

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		<i>p</i> value
	(-)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years ≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years ≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4/\text{mm}^3$)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15 ≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2 ≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non-SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥ 60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/\text{mm}^3$ vs. $15 \times 10^4/\text{mm}^3$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, $p = 0.0037$) between SVR and non-SVR patients. The distribution of fatty change in liver tissue ($\leq 10\%$ vs. 11–33% vs. 34% \leq ; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/\text{mm}^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/\text{mm}^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT

count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRD), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved $\geq 80\%$ adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon- γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

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References

- Manns MP, McHutchinson JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet*. 2001;358:958–65.
- Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonzales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med*. 2002;347:975–82.
- Hadziyannis S, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alfa-2a plus ribavirin combination therapy in chronic hepatitis C. a randomized study of treatment duration and ribavirin dose. *Ann Intern Med*. 2004;40:346–55.
- Hiramatsu N, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, et al. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. *Hepatol Res*. 2008;38:52–9.
- Honda T, Katano Y, Urano F, Murayama M, Hayashi K, Ishigami M, et al. Efficacy of ribavirin plus interferon- α in patients aged 60 years with chronic hepatitis C. *J Gastroenterol Hepatol*. 2007;22:989–95.
- Sezaki H, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, et al. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral load. *Dig Dis Sci* 2009;54:1317–24
- Puoti C, Castellacci R, Montagness F, Zaltron S, Stornaiuolo G, Bergami N, et al. Histological and virological features and follow-up of HCV carriers with normal aminotransferase levels: the Italian Study of the Asymptomatic C Carriers (ISACC). *J Hepatol*. 2002;37:117–23.
- Hui CK, Belaye T, Montegrande K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. *J Hepatol*. 2003;38:511–7.
- Okanoue T, Makiyama A, Nakayama M, Sumida Y, Mitsuyoshi H, Nakajima T, et al. A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. *J Hepatol*. 2005;43:599–605.
- Bressler BL, Guindi M, Tomlinson G, Heathcote J. High body mass index in an independent risk factor for non response to antiviral treatment in chronic hepatitis C. *Hepatology*. 2003;38:639–44.
- Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdi DM, Clouston AD, et al. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signaling 3 in patients with chronic hepatitis C, viral genotype 1. *Gut*. 2006;55:604–9.
- Patton HM, Patel K, Behling C, Bylund C, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. *J Hepatol*. 2004;40:484–90.
- Asselah T, Rubbia-Brandt L, Marcellin M, Negro F. Steatosis in chronic hepatitis C: why does it really matter? *Gut*. 2006; 55:123–30.
- Romero-Gomez M, Del Mar Vilorio M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, et al. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology*. 2005;128: 636–41.
- Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Cammozzi M, et al. Peginterferon alfa-2b plus ribavirin for native patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J Hepatol*. 2004;41:474–81.
- Everson GT, Hoefs JC, Seeff LB, Bonkovsky HL, Naishadham D, Shiffman ML, et al. Impact of disease severity on outcome of antiviral therapy for chronic hepatitis C: lessons from the HALT-C trial. *Hepatology*. 2006;44:1675–84.
- McHutchinson JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology*. 2002;123:1061–9.
- Jeffers LJ, Cassidy W, Howell CD, Hu S, Reddy R. Peginterferon alfa-2a (40kd) and ribavirin for black American patients with chronic HCV genotype 1. *Hepatology*. 2004;39:1702–6.
- Muir AJ, Bornstein JD, Killenberg PG, Atlantic Coast Hepatitis Treatment Group. Peginterferon alfa 2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *N Engl J Med*. 2004;350:2265–71.
- Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomized trial of interferon alpha 2b plus ribavirin for 48 weeks or 24 weeks versus interferon alpha 2b plus placebo for 48 weeks for treatment for chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet*. 1998;352:1426–32.
- Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5 A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med*. 1996;334:77–81.
- Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response in interferon-ribavirin combination therapy. *Intervirology*. 2005;48:372–80.
- Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the

- full-length hepatitis C virus open reading frame correlate with early response to therapy. *J Virol*. 2007;81:8211–24.
24. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology*. 2003;38:645–52.
 25. Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncalves FL, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a ribavirin. *J Hepatol*. 2005;43:425–33.
 26. Moucari R, Ripault M-P, Oules V, Martinot-Peignoux M, Asselah T, Boyer N, et al. High predictive value of early viral kinetics in retreatment with peginterferon and ribavirin of chronic hepatitis C patients non-responders to standard combination therapy. *J Hepatol*. 2007;46:596–604.
 27. 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 1a: amino acid substitutions in the core region and low-density lipoprotein cholesterol level. *J Hepatol*. 2007;46:403–10.
 28. Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: grading and staging. *Hepatology*. 1994;19:1513–20.
 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 USA*. 1990;87:9524–8.
 30. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. *J Hepatol*. 1999;30:653–9.
 31. Okanoue T, Itoh Y, Minami M, Hashimoto H, Yasui K, Yotsuyanagi H, et al. Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet count. *Hepatol Res*. 2008;38:27–36.
 32. Iwasaki Y, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, et al. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis. *Hepatology*. 2006;43:54–63.
 33. 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.
 34. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. *J Hepatol*. 2004;40:484–90.
 35. Lagging M, Romero A, Westin J, Norrkans G, Dhillon AP, Palwlosky JM, et al. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. *Hepatology*. 2006;44:1617–25.
 36. Pilli M, Zerbini A, Penna A, Orlandini A, Lukasiewicz E, Pawlowsky JM, et al. HCV-specific T-cell response in relation to viral kinetics and treatment outcome (DITTO-HCV Project). *Gastroenterology*. 2007;133:1132–43.
 37. Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology*. 2008;48:1753–60.

Genome-wide association of *IL28B* with response to pegylated interferon- α and ribavirin therapy for chronic hepatitis C

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The recommended treatment for patients with chronic hepatitis C, pegylated interferon- α (PEG-IFN- α) plus ribavirin (RBV), does not provide sustained virologic response (SVR) in all patients. We report a genome-wide association study (GWAS) to null virological response (NVR) in the treatment of patients with hepatitis C virus (HCV) genotype 1 within a Japanese population. We found two SNPs near the gene *IL28B* on chromosome 19 to be strongly associated with NVR (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, 3.11×10^{-15}). We replicated these associations in an independent cohort (combined P values, 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively). Compared to NVR, these SNPs were also associated with SVR (rs12980275, $P = 3.99 \times 10^{-24}$, and rs8099917, $P = 1.11 \times 10^{-27}$). In further fine mapping of the region, seven SNPs (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668) located in the *IL28B* region showed the most significant associations ($P = 5.52 \times 10^{-28}$ – 2.68×10^{-32} ; OR = 22.3–27.1). Real-time quantitative PCR assays in peripheral blood mononuclear cells showed lower *IL28B* expression levels in individuals carrying the minor alleles ($P = 0.015$).

Hepatitis C is a global health problem that affects a significant proportion of the world's population. The World Health Organization

estimated that in 1999, there were 170 million HCV carriers worldwide, with 3–4 million new cases appearing each year. HCV infection affects more than 4 million people in the United States, where it represents the leading cause of cirrhosis and hepatocellular carcinoma as well as the leading cause of liver transplantation¹. The American Gastroenterological Association estimated that drugs are the largest direct costs of hepatitis C¹.

The most effective current standard of care in patients with chronic hepatitis C, a combination of PEG-IFN- α with ribavirin, does not produce SVR in all patients treated. Large-scale studies on 48-week-long PEG-IFN- α /RBV treatment in the United States and Europe showed that 42–52% of patients with HCV genotype 1 achieved SVR^{2–4}, and similar results were found in Japan. However, older patients (greater than 50 years of age) had a significantly lower rate of SVR due to poor adherence resulting from adverse events and laboratory-detectable abnormalities such as neutropenia and thrombocytopenia^{5,6}. Specifically, various well-described side effects (such as a flu-like syndrome, hematologic abnormalities and adverse neuropsychiatric events) often necessitate dose reduction, and 10–14% of patients require premature withdrawal from interferon-based therapy⁷. To avoid these side effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of PEG-IFN- α /RBV treatment, it would be useful to be able to predict an individual's response before or early in treatment. Several viral factors, such as genotype 1, high baseline viral load, viral

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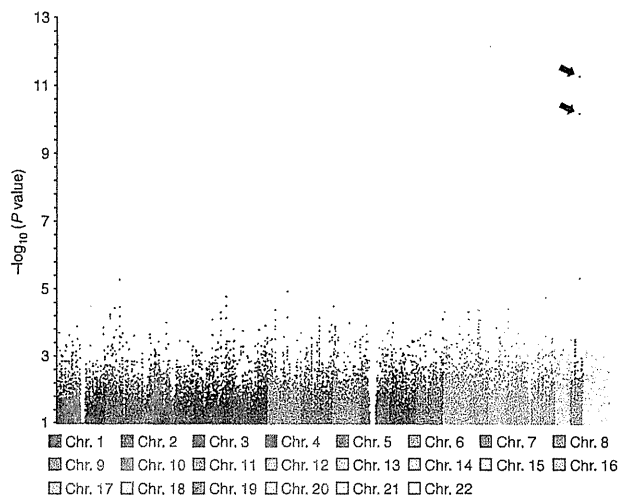


Figure 1 Genome-wide association results with PEG-IFN- α /RBV treatment in 142 Japanese patients with HCV (78 NVR and 64 VR samples). P values were calculated by using a χ^2 test for allele frequencies. The dots with arrows for chromosome 19 denote SNPs that showed significant genome-wide associations ($P < 8.05 \times 10^{-8}$) with response to PEG-IFN- α /RBV treatment.

kinetics during treatment, and amino acid pattern in the interferon sensitivity-determining region, have been reported to be significantly associated with the treatment outcome in a number of independent studies^{8–10}. Studies have also provided strong evidence that ~20% of patients with HCV genotype 1 and 5% of patients with genotype 2 or 3 have a null response to PEG-IFN- α /RBV. No definite predictor of this resistance is currently available that make it possible to bypass the initial 12–24 weeks' treatment before deciding whether treatment should be continued. If a reliable predictor of non-response were identified for use in patients before treatment initiation, then an estimated 20%, including those who have little or no chance to achieve SVR, could be spared the side effects and cost of treatment.

