

Table 3. Factors associated with RVR analyzed by univariate and multivariate logistic regression analysis. (n = 103)

		Univariate			Multivariate		
		Odds ratio	95% CI	P-Value	Odds ratio	95% CI	P-Value
Age	60 years ≤	0.7	0.18 - 2.59	0.57			
Gender	Male	1.5	0.36 - 6.07	0.59			
ISDR ²²²⁴⁻²²⁴⁸	1 ≤	24.6	4.70 - 129	8.5E-07 **	14.7	1.10 - 198	0.04 *
IRRDR ²³⁴⁰⁻²³⁸²	4 ≤	6.2	0.76 - 51.1	0.06			
Core 70	Arg	0.7	0.18 - 3.07	0.68			
Fibrosis	<2	3.6	0.72 - 17.8	0.10			
HCV RNA	<600KIU/mL	74.7	8.55 - 653	8.3E-10 **	51.2	3.97 - 662	0.003 **
BMI	<23	1.3	0.34 - 4.87	0.71			
ALB	4.1g/dL ≤	1.1	0.30 - 4.28	0.85			
γGTP	50 IU/mL ≤	0.9	0.24 - 3.49	0.91			
ALT	60 IU/mL <	0.9	0.25 - 3.59	0.94			
T-Chol	<170 mg/dL	1.2	0.33 - 4.67	0.76			
WBC	4700/μL ≤	1.9	0.47 - 7.89	0.36			
Hb	14 g/dL ≤	1.5	0.37 - 6.35	0.55			
PLT	150000/μL ≤	1.8	0.48 - 6.88	0.37			
AFP	10 ng/mL ≤	0.3	0.03 - 2.37	0.22			
PEG-IFN dose (%)	80 ≤	1.3	0.33 - 5.55	0.68			
Ribavirin dose (%)	80 ≤	3.0	0.79 - 11.4	0.09			

NOTE: Since all the RVR patients possessed IL28B TT alleles and odds ratio calculation was impossible, IL28B SNPs was excluded from the analysis.

** : <0.01 * : <0.05

Abbreviations: RVR, rapid viral response; ISDR, interferon sensitivity-determining region; IRRDR, IFN/RBV resistance-determining region; IL28B, interleukin 28B.

Table 4. Factors associated with nEVR analyzed by univariate and multivariate logistic regression analysis. (n = 103)

		Univariate			Multivariate		
		Odds ratio	95% CI	P-Value	Odds ratio	95% CI	P-Value
Age	60 years ≤	1.18	0.42 - 3.30	0.75			
Gender	Male	0.86	0.31 - 2.38	0.77			
ISDR ²²²⁴⁻²²⁴⁸	1 ≤	0.97	0.29 - 3.28	0.96			
IRRDR ²³⁴⁰⁻²³⁸²	4 ≤	0.25	0.09 - 0.69	5.0E-03 **	0.21	0.03 - 1.33	0.1
Core 70	Arg	0.03	0.01 - 0.16	2.0E-08 **	0.04	0.00 - 0.04	0.008 **
IL28B [†]	Major allele	0.05	0.01 - 0.17	5.4E-08 **	0.1	0.01 - 0.57	0.011 *
Fibrosis	<2	0.28	0.08 - 1.0	0.04 *	0.5	0.03 - 0.57	0.55
HCV RNA	<600KIU/mL	0.19	0.02 - 1.5	0.08			
BMI	<23	0.97	0.36 - 2.58	0.95			
ALB	4.1g/dL ≤	0.69	0.26 - 1.85	0.46			
γGTP	50 IU/mL ≤	1.95	0.73 - 5.22	0.18			
ALT	60 IU/mL <	0.38	0.14 - 1.03	0.05			
T-Cho	<170 mg/dL	0.34	0.11 - 1.03	0.06			
WBC	4700/μL ≤	0.64	0.23 - 1.76	0.38			
Hb	14 g/dL ≤	0.82	0.29 - 2.26	0.70			
PLT	150000/μL ≤	0.42	0.15 - 1.19	0.10			
AFP	10 ng/mL ≤	5.12	1.82 - 14.4	0.001 **	3.5	0.52 - 23.2	0.20
PEG-IFN dose (%)	80 ≤	0.37	0.14 - 1.01	0.048 *	0.9	0.13 - 5.93	0.89
Ribavirin dose (%)	80 ≤	0.38	0.12 - 1.23	0.10			

†; n=89

** : <0.01 * : <0.05

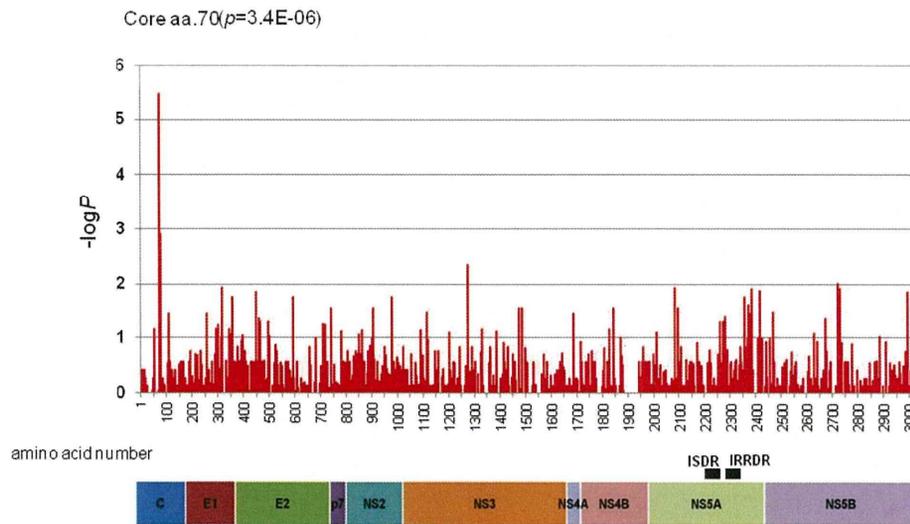
Abbreviations: nEVR, non early viral response; ISDR, interferon sensitivity-determining region; IRRDR, IFN/RBV resistance-determining region; IL28B, interleukin 28B.

