25	0.11				
AST (IU/l)	783	0.933	0.6316				
ALT (IU/l)	840	0.972	0.837				
WBC (/mm^3)	836	1.55	0.001831**				
Hemoglobin (g/dl)	838	1.34	0.00276**				
Platelets ($\times 10^4$/mm^3)	838	1.74	7.92×10^{-5}***				
Gamma-GTP (IU/l)	823	0.735	0.0288*	517	0.656	(0.43–0.99)	0.04588*
Albumin (g/dl)	809	1.41	0.01699*				
Ferritin (μg/l)	532	0.898	0.5404				
Treatment period (weeks)	840	1.02	0.6095				

Simple and multiple logistic regression was used to examine the association between SVR and patient and viral factors. Factors with $P < 0.05$ were considered for inclusion in the multiple regression model and the best model selected by backwards stepwise selection using AIC

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

IFN interferon, *OR* odds ratio, *CI* confidence interval, *AIC* akaike information criterion

of treatment included age, sex, viral load, core aa70, LDL, platelets, and white blood cell counts, whereas for 72 weeks of treatment only age, ISDR, and prior IFN treatment were significant, although LDL cholesterol was marginally significant (Table 3).

Among patients who underwent 48 weeks of therapy, 61% of patients with core aa 70 wild-type achieved an SVR compared to only 44% of patients with mutant core aa 70 ($P = 1.8 \times 10^{-5}$, Fig. 1a), whereas for 72-week patients, the ratio was 1:1 (Fig. 3a). Conversely, in the 48-week group, 71% of patients with two or more mutations in the ISDR were able to achieve an SVR compared to 52% with the wild-type ISDR, and in the 72-week group (Fig. 1b), 80% of patients with two or more ISDR mutations achieved an SVR compared to 54% with zero or one ISDR mutations (Fig. 3b). Median baseline viral load was significantly lower in 48-week SVR patients compared to that in non-SVR patients ($P = 0.001$, Fig. 1c), whereas there was no significant difference between viral load and SVR in 72-week therapy patients ($P = 0.625$, Fig. 4c). There was a significant effect of age and treatment outcome among 48-week patients ($P = 9.3 \times 10^{-6}$, Fig. 2), but the difference was not significant among 72-week therapy patients. However, the proportion of patients achieving an SVR tended to decrease with age in both groups, particularly in females over age 70 years in the 72-week group (Figs. 2, 4).

Table 3 Independent factors associated with sustained virological response to 48- and 72-week peg-interferon plus ribavirin combination therapy

Variable	48 Weeks			72 Weeks			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age	535	0.642	0.0165*	133	0.4	(0.176–0.91)	0.02877*
Sex (male vs. female)	535	0.481	0.000284**				
Viral load (Log IU/ml)	535	0.738	0.01033*				
Core aa70 substitution	535	0.454	9.95×10^{-5}**				
ISDR (0—1 vs. ≥2)	535	2.75	0.000358**	133	7	(1.35–36.2)	0.02047*
Fibrosis (F0–1 vs. F2–4)	535	0.66	0.03954*				
Prior IFN treatment				133	2.67	(1.22–5.85)	0.01431*
LDL cholesterol (mg/dl)				133	2.04	(0.952–4.35)	0.06673
WBC (/mm^3)	535	1.53	0.03342*				
Platelets ($\times 10^4$/mm^3)	535	1.54	0.03707*				

Simple and multiple logistic regression analysis was used to examine the association between SVR and patient/viral factors separately for patients receiving 48 and 72 weeks of treatment