Host factors, including age, sex, race, liver fibrosis and obesity, have also been reported to be associated with PEG-IFN- α /RBV therapy outcome^{11,12}. However, little is known about the host genetic factors that might be associated with the response to therapy: thus far only

a few candidate genes, including those encoding type I interferon receptor-1 (*IFNAR1*) and mitogen-activated protein kinase-activated protein kinase 3 (*MAPKAPK3*), have been reported to be associated with treatment response^{13,14}. We describe here a GWAS for response to PEG-IFN- α /RBV treatment.

We conducted this GWAS to identify host genes associated with response to PEG-IFN- α /RBV treatment in 154 Japanese patients with HCV genotype 1 (82 with NVR and 72 with virologic response (VR), based on the selection criteria as described in Online Methods). We used the Affymetrix SNP 6.0 genome-wide SNP typing array for 900,000 SNPs. A total of 621,220 SNPs met the following criteria: (i) SNP call rate $\geq 95\%$, (ii) minor allele frequency (MAF) $\geq 1\%$ and (iii) deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in VR samples. After excluding 4 NVR and 8 VR samples that showed quality control (QC) call rates of $< 95\%$, 78 NVR and 64 VR samples were included in the association analysis. **Figure 1** shows a genome-wide view of the single-point association data based on allele frequencies. Two SNPs located close to *IL28B* on chromosome 19 showed strong associations, with a minor allele dominant model (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, $P = 3.11 \times 10^{-15}$, respectively), with NVR to PEG-IFN- α /RBV treatment (**Table 1**). The rs8099917 lies between *IL28B* and *IL28A*, ~8 kb downstream from *IL28B* and ~16 kb upstream from *IL28A*. These associations reached genome-wide levels of significance for both SNPs in this initial GWAS cohort (Bonferroni criterion $P < 8.05 \times 10^{-8}$ (0.05/621,220)). The frequencies of minor allele-positive patients were much higher in the NVR group than in the VR group for both SNPs (74.3% in NVR, 12.5% in VR for rs12980275; 75.6% in NVR, 9.4% in VR for rs8099917). Notably, individuals homozygous for the minor allele were observed only in the NVR group. The VR group, as compared to the NVR group, showed genotype frequencies closer to those in the healthy Japanese population¹⁵, yet the minor allele frequencies were slightly higher in the transient virologic response (TVR) group (23.1%, 15.4%) than in the SVR group (9.8%, 7.8%) (**Table 1**). We applied the Cochran-Armitage test on all the SNPs and found a genetic inflation factor, λ , of 1.029 for the GWAS stage (**Supplementary Fig. 1**). We also carried out principal component analysis in 142 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (**Supplementary Fig. 2**); this suggested that the effect of population stratification was negligible.

Table 1 Significant association of two SNPs (rs12980275 and rs8099917) with response to PEG-IFN- α /RBV treatment

dbSNP rsID	Nearest gene	MAF ^b (allele)	Allele (1/2)	Stage	Null responder (NVR ^a , n = 128)			Responder (VR ^a , n = 186)			Responder (SVR ^a , n = 140)			NVR vs. VR		NVR vs. SVR	
					11	12	22	11	12	22	11	12	22	OR (95% CI) ^c	P value ^d	OR (95% CI) ^c	P value ^d
rs12980275	<i>IL28B</i>	0.15 (G)	A/G	GWAS	20	54	4	56	8	0	46	5	0	20.3	1.93×10^{-13}	26.7	7.41×10^{-13}
					(25.6)	(69.2)	(5.1)	(87.5)	(12.5)	(0.0)	(90.2)	(9.8)	(0.0)	(8.3–49.9)		(9.3–76.5)	
					10	37	3	101	21	0	73	16	0	19.2	5.46×10^{-15}	18.3	8.37×10^{-13}
				Replication	(20.0)	(74.0)	(6.0)	(82.8)	(17.2)	(0.0)	(82.0)	(18.0)	(0.0)	(8.3–44.4)		(7.6–44.0)	
				Combined	30	91	7	157	29	0	119	21	0	17.7	2.84×10^{-27}	18.5	3.99×10^{-24}
					(23.4)	(71.1)	(5.5)	(84.4)	(15.6)	(0.0)	(85.0)	(15.0)	(0.0)	(10.0–31.3)		(10.0–34.4)	
rs8099917	<i>IL28B</i>	0.12 (G)	T/G	GWAS	19	56	3	58	6	0	47	4	0	30.0	3.11×10^{-15}	36.5	5.00×10^{-14}
					(24.4)	(71.8)	(3.8)	(90.6)	(9.4)	(0.0)	(92.2)	(7.8)	(0.0)	(11.2–80.5)		(11.6–114.6)	
					11	37	2	108	14	0	78	11	0	27.4	9.47×10^{-18}	25.1	1.00×10^{-14}
				Replication	(22.0)	(74.0)	(4.0)	(88.5)	(11.5)	(0.0)	(87.6)	(12.4)	(0.0)	(11.5–65.3)		(10.0–63.1)	
				Combined	30	93	5	166	20	0	125	15	0	27.1	2.68×10^{-32}	27.2	1.11×10^{-27}
					(23.4)	(72.7)	(3.9)	(89.2)	(10.8)	(0.0)	(89.3)	(10.7)	(0.0)	(14.6–50.3)		(13.9–53.4)	

^aNVR, null virologic response; VR, virologic response; SVR, sustained virologic response. The 186 VRs consisted of 46 transient virologic response (TVRs) and 140 SVRs. ^bMinor allele frequency and minor allele in 184 healthy Japanese individuals¹⁵. The MAF of the SNPs in SVR is similar to that of TVR group, whereas that of NVR is much higher (76.6%). ^cOdds ratio for the minor allele in a dominant model. ^d P value by χ^2 test for the minor allele dominant model.



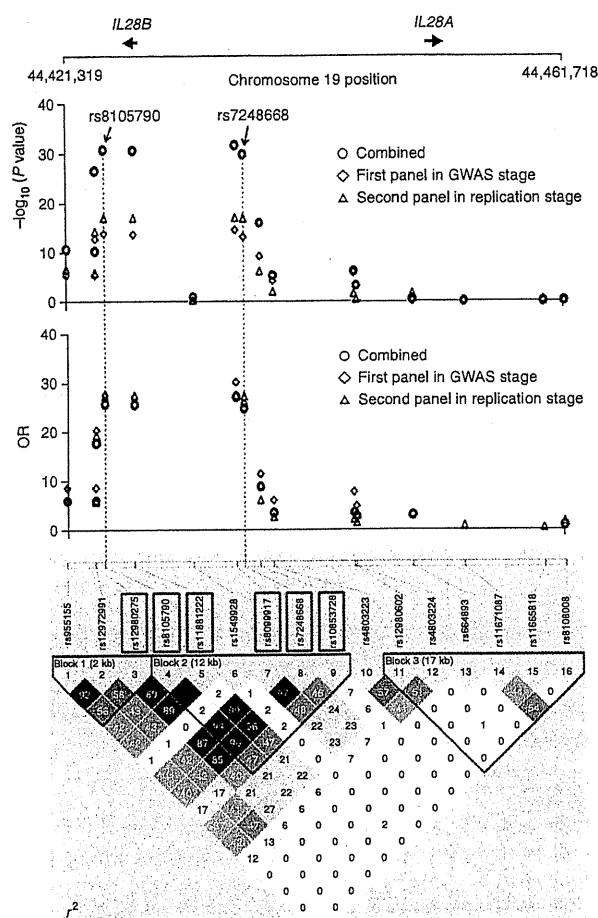


Figure 2 Genomic structure, P value and OR plots in association analysis and LD map around *IL28B* and *IL28A* (chr.19, nucleotide positions 44421319–44461718; build 35). P values by the χ^2 test for minor allele dominant effect model are shown for the first panel of 142 samples in the GWAS stage, the second panel of 172 samples in the replication stage, and the combined analysis. Below are estimates of pairwise r^2 for 16 SNPs selected in the replication study using a total of 314 Japanese patients with HCV treated with PEG-IFN- α /RBV. Boxes indicate the significantly associated SNPs with response to PEG-IFN- α /RBV treatment both in the GWAS stage and in the replication stage. Dotted lines indicate the region with the strongest associations from the positions of rs8105790 to rs7248668.

OR = 27.4 for rs8099917; Table 1). The combined P values for both stages reached 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively (Table 1). Notably, when we compared the SVR ($n = 140$) with the NVR group ($n = 128$), the original two SNPs (rs12980275 and rs8099917) again showed strong associations: both P values and ORs were similar to those observed in the comparison between VR and NVR, and the combined P values for both stages reached 3.99×10^{-24} (OR = 18.5; 95% CI = 10.0–34.4) and 1.11×10^{-27} (OR = 27.2; 95% CI = 13.9–53.4), respectively (Table 1). Comparing SVR ($n = 140$) versus NVR plus TVR ($n = 174$), we again found that these SNPs were significantly associated ($P = 1.71 \times 10^{-16}$, OR = 8.8; 95% CI 5.1–15.4 for rs12980275; $P = 1.18 \times 10^{-18}$, OR = 12.1; 95% CI 6.5–22.4 for rs8099917, Supplementary Table 2), suggesting that these SNPs would predict NVR as well as SVR before PEG-IFN- α /RBV therapy.