Table 5. Factors associated with SVR analyzed by univariate and multivariate logistic regression analysis. (n = 97)

		Univariate			Multivariate		
		Odds ratio	95% CI	P-Value	Odds ratio	95% CI	P-Value
Age	60 years ≤	0.8	0.34 - 1.78	0.55			
Gender	Male	1.4	0.61 - 3.22	0.43			
ISDR ₂₂₂₄₋₂₂₄₈	1 ≤	6.3	1.98 - 20.26	0.001 *	13.4	1.86 - 96.5	0.010 *
IRRDR ₂₃₄₀₋₂₃₈₂	4 ≤	11.1	4.07 - 30.54	4.08E-07 **	13.8	3.31 - 57.4	0.0003 **
Core 70	Arg	3.2	1.37 - 7.59	0.007 **	2.2	0.43 - 11.7	0.34
IL28B	Major allele	9.6	2.92 - 31.34	0.00003 **	16.8	2.04 - 139	0.009 **
Fibrosis	<2	3.1	1.33 - 7.23	0.008 **	1.4	0.31 - 6.64	0.65
HCV RNA	<600KIU/mL	3.5	1.39 - 9.02	0.007 **	3.5	0.72 - 17.3	0.12
BMI	<23	1.0	0.44 - 2.20	0.97			
ALB	4.1g/dL ≤	0.9	0.39 - 1.96	0.75			
γGTP	<50 IU/mL	2.6	1.13 - 5.88	0.02 *	3.5	0.90 - 13.47	0.07
ALT	≤60 IU/mL	0.8	0.35 - 1.77	0.57			
T-Cho	<170 mg/dL	1.7	0.71 - 3.94	0.24			
WBC	<4700/μL	0.8	0.36 - 1.87	0.64			
Hb	<14 g/dL	0.9	0.35 - 2.13	0.75			
PLT	150000/μL ≤	2.6	1.06 - 6.56	0.03 *	3.5	0.71 - 16.8	0.20
AFP	<10 ng/mL	3.7	1.49 - 9.29	0.004 **	3.4	0.54 - 21.2	0.20
PEG-IFN dose (%)	80 ≤	2.2	0.96 - 5.13	0.06			
Ribavirin dose (%)	80 ≤	0.8	0.37 - 1.92	0.68			

**: <0.01 *: <0.05

Abbreviations: SVR, sustained viral response; ISDR, interferon sensitivity-determining region; IRRDR, IFN/RBV resistance-determining region; IL28B, interleukin 28B.

Maekawa et al. Supplementary Fig. 1

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Supplementary Table 1. Baseline characteristics of additional 30 patients

	Additional 30 patients
Age (years)	57 (37 - 68)
Gender :male	17 (57%)
Fibrosis : F2-F4	18 (60%)
HCV RNA (KIU/mL)	1790 (5 - 25110)
BMI	23.1 (18.1 - 26.9)
ALB (g/dL)	4.0 (3.3 - 5.0)
γ GTP (IU/mL)	43 (11 - 816)
ALT (IU/mL)	55 (15 - 301)
T-Cho (mg/dL)	157 (120 - 256)
WBC (/ μ L)	4000 (2710 - 8600)
Hb (g/dL)	14.1 (11.2 - 16.3)
PLT ($*10^4/\mu$ L)	12.7 (6.8 - 29.0)
AFP (ng/mL)	4.5 (1.5 - 137.1)
IL28B TT (%)	27 (90%)
PEG-IFN dose (%)	96 (22 - 113)
Ribavirin dose (%)	81 (60 - 102)
Standard therapy	30 (100%)
SVR rate	21 (70%)

Abbreviations: IL28B, interleukin 28B.

Supplementary Table 2. Factors associated with relapse analyzed by univariate and multivariate logistic regression analysis. (n = 78)

		Univariate			Multivariate		
		Odds ratio	95% CI	P-Value	Odds ratio	95% CI	P-Value
Age	60 years ≤	1.5	0.54 - 3.94	0.46			
Gender	Male	0.8	0.28 - 2.08	0.59			
ISDR ²²²⁴⁻²²⁴⁸	1 ≤	0.1	0.03 - 0.67	0.014 **	0.2	0.04 - 1.01	0.052
IRRDR-V3 ²³⁶⁰⁻²³⁷⁷	2 ≤	0.2	0.08 - 0.62	0.007 **	0.3	0.10 - 0.90	0.03 *
Core 70	Arg	0.6	0.22 - 1.83	0.40			
IL28B	Major allele	0.4	0.08 - 1.64	0.18			
Fibrosis	<2	0.4	0.14 - 1.05	0.06			
HCV RNA	<600KIU/mL	0.4	0.15 - 1.24	0.11			
BMI	<23	0.7	0.27 - 1.93	0.51			
ALB	4.1g/dL ≤	1.3	0.51 - 3.59	0.55			
γGTP	50 IU/mL ≤	1.6	0.57 - 4.34	0.10			
ALT	60 IU/mL <	0.9	0.35 - 2.50	0.89			
T-Cho	<170 mg/dL	1.9	0.64 - 5.54	0.24			
WBC	4700/μL ≤	1.8	0.66 - 4.72	0.25			
Hb	14 g/dL ≤	1.5	0.47 - 4.66	0.50			
PLT	150000/μL ≤	0.5	0.17 - 1.44	0.19			
AFP	10 ng/mL ≤	2.0	0.64 - 6.04	0.23			
PEG-IFN dose (%)	80 ≤	0.6	0.23 - 1.77	0.39			
Ribavirin dose (%)	80 ≤	0.8	0.27 - 2.18	0.62			

**: <0.01 *: <0.05

Abbreviations: ISDR, interferon sensitivity-determining region; IRRDR, IFN/RBV resistance-determining region; IL28B, interleukin 28B.

Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFN α) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFN α -2b/RBV and quantified expressions of viral sensors (*RIG-I*, *MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFN λ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈ 3.3 -fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈ 2.6 -fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. *IPS-1* down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of *IPS-1* protein cleavage was associated with the variable treatment response. **Conclusion:** Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFN α /RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.¹ Pegylated interferon α (PEG-IFN α) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the

natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFN α /RBV combination therapy.² In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ -GTP, γ -glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; *IPS-1*, *IFN β* promoter stimulator 1; *ISG15*, interferon-stimulated gene 15; *MDA5*, melanoma differentiation associated gene 5; NVR, nonvirological responders; PEG-IFN α , pegylated interferon α ; SNP, single nucleotide polymorphism; *RIG-I*, retinoic acid-inducible gene I; RBV, ribavirin; *RNF125*, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; *USP18*, ubiquitin-specific protease 18; VR, virological responder.

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Received May 14, 2011; accepted August 16, 2011.

Supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Japanese Ministry of Welfare, Health and Labor. The funding source had no role in the collection, analysis, or interpretation of the data, or in the decision to submit the article for publication.

(*IL28B*) that encodes for type III IFN λ 3 were shown to be strongly associated with a virological response to PEG-IFN α /RBV combination therapy.³⁻⁵ In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFN α /RBV.³ However, mechanisms involving resistance to PEG-IFN α /RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.⁶ The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.^{7,8} The IFN β promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFN β , mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in IFN β gene activation.⁹⁻¹² RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,^{8,13} a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.¹⁴ Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,¹⁵ and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.^{16,17} Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.^{9,18} Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFN α /RBV combination therapy.¹⁹ We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFN α /RBV combination therapy.¹⁹ However, association of gene expression involv-

ing innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFN α /RBV combination therapy in CH-C patients.

