

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

The innate immune system has an essential role in host antiviral defense against HCV infection.<sup>6</sup> 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.<sup>7,8</sup> The IFN $\beta$  promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFN $\beta$ , 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 $\beta$  gene activation.<sup>9-12</sup> RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,<sup>8,13</sup> 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.<sup>14</sup> Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,<sup>15</sup> and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.<sup>16,17</sup> Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.<sup>9,18</sup> 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 $\alpha$ /RBV combination therapy.<sup>19</sup> 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 $\alpha$ /RBV combination therapy.<sup>19</sup> 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 $\alpha$ /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.<sup>19</sup> 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 $\alpha$ -2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5  $\mu$ g kg<sup>-1</sup> week<sup>-1</sup> 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 $\alpha$ -2b was reduced to 0.75  $\mu$ g kg<sup>-1</sup> week<sup>-1</sup> when either neutrophil count was less than 750/mm<sup>3</sup> or platelet count was less than 80  $\times$  10<sup>3</sup>/mm<sup>3</sup>. 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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**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 <sup>2</sup>	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 <sup>3</sup> /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 <sup>6.3</sup> 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'-TCATTTCGTGCATGCTC ACTGATAA-3'; *MDA5*, 5'-ACATAACAGCAACATG GGCAGTG-3', 5'-TTTGGTAAGGCCTGAGCTGG AG-3'; *LGP2*, 5'-ACAGCCTTGCAAACAGTACAAC CTC-3', 5'-GTCCCAAATTTCCGGCTCAAC-3'; *IPS-1*, 5'-GGTGCCATCCAAAGTGCCCTACTA-3', 5'-CAGC ACGCCAGGCTTACTCA-3'; *RNF125*, 5'-AGGGCA CATAITCCGGACTTGTCA-3', 5'-CGGGTATTAAC GGCAAAGTGG-3'; *ISG15*, 5'-AGCGAAGTCTCT TTGCCAGTACA-3', 5'-CAGCTCTGACACCGACA TGGA-3'; *USP18*, 5'-TGGTTCTGCTTCAATGACT CCAATA-3', 5'-TTTGGGCATTTCCATTAGCACT C-3'; *IFNλ*: 5'-CAGCTGCAGGTGAGGGA-3', 5'-G GTGGCCTCCAGAACCCTT-3'; *GAPDH*, 5'-GCACC GTCAAGGCTGAGAAC-3', 5'-ATGGTGGTGAAGA CGCCAGT-3'; *HMBS*, 5'-AAGCGGAGCCATGTCT GGTAAC-3', 5'-GTACCCACGCCAATCACTCTCA-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'-ATCCTCTCTCATCCCTCA 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.<sup>19</sup> In brief, liver biopsy specimens of

approximately 10 mg were homogenized in 100  $\mu$ L of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30  $\mu$ 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- $\beta$ -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 replicon<sup>20</sup> 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 $\alpha$ -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.<sup>3</sup> *IL28B* minor patients were significantly associated with a higher  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -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 $\lambda$  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 $\lambda$*  (*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 $\lambda$*  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 $\delta$*  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

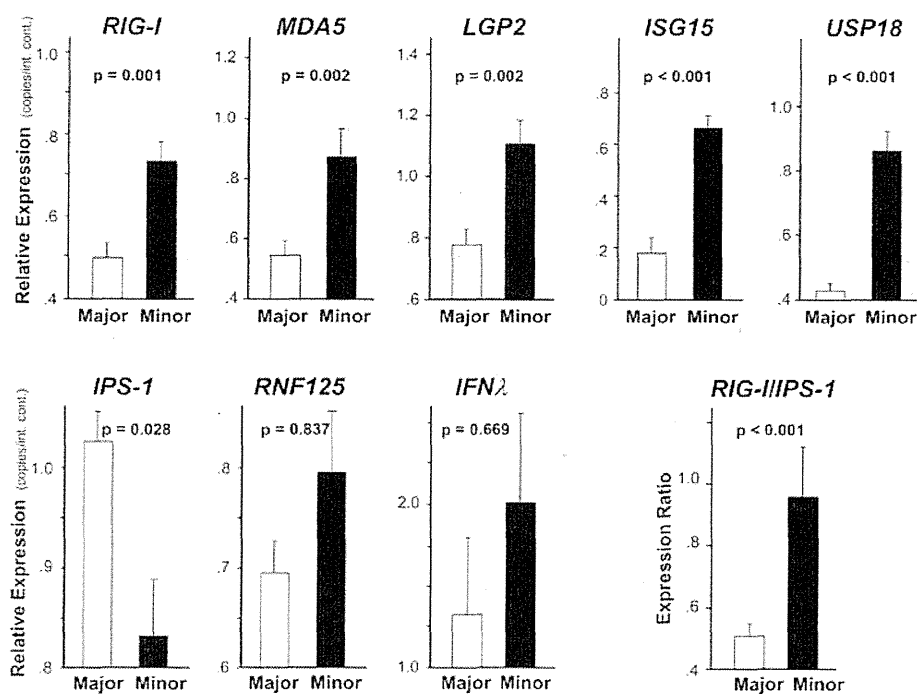


Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), 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