** $P < 0.001$, * $P < 0.05$

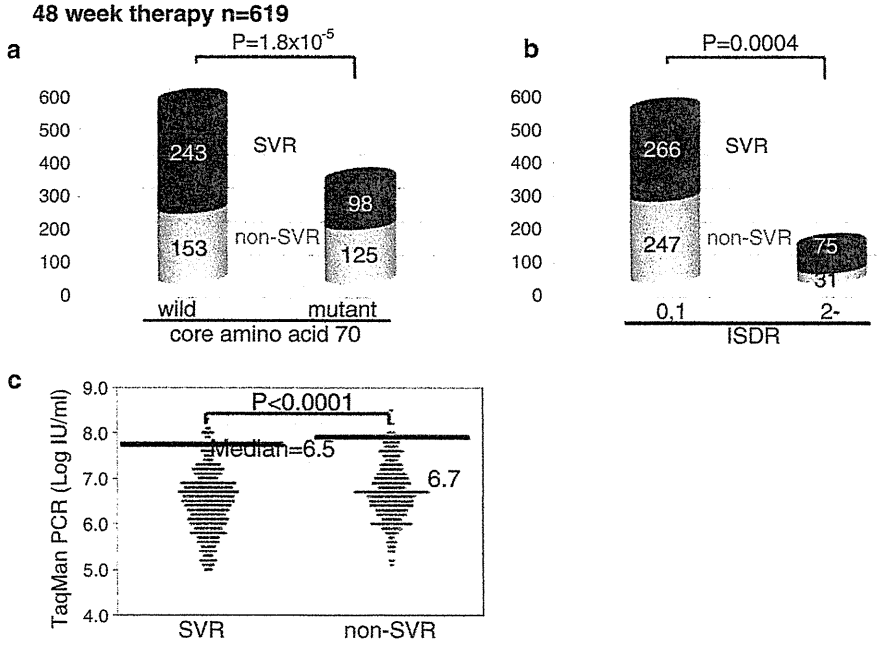
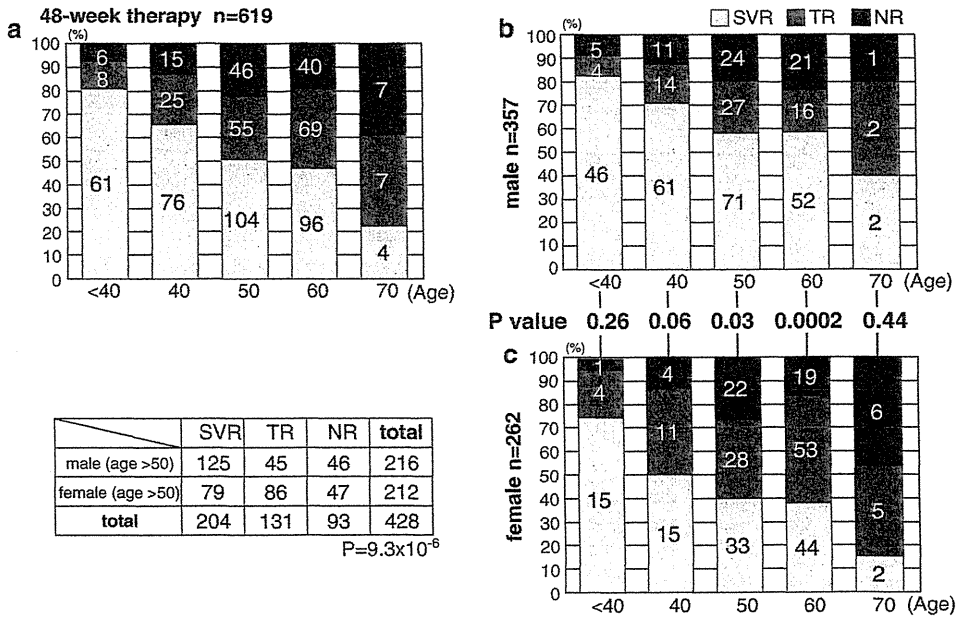


Fig. 1 Viral factors for 48-week treatment. Relationships between sustained virological response (*SVR*) and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients treated for 48 weeks. *PCR* Polymerase chain reaction

CART analysis

Figure 5 shows the decision tree generated by CART analysis. All variables were included during model construction, and the SimpleCart algorithm generated a tree based on the following fields: age, cholesterol, sex, γGTP, 48 versus 72 weeks of treatment, and aa substitutions in the ISDR and at core aa70. Age was used as the first cutoff, and patients younger than 46.5 years were classified as having a high probability for SVR (78%). Total cholesterol was identified as the next decision point, and patients with cholesterol higher than 211.5 mg/dl were classified as SVR if they were younger than 62.5 years (84%) and NR (65%) otherwise. Patients with cholesterol lower than 211.5 mg/dl were subdivided next by sex. Females who received 48 weeks of treatment were classified as NR (71%), whereas females receiving 72 weeks of treatment were classified as SVR if they were younger than 58.5 years (71%) or NR otherwise (64%). Males who were infected with aa70 wild-type were classified as SVR (62%), whereas males with aa70 substitutions were classified as NR if total cholesterol was less than 130 mg/dl (97%). Males with ISDR substitutions were classified as SVR (75%), and those with wild-type ISDR were classified



	SVR	TR	NR	total
male (age >50)	125	45	46	216
female (age >50)	79	86	47	212
total	204	131	93	428

$P=9.3\times10^{-6}$

Fig. 2 Relationship between age and response to treatment for 48-week therapy. Treatment outcomes by age in 10-year intervals are shown for **a** all patients, **b** males only, and **c** females only. *NR* non-viral response