Patients and Methods

Patients. Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.¹⁹ Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment Protocol. The patients were administered subcutaneous injections of PEG-IFN α -2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5 μ g kg⁻¹ week⁻¹ for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFN α -2b was reduced to 0.75 μ g kg⁻¹ week⁻¹ when either neutrophil count was less than 750/mm³ or platelet count was less than 80 \times 10³/mm³. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

Measurement of Hepatic Gene Expression. Liver biopsy was performed immediately before initiating

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DOI 10.1002/hep.24623

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Table 1. Patient Characteristics and *IL28B* Genotype

	<i>IL28B</i> Major*	<i>IL28B</i> Minor†	P-value‡
Patients, n	54	34	
Age (SD), year	58.8 (10.0)	59.1 (10.3)	0.918§
Sex, n (%)			0.051
Male	13 (24.1)	15 (44.1)	
Female	41 (75.9)	19 (55.9)	
BMI (SD), kg/m ²	22.7 (3.5)	23.5 (3.6)	0.193§
ALT (SD), IU/L	61.3 (50.7)	62.4 (44.7)	0.962§
γ-GTP (SD), IU/L	36.7 (25.9)	57.3 (52.4)	0.010§
LDL-cholesterol (SD), mg/dL	103.3 (29.8)	91.8 (26.9)	0.067§
Hemoglobin (SD), g/dL	14.1 (1.4)	14.4 (1.3)	0.186§
Platelet count (SD), ×10 ³ /μL	161 (6.4)	163 (4.4)	0.489§
Fibrosis stage, n (%)			0.532
F1, 2	38 (70.4)	26 (76.5)	
F3, 4	16 (29.6)	8 (23.5)	
Viral load (SD), ×10 ^{6.3} IU/mL	1.7 (1.4)	1.9 (2.0)	0.788§
%HCV core 70 & 91 a.a. double mutation¶	8.9	43.5	0.001
%ISDR wild**	43.5	51.7	0.486
Viral response, n (%)			<0.001
SVR	17 (31.5)	13 (38.2)	
TVR	26 (48.1)	3 (8.8)	
NVR	11 (20.4)	18 (52.9)	

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

*rs8099917 TT and rs12979860 CC.

†rs8099917 TG and rs12979860 CT.

BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; SVR, sustained virological response; TVR, transient virological response; NVR, nonvirological response.

‡Comparison between *IL28B* major and minor genotypes.

§Mann-Whitney *U* test.

||Chi-square test.

¶HCV core mutation was determined in 68 patients.

**ISDR was determined in 75 patients.

the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of the positive and negative cytoplasmic viral sensor (*RIG-I*, *MDA5*, and *LGP2*), the adaptor molecule (*IPS-1*), the related ubiquitin E3-ligase (*RNF125*), the modulators of these molecules (*ISG15* and *USP18*), and *IFNλ* (*IL28A/B*) was quantified by real-time quantitative polymerase chain reaction (PCR) using target gene-specific primers. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen reagent (Nippon Gene, Toyama, Japan) from the liver biopsy specimen, which was 0.2–0.4 cm in length and 13G in diameter. Complementary DNA (cDNA) was transcribed from 2 μg of total RNA template in a 140-μL reaction mixture using the SYBR RT-PCR Kit (Takara Bio, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio) with the SYBR RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Assays were performed in duplicate and the expression levels

of target genes were normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and hydroxymethylbilane synthase (*HMBS*), an enzyme that is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two housekeeping genes was used in the present study. Sequences of the primer sets were as follows: *RIG-I*, 5'-AAAGCATGCA TGGTGTTCAG-3', 5'-TCATTCGTGCATGCTC ACTGATAA-3'; *MDA5*, 5'-ACATAACAGCAACATG GGCAGTG-3', 5'-TTTGGTAAGGCCTGAGCTGG AG-3'; *LGP2*, 5'-ACAGCCTTGCAAACAGTACAAC CTC-3', 5'-GTCCCAAATTTCCGGCTCAAC-3'; *IPS-1*, 5'-GGTGCCATCCAAAGTGCCTACTA-3', 5'-CAGC ACGCCAGGCTTACTCA-3'; *RNF125*, 5'-AGGGCA CATATTCGGACTTGTC-3', 5'-CGGGTATTAAC GGCAAAGTGG-3'; *ISG15*, 5'-AGCGAACTCATCT TTGCCAGTACA-3', 5'-CAGCTCTGACACCGACA TGGA-3'; *USP18*, 5'-TGGTCTGCTTCAATGACT CCAATA-3', 5'-TTTGGGCATTTCCATTAGCACT C-3'; *IFNλ*: 5'-CAGCTGCAGGTGAGGGA-3', 5'-G GTGGCCTCCAGAACCTT-3'; *GAPDH*, 5'-GCACC GTCAAGGCTGAGAAC-3', 5'-ATGGTGGTGAAGA CGCCAGT-3'; *HMBS*, 5'-AAGCGGAGCCATGTCT GGTAAC-3', 5'-GTACCCACGCGAATCACTCTCA-3'.

Genotyping for *IL28B* (rs8099917 and rs12979860) Polymorphism. Genetic polymorphism in a tagged SNP located near the *IL28B* gene (rs8099917 and rs12979860) was determined by direct sequencing of PCR-amplified DNA. In brief, after extraction from whole blood samples, genomic DNA was amplified by PCR. Sequences of the primer sets were: rs8099917, 5'-ATCCTCCTCTCATCCCTCA TC-3', 5'-GGTATCAACCCACCTCAAAT-3'; rs129 79860, 5'-GGACGAGAGGGCGTTAGAG-3', 5'-AG GGACCGCTACGTAAGTCAC-3'.

Both strands of the PCR products were sequenced by the dye terminator method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Chiba, Japan); nucleotide sequences were determined by a capillary DNA sequencer ABI3730xl (Applied Biosystems). Homozygosity (rs8099917 GG and rs12979860 TT) or heterozygosity (rs8099917 TG and rs12979860 CT) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (rs8099917 TT and rs12979860 CC) was defined as having the *IL28B* major allele.

Western Blotting. Western blotting was performed using samples from 14 patients (six from *IL28B* major patients and eight from *IL28B* minor patients) as described.¹⁹ In brief, liver biopsy specimens of

approximately 10 mg were homogenized in 100 μ L of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30 μ g of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti- β -actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon²⁰ were used for a positive control for cleaved IPS-1.

Definitions of Response to Therapy. A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFN α -2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

Statistical Analysis. Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney *U* test for two groups. All tests of significance were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics and IL28B Genotype. Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report

of the rarity of a minor homozygote in Japanese patients.³ *IL28B* minor patients were significantly associated with a higher γ -glutamyl transpeptidase (γ -GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

Gene Expression Involving Innate Immunity and IFN λ in the Liver. Hepatic expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFN λ* (*IL28A/B*) expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFN λ* based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the *ISG15/USP18* system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFN δ* was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the *RIG-I/IPS-1* and *ISG15/USP18* systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