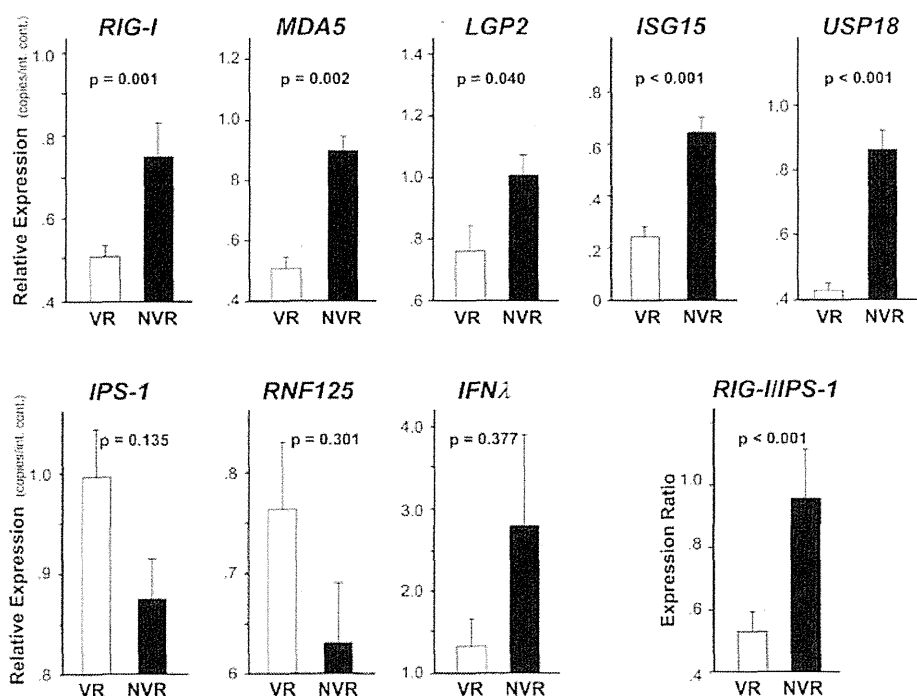


Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) 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.

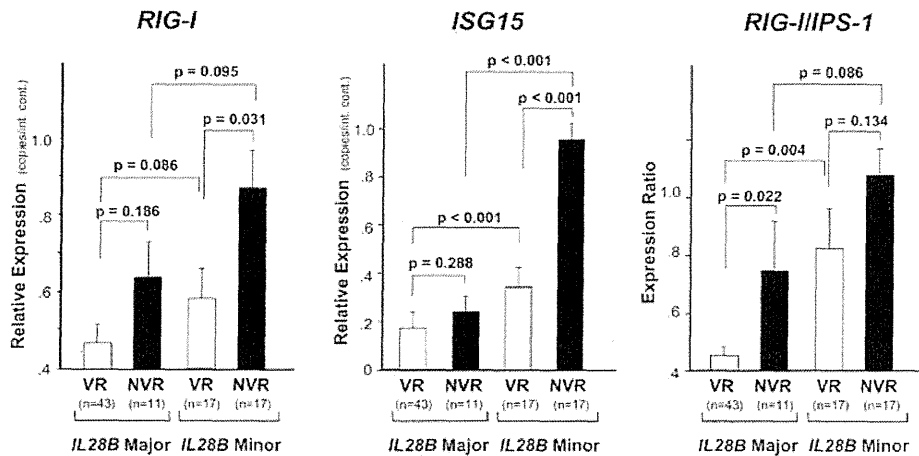


Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* 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-IIIIPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIG-IIIIPS-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-IIIIPS-1* ratio were significantly

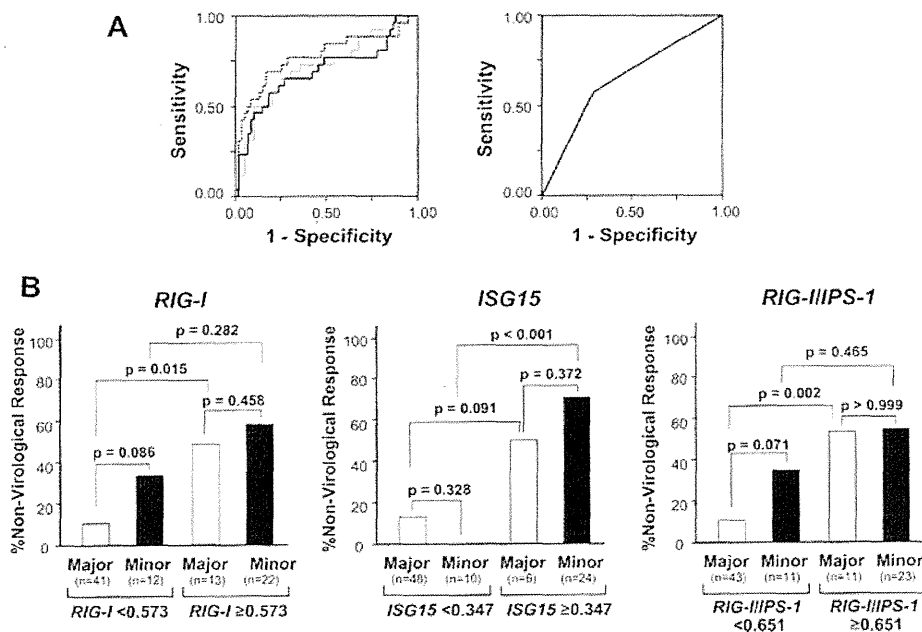


Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological 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/ISG15* 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 <sup>2</sup> )	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 <sup>6</sup> /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.