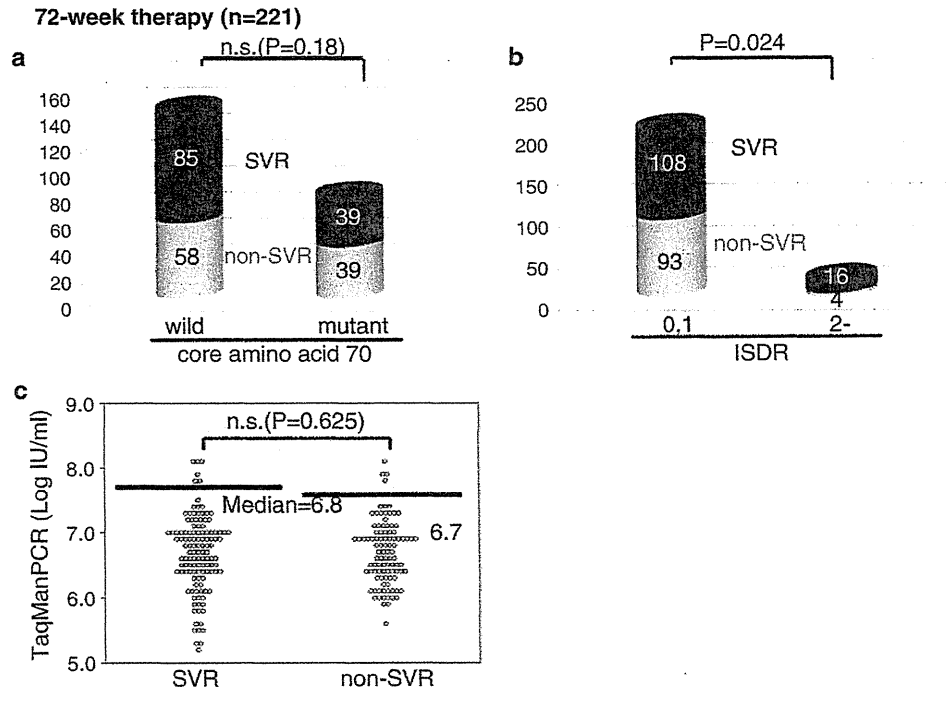
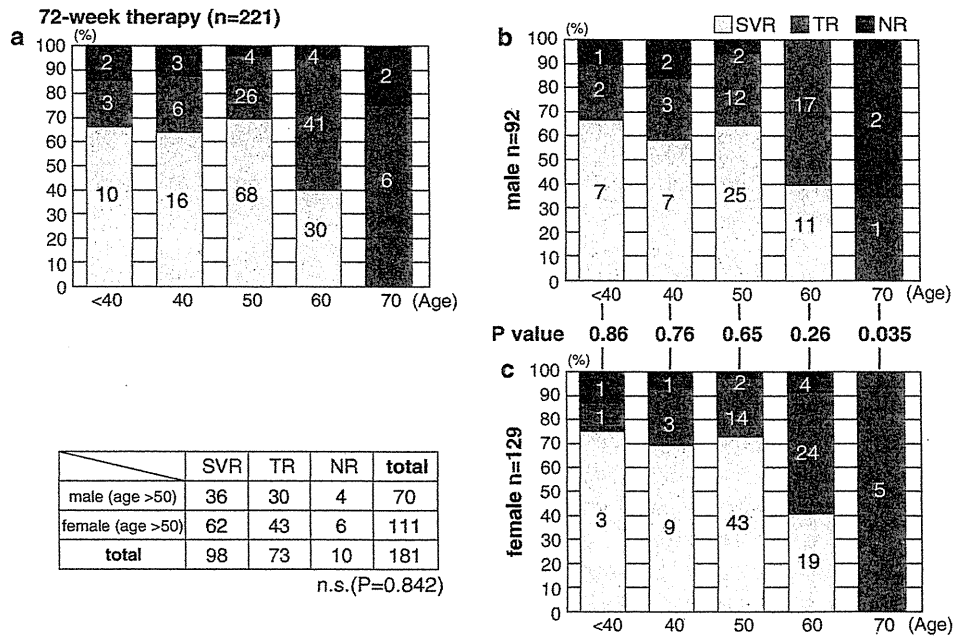


Fig. 3 Viral factors for 72-week treatment. Relationships between sustained virological response and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients treated for 72 weeks. *n.s.*, Not significant

as SVR if γGTP was less than 48.5 IU/l (57%) and NR otherwise (77%).

All factors selected during tree construction were found to be significant in univariate analysis, except for treatment length and cholesterol, and each remained significant in multivariate logistic regression. Although LDL was included in the multivariate logistic model, it was not selected during tree construction. Tenfold cross-validation resulted in 65.2% correctly classified instances with a kappa statistic of 0.29. The true positive rate was 69.2%, the false positive rate was 39.7%, and precision was 68.4%.

To compare the performance of SVR prediction between the logistic and CART models, the WEKA Logistic classifier was used to perform tenfold validation based on the



	SVR	TR	NR	total
male (age >50)	36	30	4	70
female (age >50)	62	43	6	111
total	98	73	10	181

n.s.(P=0.842)

Fig. 4 Relationship between age and response to treatment for 72-week therapy. Treatment outcomes by age in 10-year intervals are shown for **a** all patients, **b** males only, and **c** females only