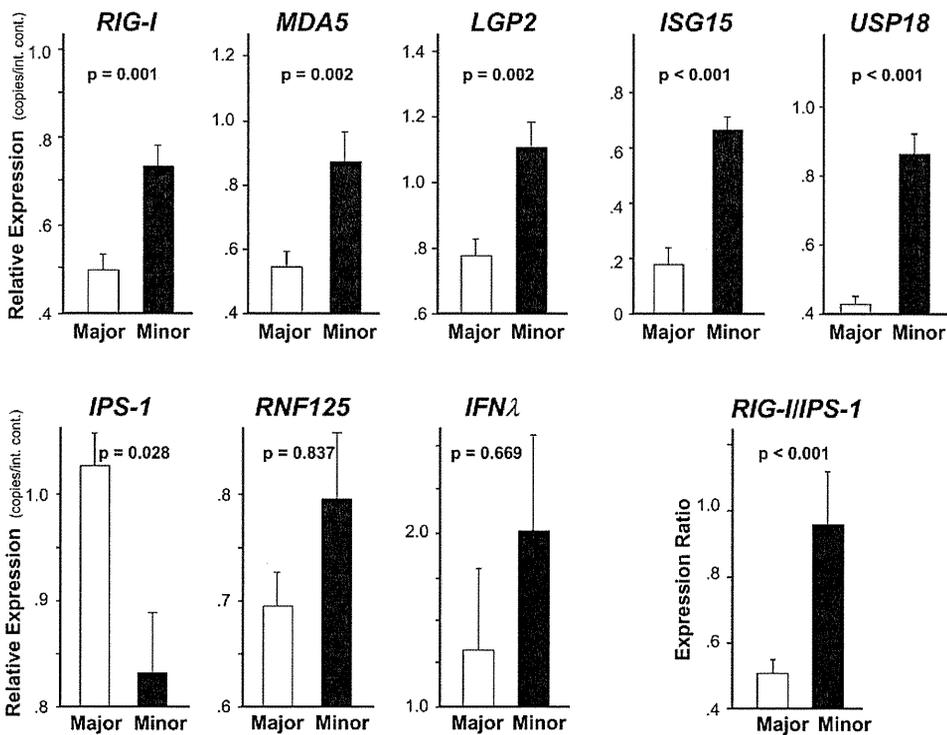


Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The P-values were determined by the Mann-Whitney U test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

Receiver Operator Characteristic (ROC) Analysis. To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

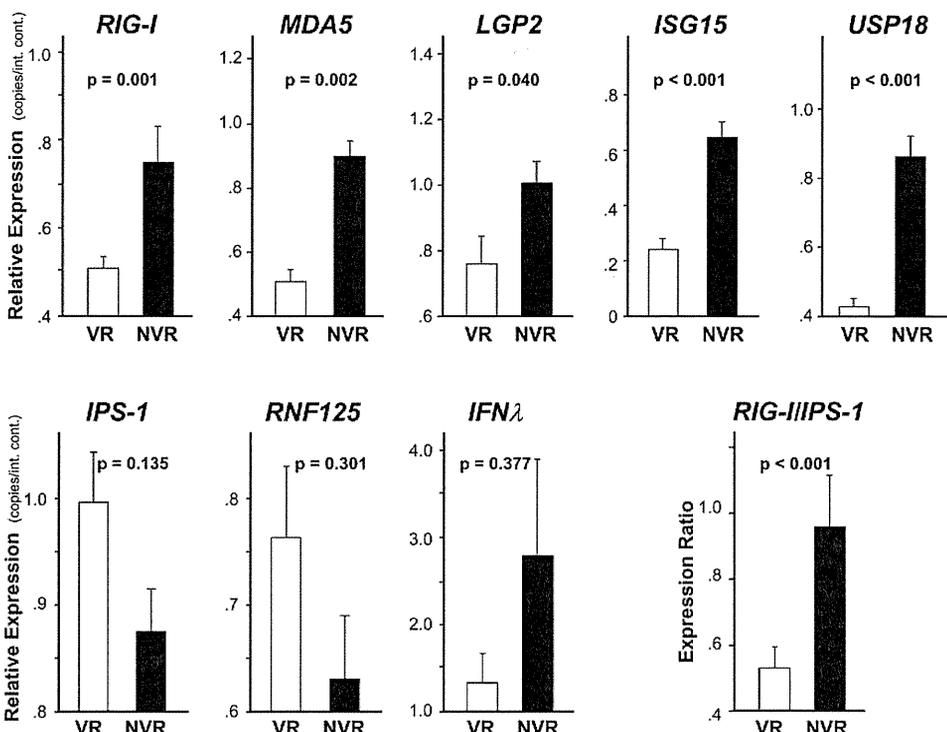


Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The P-values were determined by the Mann-Whitney U test.

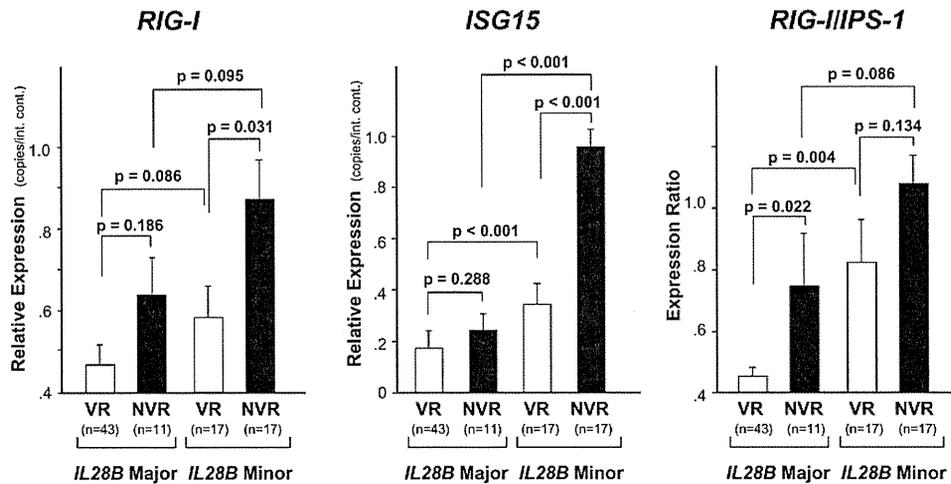


Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio, no statistically significant difference was found in

NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

Factors Associated with NVR. In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic expressions of *RIG-I*, *MDA5*, *LGP2*, *ISG15*, and *USP18*, and *RIG-I/IPS-1* ratio were significantly

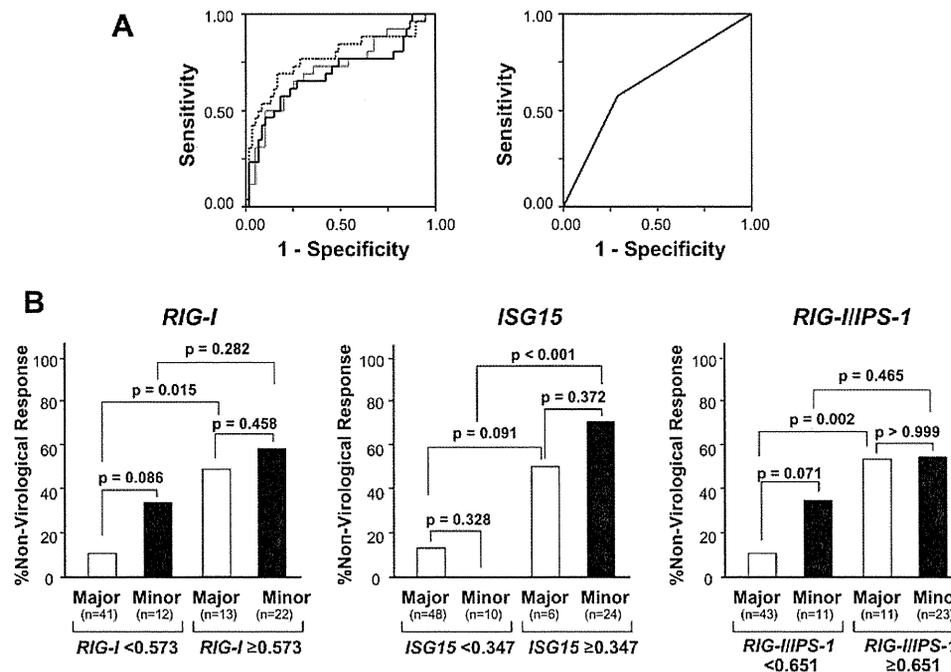


Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
<i>RIG-I</i> (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
<i>ISG15</i> (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
<i>RIG-I/IPS-1</i> (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
<i>IL28B</i> genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

*Genotype at rs8099917.