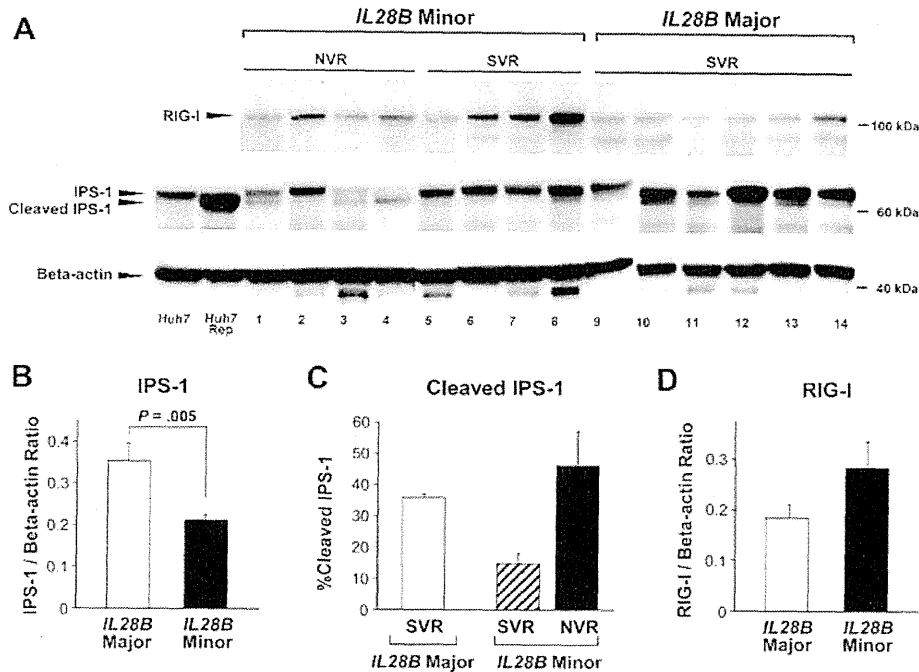


Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and  $\beta$ -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  $\beta$ -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  $\beta$ -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 $\alpha$ /RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFN $\alpha$ /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.<sup>7,8</sup> 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,<sup>19</sup> 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 $\alpha$ /RBV response as well as with *IL28B* genotype.<sup>21-23</sup>

In contrast to cytoplasmic viral sensor (*RIG-I*, *MDA5*, and *IIGP2*) 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,<sup>9,18</sup>

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  $\gamma$ -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 $\alpha$ /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 $\alpha$ /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 $\lambda$*  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 $\lambda$*  (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFN $\lambda$*  in relation to treatment response need further clarification by specifying type of *IFN $\lambda$*  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.<sup>19</sup> 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.<sup>24</sup> 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.<sup>25</sup> Because *IL28B* polymorphism strongly influences treatment responses within each population group,<sup>5</sup> 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.<sup>5</sup> 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 $\alpha$ /RBV was only 27.6% in *IL28B* minor patients.<sup>26</sup> Because new anti-HCV therapy should still contain PEG-IFN $\alpha$ /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 $\alpha$ /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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## Inhibition of hepatocellular carcinoma by PegIFN $\alpha$ -2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study

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### Abstract

**Background** We investigated whether the administration of maintenance doses of interferon prevented hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.

**Methods** Study 1: A multicenter, retrospective, cooperative study was carried out to determine whether long-term administration of low-dose peginterferon alpha-2a

(PegIFN $\alpha$ -2a) prevented HCC development in patients with chronic hepatitis C. In total, 594 chronic hepatitis C patients without a history of HCC were enrolled and treated with 90  $\mu$ g PegIFN $\alpha$ -2a administered weekly or bi-weekly for at least 1 year. Study 2: HCC developed in 16 of 99 additional patients without PegIFN $\alpha$ -2a treatment during 3.8 years of observation. A propensity-matched control study was then carried out to compare the incidence of

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HCC between the 59 patients who received low-dose PegIFN $\alpha$ -2a (PegIFN $\alpha$ -2a group) and 59 patients who did not receive PegIFN $\alpha$ -2a treatment (control group), matched for sex, age, platelet count, and total bilirubin levels.

**Results** Study 1: HCC developed in 49 patients. The risk of HCC was lower in patients with undetectable hepatitis C virus RNA,  $\leq 40$  IU/L alanine aminotransferase (ALT), or  $\leq 10$  ng/L alpha-fetoprotein (AFP) 24 weeks after the start of therapy. Study 2: The incidence of HCC was significantly lower in the PegIFN $\alpha$ -2a group than in the control group.

**Conclusions** Low-dose and long-term maintenance administration of PegIFN $\alpha$ -2a decreased the incidence of HCC in patients with normalized ALT and AFP levels at 24 weeks compared with patients without normal ALT and AFP levels.

**Keywords** Chronic hepatitis C · Hepatocellular carcinoma · Peginterferon

## Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer worldwide, often develops because of long-term hepatitis B or C virus infection [1, 2]. In particular, chronic hepatitis C and hepatic cirrhosis increase the risk of HCC; the annual incidence of tumor development in such patients may be as high as 2–4 % [3–5]. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment, although the incidence remains high in non-SVR patients [6–9]. A detailed analysis of HCC development revealed that chronic hepatitis C patients aged 65 years or more, especially those with advanced fibrosis of the liver, were at an increased risk of developing HCC [10]. For patients

65 years or older with advanced liver fibrosis, the dose of ribavirin is often reduced or the agent is discontinued, resulting in lower SVR rates in those with discontinuation of ribavirin. Establishing an effective treatment strategy for preventing the development of HCC is important for these high-risk patients.

Factors related to the development of HCC have been analyzed in patients who did not achieve an SVR even after IFN treatment; advanced fibrosis of the liver and high levels of serum alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) are risk factors for HCC development [11, 12]. A randomized controlled trial was conducted in Western countries to determine whether combined peginterferon and ribavirin treatment with weekly administration of 90  $\mu$ g peginterferon alpha-2a (PegIFN $\alpha$ -2a) could prevent HCC in non-responders. A 3.5-year follow up showed that administration of a maintenance dose of PegIFN $\alpha$ -2a did not reduce tumor incidence in these patients [13]. However, after 8.5 years of observation, the incidence of HCC was decreased among those in the PegIFN $\alpha$ -2a group with cirrhosis [14]. Meanwhile, Bruix et al. [15] reported that maintenance therapy with PegIFN $\alpha$ -2b did not prevent HCC in chronic hepatitis C patients with cirrhosis. In Japan, long-term low-dose administration of natural IFN has been reported to decrease the incidence of HCC [16]. In light of these conflicting results, investigations should be carried out in a large number of patients with chronic hepatitis C to resolve the question of whether IFN treatment prevents the development of HCC.

We carried out a multicenter retrospective cooperative study of patients with chronic hepatitis C to determine whether those treated with 90  $\mu$ g PegIFN $\alpha$ -2a without ribavirin had a reduced incidence of HCC compared with those not treated with IFN.