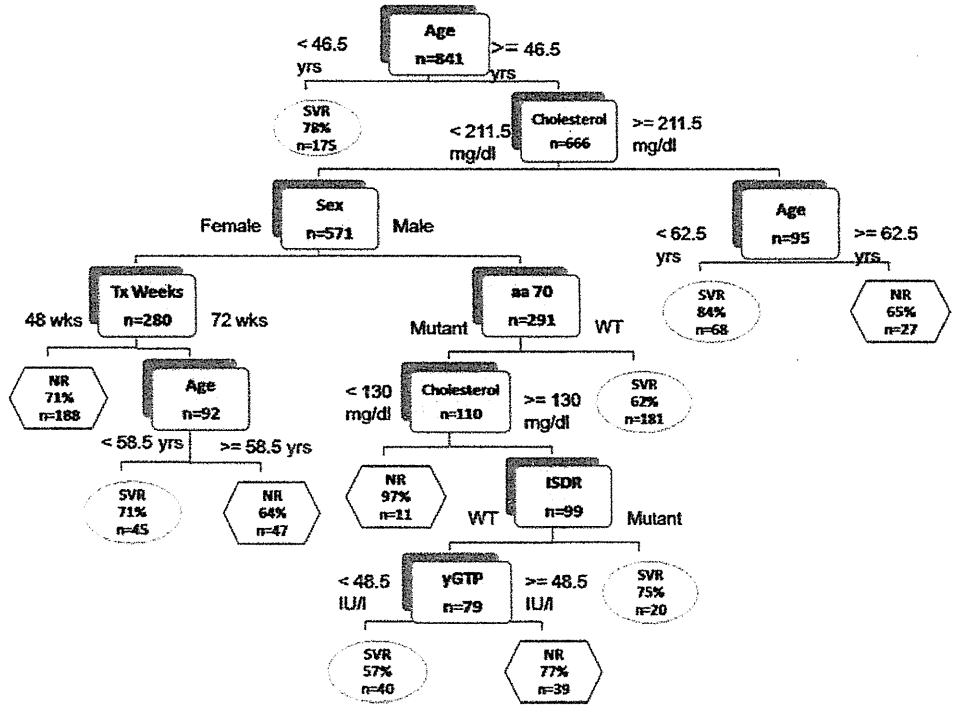


Fig. 5 Decision tree for SVR prediction. *Boxes* represent branch points based on cutoff values for factors determined by the tree generation algorithm. Each branch contains two choices, and each path ends in a prediction for either SVR or NR with an associated probability. *yrs* Years, *Tx* treatment, *ISDR* interferon sensitivity determining region, *aa* amino acid, *WT* wild-type, *γGTP* γ-glutamyl transpeptidase

multivariate logistic regression model above. The true positive rate for the logistic classifier was somewhat higher, at 73.1%, but with a slightly worse false-positive rate of 48%, and 63.7% correctly classified instances with a kappa statistic of 0.25 and precision 0.65. Receiver operating characteristic (ROC) curves were very similar, and the area under the curve was 0.677 for the CART model and 0.696 for the logistic model.

Discussion

Using two complementary approaches we identified several pretreatment factors predictive for SVR in patients treated for 48 and 72 weeks. Logistic regression and CART analysis both suggest that sex, age, cholesterol, and substitutions at core aa70 and ISDR are associated with SVR in patients with a high viral load of genotype 1b. Based on

the decision tree topology and a significant interaction between sex and treatment duration, it appears that 72 weeks of treatment may be most beneficial in women between the ages of 46 and 58 years who have low cholesterol. In general, patients who are younger, male, have cholesterol over 130 mg/dl, or who have wild-type core aa70 or mutant ISDR are the most likely to achieve an SVR.

Because each of the above values can be determined prior to treatment and are interpretable by clinicians, they may be useful as a guide when establishing a treatment regimen in the case of potentially difficult-to-treat patients. Once IFN treatment has been started, early and/or rapid viral response is likely to be the strongest predictor of SVR [33], and slow responders have been shown to be the most likely to benefit from extended treatment [34, 35]. However, because of the expense, low success rate, and potential side effects of IFN-based therapy, predictors available prior to treatment are also needed. Factors predictive of NR may help guide the decision to avoid or discontinue IFN therapy in patients with a low probability of SVR, and factors predictive of SVR may help identify subsets of patients who are likely to achieve an SVR if treated longer than the standard 48-week regimen.

Several other recent studies have examined predictors for SVR for 72 weeks of treatment, although nearly all focus on on-treatment predictors and conclude that 72-week therapy significantly improves SVR rates in slow responders [9, 10, 35]. Ferenci et al. [11] also showed that extension to 72 weeks decreased the relapse rate among early viral responders. In a large retrospective cohort study, Watanabe et al. [36] dissected a complex relationship between SVR and age, sex, and viral load similar to that reported here, although results are difficult to compare because they did not measure cholesterol or viral substitutions. While they recommend 72-week therapy for all slow-responding patients regardless of sex or age, they note that the SVR rate was surprisingly high among elderly female patients following 72-week treatment, noting that the SVR for 48-week treatment was typically low among older female patients in Japan, which they suggest could be related to the development of insulin resistance associated with menopause [36]. Other studies discourage the use of 72-week therapy for all patients except in the specific case of slow responders [8]. Moreover, in a large prospective study, Buti et al. [34] conclude that 48-week combination therapy should remain the standard of care even for slow responders, due to the increased cost and incidence of adverse events relative to a modest increase in the SVR rate. They clarify, however, that patients with a less than 2 log decline at week 8 and undetectable HCV RNA at week 24 are the most likely to benefit from 72-week treatment. Unfortunately they did not examine other predictors in a multivariate analysis. Because each of these studies hinges on rapid versus slow viral response and an on-treatment predictor requiring up to 24 weeks of treatment to establish, pretreatment predictors of early viral kinetics, including those presented here (e.g., viral substitutions and baseline cholesterol levels [12]), may be useful for predicting the outcome of extended therapy prior to treatment [17].