†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

IPS-1 and RIG-I Protein Expression in the Liver. Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

Table 3. Factors Associated with Nonvirological Response

Factors	Univariate Analysis		Multivariate Analysis*	
	Risk Ratio (95% CI)	P-value	Risk Ratio (95% CI)	P-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m ²)	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10 ⁴ /μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
<i>IL28B</i> genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/int. control)				
<i>RIG-I</i>	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
<i>MDA5</i>	1.53 (1.12-2.00)	0.001		
<i>LGP2</i>	1.34 (1.04-1.74)	0.026		
<i>IPS-1</i>	0.90 (0.78-1.04)	0.143		
<i>RNF125</i>	0.93 (0.83-1.04)	0.204		
<i>ISG15</i>	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
<i>USP18</i>	1.67 (1.27-2.20)	<0.001		
<i>IFNλ</i>	1.02 (0.99-1.05)	0.170		
<i>RIG-I/IPS-1</i> ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.

*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5*, *LGP2*, *USP18*, and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.

†rs8099917 TT and rs12979860 CC.

‡rs8099917 TG and rs12979860 CT.

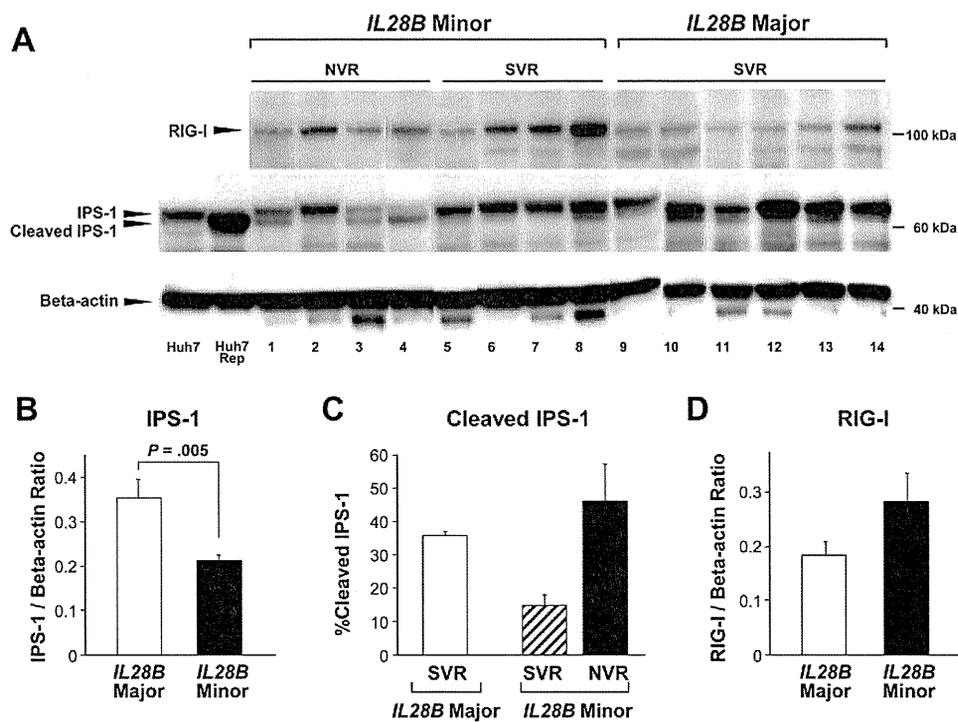


Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β -actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β -actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β -actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFN α /RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFN α /RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a

pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.^{7,8} Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,¹⁹ suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFN α /RBV response as well as with *IL28B* genotype.²¹⁻²³

In contrast to cytoplasmic viral sensor (*RIG-I*, *MDA5*, and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,^{9,18}

transcriptional and translational *IPS-1* expression are probably suppressed by HCV with resistant phenotype, which may be more adaptive in *IL28B* minor patients than in *IL28B* major patients. When we analyzed the proportion of full-length or cleaved IPS-1 to the total IPS-1 protein in a subgroup of *IL28B* minor patients, cleaved IPS-1 product was less dominant in SVR than in NVR, whereas uncleaved full-length IPS-1 protein was more dominant in SVR than in NVR. Therefore, the ability of HCV to evade host innate immunity by cleaving IPS-1 protein and/or host capability of protection from IPS-1 cleavage is probably responsible for the variable treatment responses in *IL28B* minor patients.

Our results indicated a close association between *IL28B* minor patients with higher γ -GTP level and higher frequency of HCV core double mutants, which are known factors for NVR. In contrast, no significant association was observed between *IL28B* genotype and age, gender, or liver fibrosis, which are also known to be unfavorable factors for virological response to PEG-IFN α /RBV. Therefore, certain factors other than the *IL28B* genotype may independently influence virological response. To elucidate whether gene expression involving innate immunity independently associates with a virological response from the *IL28B* genotype, we performed further analysis in a subgroup and conducted a multivariate regression and ROC analyses. Our multivariate and ROC analyses demonstrate that higher expressions of *RIG-I* and *ISG15* as well as a higher ratio of *RIG-I/IPS-1* are independently associated with NVR, and quantification of these values is more useful in predicting final virological response to PEG-IFN α /RBV than determination of *IL28B* genotype in each individual patients. However, the SVR rates in our patients were similar among *IL28B* genotypes, which suggests more SVR patients with the *IL28B* minor allele were included in the present study than those in the general CH-C population. Hence, our data did not necessarily exclude the possibility of the *IL28B* genotype in predicting NVR, although our multivariate analysis could not identify the *IL28B* minor allele as an independent factor for NVR. Interestingly, an association between *IL28B* genotype and expressions of *RIG-I* and *ISG15* as well as *RIG-I/IPS-1* expression ratio is still observed even in patients with the same subgroup of virological response (Fig. 3).

In the present study, although hepatic *IFN λ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of

IL28B from that of *IL28A*, and moreover, we could not specify which cell expresses *IFN λ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFN λ* in relation to treatment response need further clarification by specifying type of *IFN λ* and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I/IPS-1* ratio was significantly higher in NVR with HCV genotype 2.¹⁹ However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.²⁴ The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

Differences in allele frequency for *IL28B* SNPs among the population groups has been reported. The frequency of *IL28B* major allele among patients with Asian ancestry is higher than that among patients with European and African ancestry.²⁵ Because *IL28B* polymorphism strongly influences treatment responses within each population group,⁵ our data obtained from Japanese patients can be applied to other population groups. However, the rate of SVR having African ancestry was lower than that having European ancestry within the same *IL28B* genotype.⁵ Hence, further study is required to clarify whether this difference among the population groups with the same *IL28B* genotype could be explained by differences in expression of genes involved in innate immunity.