Among the newly analyzed SNPs in the replication study, six (rs12980275, rs8105790, rs11881222, rs8099917, rs7248668 and rs10853728) showed significant associations both in the GWAS stage ($P < 8.05 \times 10^{-8}$) and in the replication stage ($P < 0.0031$ (0.05/16)) after Bonferroni correction. These SNPs are located within a 15.7-kb region that includes *IL28B* (Fig. 2 and Supplementary Table 1). In particular, the strongest associations with NVR were observed for four SNPs, rs8105790, rs11881222, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron and the upstream flanking region of *IL28B*. The combined P values for these polymorphisms were 1.98×10^{-31} (OR = 25.7; 95% CI = 13.9–47.6), 2.84×10^{-31} (OR = 25.6; 95% CI = 13.8–47.3), 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3) and 1.84×10^{-30} (OR = 24.7; 95% CI = 13.3–45.8), respectively (Supplementary Table 1). We then sequenced this region to identify further variants and found three SNPs (rs8103142, rs28416813 and rs4803219) located in the third exon, the first intron and the upstream flanking region of *IL28B*, and a few infrequent variations. These SNPs also showed strong associations in the combined dataset of 128 NVR and 186 VR samples ($P = 1.40 \times 10^{-29}$, OR = 26.6 for rs8103142; $P = 5.52 \times 10^{-28}$, OR = 22.3 for rs28416813; $P = 2.45 \times 10^{-29}$, OR = 23.3 for rs4803219; Supplementary Table 3). We also performed LD and haplotype analyses with seven SNPs. These SNPs were in strong LD, and the risk haplotype showed a level of association similar to those of individual SNPs ($P = 1.35 \times 10^{-25}$, OR = 11.1; 95% CI = 6.6–18.6) (Table 2). These results suggest that the association with NVR was primarily driven by one of these SNPs.

We analyzed the region of ~40 kb (chr. 19, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) using Haploview software for linkage disequilibrium (LD) and haplotype structure based on the HapMap data for individuals of Japanese ancestry. The LD blocks were analyzed using the four-gamete rule, and four blocks were observed (Supplementary Fig. 3). We selected 16 SNPs for both replication study and high-density association mapping, including tagging SNPs estimated on the basis of the haplotype blocks, one SNP located within *IL28B* (rs11881222) and the significantly associated SNPs from the GWAS stage (rs12980275 and rs8099917) (Supplementary Table 1).

To validate the results of the GWAS stage, 16 SNPs selected for the replication stage, including the original SNPs, were genotyped using the DigiTag2 assay in an independent set of 172 Japanese patients with HCV treated with PEG-IFN- α /RBV treatment (50 NVR and 122 VR samples), together with the first panel of 142 samples analyzed in the GWAS stage (Supplementary Table 1). The associations of the original SNPs were replicated in the replication cohort of 172 patients ($P = 5.46 \times 10^{-15}$, OR = 19.2 for rs12980275; $P = 9.47 \times 10^{-18}$,

Table 2 Association analysis of response to treatment by *IL28B* haplotype

SNP							Frequencies		P value	OR (95% CI)
rs8105790	rs11881222	rs8103142	rs28416813	rs4803219	rs8099917	rs7248668	NVR group	VR group		
T	A	T	C	C	T	G	0.543	0.942	1.81×10^{-32}	0.1 (0.04–0.12)
C	G	C	G	T	G	A	0.387	0.054	1.35×10^{-25}	11.1 (6.6–18.6)

Association analysis of haplotypes consisting of seven SNPs with response to PEG-IFN- α /RBV treatment in 314 Japanese patients with HCV. Boldface letters: rs11881222 (third intron); rs8103142 (third exon).



LETTERS

Table 3 Factors associated with NVR by logistic regression model

Factors	Odds ratio	95% CI	P value
rs8099917 (G allele)	37.68	16.71–83.85	<0.0001
Age	1.02	0.98–1.07	0.292
Gender (Female)	3.32	1.49–7.39	0.003
Re-treatment ^a	1.12	0.55–2.33	0.750
Platelet count	0.93	0.87–1.01	0.080
Aminotransferase level	1.00	0.99–1.00	0.735
Fibrosis stage ²⁰	1.10	0.73–1.66	0.658
HCV-RNA level	1.01	0.99–1.02	0.139

^aRe-treatment, non-response to previous treatment with interferon- α (plus RBV).

To examine the relative contribution of factors associated with NVR, we used a logistic regression model. One tagging SNP located within *IL28B* (minor allele of rs8099917) was the most significant factor for predicting NVR, followed by gender (Table 3). Clinically, viral factors such as HCV genotype and HCV RNA level are important for the outcome of PEG-IFN- α /RBV therapy. Indeed, mean HCV-RNA level was significantly lower in SVR (SVR versus TVR, $P = 0.002$; SVR versus NVR, $P = 0.016$; Supplementary Table 4). Mean platelet count and the proportion of mild fibrosis (F1–F2) were significantly higher in SVR than in NVR.

Real-time quantitative PCR assays in peripheral blood mononuclear cells revealed a significantly lower level of *IL28* mRNA expression in individuals with the minor alleles (Fig. 3), suggesting that variant(s) regulating *IL28* expression is associated with a response to PEG-IFN- α /RBV treatment. *IL28B* encodes a cytokine distantly related to type I (α and β) interferons and the interleukin (IL)-10 family. This gene and *IL28A* and *IL29* (encoding IL-28A and IL-29, respectively) are three closely related cytokine genes that encode proteins known as type III IFNs (IFN- λ s) and that form a cytokine gene cluster at chromosomal region 19q13 (ref. 16). The three cytokines are induced by viral infection and have antiviral activity^{16,17}. All three interact with a heterodimeric class II cytokine receptor that consists of IL-10 receptor beta (IL10R β) and IL-28 receptor alpha (IL28R α , encoded by *IL28RA*)^{16,17}, and they may serve as an alternative to type I IFNs in providing immunity to viral infection.

Notably, a recent report showed that the strong antiviral activity evoked by treating mice with TLR3 or TLR9 agonists was significantly reduced in both *IL28RA*^{-/-} and *IFNAR*^{-/-} mice, indicating that IFN- λ is important in mediating antiviral protection by ligands for TLR3 and TLR9 (ref. 18). IFN- λ induced a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN- α induced the same genes with more rapid and transient kinetics¹⁹. Therefore, it is possible that IFN- λ induces a slower but more sustained response that is important for TLR-mediated antiviral protection. This might be one of the ways that a genetic variant regulating *IL28* expression influences the response to PEG-IFN- α /RBV treatment. Further research will be required to fully understand the specific mechanism by which a genotype might affect the response to treatment.

In conclusion, the strongest associations with NVR were observed for seven SNPs, rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron, the third exon, the first intron and the upstream flanking region of *IL28B*. Further studies following our report of this robust genetic association to NVR may make it possible to develop a pre-treatment predictor of which individuals are likely to respond to PEG-IFN- α /RBV treatment. This would remove the need for the initial 12–24 weeks of treatment that is currently used as a basis for a clinical decision about whether treatment should be continued. That would allow better targeting of PEG-IFN- α /RBV

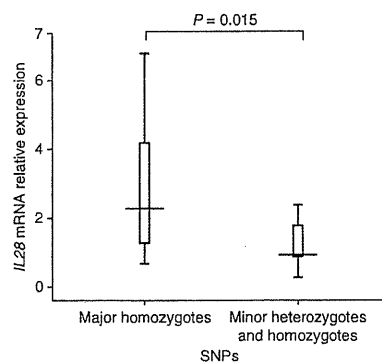


Figure 3 Quantification of *IL28* mRNA expression. The expression level of *IL28* genes was determined by real-time quantitative RT-PCR using RNA purified from peripheral blood mononuclear cells. Distribution of relative gene expression levels was compared between the individuals homozygous for major alleles ($n = 10$) and the heterozygous or homozygous individuals carrying minor alleles ($n = 10$) of rs8099917 by using the Mann-Whitney U -test. The bars indicate the median. All samples were obtained from HCV-infected patients before PEG-IFN- α /RBV therapy.

treatment, avoiding the unpleasant side effects that commonly accompany the treatment where it is unlikely to be beneficial, and reduce overall treatment costs. Because of the small number of samples in this study, we plan to conduct a further prospective multicenter study to establish these SNPs as a clinically useful marker.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturegenetics/>.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study design and discussion: Y.T., N.N., N.M., K.T., M.M.; sample collection: Y.T., M.K., K.M., N.S., M.N., M.K., K.H., S.H., Y.I., E.M., E.T., S.M., Y.M., M.H., A.S., Y.H., S.N., I.S., M.I., K.I., K.Y., F.S., N.I.; genotyping: N.N.; statistical analysis: N.N., A.K., K.I.; quantitative RT-PCR: M.S.; manuscript writing: Y.T., N.N., K.T., M.M.

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1. Ray Kim, W. Global epidemiology and burden of hepatitis C. *Microbes Infect.* **4**, 1219–1225 (2002).
2. Manns, M.P. *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* **358**, 958–965 (2001).



3. Fried, M.W. *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* **347**, 975–982 (2002).
4. Hadziyannis, S.J. *et al.* 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 (2004).
5. Bruno, S. *et al.* Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J. Hepatol.* **41**, 474–481 (2004).
6. Sezaki, H. *et al.* Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. *Dig. Dis. Sci.* **54**, 1317–1324 (2009).
7. Fried, M.W. Side effects of therapy of hepatitis C and their management. *Hepatology* **36**, S237–S244 (2002).
8. Pascu, M. *et al.* Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. *Gut* **53**, 1345–1351 (2004).
9. Shirakawa, H. *et al.* Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* **48**, 1753–1760 (2008).
10. Akuta, N. *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.* **46**, 403–410 (2007).
11. Walsh, M.J. *et al.* Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* **55**, 529–535 (2006).
12. Gao, B., Hong, F. & Radaeva, S. Host factors and failure of interferon-alpha treatment in hepatitis C virus. *Hepatology* **39**, 880–890 (2004).
13. Matsuyama, N. *et al.* The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. *Hepatol. Res.* **25**, 221–225 (2003).
14. Tsukada, H. *et al.* A polymorphism in MAPKAPK3 affects response to interferon therapy for chronic hepatitis C. *Gastroenterology* **136**, 1796–1805 (2009).
15. Nishida, N. *et al.* Evaluating the performance of Affymetrix SNP Array 6.0 platform with 400 Japanese individuals. *BMC Genomics* **9**, 431 (2008).
16. Kottenko, S.V. *et al.* IFN- λ s mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat. Immunol.* **4**, 69–77 (2003).
17. Sheppard, P. *et al.* IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat. Immunol.* **4**, 63–68 (2003).
18. Ank, N. *et al.* An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J. Immunol.* **180**, 2474–2485 (2008).
19. Marcello, T. *et al.* Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* **131**, 1887–1898 (2006).
20. Desmet, V.J., Gerber, M., Hoofnagle, J.H., Manns, M. & Scheuer, P.J. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* **19**, 1513–1520 (1994).