## Patients and methods

**Study 1: analysis of risk factors for HCC in patients treated with long-term low-dose-PegIFN $\alpha$ -2a**

In total, at 21 hepatitis centers throughout Japan, 743 patients with hepatitis C who had received 90  $\mu$ g of PegIFN $\alpha$ -2a therapy weekly or bi-weekly for 1 year or more without having received the full dose (180  $\mu$ g) since December 2003 were examined retrospectively for the development of HCC. The end of enrollment in this study was the end of December 2008 and the end of follow up was the end of December 2010. Patients with a history of HCC before the start of therapy and those with a therapy period of less than 48 weeks were excluded, leaving 594 patients who had undergone long-term administration of PegIFN $\alpha$ -2a for analysis. At the 21 centers involved in this

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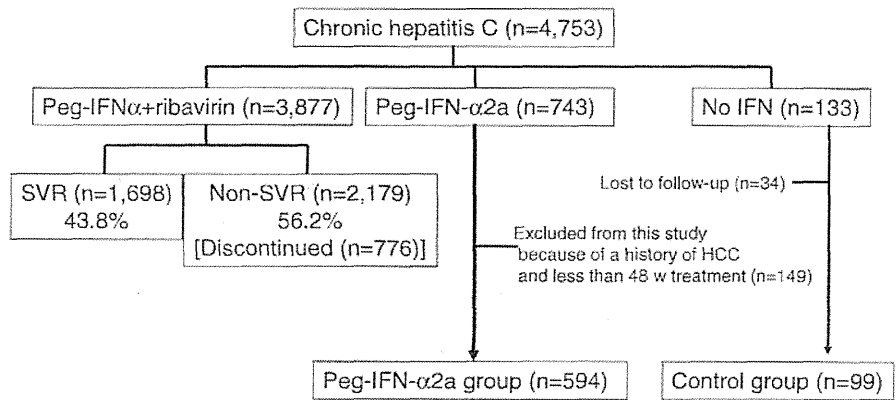
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**Fig. 1** Flow diagram of the patients' enrollment in the study. *Peg-IFN $\alpha$*  pegylated interferon  $\alpha$ . *SVR* sustained viral response. *HCC* hepatocellular carcinoma, *w* week



study, 4,753 patients with chronic hepatitis C had been treated; Peg-IFN and ribavirin combination treatment had been administered to 3,877 patients, 743 patients had received Peg-IFN alone, and 133 patients had not agreed to receive IFN (a flow diagram of the enrollment of patients in this study is shown in Fig. 1). In the patients with Peg-IFN and ribavirin combination treatment, the SVR rate was 43.8 %; SVR was not achieved in 2,179 patients, and in 776 of these patients, the combination therapy was discontinued owing to adverse events or the patient's choice. Patients who failed to achieve an SVR were not included in this study, because the incidence of HCC is known to be reduced even in non-responders to IFN [17].

The backgrounds of the 594 patients studied are shown in Table 1. Findings from the liver biopsies of the patients were classified according to international standards [18]. Long-term PegIFN $\alpha$ -2a treatment is approved by the Japanese Medical Insurance system. Written informed consent was obtained from all patients prior to participation in this study. The study design was approved by the regional ethics committees of the 21 centers involved in this study, including the Musashino Red Cross Hospital, in accordance with the Helsinki Declaration. The 743 patients treated with PegIFN $\alpha$ -2a alone were not indicated for Peg-IFN $\alpha$  and ribavirin combination therapy because of anemia or heart disease. The 133 patients who did not agree to receive IFN served as the control group (see Fig. 1). A large proportion of the 594 study patients had advanced fibrosis of the liver and active inflammation. A dose of 90  $\mu$ g PegIFN $\alpha$ -2a was administered to 512 and 82 patients weekly and biweekly, respectively, according to the patients' wishes. There were no significant differences between the weekly and biweekly groups in the patients' background data (data not shown).

The median duration of follow up in the PegIFN $\alpha$ -2a group was 1,273 days (range 228–2,768 days) and HCC was observed in 49 of the 594 patients (Table 1). Pre-treatment and on-treatment factors associated with the development of HCC were analyzed by Student's *t*-test, the

**Table 1** Background data of patients treated with PegIFN $\alpha$ -2a (*n* = 594)

	<i>n</i> = 594
Age (years)	61.7 $\pm$ 11.7
Sex (male/female)	258/336
BMI	23.2 $\pm$ 3.3
Genotype (1/2)	443/151
Diagnosis (ASC/CH/LC)	4/460/130
History of excess alcohol consumption ( $\geq$ 60 g/day; yes/no)	118/376
Fibrosis (F0, 1, 2/F3, 4)	443/151
Inflammatory activity (A0, 1/A2, 3)	469/125
Diabetes mellitus (no/yes)	499/95
LDL cholesterol (mg/dL)	94.2 $\pm$ 31.1
Fasting blood sugar (mg/dL)	106.3 $\pm$ 28.5
White blood cell count (/mm <sup>3</sup> )	4,360 $\pm$ 1,470
Red blood cell count ( $\times 10^6/\mu$ L)	423.8 $\pm$ 56.4
Hemoglobin (g/dL)	13.3 $\pm$ 1.8
Platelet count ( $\times 10^3/\mu$ L)	137 $\pm$ 56
Albumin (g/dL)	4.0 $\pm$ 0.5
Total bilirubin (mg/dL)	0.8 $\pm$ 0.6
AST (IU/L)	65.8 $\pm$ 47.8
ALT (IU/L)	72.1 $\pm$ 68.0
Gamma-GTP (IU/L)	55.2 $\pm$ 51.3
Esophageal varices (no/yes)	344/31
Alpha fetoprotein (ng/L)	6.9 (4.2–13.8)
Once weekly or biweekly PegIFN $\alpha$ -2a	512:82
Baseline HCV RNA (KIU/mL)	1,024 (73–2,130)
Development of HCC (no/yes)	545/49