In a recent report, an SVR rate of telaprevir with PEG-IFN α /RBV was only 27.6% in *IL28B* minor patients.²⁶ Because new anti-HCV therapy should still contain PEG-IFN α /RBV as a platform for the therapy, our findings regarding innate immunity in addressing the mechanism of virological response and predicting NVR remain important in this new era of directly acting anti-HCV agents, such as telaprevir and boceprevir.

In conclusion, this clinical study in humans demonstrates the potential relevance of the molecules involved in innate immunity to the genetic variation

of *IL28B* and clinical response to PEG-IFN α /RBV. Both the *IL28B* minor allele and higher expressions of *RIG-I* and *ISG15* as well as higher *RIG-I/IPS-1* ratio are independently associated with NVR. Innate immune responses in *IL28B* minor patients may have adapted to a different equilibrium compared with that in *IL28B* major patients. Our data will advance both understanding of the pathogenesis of HCV resistance and the development of new antiviral therapy targeted toward the innate immune system.

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Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

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Background & Aims: Pegylated interferon and ribavirin (PEG-IFN/RBV) therapy for chronic hepatitis C virus (HCV) genotype 1 infection is effective in 50% of patients. Recent studies revealed an association between the *IL28B* genotype and treatment response. We aimed to develop a model for the pre-treatment prediction of response using host and viral factors.

Methods: Data were collected from 496 patients with HCV genotype 1 treated with PEG-IFN/RBV at five hospitals and universities in Japan. *IL28B* genotype and mutations in the core and IFN sensitivity determining region (ISDR) of HCV were analyzed to predict response to therapy. The decision model was generated by data mining analysis.

Results: The *IL28B* polymorphism correlated with early virological response and predicted null virological response (NVR) (odds ratio = 20.83, $p < 0.0001$) and sustained virological response (SVR) (odds ratio = 7.41, $p < 0.0001$) independent of other covariates. Mutations in the ISDR predicted relapse and SVR independent of *IL28B*. The decision model revealed that patients with the minor *IL28B* allele and low platelet counts had the highest NVR (84%) and lowest SVR (7%), whereas those with the major *IL28B* allele and mutations in the ISDR or high platelet counts had the lowest NVR (0–17%) and highest SVR (61–90%). The model had high reproducibility and predicted SVR with 78% specificity and 70% sensitivity.

Conclusions: The *IL28B* polymorphism and mutations in the ISDR of HCV were significant pre-treatment predictors of response to PEG-IFN/RBV. The decision model, including these host and viral factors may support selection of optimum treatment strategy for individual patients.

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Introduction

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the most effective standard of care for chronic hepatitis C but the rate of SVR is around 50% in patients with HCV genotype 1 [2,3], the most common genotype in Japan, Europe, the United States, and many other countries. Moreover, 20–30% of patients with HCV genotype 1 have a null virological response (NVR) to PEG-IFN/RBV therapy [4]. The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment [5] but there is no established method for prediction before treatment. Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects such as flu-like symptoms, depression and hematological abnormalities, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN/RBV therapy in

Keywords: *IL28B*; ISDR; Peg-interferon; Ribavirin; Data mining; Decision tree.

Received 14 March 2010; received in revised form 22 June 2010; accepted 7 July 2010

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Journal of Hepatology 2010 vol. xxx | xxx-xxx

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Table 1. Baseline characteristics of all patients, and patients assigned to the model building or validation groups.

	All patients n = 496	Model group n = 331	Validation group n = 165
Gender: male	250 (50%)	170 (51%)	80 (48%)
Age (years)	57.1 ± 9.9	56.8 ± 9.7	57.5 ± 10.2
ALT (IU/L)	78.6 ± 60.8	78.1 ± 61.4	79.7 ± 59.6
GGT (IU/L)	59.3 ± 63.6	58.9 ± 62.0	60.2 ± 66.9
Platelets (10 ⁹ /L)	154 ± 53	153 ± 52	154 ± 56
Fibrosis: F3-4	121 (24%)	80 (24%)	41 (25%)
HCV-RNA: >600,000 IU/ml	409 (82%)	273 (82%)	136 (82%)
ISDR mutation: ≤1	220 (88%)	290 (88%)	145 (88%)
Core 70 (Arg/Gln or His)	293 (59%)/203 (41%)	197 (60%)/134 (40%)	96 (58%)/69 (42%)
Core 91 (Leu/Met)	299 (60%)/197 (40%)	200 (60%)/131 (40%)	99 (60%)/66 (40%)
<i>IL28B</i> : Minor allele	151 (30%)	101 (31%)	50 (30%)
SVR	194 (39%)	129 (39%)	65 (39%)
Relapse	152 (31%)	103 (31%)	49 (30%)
NVR	150 (30%)	99 (30%)	51 (31%)

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine; Minor, heterozygote or homozygote of minor allele; SVR, sustained virological response; NVR, null virological response.

Japanese [6], European [7], and a multi-ethnic population [8,9]. The last three studies focused on the association of SNPs in the *IL28B* region with SVR [7–9] but we found a stronger association with NVR [6]. In addition to these host genetic factors, we have reported that mutations within a stretch of 40 amino acids in the NS5A region of HCV, designated as the IFN sensitivity determining region (ISDR), are closely associated with the virological response to IFN therapy: a lower number of mutations is associated with treatment failure [10–13]. Amino acid substitutions at positions 70 and 91 of the HCV core region (Core70, Core91) also have been reported to be associated with response to PEG-IFN/RBV therapy: glutamine (Gln) or histidine (His) at Core70 and methionine (Met) at Core91 are associated with treatment resistance [4,14]. The importance of substitutions in the HCV core and ISDR was confirmed recently by a Japanese multicenter study [15]. How these viral factors contribute to response to therapy is yet to be determined. For general application in clinical practice, host genetic factors and viral factors should be considered together.

Data mining analysis is a family of non-parametric regression methods for predictive modeling. Software is used to automatically explore the data to search for optimal split variables and to build a decision tree structure [16]. The major advantage of decision tree analysis over logistic regression analysis is that the results of the analysis are presented in the form of flow chart, which can be interpreted intuitively and readily made available for use in clinical practice [17]. The decision tree analysis has been utilized to define prognostic factors in various diseases [18–25]. We have reported recently its usefulness for the prediction of an early virological response (undetectable HCV-RNA within 12 weeks of therapy) to PEG-IFN/RBV therapy in chronic hepatitis C [26].

This study aimed to define the pre-treatment prediction of response to PEG-IFN/RBV therapy through the integrated analysis of host factors, such as the *IL28B* genetic polymorphism and various clinical covariates, as well as viral factors, such as mutations in the HCV core and ISDR and serum HCV-RNA load. In addition,

for the general application of these results in clinical practice, decision models for the pre-treatment prediction of response were determined by data mining analysis.