ONLINE METHODS

Study cohorts. From April 2007 to April 2009, samples were obtained from 314 patients with chronic HCV (genotype 1) infection who were treated at 15 multicenter hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN- α 2b (1.5 μ g per kg body weight (μ g/kg) subcutaneously once a week) or PEG-IFN- α 2a (180 μ g/kg once a week) plus RBV (600–1,000 mg daily depending on body weight). As a reduction in the dose of PEG-IFN- α and RBV can contribute to a less sustained virological response²¹, only patients with an adherence of >80% dose for both drugs during the first 12 weeks were included in this study. HBsAg-positive and/or anti-HIV–positive individuals were excluded from this study.

NVR (seen in ~20% of total treated patients) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks and detectable viremia 24 weeks after treatment. VR was defined as the achievement of SVR or transient TVR in this study; SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or on completion of the therapy. Of 878 patients with HCV genotype 1 treated by PEG-IFN- α /RBV at 14 hospitals, only 114 (13.0%) met the criteria for NVR in this study. For the GWAS stage of the study, a case-control study was conducted comparing individuals with NVR (82 individuals) and VR (72 individuals). For the replication stage, an independent cohort of samples from 172 Japanese patients with HCV genotype 1, including 50 with NVR and 122 with VR, was obtained from an independent cohort study at Tokyo Medical and Dental University Hospital (Ochanomizu Liver Conference Study Group) and Musashino Red Cross Hospital. Clinical data from the combined cohorts, with a total of 140 SVR, 46 TVR and 128 NVR patients, are shown in Supplementary Table 4.

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

SNP genotyping and data cleaning. In the GWAS stage, we genotyped 154 Japanese patients with HCV receiving PEG-IFN- α /RBV treatment using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. After exclusion of 4 NVR samples and 8 SVR samples with QC call rates <95%, the remaining 142 samples were recalled using the Birdseed version 3 software (Affymetrix). The average overall call rate of 78 NVR and 64 VR samples reached 99.46% and 99.46%, respectively. We then applied the following thresholds for QC in data cleaning: SNP call rate \geq 95% for all samples, MAF \geq 1% for all samples and HWE P value \geq 0.001 for VR group^{22,23}. A total of 621,220 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots for the SNPs showing $P < 0.001$ in association analyses by comparing allele frequencies in NVR and VR groups were checked by visual inspection. SNPs with ambiguous genotype calls were excluded. Supplementary Table 5 shows SNPs that might be weakly associated with NVR ($P < 10^{-4}$).

Although the 12 samples noted above were excluded from the GWAS stage by data cleaning, their quality was good enough for the SNP typing in the replication study, and thus they were included in the replication stage. In the subsequent replication stage with high-density association mapping, SNP genotyping in the independent set of 172 patients was completed using the DigiTag2 assay²⁴ and direct sequencing using the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). In addition, strongly associated SNPs identified in the GWAS stage were also genotyped for the GWAS samples using the DigiTag2 assay, and the results were 100% concordant to those from the GWAS platform.

Screening for new polymorphisms. To determine possible genomic variants in the region of *IL28B* and its promoter, we sequenced the 3.3-kb region in a total of 48 Japanese patients with HCV (28 NVR and 20 VR). We selected 7 samples from NVR patients who were minor allele homozygotes for 2 SNPs (rs12980275 and rs8099917), 11 samples from NVR and 10 samples from VR heterozygotes, and 10 samples from NVR and 10 samples from VR major

allele homozygotes. The sequencing primers were designed using the Visual OMP Nucleic Acid software (Supplementary Table 6). PCR was carried using TaKaRa LA *Taq* polymerase (Takara Biochemicals) under the following thermal cycler conditions: stage 1, 94 °C for 1 min; stage 2, 98 °C for 10 s, 68 °C for 15 min, for a total of 30 cycles; stage 3, 72 °C for 10 min. A 50- μ l PCR analysis was performed using 2.5 U TaKaRa LA *Taq* with 1 \times LA PCR buffer II, 0.4 mM dNTP, 10 pmol of each primer and 10 ng of genomic DNA. For sequencing, 7.0 μ l of the PCR products were incubated with 3 μ l of Exonuclease I/Shrimp Alkali Phosphatase (Takara Biochemicals) first for 90 min at 37 °C and then for another 10 min at 80 °C. Sequencing reactions were performed with the use of a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). After purification with MultiScreen-HV (Millipore) and Sephadex G-50 Fine (GE Healthcare UK Ltd.), the reaction products were applied to the Applied Biosystems 3730 DNA Analyzer.

In the variation screening, three SNPs (rs8103142, rs28416813 and rs4803219) and a few infrequent variations were detected. We then typed these SNPs in all of the 314 patients.

Statistical analysis. The observed association between a SNP and response to PEG-IFN- α /RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on the X chromosome were removed because gender was not matched between the NVR group and the VR group. A total of 621,220 SNPs passed the QC filters in the GWAS stage; therefore, significance levels after the Bonferroni correction for multiple testing were $P = 8.05 \times 10^{-8}$ (0.05/621,220) in the GWAS stage and $P = 0.0031$ (0.05/16) in the replication stage. None of the 16 markers genotyped in the replication stage showed deviations from Hardy-Weinberg equilibrium in the VR group ($P > 0.05$).

The inflation factor λ was estimated based on the median χ^2 and revealed to be 1.029 (median) and 1.011 (mean), suggesting that the population substructure should not have any substantial effect on the statistical analysis (Supplementary Fig. 1). In addition, the principal component analysis on the 142 patients (78 NVR samples and 64 VR samples) analyzed in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Supplementary Fig. 2).

For the replication study and the high-density association mapping, 16 SNPs were selected from the region of ~40 kb (chr. 9, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) in the GWAS stage by analyzing, using Haploview software, LD and haplotype structure based on the HapMap data for individuals of Japanese descent. These SNPs included tagging SNPs estimated on the basis of haplotype blocks, SNPs located within the *IL28B* and *IL28A* genes (rs11881222 and rs576832, respectively) and the significantly associated SNPs identified in the GWAS stage (Supplementary Table 1). On the basis of the genotype data from the total of 314 patients in the GWAS stage and replication stages, haplotype blocks were estimated using the four-gamete rule, and three blocks were observed (Fig. 2). Association of haplotype with response to PEG-IFN- α /RBV treatment was analyzed using Haploview software.

The logistic regression model was used to assess the factors associated with NVR. STATA 10 (Statacorp LP) was used for all analysis. Age, platelet count, and aminotransferase (ALT) and HCV-RNA levels were applied as continuous variables.

Real-time quantitative RT-PCR for *IL28* gene. A layer of mononuclear cells was collected via Ficoll from peripheral blood. Total RNA was isolated using the RNeasy Mini Kit and the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript II reverse transcriptase with Oligo (dT)_{12–18} primer (Invitrogen). The relative quantification of the target gene was determined using Custom TaqMan Gene Expression Assays, and the expression of glyceraldehyde-3-phosphate dehydrogenase was used to normalize the gene expression level (Applied Biosystems) according to the manufacturer's protocol. The data were analyzed by the 2^[- $\Delta\Delta C_t$] method using Sequence Detector version 1.7 software (Applied Biosystems). A standard curve was prepared by serial tenfold dilutions of

human cDNA. The curve was linear over 7 logs with a correlation coefficient of 0.998. The specific detection of *IL28B* in real-time PCR is hard to establish, because the nucleotide differences between *IL28A* and *IL28B* consist of only 9 nucleotides scattered throughout the gene. Primers and probes are designed for the *IL28* gene (Supplementary Table 6).

URLs. The results of the present GWAS have been registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

21. McHutchison, J.G. *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* **123**, 1061–1069 (2002).
22. Miyagawa, T. *et al.* Variant between *CPT1B* and *CHKB* associated with susceptibility to narcolepsy. *Nat. Genet.* **40**, 1324–1328 (2008).
23. Miyagawa, T. *et al.* Appropriate data cleaning methods for genome-wide association study. *J. Hum. Genet.* **53**, 886–893 (2008).
24. Nishida, N., Tanabe, T., Takasu, M., Suyama, A. & Tokunaga, K. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* **131**, 1887–1898 (2006).



Malnutrition Impairs Interferon Signaling Through mTOR and FoxO Pathways in Patients With Chronic Hepatitis C

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BACKGROUND & AIMS: Patients with advanced chronic hepatitis C (CH-C) often are malnourished, but the effects of malnutrition on interferon (IFN) signaling and response to treatment have not been determined. We assessed the importance of the nutritional state of the liver on IFN signaling and treatment response. **METHODS:** We studied data from 168 patients with CH-C who were treated with the combination of pegylated-IFN and ribavirin. Plasma concentrations of amino acids were measured by mass spectrometry. Liver gene expression profiles were obtained from 91 patients. Huh-7 cells were used to evaluate the IFN signaling pathway, mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO). Antiviral signaling induced by branched-chain amino acids (BCAAs) was determined using the in vitro hepatitis C virus replication system. **RESULTS:** Multivariate logistic regression analysis showed that Fischer's ratio was associated significantly with nonresponders, independent of interleukin-28B polymorphisms or the histologic stage of the liver. Fischer's ratio was correlated inversely with the expression of BCAA transaminase 1, and was affected by hepatic mTORC1 signaling. IFN stimulation was impaired substantially in Huh-7 cells grown in medium that was low in amino acid concentration, through repressed mTORC1 signaling, and increased Socs3 expression, which was regulated by Foxo3a. BCAA could restore impaired IFN signaling and inhibit hepatitis C virus replication under conditions of malnutrition. **CONCLUSIONS:** Malnutrition impaired IFN signaling by inhibiting mTORC1 and activating Socs3 signaling through Foxo3a. Increasing BCAAs to up-regulate IFN signaling might be used as a new therapeutic approach for patients with advanced CH-C.