*PegIFN* pegylated interferon, *BMI* body mass index, *ASC* asymptomatic carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *LDL* low-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GTP* guanosine triphosphate, *HCV* hepatitis C virus. *HCC* hepatocellular carcinoma

Values are means  $\pm$  SD, with ranges in parentheses

Mann–Whitney *U*-test, and the  $\chi^2$  test (Table 2). Independent factors for the development of HCC were assessed by multivariate analysis using logistic regression. The

incidence of HCC was analyzed according to the ALT, AFP, and hepatitis C virus (HCV) RNA levels 24 weeks after the start of PegIFN $\alpha$ -2a administration by using the Kaplan–Meier method. The risk of HCC was analyzed, using the Kaplan–Meier method, only in the non-responders with detectable HCV RNA during PegIFN $\alpha$ -2a administration by dividing them according to the ALT and AFP levels 24 weeks after the start of therapy. The incidence of HCC was compared between the patients with ALT levels of <41 IU/L and those with levels of  $\geq$ 41 IU/L, and between patients with serum AFP levels of <10 ng/L and those with levels of  $\geq$ 10 ng/mL at 24 weeks after starting treatment, because at most of the centers participating in the this study, the upper normal range of serum ALT is set at 40 IU/L, and the most significant difference in the incidence of HCC was observed between the PegIFN $\alpha$ -2a and control group with the cut-off serum ALT set at 41 IU/L and cutoff serum AFP set at 10 ng/mL, 24 weeks after starting treatment. The HCV RNA level was measured using the Amplicor Monitor method with a lower detection limit of 50 IU/L (Roche Diagnostics, Tokyo, Japan). A history of excess alcohol consumption was determined as >60 g alcohol per day in order to exclude alcoholic liver disease.

An asymptomatic carrier was defined as a patient with a serum ALT level within the normal range and minimal inflammation or fibrosis in the biopsied tissues of the liver. Chronic hepatitis was defined as mild-to-severe fibrosis of the liver according to liver biopsy [18]. The diagnosis of liver cirrhosis was based on the results of histological examination of the biopsied liver tissues.

**Study 2: incidence of HCC in the PegIFN $\alpha$ -2a therapy and non-administration (control) groups in comparison with propensity-matched controls**

Ninety-nine of the 133 chronic hepatitis C patients who had not received IFN were examined as controls; patients in this group received liver-protective agents such as glycyrrhizin or were untreated, and the group was observed for more than 1 year. None of the individuals in the control groups had received IFN alone or PegIFN $\alpha$  and ribavirin combination treatment. They were treated for a median of 1,395 days (range 75–6,556 days). Fifty-nine of these patients underwent liver biopsy before the treatment and were considered the control group for the propensity-matched study. For the propensity-matched study, 59 patients were selected from the PegIFN $\alpha$ -2a group according to their age, sex, platelet count, and total bilirubin levels, which had been identified as independent pretreatment risk factors for the development of HCC in Study 1. The rates of HCC were analyzed using the Kaplan–Meier method, and the risk of HCC was analyzed particularly in patients with advanced fibrosis of the liver (F3 and F4).

**Table 2** Comparison of HCC and non-HCC patients with long-term PegIFN $\alpha$ -2a administration ( $n = 594$ )

	Patients with or without development of HCC		<i>p</i> value
	With HCC ( $n = 49$ )	Without HCC ( $n = 545$ )	
Pretreatment parameters			
Age (years)	63.8 $\pm$ 1.7	61.3 $\pm$ 0.5	<0.05
Sex (male/female)	32/17	226/319	<0.01
BMI	24.0 $\pm$ 0.5	23.1 $\pm$ 0.2	n.s.
Genotype (1/2)	47/6	397/148	n.s.
History of excess alcohol consumption ( $\geq$ 60 g/day: yes/no)	11/38	107/338	n.s.
Fibrosis (F0, 1, 2/F3, 4)	25/24	418/127	<0.001
Inflammatory activity (A0, 1/A2, 3)	7/42	462/83	<0.001
Diabetes mellitus (no/yes)	38/11	461/84	n.s.
LDL cholesterol (mg/dL)	88.2 $\pm$ 9.0	94.7 $\pm$ 2.6	n.s.
White blood cell count (/mm <sup>3</sup> )	4,355 $\pm$ 210	4,360 $\pm$ 64	n.s.
Red blood cell count ( $\times 10^6/\mu$ L)	420.8 $\pm$ 8.1	424.1 $\pm$ 2.6	n.s.
Hemoglobin (g/dL)	13.6 $\pm$ 0.3	13.3 $\pm$ 0.1	n.s.
Platelet count ( $\times 10^3/\mu$ L)	106 $\pm$ 8	140 $\pm$ 2	<0.001
Albumin (g/dL)	3.8 $\pm$ 0.1	4.0 $\pm$ 0.1	<0.001
Total bilirubin (mg/dL)	1.2 $\pm$ 0.1	0.8 $\pm$ 0.1	<0.001
AST (IU/L)	78.1 $\pm$ 6.8	64.6 $\pm$ 2.1	n.s.
ALT (IU/L)	72.8 $\pm$ 9.7	72.0 $\pm$ 2.9	n.s.
Gamma-GTP (IU/L)	68.7 $\pm$ 7.5	53.9 $\pm$ 2.3	n.s.
Alpha fetoprotein (ng/L)	17.1 (4.4–36.8)	16.7 (4.1–23.1)	n.s.
Esophageal varices	29.0 % (9/31)	6.4 % (22/344)	<0.01
On-treatment parameters			
ALT (IU/L)	59.4 $\pm$ 5.7	44.6 $\pm$ 1.8	<0.05
Alpha fetoprotein (ng/L)	9.8 (4.6–17.4)	5.5 (3.7–11.1)	<0.01
HCV RNA level (KIU/mL)	236 (<0.5–2,210)	21 (<0.5–1,780)	<0.05

n.s. not significant

### Statistical analysis

Categorical data were compared using the  $\chi^2$  test or Fisher's exact test. The distributions of continuous variables were analyzed using Student's *t*-test and the Mann–Whitney *U*-test for two groups. Multivariate analysis was

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at  $p < 0.05$ . Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFN $\alpha$ -2a group were included.