Materials and methods

Patients

This was a multicentre retrospective study supported by the Japanese Ministry of Health, Labor and Welfare. Data were collected from a total of 496 chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. Of these, 98 patients also were included in the original GWAS analysis [6]. The inclusion criteria in this study were as follows (1) infection by genotype 1b, (2) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (3) lack of other causes of liver disease, such as autoimmune hepatitis, and primary biliary cirrhosis, (4) completion of at least 24 weeks of therapy, (5) adherence of more than 80% to the planned dose of PEG-IFN and RBV for the NVR patients, (6) availability of DNA for the analysis of the genetic polymorphism of *IL28B*, and (7) availability of serum for the determination of mutations in the ISDR and substitutions of Core70 and Core91 of HCV. Patients received PEG-IFN alpha-2a (180 µg) or 2b (1.5 µg/kg) subcutaneously every week and were administered a weight adjusted dose of RBV (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily) which is the recommended dosage in Japan. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee. The baseline characteristics are listed in Table 1. For the data mining analysis, 67% of the patients (331 patients) were assigned randomly to the model building group and 33% (165 patients) to the validation group. There were no significant differences in the clinical backgrounds between these two groups.

Laboratory and histological tests

Blood samples were obtained before therapy and were analyzed for hematologic tests and for blood chemistry and HCV-RNA. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction as reported previously [4,11]. Genetic polymorphism in one tagging SNP located near the *IL28B* gene (rs8099917) was determined by the GWAS or DigiTag2 assay [27]. Homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (TT) was

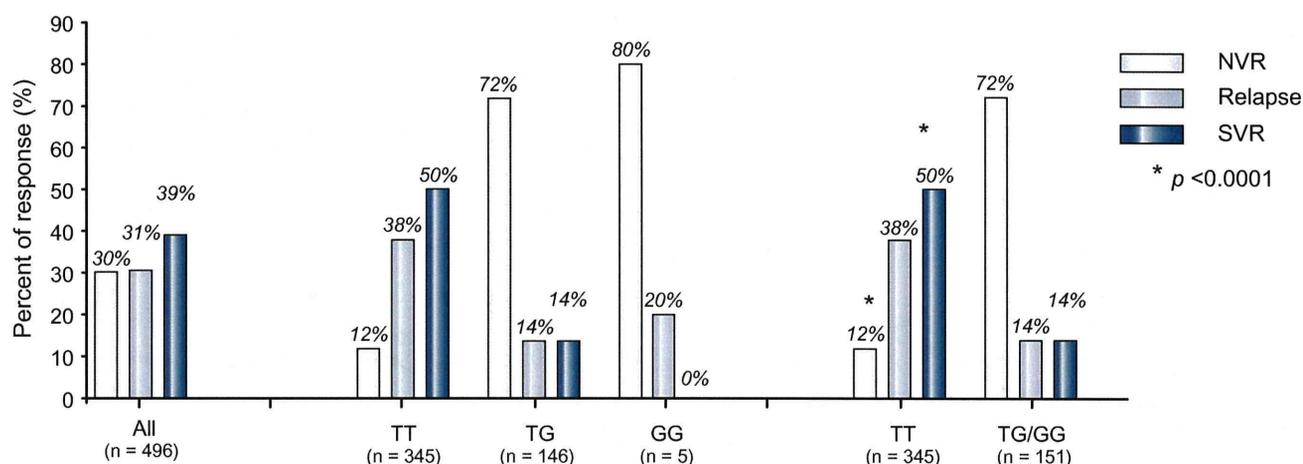


Fig. 1. Association between the IL28B genotype (rs8099917) and treatment response. The rates of response to treatment are shown for each rs8099917 genotype. The rate of null virological response (NVR), relapse, and sustained virological response (SVR) is shown. The p values are from Fisher's exact test. The rate of NVR was significantly higher ($p < 0.0001$) and the rate of SVR was significantly lower ($p < 0.0001$) in patients with the IL28B minor allele compared to those with the major allele. [This figure appears in colour on the web.]

defined as having the IL28B major allele. In this study, NVR was defined as a less than 2 log reduction of HCV-RNA at week 12 and detectable HCV-RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic systems, CA) at week 24 during therapy. RVR (rapid virological response) and complete early virological response (cEVR) were defined as undetectable HCV-RNA at 4 weeks and 12 weeks during therapy and SVR was defined as undetectable HCV-RNA 24 weeks after the completion of therapy. Relapse was defined as reappearance of HCV-RNA after the completion of therapy. The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Percentage of steatosis was quantified in 111 patients by determining the average proportion of hepatocytes affected by steatosis.

Statistical analysis

Associations between pre-treatment variables and treatment response were analyzed by univariate and multivariate logistic regression analysis. Associations between the IL28B polymorphism and sequences of HCV were analyzed by Fisher's exact test. SPSS software v.15.0 (SPSS Inc., Chicago, IL) was used for these analyses. For the data mining analysis, IBM-SPSS Modeler version 13.0 (IBM-SPSS Inc., Chicago, IL) software was utilized as reported previously [26]. The patients used for model building were divided into two groups at each step of the analysis based on split variables. Each value of each variable was considered as a potential split. The optimum variables and cut-off values were determined by a statistical search algorithm to generate the most significant division into two prognostic subgroups that were as homogeneous as possible for the probability of SVR. Thereafter, each subgroup was evaluated again and divided further into subgroups. This procedure was repeated until no additional significant variable was detected or the sample size was below 15. To avoid over-fitting, 10-fold cross validation was used in the tree building process. The reproducibility of the resulting model was tested with the data from the validation patients.

Results

Association between the IL28B (rs8099917) genotype and the PEG-IFN/RBV response

The rs8099917 allele frequency was 70% for TT ($n = 345$), 29% for TG ($n = 146$), and 1% for GG ($n = 5$). We defined the IL28B major allele as homozygous for the major sequence (TT) and the IL28B minor allele as homozygous (GG) or heterozygous (TG) for the minor sequence. The rate of NVR was significantly higher (72% vs. 12%, $p < 0.0001$) and the rate of SVR was significantly lower (14% vs. 50%, $p < 0.0001$) in patients with the IL28B minor allele compared to those with the major allele (Fig. 1).

Effect of the IL28B polymorphism, substitutions in the ISDR, Core70, and Core91 of HCV on time-dependent clearance of HCV

Patients were stratified according to their IL28B allele type, the number of mutations in the ISDR, the amino acid substitutions in Core70 and Core91, and the rate of undetectable HCV-RNA at 4, 8, 12, 24, and 48 weeks after the start of therapy was analyzed (Fig. 2A–D). The rate of undetectable HCV-RNA was significantly higher in patients with the IL28B major allele than the minor allele, in patients with two or more mutations in the ISDR compared to none or only one mutation, in patients with arginine (Arg) at Core70 rather than Gln/His, and in patients with leucine (Leu) at Core91 rather than Met. The difference was most significant when stratified by the IL28B allele type. The rate of RVR and cEVR was significantly more frequent in patients with the IL28B major allele compared with those with the IL28B minor allele: 9% vs. 3% for RVR ($p < 0.005$) and 57% vs. 11% for cEVR ($p < 0.0001$). These findings suggest that IL28B has the greatest impact on early virological response to therapy.

Association between substitutions in the ISDR and relapse after the completion of therapy

Patients were stratified according to the IL28B allele, number of mutations in the ISDR, and amino acid substitutions of Core70 and Core91, and the rate of relapse was analyzed (Fig. 3A and B). Among patients who achieved cEVR, the rate of relapse was significantly lower in patients with two or more mutations in the ISDR compared to those with only one or no mutations (15% vs. 31%, $p < 0.005$) (Fig. 3 B). On the other hand, the relapse rate was not different between the IL28B major and minor alleles within patients who achieved RVR (3% vs. 0%) or cEVR (28% vs. 29%) (Fig. 3A). Amino acid substitutions of Core70 and Core91 were not associated with the rate of relapse (data not shown).