Keywords: HCV; Liver Disease; Therapy; Diet.

Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C), but approximately 50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.¹

Recent landmark studies of genome-wide associations identified genomic loci associated with treatment responses to pegylated (Peg)-IFN and RBV combination therapy,^{2,3} and a polymorphism in the interleukin (IL)-28B gene was found to predict hepatitis C treatment-induced viral clearance. Moreover, we previously showed that expression of hepatic IFN-stimulated genes (ISGs) was associated with the IL-28B polymorphism and might contribute to the treatment response.⁴ In addition to the IL-28B polymorphism, host factors such as fibrosis stage and metabolic status of the liver might be associated with the treatment outcome^{4,5}; however, the significance of these factors in conjunction with the IL-28B polymorphism has not been evaluated fully.

In CH-C livers, prolonged liver cell damage, fibrosis development, and microcirculation failure can lead to a state of malnutrition in hepatocytes, resulting in the impairment of multiple metabolic pathways. In patients with advanced stage CH-C, hypoalbuminemia and decreased plasma values for the Fischer's ratio of branched-amino acids (BCAA; leucine, isoleucine, and valine) to aromatic amino acids (tyrosine and phenylalanine) commonly are observed. BCAA are the essential amino acids necessary for ammonium metabolism in muscle when the liver is unable to perform this function. Recent reports have shown that BCAA activates albumin synthesis in rat

Abbreviations used in this paper: BCAA, branched-chain amino acid; BCAT1, branched chain amino-acid transaminase 1; CH-C, chronic hepatitis C; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBE, Foxo binding element; FBEmut, Foxo binding element mutant; FoxO, forkhead box, subgroup O; GLuc, Gaussia luciferase; IFN, interferon; IL, interleukin; ISG, interferon-stimulated genes; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NR, no response; PCR, polymerase chain reaction; Peg, pegylated; p-mTOR, phosphorylated form of mammalian target of rapamycin; pS6K, phosphorylated form of p70 S6 protein kinase; pSTAT1, phosphorylated form of signal transducer and activator of transcription 1; Raptor, regulatory associated protein of mTOR; RBV, ribavirin; S6K, p70 S6 protein kinase; siRNA, small interfering RNA; SVR, sustained viral response; TR, transient response.

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primary hepatocytes⁶ and cirrhotic rat liver⁷ through mammalian target of rapamycin (mTOR) signaling, a central regulator of protein synthesis, by sensing nutrient conditions.⁸ Thus, peripheral amino acid composition is closely related to signaling pathways in the liver.

In addition to metabolic aspects, recent reports have elucidated new functional roles for mTOR in the IFN signaling pathway. Targeted disruptions of tuberous sclerosis 2 and eukaryotic translation initiation factor 4E binding protein 1, which both inhibit mTOR complex 1 (mTORC1) signaling, substantially enhanced IFN- α -dependent antiviral responses.^{9,10} Therefore, mTORC1 signaling might be involved in the antiviral response as well as in metabolic processes. However, these issues have not yet been addressed in terms of IFN treatment for CH-C. In the present study, therefore, we evaluated the clinical relevance of the nutritional state of the liver, as estimated by the plasma Fischer's ratio, on Peg-IFN and RBV combination therapy. We also evaluated antiviral signaling induced by BCAA using an in vitro HCV replication system.

Materials and Methods

Patients

A total of 168 patients with CH-C at the Graduate School of Medicine at Kanazawa University Hospital (Kanazawa, Japan) and its related hospitals in Japan (Table 1, Supplementary Table 1) were evaluated in the present study. The clinical characteristics of these patients have been described previously.⁴ All patients were administered Peg-IFN- α 2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks. The definition of the treatment response was as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapse during the follow-up period; and no response (NR). Genetic variation of the IL-28B polymorphism at rs8099917 was evaluated in all patients using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) as described previously.⁴ Gene expression profiling in the liver was performed in 91 patients using the Affymetrix Human 133 Plus 2.0 microarray chip (Affymetrix, Santa Clara, CA) as described previously (Supplementary Table 1).⁴

Plasma Amino Acid Analysis

Amino acid concentrations in plasma samples were measured by high-performance liquid chromatography-electrospray ionization-mass spectrometry, followed by derivatization.¹¹ Detailed experimental procedures are described in the Supplementary Materials and Methods section.

Culture Medium

Huh-7 and Huh-7.5 cells (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10%

fetal bovine serum and 1% penicillin/streptomycin (normal medium). Amino acid-free medium (ZERO medium) was prepared by mixing 5.81 g nutrition-free DMEM (Nacalai Tesque, Kyoto, Japan), 1.85 g NaHCO₃, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22- μ m filter (Millipore, Billerica, MA). Low amino acid media ($\times 1/5$, $\times 1/10$, $\times 1/30$, and $\times 1/100$ DMEM) were prepared by diluting $\times 1$ DMEM with ZERO medium. Powdered BCAA (leucine-isoleucine-valine, 2:1:1.2) (Ajinomoto Pharma, Tokyo, Japan) was freshly dissolved with distilled water at 100 mmol/L, then applied to cultured medium at 2 mmol/L, 4 mmol/L, or 8 mmol/L.

Western Blotting and Immunofluorescence Staining

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium and maintained for up to 24 hours. Western blotting was performed as previously described.¹² Cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer containing complete Protease Inhibitor Cocktail and PhosSTOP (Roche Applied Science, Indianapolis, IN). The membranes were blocked in Blocking One-P (Nacalai Tesque). The antibodies used for Western blotting are summarized in the Supplementary Materials and Methods section.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton-X 100 in PBS. The primary anti-forkhead box O (Foxo)3a antibody (Abcam, Cambridge, MA) was used at a final concentration of 2 μ g/mL in PBS containing 2% fetal bovine serum at 4°C for 16 hours. Incubation with the Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA) at a 500-fold dilution in PBS containing 3% fetal bovine serum antibody was performed for 4 hours, and cells were stained with Hoechst 33258 to visualize nuclear DNA (Vector Laboratories, Burlingame, CA).

Quantitative Real-Time Detection Polymerase Chain Reaction

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium, to which IFN- α and/or BCAA was added, and maintained for 24 hours. Rapamycin treatment (100 nmol/L) was performed for 30 minutes in normal medium before a medium change. RNA was isolated using TriPure isolation reagent (Roche Applied Science), and complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time detection polymerase chain reaction (PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) containing specific primers according to the manufacturer's

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR	NR	Univariate P value	Multivariate odds (95% CI)	Multivariate P value
Patients, n	125	43		—	
Age and sex					
Age, y	57 (30–72)	56 (30–73)	.927	—	
Sex, male vs female	68 vs 57	24 vs 19	.872	—	
Liver histology					
F stage (F1–2 vs F3–4)	95 vs 30	20 vs 23	.001	6.35 (2.02–23.7)	.001
A grade (A0–1 vs A2–3)	68 vs 57	19 vs 24	.248	—	
Host gene factors					
IL-28B (TT vs TG/GG) ^a	109 vs 12	12 vs 31	<.001	19.7 (5.74–82.7)	<.001
ISGs (Mx, IFI44, IFIT1), (<3.5 vs ≥3.5)	103 vs 22	12 vs 31	<.001	5.26 (1.65–17.6)	.005
Metabolic factors					
BMI, kg/m ²	23.2 (16.3–34.7)	23.4 (19.5–40.6)	.439	—	
TG, mg/dL	98 (30–323)	116 (45–276)	.058	—	
T-Chol, mg/dL	167 (90–237)	160 (81–214)	.680	—	
LDL-Chol, mg/dL	82 (36–134)	73 (29–123)	.019	—	
HDL-Chol, mg/dL	42 (20–71)	47 (18–82)	.098	—	
FBS, mg/dL	94 (60–291)	96 (67–196)	.139	—	
Insulin, μU/mL	6.6 (0.7–23.7)	6.8 (2–23.7)	.039	—	
HOMA-IR	1.2 (0.3–11.7)	1.2 (0.4–7.2)	.697	—	
Fischer ratio	2.3 (1.5–3.3)	2.1 (1.5–2.8)	.005	8.91 (1.62–55.6)	.011
Other laboratory parameters					
AST level, IU/L	46 (18–258)	64 (21–283)	.017	—	
ALT level, IU/L	60 (16–376)	82 (18–345)	.052	—	
γ-GTP level, IU/L	36 (4–367)	75 (26–392)	<.001	—	
WBC, /mm ³	4800 (2100–11100)	4800 (2500–8200)	.551	—	
Hb level, g/dL	14 (9.3–16.6)	14.4 (11.2–17.2)	.099	—	
PLT, ×10 ⁴ /mm ³	15.7 (7–39.4)	15.2 (7.6–27.8)	.378	—	
Viral factors					
ISDR mutations ≤1 vs ≥2	80 vs 44	34 vs 9	.070	4.12 (1.25–15.9)	.019
HCV-RNA, KIU/mL	2300 (126–5000)	1930 (140–5000)	.725	—	
Treatment factors					
Total dose administered					
Peg-IFN, μg	3840 (960–7200)	3840 (1920–2880)	.916	—	
RBV, g	202 (134–336)	202 (36–336)	.531	—	
Achieved administration rate					
Peg-IFN, %					
≥80%	84	28	.975	—	
<80%	42	14			
RBV (%)					
≥80%	76	24	.745	—	
<80%	50	18			
Achievement of EVR	101/125 (81%)	0/43 (0%)	<.001	—	

BMI, body mass index; CI, confidence interval; FBS, fasting blood sugar; γ-GTP, gamma-glutamyl transpeptidase; Hb, hemoglobin; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; PLT, platelets; T-chol, total cholesterol; TG, triglycerides; WBC, leukocytes.