The combination of multiple approaches to identify predictive factors should help improve confidence in the results and partially protect against the bias inherent in any single approach. Comparing the results of a standard analysis with an alternative technique may reveal which variables are robust and which are sensitive to methodological differences. There are many different classification tools, including neural networks, Bayesian networks, and support vector machines, but models based on these may be more difficult to interpret or apply in clinical practice. On the other hand, decision tree approaches such as C4.5 and CART are widely used in biomedical studies [37–39] and provide a simple and intuitive hierarchical format that in many cases can be used without a computer.

The lack of randomized assignment of patients to duration of treatment limits the conclusions that can be drawn from the present study, and additional predictive factors, particularly interleukin (IL) 28B single-nucleotide polymorphism (SNP) genotype and viral kinetics, should be included in future prospective studies. Comparison of ROC curves suggests that the performance of the two models in the present study is similar, although neither is sufficiently sensitive or specific for accurate clinical prediction based on the number of patients analyzed. Nonetheless the strong overlap between the variables selected by each method suggests that several patient factors, including age, sex, and cholesterol level, as well as several viral factors, including core aa70 and ISDR substitutions, are robust predictors for SVR. Differences in the variables selected between the two approaches suggest that several models with similar predictive ability are also possible. In the regression model, LDL cholesterol but not total cholesterol was an independent factor associated with SVR, whereas in the CART analysis total cholesterol was selected instead. This may be due to the hierarchical nature of decision tree models, which may yield better results in the face of missing data, higher-order interactions, or nonlinear relationships. Comparison of separate models for 48 and 72 weeks also suggests that age and ISDR substitutions are important predictors of SVR for patients undergoing 72 weeks of treatment, whereas the decision tree suggests that the 72-week treatment length is important mainly for a subgroup of female patients. Without greater understanding of the role of HCV core and ISDR substitutions, it is difficult to interpret the role of these predictors, as well as

potential interactions with cholesterol level and other clinical factors. Further studies should be performed to investigate these interactions and to better characterize the subgroup of patients who are most likely to respond to long-term IFN therapy.

Acknowledgments This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education, Culture, Sports, Science and Technology, Government of Japan. We thank Sakura Akamatsu and Mika Tsuzuno for their assistance.

Conflict of interest None of the authors have conflicts of interest to declare.

References

1. Hoofnagle JH. Hepatitis C: the clinical spectrum of disease. Hepatology. 1997;26:15S–20S.
2. Di Bisceglie AM. Hepatitis C. Lancet. 1998;351:351–5.
3. Marcellin P. Hepatitis C: the clinical spectrum of the disease. J Hepatol. 1999;31(Suppl 1):9–16.
4. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001;358:958–65.
5. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
6. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–55.
7. Jensen DM, Marcellin P, Freilich B, Andreone P, Di Bisceglie A, Brandao-Mello CE, et al. Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. Ann Intern Med. 2009;150:528–40.
8. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
9. Sanchez-Tapias JM, Diago M, Escartin P, Enriquez J, Romero-Gomez M, Barcena R, et al. Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. Gastroenterology. 2006;131:451–60.
10. Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis C genotype 1-infected slow responders. Hepatology. 2007;46:1688–94.
11. Ferenci P, Laferl H, Scherzer TM, Maieron A, Hofer H, Stauber R, et al. Peginterferon alfa-2a/ribavirin for 48 or 72 weeks in hepatitis C genotypes 1 and 4 patients with slow virologic response. Gastroenterology. 2010;138:503–12. 12 e1.
12. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;46:403–10.
13. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol. 2006;78:83–90.
14. Okanoue T, Itoh Y, Hashimoto H, Yasui K, Minami M, Takehara T, et al. Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. J Gastroenterol. 2009;44:952–63.
15. Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka T, Takaki S, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol. 2009;81:640–9.
16. Ishii K, Shinohara M, Sawa M, Kogame M, Higami K, Sano M, et al. Interferon alpha receptor 2 expression by peripheral blood monocytes in patients with a high viral load of hepatitis C virus genotype 1 showing substitution of amino acid 70 in the core region. Intervirology. 2010;53:105–10.
17. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. J Med Virol. 2009;81:452–8.
18. Sarrazin C, Herrmann E, Bruch K, Zeuzem S. Hepatitis C virus nonstructural 5A protein and interferon resistance: a new model for testing the reliability of mutational analyses. J Virol. 2002;76:11079–90.
19. Jenke AC, Moser S, Orth V, Zilbauer M, Gerner P, Wirth S. Mutation frequency of NS5A in patients vertically infected with HCV genotype 1 predicts sustained virological response to peginterferon alfa-2b and ribavirin combination therapy. J Viral Hepat. 2009;16:853–9.
20. Layden-Almer JE, Kuiken C, Ribeiro RM, Kunstman KJ, Perelson AS, Layden TJ, et al. Hepatitis C virus genotype 1a NS5A pretreatment sequence variation and viral kinetics in African American and white patients. J Infect Dis. 2005;192:1078–87.
21. Vuillermoz I, Khattab E, Sablon E, Ottevaere I, Durantel D, Vieux C, et al. Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon-ribavirin therapy. J Med Virol. 2004;74:41–53.
22. Puig-Basagoiti F, Forns X, Furcic I, Ampurdanes S, Gimenez-Barcons M, Franco S, et al. Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. J Gen Virol. 2005;86:1067–75.
23. Wohnsland A, Hofmann WP, Sarrazin C. Viral determinants of resistance to treatment in patients with hepatitis C. Clin Microbiol Rev. 2007;20:23–38.
24. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48:38–47.
25. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol. 2007;51:471–82.
26. Yang SS, Lai MY, Chen DS, Chen GH, Kao JH. Mutations in the NS5A and E2-PePHD regions of hepatitis C virus genotype 1b and response to combination therapy of interferon plus ribavirin. Liver Int. 2003;23:426–33.

27. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis—diagnosis, grading and staging. Hepatology. 1994;19:1513–20.
28. Chayama K, Suzuki F, Tsubota A, Akuta N, Someya T, Kobayashi M, et al. Evaluation of quantitative measurements of hepatitis C virus RNA to predict sustained response to interferon by genotype. J Virol Methods. 2001;95:33–45.
29. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A. 1990;87:9524–8.
30. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis-C virus 1b – sensitivity to interferon is conferred by amino-acid substitutions in the NS5A region. J Clin Invest. 1995;96:224–30.
31. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334:77–81.
32. Hall M, Frank E, Holmes G, Pfahringer B, Reutemann P, Witten IH. The WEKA data mining software: an update. SIGKDD Explor. 2009;11:10–8.
33. Zeuzem S, Berg T, Moeller B, Hinrichsen H, Mauss S, Wedemeyer H, et al. Expert opinion on the treatment of patients with chronic hepatitis C. J Viral Hepat. 2009;16:75–90.
34. Buti M, Lurie Y, Zakharova NG, Blokhina NP, Horban A, Teuber G, et al. Randomized trial of peginterferon alfa-2b and ribavirin for 48 or 72 weeks in patients with hepatitis C virus genotype 1 and slow virologic response. Hepatology. 2010;52:1201–7.
35. Farnik H, Lange CM, Sarrazin C, Kronenberger B, Zeuzem S, Herrmann E. Meta-analysis shows extended therapy improves response of patients with chronic hepatitis C virus genotype 1 infection. Clin Gastroenterol Hepatol. 2010;8:884–90.
36. Watanabe S, Enomoto N, Koike K, Izumi N, Takikawa H, Hashimoto E, et al. Prolonged treatment with pegylated interferon alpha 2b plus ribavirin improves sustained virological response in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan. Hepatol Res. 2010;40:135–44.
37. Kurosaki M, Matsunaga K, Hirayama I, Tanaka T, Sato M, Yasui Y, et al. A predictive model of response to peginterferon ribavirin in chronic hepatitis C using classification and regression tree analysis. Hepatol Res. 2010;40:251–60.
38. El Malki HO, El Mejdoubi Y, Souadka A, Mohsine R, Ifrine L, Abouqal R, et al. Predictive model of biliocystic communication in liver hydatid cysts using classification and regression tree analysis. BMC Surg. 2010;10:16.
39. Augustin S, Muntaner L, Altamirano JT, Gonzalez A, Saperas E, Dot J, et al. Predicting early mortality after acute variceal hemorrhage based on classification and regression tree analysis. Clin Gastroenterol Hepatol. 2009;7:1347–54.

Journal of General Virology (2011), 92, 1071–1081 DOI 10.1099/vir.0.029124-0

IL-28B predicts response to chronic hepatitis C therapy – fine-mapping and replication study in Asian populations