**Results**

**Study 1**

We analyzed the factors involved in the development of HCC in patients who received 90  $\mu$ g PegIFN $\alpha$ -2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFN $\alpha$ -2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pretreatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFN $\alpha$ -2a maintenance treatment (Table 3).

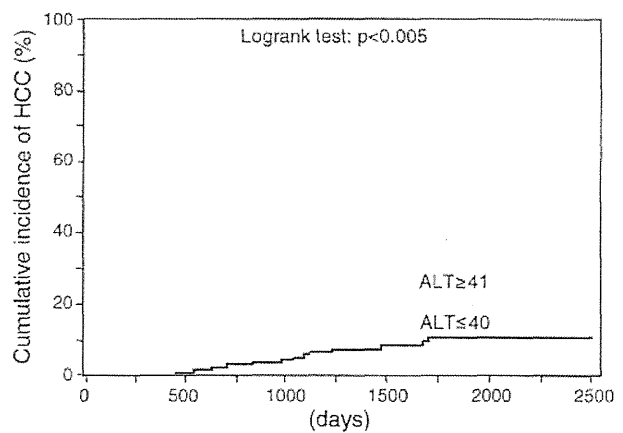
Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of  $\geq 41$  IU/L and AFP levels of  $\geq 10$  ng/L 24 weeks after the start of the PegIFN $\alpha$ -2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of  $\leq 40$  IU/L than in those with ALT levels of  $\geq 41$  IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of  $< 10$  ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of

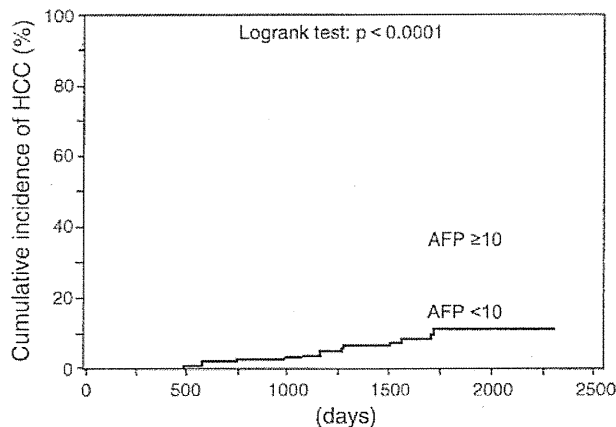
$\geq 10$  ng/mL (Fig. 3). The dose of PegIFN $\alpha$ -2a was reduced to 45  $\mu$ g in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFN $\alpha$ -2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

**Table 3** Independent risk factors for HCC development in patients treated with 90  $\mu$ g PegIFN $\alpha$ -2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	<i>p</i>
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count ( $< 120 \times 10^3/\mu$ L vs. $\geq 120 \times 10^3/\mu$ L)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) ( $\geq 41$ vs. $< 40$ IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) ( $\geq 10$ vs. $< 10$ ng/L)	3.78	1.92–11.8	<0.01



**Fig. 2** Comparison of HCC rates in patients administered with PegIFN $\alpha$ -2a ( $n = 594$ ) with respect to alanine aminotransferase (ALT) levels 24 weeks after the start of therapy. *Black line* patients with ALT  $\geq 41$  IU/L in the first 24 weeks, *gray line* patients with ALT  $\leq 40$  IU/L in the first 24 weeks



**Fig. 3** Comparison of HCC rates in patients administered PegIFN $\alpha$ -2a ( $n = 594$ ) with respect to alpha-fetoprotein (AFP) levels in the first 24 weeks after the start of therapy. *Black line* patients with AFP  $\geq 10$  ng/mL at 24 weeks, *gray line* patients with AFP  $< 10$  ng/mL at 24 weeks

## Study 2

We compared the incidence of HCC between 59 patients in the control group and the same number of patients in the PegIFN $\alpha$ -2a group using the matched-pair test. The backgrounds of the patients are shown in Table 4. The PegIFN $\alpha$ -2a group had higher rates of advanced fibrosis (F3 and F4) and active inflammation (A2 and A3). No other differences were found between the two groups, except for the white blood cell count (Table 4).

Development of HCC was observed in 2 patients in the PegIFN $\alpha$ -2a group and 8 in the control group. The incidence of HCC was compared between the two groups, using the Kaplan–Meier method. The incidence of HCC in the PegIFN $\alpha$ -2a group was significantly lower than that in the control group (log-rank test,  $p = 0.0187$ ; Fig. 4). Among the patients with advanced fibrosis of the liver (F3 and F4), those in the PegIFN $\alpha$ -2a group had a lower incidence of HCC than those in the control group. The independent risk factors for the development of HCC were analyzed using the stepwise Cox proportional hazard model. Only PegIFN $\alpha$ -2a administration and age were identified as independent risk factors for the development of HCC (Table 5).