^aIL-28B SNP at rs8099917.

instructions. The primer sequence for real-time detection PCR is given in the Supplementary Materials and Methods section. HCV RNA was detected as described previously¹² and expression was standardized to that of glyceraldehyde-3-phosphate dehydrogenase.

Reporter Assay

Construction of the interferon stimulated response element (ISRE)-luc reporter plasmid and Socs3-luc or Socs3 (FoxO binding element mutant [FBEmut])-luc reporter plasmids is described in the Supplementary Materials and Methods section.

Huh-7 cells were transfected with the ISRE-luc reporter plasmid 24 hours before IFN-alfa treatment. Cells were

treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid media. After 24 hours, luciferase activities were measured using the Dual Luciferase assay system (Promega, Madison, WI). For Socs3 promoter activities, Huh-7 cells were transfected with Socs3-luc or Socs3 (FBEmut)-luc reporter plasmids together with the Foxo3a expression plasmid, and luciferase activities were measured after 24 hours. Values were normalized to the luciferase activity of the co-transfected pGL4.75 Renilla luciferase-expressing plasmid (Promega).

Knockdown Experiments

Huh-7 cells were transfected with Ctrl (Stealth RNAi Negative Control Low GC Duplex #2; Invitrogen) or

targets (regulatory associated protein of mTOR [Raptor] and Foxo3a) (Supplementary Materials and Methods) small interfering RNA (siRNA) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, cells were cultured in normal or low-amino-acid media for a further 24 hours. The knockdown effect was confirmed by Western blotting.

Chromatin Immunoprecipitation Assay

Detailed experimental procedures are described in the Supplementary Materials and Methods section.

HCV Replication Analysis

pH77S3 is an improved version of pH77S, a plasmid containing the full-length sequence of the genotype 1a H77 strain of HCV with 5 cell culture-adaptive mutations that promote its replication in Huh-7 hepatoma cells.¹³ pH77S.3/Gaussia luciferase (GLuc)2A is a related construct in which the GLuc sequence, fused to the 2A autocatalytic protease of foot-and-mouth virus RNA, was inserted in-frame between p7 and NS2¹⁴ (Supplementary Materials and Methods). A signal sequence in GLuc directs its secretion into cell culture media, allowing real-time, dynamic measurements of GLuc expression to be performed without the need for cell lysis.

A 10- μ g aliquot of synthetic RNA transcribed from pH77S.3/GLuc2A was used for electroporation. Cells were pulsed at 260 V and 950 μ F using the Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA) and plated in fresh normal medium for 12 hours to recover. Cell medium was changed to $\times 1$ DMEM without serum for 8 hours, then changed to low-amino-acid medium containing 0–8 mmol/L BCAA for a further 24 hours. Cells and culture medium were collected and used for GLuc assays, real-time detection PCR, and Western blotting. The number of viable cells was determined by a (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) assay (Promega).

Continuously JFH-1-infecting Huh-7 cells were obtained by the infection of Huh-7 cells with JFH-1 cell culture-derived HCV at a multiplicity of infection of 0.01. Cells were maintained in normal medium by passaging every 3–4 days for approximately 6 months. About 20%–30% of the cells consistently were positive for HCV core protein (Supplementary Figure 4). Culture medium of JFH-1-infecting Huh-7 cells was changed to the low-amino-acid medium containing 0–8 mmol/L BCAA for 24 hours. Cells then were collected and used for assays.

Statistical Analysis

Results are expressed as mean \pm standard deviation. Significance was tested by 1-way analysis of variance with the Bonferroni method, and differences were considered statistically significant at a *P* value of less than .05.

Results

Fischer's Ratio as a Predictive Factor for Treatment Response

The clinical characteristics of patients who received Peg-IFN and RBV combination therapy are shown in Table 1 and Supplementary Table 1, and explanations of these characteristics have been described previously.⁴ All patients were infected with HCV genotype 1b and had a high viral load (>100 IU/mL). We compared patients with SVR + TR against those with NR, as assessed by the overall plausibility of treatment response groups using Fisher's C statistic as previously described.⁴ We included data on the IL-28B polymorphism and plasma amino acid composition (aminogram).

Univariate regression analysis showed that no single amino acid was associated significantly with treatment response; however, using Fischer's ratio, the BCAA (Ile+Leu+Val)/aromatic amino acids (Phe+Tyr) ratio was associated significantly with treatment response ($P = .005$) (Table 1). Of the 121 patients with IL-28B major type, SVR, TR, and NR were observed in 53%, 37%, and 10%, respectively, and among 33 patients with IL-28B minor type, SVR, TR, and NR were observed in 15%, 17%, and 68%, respectively ($P < .001$) (data not shown). Fischer's ratio of SVR, TR, and NR was 2.35 ± 0.38 , 2.30 ± 0.29 , and 2.10 ± 0.31 , respectively ($P < .015$) (data not shown).

We selected IL-28B polymorphism, hepatic ISG expression, fibrosis stage, HCV RNA, interferon sensitivity determining region mutation, and Fischer's ratio as factors for multivariate analysis. Multivariate analysis revealed that the minor type of IL-28B polymorphism (TG or GG at rs8099917) (odds ratio, 19.7; $P < .001$), advanced fibrosis stage of the liver (F3–4) (odds ratio, 6.35; $P = .001$), high hepatic ISGs (≥ 3.5) (odds ratio, 5.26; $P = .005$), low Fischer's ratio (continuous range, 1.5–3.3) (unit odds, 8.91; $P = .011$), and presence of ISDR mutation (≤ 1) (odds ratio, 4.12; $P = .019$) independently contributed to NR (Table 1).

The distribution of the Fischer's ratio according to fibrosis stage is shown in Supplementary Figure 1. The ratio decreased significantly in advanced fibrosis stage (F3–4) compared with early fibrosis stage (F1). No significant association between major or minor type of IL-28B polymorphism and different fibrosis stages of the liver was observed (Supplementary Figure 1A). In early fibrosis (F1–2) (Supplementary Figure 1B), 90% (80 of 89) of SVR+TR cases had the major type of IL-28B polymorphism, and 94% (16 of 17) of NR cases had the minor type. However, in the advanced fibrosis stage of the liver (F3–4) (Supplementary Figure 1C), 85% (23 of 27) of SVR+TR cases had the major type of IL-28B polymorphism and 50% (10 of 20) of NR cases had the minor type. Thus, in advanced fibrosis stages, factors other than the IL-28B polymorphism appear to contribute to NR. Interestingly, the Fischer's ratio was significantly lower in NR patients than SVR+TR pa-

tients in the advanced fibrosis stage of the liver. Therefore, Fischer's ratio could be an important predictor for NR that is independent of IL-28B polymorphism and histologic stage of the liver.

Fischer's Ratio and mTORC1 Signaling in CH-C Livers

Hepatic gene expression in 91 of 168 patients (Supplementary Table 1) was obtained using Affymetrix genechip analysis as described previously.⁴ To examine the relationship between the plasma Fischer's ratio and mTORC1 signaling in the liver we evaluated the expression of key regulatory genes related to mTORC1 signaling. We found that expression of branched chain amino acid transaminase 1 (BCAT1), an important catalytic enzyme of BCAA, was significantly negatively correlated with Fischer's ratio (Figure 1A). This indicates that the plasma Fischer's ratio is regulated in the liver as well as in peripheral muscle. Interestingly, the expression of *c-myc*, a positive regulator of BCAT1 (Figure 1C),¹⁵ was correlated negatively with the Fischer's ratio (Figure 1B). The expression of PDCD4, a negative transcriptional target of ribosomal p70 S6 protein kinase (S6K), downstream of mTORC1, was correlated significantly with BCAT1 (Figure 1D and E). Thus, in CH-C livers, BCAT1 is induced with progressive liver disease and mTORC1 signaling is repressed, a process that might involve *c-myc*. Fischer's ratio of the plasma therefore can be seen to reflect mTORC1 signaling in the liver.

Impaired IFN Signaling in Huh-7 Cells Grown in Low-Amino-Acid Medium

Recent reports have shown the functional relevance of mTOR on IFN signaling and antiviral responses.^{9,10} To evaluate IFN- α signaling and the mTOR pathway, we used Huh-7 cells grown in different amino acid conditions ($\times 1$ DMEM, $\times 1/5$ DMEM, $\times 1/30$ DMEM, and $\times 1/100$ DMEM). The phosphorylated forms of mTOR (p-mTOR) and S6K (pS6K), an important downstream regulator of mTORC1 signaling, were decreased substantially in $\times 1/30$ DMEM and $\times 1/100$ DMEM (Figure 2A). Interestingly, the expression of the phosphorylated form of signal transducer and activator of transcription 1 (pSTAT1), an essential transducer of type 1 IFN signaling, also was decreased in these conditions (Figure 2A). Similarly, the expression of p-mTOR and pSTAT1 was repressed significantly in CH-C livers with a low Fischer's ratio compared with those with a high Fischer's ratio (Supplementary Figure 2, Supplementary Table 2).

To examine whether decreased pSTAT1 expression might be owing to repressed mTORC1 signaling, we knocked down the expression of Raptor, a specific subunit of mTORC1. We achieved more than 50% knockdown of Raptor by specific siRNA (Figure 2B). Under these conditions, the expression of p-mTOR and pS6K were repressed, which is consistent with previous reports.¹⁶ The expression of pSTAT1 also was repressed after Raptor knockdown (Figure 2B).