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Type I interferon (IFN) is used for the treatment of chronic hepatitis C virus (HCV) infection. Despite advances in antiviral therapy, a large proportion of patients remain infected following current therapies. Through a genome-wide scan, we found two variants (rs8099917 and rs12979860) in the IL-28B locus that affect the outcome of PEG-IFN and ribavirin combination therapy, consistent with recent studies ($P=6.52\times10^{-8}$; odds ratio 2.46 and $P=8.63\times10^{-8}$, odds ratio 2.40, respectively). Significant associations were also observed in the case of IFN monotherapy for HCV genotypes 1b and 2a. With rs8099917, HCV genotype 1b patients had a significantly lower frequency of the favourable genotype (86.6 %) compared with healthy controls (91.7 %), and HCV genotype 2a patients had an intermediate frequency (89.9 %). Similar results were found for rs12979860. Fine-mapping analysis revealed that rs8099917 had the strongest association with treatment outcome and 14 others, including four novel single nucleotide polymorphisms, had comparable associations. Haplotype analysis revealed that none of the haplotypes showed stronger association than any single marker. Early non-responders who could not achieve 2 log viral decline during the first 12 weeks of treatment had higher odds ratios for these two variants. The favourable allele of rs8099917 is also associated with initial viral decline at 2 and 4 weeks following the start of therapy. Multivariate analysis of PEG-IFN and ribavirin-treated patients showed that rs8099917 genotype, viral load, fibrosis and age were significant predictors of response to therapy. Common variation at the IL-28B locus is predictive of various IFN-based therapies for HCV independent of regimen or HCV genotype.

Received 22 November 2010
Accepted 10 January 2011

INTRODUCTION

Hepatitis C virus (HCV) is one of the major causes of liver cirrhosis and hepatocellular carcinoma (Barrera *et al.*, 1995; Welzel *et al.*, 2009). The only drug that can currently lead to the eradication of HCV is interferon (IFN). Sustained viral response (SVR), defined as HCV RNA negative 24 weeks after cessation of therapy, can be achieved by the current treatment regimen of pegylated-interferon (PEG-IFN) combined with ribavirin, but this can only be attained in less than 50 % of patients infected with genotype 1 HCV, in contrast to the higher eradication rates for other HCV subtypes (Hadziyannis *et al.*, 2004; Manns *et al.*, 2001). Both viral [e.g. HCV genotype, amino acid substitutions in the NS5A (Enomoto *et al.*, 1996) and core region (Akuta *et al.*, 2007)] and host factors [young age (Manns *et al.*, 2001), body mass index (Bressler *et al.*, 2003) and insulin resistance (Romero-Gómez *et al.*, 2005)] influence the outcome of IFN therapy. Viral load and the stage of liver fibrosis, which vary among patients even when they have been infected by the same donor (Casiraghi *et al.*, 2004; Kenny-Walsh & Irish Hepatology Research Group, 1999), have also been reported to influence the outcome of IFN therapy (Tsubota *et al.*, 1994). Different disease outcomes and effects of IFN therapy may also partly depend on host genetic factors, and different responses among ethnic groups have been reported (Conjeevaram *et al.*, 2006; Welzel *et al.*, 2009). To date, polymorphisms in myxovirus resistance protein A (Hijikata *et al.*, 2000; Knapp *et al.*, 2003), IFN-α-receptor 1 (Matsuyama *et al.*, 2003), osteopontin (Naito *et al.*, 2005) and mitogen-activated protein kinase-activated protein kinase 3 (Tsukada *et al.*, 2009) have been reported to be associated with IFN response by candidate gene approach. However, most of these studies were limited by their relatively small sample sizes and lack of validation. Thus, the host genetic factors that influence IFN responsiveness in HCV-infected patients have not been fully explored. Recently, Ge *et al.* reported an association between variation at the IL-28B locus and the outcome of PEG-IFN and ribavirin therapy among genotype 1b-infected European Americans, African Americans and Hispanics (Ge *et al.*, 2009). The association was also independently reported by two other research groups (Suppiah *et al.*, 2009; Tanaka *et al.*, 2009).

In this report, we analysed 2028 Japanese and 73 Taiwanese patients treated with IFN for HCV infection and compared them with 282 Japanese healthy control subjects. Our results not only replicate recent findings regarding the predictive effect of IL-28B variation on the outcome of PEG-IFN and ribavirin therapy against HCV genotype 1b, but also showed similar association in HCV patients with genotype 1b and genotype 2a under various therapeutic regimes. We also provide important resequencing data for the IL-28B locus.

RESULTS

IL-28 locus single nucleotide polymorphism (SNP) genotypes and the effect of therapy

SNPs (510 537) on the Illumina chips passed quality-control filters. During the quality control check, one subject from each of the 11 close relative pairs was removed from the association analysis according to PI_HAT value. Subsequent principal component analysis identified no outliers from the JPT/CHB clusters. Finally, 304 sustained responders (SRs) and 279 non-responders (NRs) treated with PEG-IFN plus ribavirin were retained and tested for association with the outcome of IFN therapy. Since the genomic control inflation factor was 0.945, indicating that population substructure effects were negligible, we did not correct for genomic control in the genome-wide association analysis. Two SNPs (rs8099917 and rs12979860) located upstream of IL-28B on chromosome 19 showed strong associations with the response to IFN therapy (uncorrected $P=2.7\times10^{-9}$, 3.9×10^{-9}, respectively). We conclude that these top two SNPs are in strong linkage disequilibrium and that we are detecting essentially the same signal ($r^2=0.95$). Thus, detailed results of only rs8099917 are shown (Table 1). The calculated odds ratio (OR) of the most significant SNP (rs8099917) for IFN response was 2.7 (95 % CI, 1.9–3.8) using the allele model. There was no significant association between SNPs and the treatment outcome in any other region.