Factors associated with response by multivariate logistic regression analysis

By univariate analysis, the minor allele of IL28B ($p < 0.0001$), one or no mutations in the ISDR ($p = 0.03$), high serum level of

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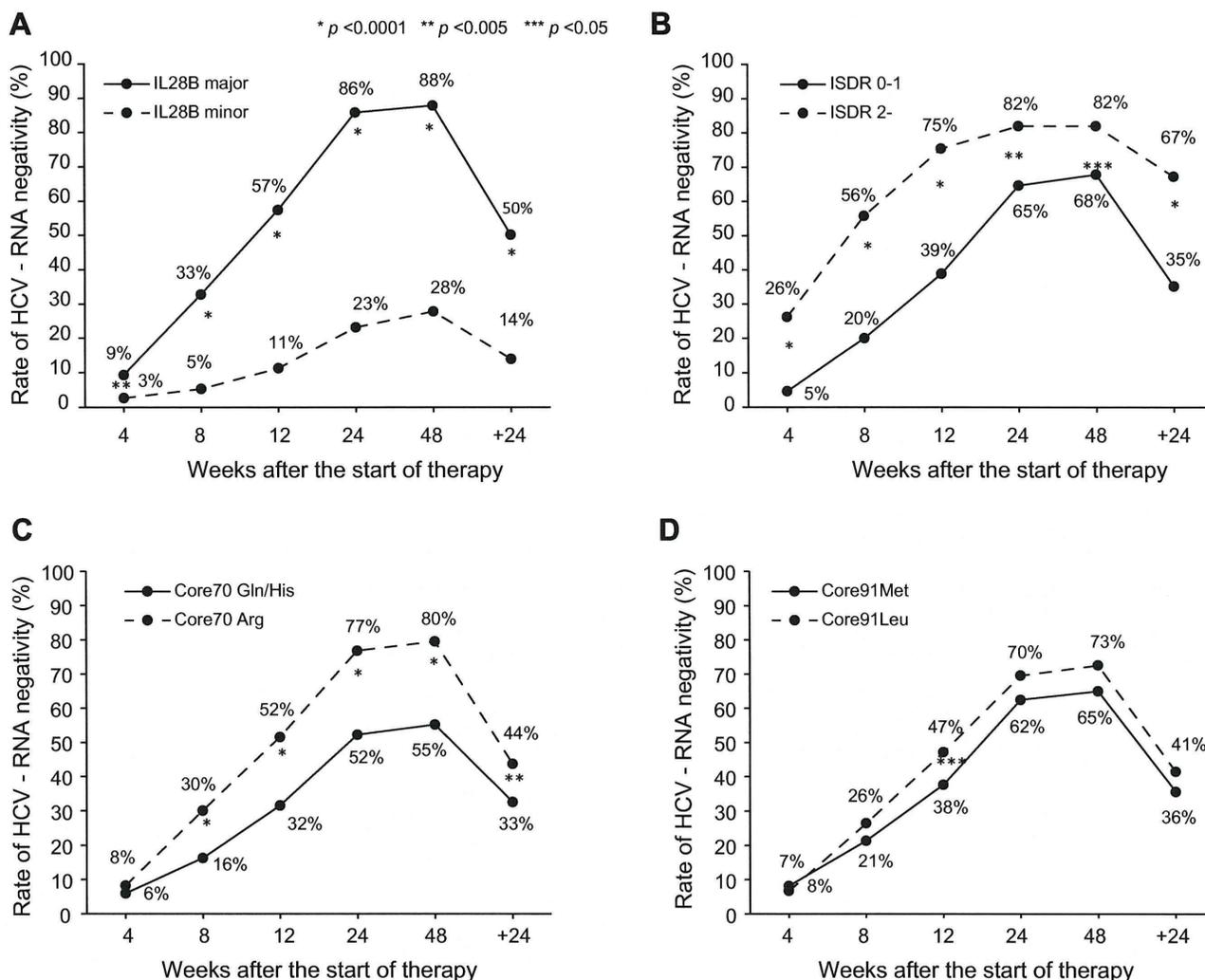


Fig. 2. Effect of *IL28B* mutations in the ISDR, Core70 and Core91 of HCV on time-dependent clearance of HCV. The rate of undetectable HCV-RNA was plotted for serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks) and for 24 weeks after the completion of therapy. Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele), (B) the number of mutations in the ISDR (0–1 mutation vs. 2 or more mutations), amino acid substitutions of (C) Core70 (Gln/His vs. Arg), and (D) Core91 (Met vs. Leu). The *p* values are from Fisher's exact test.

HCV-RNA ($p = 0.035$), Gln or His at Core70 ($p < 0.0001$), low platelet counts ($p = 0.009$), and advanced fibrosis ($p = 0.0002$) were associated with NVR. By multivariate analysis, the minor allele of *IL28B* (OR = 20.83, 95%CI = 11.63–37.04, $p < 0.0001$) was associated with NVR independent of other covariates (Table 2). Notably, mutations in the ISDR ($p = 0.707$) and at amino acid Core70 ($p = 0.207$) were not significant in multivariate analysis due to the positive correlation with the *IL28B* polymorphism ($p = 0.004$ for ISDR and $p < 0.0001$ for Core70, Fig. 4).

Genetic polymorphism of *IL28B* also was associated with SVR (OR = 7.41, 95% CI = 4.05–13.57, $p < 0.0001$) independent of other covariates, such as platelet counts, fibrosis, and serum levels of HCV-RNA. Mutation in the ISDR was an independent predictor of SVR (OR = 2.11, 95% CI = 1.06–4.18, $p = 0.033$) but the amino acid at Core70 was not (Table 3).

Factors associated with the *IL28B* polymorphism

Patients with the *IL28B* minor allele had significantly higher serum level of gamma-glutamyltransferase (GGT) and a higher

frequency of hepatic steatosis (Table 4). When the association between the *IL28B* polymorphism and HCV sequences was analyzed, Gln or His at Core70, that is linked to resistance to PEG-IFN and RBV therapy [4,14,15], was significantly more frequent in patients with the minor *IL28B* allele than in those with the major allele (67% vs. 30%, $p < 0.0001$) (Fig. 4). Other HCV sequences with an IFN resistant phenotype also were more prevalent in patients with the minor *IL28B* allele than those with the major allele: Met at Core91 (46% vs. 37%, $p = 0.047$) and one or no mutations in the ISDR (94% vs. 85%, $p = 0.004$) (Fig. 4).

Data mining analysis

Data mining analysis was performed to build a model for the prediction of SVR and the result is shown in Fig. 5. The analysis selected four predictive variables, resulting in six subgroups of patients. Genetic polymorphism of *IL28B* was selected as the best predictor of SVR. Patients with the minor *IL28B* allele had a lower probability of SVR and a higher probability of NVR than those with the major *IL28B* allele (SVR: 14% vs. 50%, NVR: 72% vs.