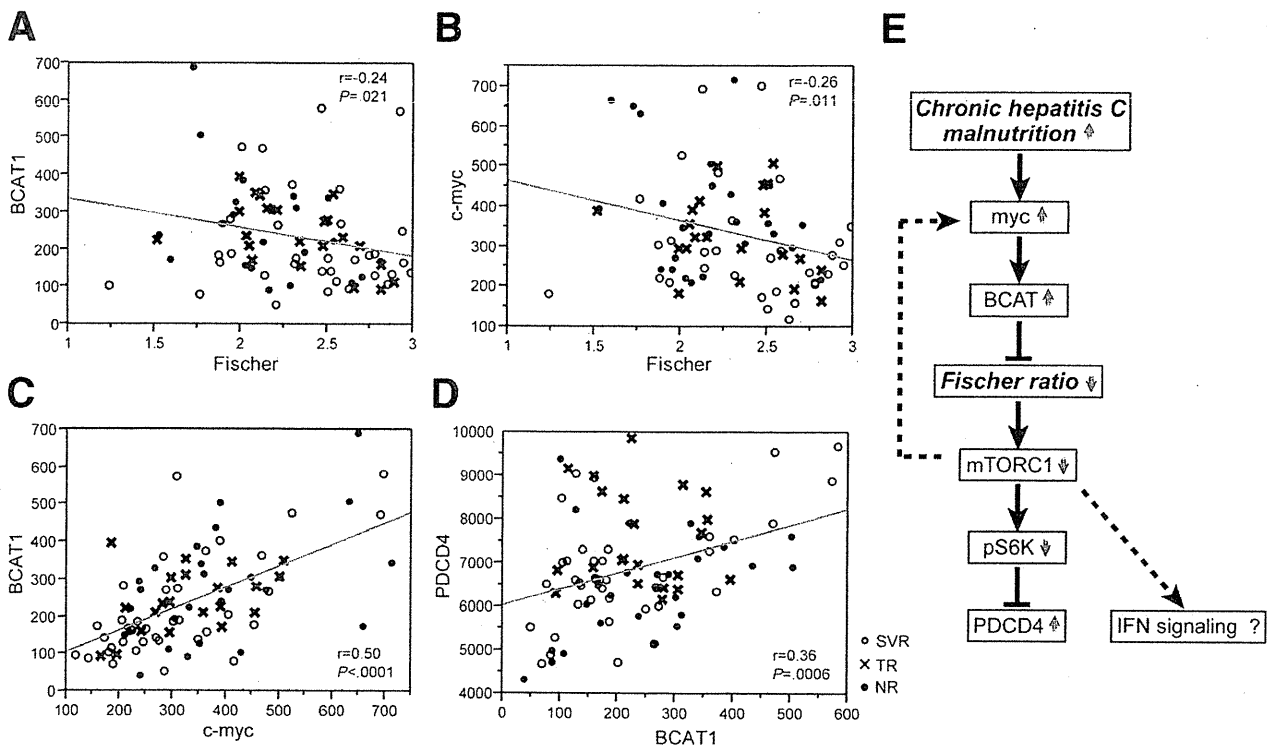


Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (A) BCAT1 and Fischer's ratio. (B) *c-myc* and Fischer's ratio. (C) BCAT1 and *c-myc*. (D) PDCD4 and BCAT1. (E) Putative signaling of mTORC1-related genes in CH-C.

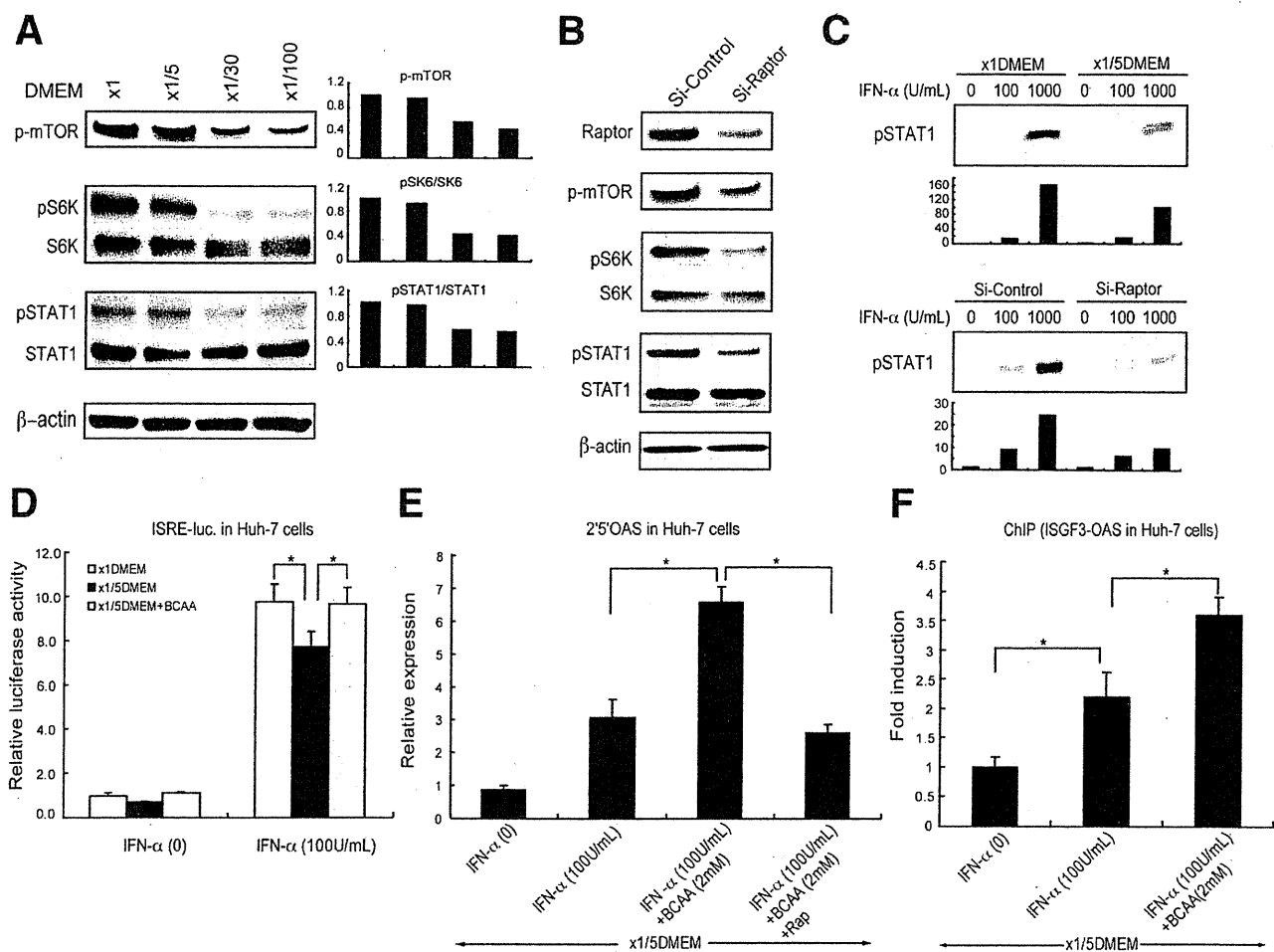


Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid media. (A) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (B) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (C) IFN- α stimulation and pSTAT1 expression in low-amino-acid media or under Raptor knock-down conditions. (D) IFN- α stimulation and ISRE reporter activities in normal and low-amino-acid media. (E) IFN- α stimulation and 2'5'OAS expression supplemented with BCAA or rapamycin in low-amino-acid media. (F) Chromatin immunoprecipitation of 2'5'OAS promoter region by ISGF3 γ .

The induction of pSTAT1 by IFN- α (1000 U/mL) stimulation was impaired in $\times 1/5$ DMEM or in Raptor knocked-down condition, compared with the control (Figure 2C). Consistent with these results, IFN- α -induced ISRE-dependent transcriptional activity, as measured using an ISRE-luciferase reporter assay, was impaired significantly in $\times 1/5$ DMEM compared with $\times 1$ DMEM (Figure 2D). However, this activity could be rescued by the addition of 2 mmol/L BCAA (Figure 2D). These results were confirmed by determining the expression of the endogenous IFN- α responsive gene, 2'5'OAS, using quantitative reverse-transcription PCR. Figure 2E shows that BCAA treatment augmented 2'5'OAS expression in low levels of amino acids, and that this could be reversed by the addition of rapamycin, an inhibitor of mTORC1 (Figure 2E). Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that transcriptional augmentation by BCAA was mediated by the binding of the IFN- α -inducible transcription factor, ISGF3 γ , to the promoter region of 2'5'OAS (Figure 2F). These results indicate that

amino acids in culture media play an essential role in IFN- α signaling through mTORC1 signaling, and that the addition of BCAA can overcome impaired IFN- α signaling in Huh-7 cells.

Induction of *Socs3* in Low-Amino-Acid Medium in Huh-7 Cells

Besides being involved in mTOR signaling, Foxo transcriptional factors mediate another important branch of nutrition-sensing signaling pathway.¹⁷ Therefore, we evaluated forkhead box O3A (Foxo3a) expression in low-amino-acid conditions in Huh-7 cells. After 6 hours culture in $\times 1/5$, $\times 1/30$, and $\times 1/100$ DMEM, expression of the phosphorylated form of Foxo3a (pFoxo3a) decreased, whereas that of total Foxo3a increased in $\times 1/5$ and $\times 1/30$ DMEM, and the ratio of pFoxo3a to Foxo3a (pFoxo3a/Foxo3a) substantially decreased (Figure 3A and B). It has been reported that dephosphorylated Foxo3a is translocated to the nucleus before activation of its target genes.¹⁸ In the present study, immunofluorescent staining