As shown in Table 1, the other sets, including genotype 1b- as well as genotype 2a-infected patients, also showed statistically significant associations. A combined analysis of genotype 1b or genotype 2a sets also provided strong evidence of an association between treatment outcome and polymorphism at the IL-28B locus.

Genotype frequencies of the IL-28B polymorphisms in HCV genotypes 1b and 2a were compared with healthy control subjects (Table 2). Genotype 1b-infected patients had a significantly lower frequency of the favourable rs8099917 T allele (86.8 %; $P=2.9\times10^{-3}$) compared with healthy control (91.7 %), and genotype 2a-infected patients had an intermediate frequency (89.9 %; $P=0.41$). Reflecting their strong linkage disequilibrium ($r^2=0.98$), genotype frequencies of rs8099917 in each group showed the same tendency as those of rs12979860 C allele (genotype 1b; 86.6 %; $P=1.7\times10^{-3}$, genotype 2a; 89.9 %; $P=0.45$) compared to healthy control (91.5 %).

Resequencing and fine-mapping

We resequenced the IL-28B region surrounding the marker SNP associated with IFN treatment outcome. We used Phase II HapMap JPT genotype data and the HAPLOVIEW program (http://www.broadinstitute.org/haploview) to define a linkage disequilibrium (LD) block containing the landmark SNP. It should be noted that IL-28B and IL-28A genes are adjacent and are highly homologous. Therefore, although

Table 1. Results of genome-wide association analysis, validation analysis and meta-analysis

Association analysis results for rs8099917 (T/G) in six populations are provided.

Stage	Ethnicity	HCV genotype	SR			NR			Allele model			Co-dominant model*		Dominant model for allele 2		Recessive model for allele 2	
			TT	TG	GG	TT	TG	GG	OR (95 %CI)†	P‡	Phet§	OR (95 %CI)	P	OR (95 %CI)	P	OR (95 %CI)	P
Set-1	Japanese	1b	247 (81.8)	53 (17.5)	2 (0.7)	169 (60.1)	100 (35.6)	12 (4.3)	2.7 (1.9–3.8)	2.7×10^{-9}		2.8 (1.9–4.0)	6.6×10^{-8}	3.0 (2.0–4.3)	7.6×10^{-9}	6.7 (1.5–30.1)	5.3×10^{-3}
Set-2	Japanese	1b	59 (88.1)	8 (11.9)	0 (0.0)	81 (68.6)	33 (28.0)	4 (3.4)	3.3 (1.5–7.3)	1.9×10^{-3}		3.3 (1.5–7.3)	2.3×10^{-3}	3.4 (1.5–78)	3.1×10^{-3}	-	0.30
Set-3	Japanese	1b	200 (92.2)	16 (7.4)	1 (0.5)	394 (73.9)	129 (24.2)	10 (1.9)	3.8 (2.3–6.2)	4.1×10^{-8}		3.7 (2.3–6.2)	5.9×10^{-8}	4.2 (2.4–7.1)	2.4×10^{-8}	4.1 (0.53–32.5)	0.19
Set-4	Japanese	2a	289 (84.5)	50 (14.6)	3 (0.9)	126 (73.7)	40 (23.4)	5 (2.9)	1.9 (1.3–2.9)	1.4×10^{-3}		1.9 (1.3–2.8)	1.9×10^{-3}	1.9 (1.2–3.1)	3.3×10^{-3}	3.4 (0.80–14.4)	0.12
Set-5	Taiwanese	1b	21 (84.0)	3 (12.0)	1 (4.0)	9 (47.4)	10 (52.6)	0 (0.0)	3.2 (1.0–10.0)	4.3×10^{-2}		3.6 (1.0–12.6)	4.1×10^{-2}	5.8 (1.4–23.6)	2.0×10^{-2}	–	1
Set-5	Taiwanese	2a	24 (96.0)	1 (4.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)	16.3 (1.3–208)	4.7×10^{-2}		24.0 (1.5–395)	5.9×10^{-3}	24.0 (1.5–395)	4.2×10^{-2}	–	1
Combined analysis‖		1b							3.5 (2.6–4.6)	1.2×10^{-18}	0.66						
Combined analysis‖		2a							2.1 (1.3–3.2)	1.6×10^{-3}	0.05						
Combined analysis‖		Overall							3.0 (2.4–3.8)	1.0×10^{-20}	0.13						

*ORs and 95 % CI are calculated using logistic regression based on the co-dominant model.

†OR of minor allele from 2 × 2 allele frequency table.

‡*P*-values of Pearson's χ^2-test for the allele model.

§Results of Breslow–Day test.

‖Calculated by the Mantel–Haenszel method of combining allele-frequency counts.