## Discussion

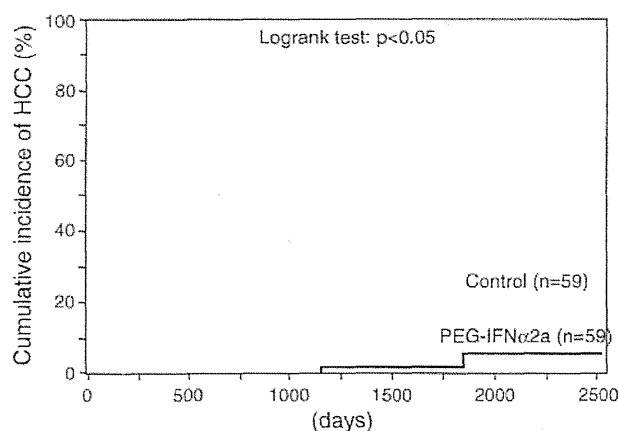
The number of HCC cases resulting from HCV infection continues to increase worldwide [19]. To date, IFN therapy is the most effective preventive measure against HCC in patients with chronic hepatitis C; furthermore, the

**Table 4** Backgrounds of the patients in the propensity-matched control study (PegIFN $\alpha$ -2a group,  $n = 59$ ; control group,  $n = 59$ )

	PegIFN $\alpha$ -2a group ( $n = 59$ )	Control group ( $n = 59$ )	$p$ value
Age (years)	60.5 $\pm$ 13.0	63.3 $\pm$ 10.5	n.s.
Gender (male/female)	24/35	25/34	n.s.
BMI	22.9 $\pm$ 3.6	22.9 $\pm$ 3.4	n.s.
Genotype (1/2)	49/10	46/13	n.s.
History of excess alcohol consumption (60 g/day; yes/no)	10/49	4/55	n.s.
Fibrosis (F0, 1, 2/F3, 4)	37/22	43/16	$< 0.05$
Development of HCC (F0–2/F3, 4)	1/1	1/7	n.s.
Inflammatory activity (A0,1/A2, 3)	19/40	30/29	$< 0.05$
Diabetes mellitus (no/yes)	57/2	56/3	n.s.
LDL cholesterol (mg/dL)	95.3 $\pm$ 23.8	117.0 $\pm$ 4.2	n.s.
White blood cell count (/mm <sup>3</sup> )	4,260 $\pm$ 1,239	5,193 $\pm$ 2,078	$< 0.05$
Red blood cell count ( $\times 10^{-4}$ / $\mu$ L)	430 $\pm$ 57.8	441 $\pm$ 44.9	n.s.
Hemoglobin (g/dL)	13.6 $\pm$ 1.5	13.6 $\pm$ 1.9	n.s.
Platelet count ( $\times 10^{-3}$ / $\mu$ L)	14.5 $\pm$ 5.7	15.8 $\pm$ 5.7	n.s.
Albumin (g/dL)	4.1 $\pm$ 0.5	4.1 $\pm$ 0.4	n.s.
Total bilirubin (mg/dL)	0.7 $\pm$ 0.5	0.9 $\pm$ 0.7	n.s.
AST (IU/L)	58.3 $\pm$ 47.7	49.7 $\pm$ 26.6	n.s.
ALT (IU/L)	63.6 $\pm$ 68.7	58.0 $\pm$ 39.2	n.s.
Gamma-GTP (IU/L)	78.3 $\pm$ 81.3	55.3 $\pm$ 75.1	n.s.
Baseline alpha-fetoprotein (AFP) (ng/L)	7.2 (4.3–14.2)	7.7 (3.9–13.8)	n.s.
Baseline HCV RNA level (KIU/mL)	1,230 (24–3,870)	1,024 (38–3,110)	n.s.

incidence of HCC is reduced in patients who achieve an SVR to IFN [6–8]. Therefore, achieving an SVR is the most effective approach for reducing the risk of developing HCC. In Japan, the incidence of HCC is elevated in older patients with hepatitis C. Corroborating this finding, the results of a Japanese study show a higher risk of HCC in patients aged 65 years and more [10]. Therefore, prevention of HCC in aged patients is an important challenge.

In the present multicenter, cooperative, retrospective study conducted in Japan, the incidence of HCC was reduced in patients who received 90  $\mu$ g PegIFN $\alpha$ -2a weekly or biweekly and had AFP values of  $< 10$  ng/mL and ALT values of  $\leq 40$  IU/L 24 weeks after the start of the treatment. The results of the matched case–control study of the PegIFN $\alpha$ -2a group and the non-IFN control group show that the incidence of HCC was significantly lower in the PegIFN $\alpha$ -2a group than in the control group, especially in patients with advanced fibrosis of the liver (F3 and F4). However, there could have been a selection bias between



**Fig. 4** Comparison of HCC rates between the long-term PegIFN $\alpha$ -2a administration group ( $n = 59$ ) and non-administration group ( $n = 59$ ) in the propensity-matched control study (Kaplan-Meier log-rank test,  $p = 0.019$ )

**Table 5** Risk factors for HCC in the propensity-matched control study (Cox proportional hazard model)

Variables	Risk ratio	95 % CI	$p$ value
PegIFN versus control	0.17	0.03–0.75	<0.05
Age (every 1 year)	1.12	1.02–1.25	<0.05
Fibrosis (F3, 4 vs. F0, 1, 2)	1.70	0.75–4.16	n.s.
Platelet count (every $10 \times 10^3/\mu\text{L}$ )	0.89	0.73–1.09	n.s.
Albumin (every 1.0 g/dL)	0.80	0.10–6.68	n.s.
On-treatment AFP (<10 vs. $\geq 10$ ng/L)	4.07	0.59–40.12	n.s.

the PegIFN $\alpha$ -2a group and the control group (patients who did not agree to receive IFN treatment), because this was a retrospective and non-randomized study. However, concordant with the findings of the HALT-C study [14], the present results show that PegIFN $\alpha$ -2a inhibits the development of HCC in patients with advanced fibrosis of the liver.

Recent studies show that polymorphisms in the host *IL28B* gene are important factors in the response to PegIFN $\alpha$  and ribavirin combination therapy [20, 21]. However, the mechanism of *IL28B* involvement in the response to PegIFN $\alpha$  and ribavirin has not been elucidated completely. A recent report has shown that *IL28B* is a significant factor in the development of HCC as well as in the response to IFN therapy [22]. Further studies are warranted to analyze the relationship between *IL28B* and inhibition of the development of HCC by PegIFN $\alpha$  in chronic hepatitis C.

Risk factors for the development of HCC have been discussed previously. Increased intrahepatic fat is involved in the development of HCC in chronic hepatitis C patients [23, 24]. In addition, diabetes-associated fat disorder [25,

26], hepatic iron overload [27], advanced fibrosis, older age, and fatty deposits in the liver are risk factors for HCC development [4]. Therefore, it is important to establish strategies to mitigate these risk factors to prevent the development of HCC and thus improve the outcomes of hepatitis C patients.