CLINICAL LIVER

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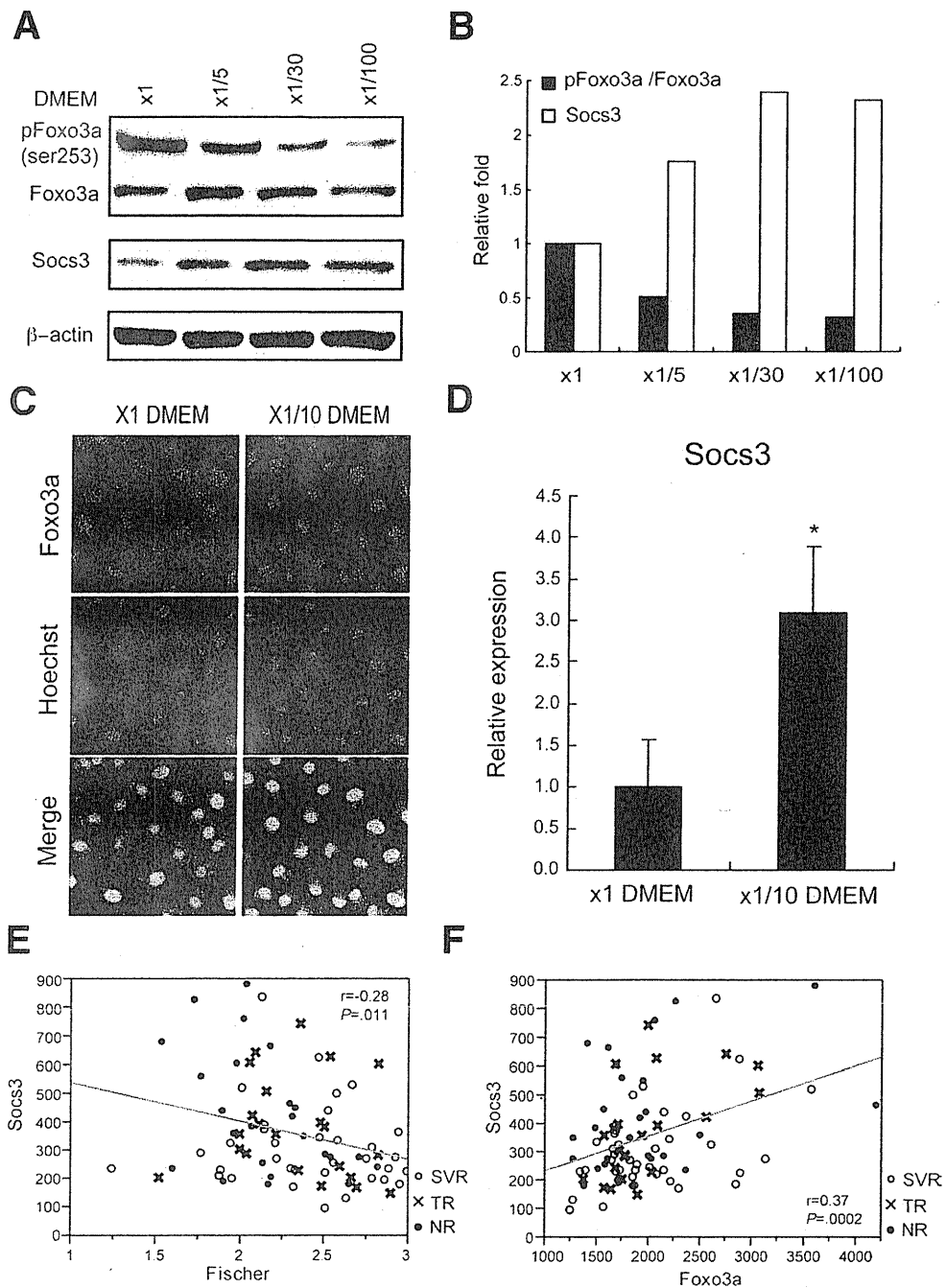


Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (A) Foxo3a and Socs3 expression in different amino acid media. (B) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (C) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal and low-amino-acid media. (D) Relative change of Socs3 messenger RNA in Huh-7 cells in normal and low-amino-acid media. (E) Regression analysis of Socs3 in liver and Fischer's ratio. (F) Regression analysis of Socs3 and Foxo3a in liver.

with an anti-Foxo3a antibody showed that Foxo3a diffused in both the cytoplasm and nucleus in normal amino acid medium, but localized in the nucleus in low-amino-acid medium ($\times 1/10$ DMEM) (Figure 3C).

Interestingly, in low-amino-acid medium, transcription and protein expression of Socs3 increased significantly (Figure 3A, B, and D). The induction of Socs3 in a state of malnutrition also was confirmed in clinical samples. In CH-C livers there was a significant negative correlation

between the plasma Fischer's ratio and Socs3 expression, implying that Socs3 expression increases during the malnutrition state induced by CH-C. There was also a significant correlation between Foxo3a and the transcriptional level of Socs3 in CH-C livers (Figure 3E and F), suggesting an *in vitro* and *in vivo* biological role for Foxo3a in the activation of Socs3 expression. These findings also were confirmed by Western blotting of CH-C livers (Supplementary Figure 2, Supplementary Table 2).

Socs3 Is a Transcriptional Target of Foxo3a

The significant correlation between *Socs3* and *Foxo3a* in CH-C livers prompted us to analyze the *Socs3* promoter sequence and, in doing so, we identified a putative *Foxo* binding element (FBE) (Figure 4A). To investigate the functional relevance of *Foxo3a* in the transcriptional regulation of *Socs3*, we constructed reporter plasmids containing a luciferase coding region fused to the *Socs3* promoter region (*Socs3-luc*). *Socs3-luc* promoter activity was increased substantially by the overexpression of *Foxo3a* (Figure 4B). The mutations introduced in the putative FBE (FBE_{mut}) in the *Socs3* promoter significantly reduced *Foxo3a*-induced *Socs3* promoter activation (Figure 4B).

Foxo3a then was knocked down by siRNA and *Socs3* induction was evaluated. After suppression of *Foxo3a* (Supplementary Figure 3), *Socs3* promoter activity was repressed significantly in low-amino-acid medium ($\times 1/10$ DMEM) (Figure 4C). Thus, *Foxo3a* appears to be indispensable for activating the *Socs3* promoter under low-amino-acid conditions. Correlating with these results, ChIP assays using an anti-*Foxo3a* antibody showed a significant increase in the association between *Foxo3a* and the FBE of the *Socs3* promoter in low-amino-acid conditions ($\times 1/10$ DMEM) (Figure 4D). Taken together, these results suggest that, besides mTORC1 signaling, the *Foxo3a*-mediated *Socs3* signaling pathway might contribute to impaired IFN signaling in a state of malnutrition in CH-C. BCAA potentially restores this signaling (Figure 4E).

Effect of BCAA on HCV Replication in Huh-7 or Huh-7.5 Cells

Based on the earlier-described results, we used 2 HCV in vitro replication systems to examine whether BCAA affects HCV replication in Huh-7 or Huh-7.5 cells. The first system used a recombinant infectious genotype 1a clone, H77S.3/GLuc2A (Supplementary Materials and Methods, Supplementary Figure 4), including reporter genes, whereas the second used continuously JFH-1-infecting Huh-7 cells (Supplementary Materials and Methods).

The synthetic RNA transcribed from pH77S.3/GLuc2A was introduced into Huh-7.5 cells and replication of H77S.3/GLuc2A was evaluated in normal or low-amino-acid medium supplemented with BCAA. H77S.3/GLuc2A increased significantly by 2.6-fold in Huh-7.5 cells grown in low-amino-acid medium ($\times 1/5$ DMEM) compared with normal amino acid medium ($\times 1$ DMEM). Interestingly, BCAA repressed H77S.3/GLuc2A replication in a dose-dependent manner (Figure 5A). In agreement with these results, the expression of *Mx-1* was increased significantly by the addition of BCAA (Figure 5B). Similar findings were observed in JFH-1-infecting Huh-7 cells (Materials and Methods, Supplementary Figure 4). Although no obvious increase in HCV replication was observed in low-amino-acid medium ($\times 1/5$ DMEM) com-

pared with normal amino acid medium ($\times 1$ DMEM), JFH-1 replication was repressed significantly by the addition of BCAA in a dose-dependent manner (Figure 5D). The expression of *Mx-1* was increased substantially by the addition of BCAA (Figure 5E), suggesting that BCAA significantly repressed HCV replication in cells with either naive or persistent HCV infection. Importantly, there were no significant differences in cell viability between the conditions (Figure 5C and F).

To validate these findings, signaling pathways in HCV replicating cells were examined (Figure 6A and B). BCAA increased pS6K in a dose-dependent manner, implying its involvement in the activation of mTORC1 signaling. Related to this, expression of pSTAT1 was shown to be increased and the ratio of pSTAT1 to total STAT1 (pSTAT1/STAT1) increased 2.5- to 3-fold after the addition of BCAA. Thus, BCAA activated mTORC1 and the JAK-STAT signaling pathway in HCV-infected cells. In addition, the expression ratio of pFoxo3a to total Foxo3a (pFoxo3a/Foxo3a) increased 3- to 4-fold, indicating an increase in the cytoplasmic form of *Foxo3a* that is exposed to proteasome degradation. Concordant with these findings, we observed a decrease in the expression of *Socs3*. In addition, expression of the HCV core protein decreased as shown in Figure 6A and B. Thus, these results clearly show that BCAA repressed HCV replication through activation of IFN signaling and repression of *Socs3*-mediated IFN inhibitory signaling, as proposed in Figure 4E.

Discussion

Thompson et al⁵ showed that the IL-28B polymorphism, HCV RNA, nationality (Caucasian/Hispanic vs African American), hepatic fibrosis stage, and fasting blood sugar level are all significant variables for achieving SVR in patients infected with genotype 1 HCV. However, the significance of variable factors for treatment response in conjunction with the IL-28B polymorphism has not been evaluated fully. In the present study, in addition to previously examined variables,⁴ we included the plasma Fischer's ratio as a nutritional parameter. Multivariate analysis showed that the minor type of IL-28B polymorphism, advanced fibrosis stage, high hepatic ISGs, low Fischer's ratio, and ISDR mutation (≤ 1) independently contributed to NR (Table 1). Interestingly, among patients of similar fibrosis stage (F3-4), the Fischer's ratio was significantly lower in NR than SVR+TR cases. Therefore, the plasma value of Fischer's ratio was associated with the treatment response that was independent of the IL-28B polymorphism and histologic stage of the liver, although patients with advanced hepatic fibrosis are likely to be nutritionally affected.

As a nutrient sensor signaling pathway, the protein kinase mTOR plays an essential role in maintaining homeostasis and regulates protein synthesis in response to nutrient conditions. mTOR is the catalytic subunit of 2 distinct complexes, mTORC1 and mTORC2. In addition