IFN therapy after HCC treatment is reported to inhibit the recurrence of tumors [28, 29], and a meta-analysis has revealed a trend toward inhibition of the recurrence of HCC [30, 31]. The prevention of HCC is an important issue that needs to be addressed to improve the survival of chronic hepatitis C patients. The findings of the present study and the HALT-C trial [14] indicate the effectiveness of long-term administration of maintenance IFN for preventing the development of HCC in chronic hepatitis C patients without an SVR. Improvement in ALT levels is also known to be an important predictor for the prevention of HCC [32]. A low AFP value during IFN administration is also recognized as a significant indicator of a lower risk of HCC [33, 34]. Recently, Osaki et al. [35] reported that a decrease of serum AFP during treatment with IFN was associated with a reduced incidence of HCC. Taking these findings and our own together, we conclude that maintenance administration of low-dose PegIFN $\alpha$ -2a weekly or biweekly to non-SVR patients with chronic hepatitis C decreases the incidence of HCC, especially in patients whose serum ALT and AFP levels are within the normal range 24 weeks after the start of treatment. The preventive effects of IFN against the development of HCC without elimination of the virus may be associated with its anticarcinogenic effects [16, 35]; however, the precise mechanism should be investigated.

The limitations of the present study are that it is retrospective and multicentric; therefore, potentially there may have been a selection bias. However, the reduction of the rate of development of HCC by maintenance administration of PegIFN $\alpha$ -2a in the patients in whom serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment may be attributable to the anticarcinogenic effects of IFN without elimination of the virus.

## Conclusion

The incidence of HCC was lower in non-SVR patients with chronic hepatitis C who were administered with maintenance low-dose PegIFN $\alpha$ -2a; especially in those whose serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment.

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**Conflict of interest** Namiki Izumi received lecture fees from Chugai Co. and MSD Co. in 2011.

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# Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean

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## Abstract

Hepatitis B virus (HBV) infection can lead to serious liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC); however, about 85–90% of infected individuals become inactive carriers with sustained biochemical remission and very low risk of LC or HCC. To identify host genetic factors contributing to HBV clearance, we conducted genome-wide association studies (GWAS) and replication analysis using samples from HBV carriers and spontaneously HBV-resolved Japanese and Korean individuals. Association analysis in the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with  $P_{meta} = 1.89 \times 10^{-12}$  for rs3077 and  $P_{meta} = 9.69 \times 10^{-10}$  for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against chronic hepatitis B (CHB) in Japanese, Korean and other Asian populations, including Chinese and Thai individuals ( $P_{meta} = 4.40 \times 10^{-19}$  for rs3077 and  $P_{meta} = 1.28 \times 10^{-15}$  for rs9277542). These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV were replicated widely in East Asian populations; however, there are no reports of GWAS in Caucasian or African populations. Based on the GWAS in this study, there were no significant SNPs associated with HCC development. To clarify the pathogenesis of CHB and the mechanisms of HBV clearance, further studies are necessary, including functional analyses of the HLA-DP molecule.

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## Introduction

Overall, one-third of the world's population (2.2 billion) is infected with hepatitis B virus (HBV), and about 15% of these are chronic carriers. About 75% of the chronic carriers live in the east-south Asia and east pacific area, and there are 1.3–1.5 million chronic carriers living in Japan [1]. Of chronic carriers, 10–15% develop liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC), and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in hepatitis B surface antigen (HBsAg) negative and hepatitis B core antibody (anti-HBc) positive, i.e. HBV-resolved individuals [2–3]. In Japan, although the major route of HBV transmission was perinatal transmission and horizontal transmission in early childhood, infant HBV carriers have successfully been reduced since 1986 through a selective vaccination policy by the Japanese government [4–7]. However, the prevalence of HBV genotype A in acute HBV (AHB) infection has increased markedly since 2000, reaching approximately 52% in 2008 due to the lack of a universal HB vaccination, and around 10% of AHB cases could be persistent infection [8–9]. Viral factors, as well as host factors, are thought to be associated with persistent HB infection.

In 2009, significant associations between chronic hepatitis B (CHB) and a region including *HLA-DPA1* and *HLA-DPB1* were identified using 786 Japanese individuals having CHB and 2,201 control individuals through a two-stage genome-wide association study (GWAS) [10]. The same group was also subjected to a second GWAS using a total of 2,667 Japanese persistent HBV infection cases and 6,496 controls, which confirmed significant associations between the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in the genetic region including the *HLA-DQ* gene [11]. The associations between *HLA-DP* variants with HBV infection were replicated in other Asian populations, including Thai and Han Chinese individuals [10,12–13]. With regard to HBV clearance, the association between the human leukocyte antigen (HLA) class II allele and clearance of HBV was confirmed by the candidate gene approach in African, Caucasian and Asian populations [14–13]. However, in a previous GWAS using samples of Japanese CHB and control individuals, the clinical data on HBV exposure in the control individuals were unknown, and this may have led to bias. Moreover, there have been no reports of GWAS using samples from HBV carriers and HBV-resolved individuals to identify host genetic factors associated with HBV clearance other than HLA class II molecules.

Here, we performed a GWAS using samples from Japanese HBV carriers, healthy controls and spontaneously HBV-resolved individuals in order to confirm or identify the host genetic factors related to CHB and viral clearance. In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls were randomly selected with clinically no evidence of HBV exposure, therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related with CHB or HBV clearance.

## Results

### Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

In this study, we conducted a GWAS using samples from 181 Japanese HBV carriers (including asymptomatic carriers (ASC), CHB cases, LC cases and HCC cases, based on the criteria described in Materials and Methods) and 184 healthy controls in

order to identify the host genetic factors related to progression of CHB. All samples were genotyped using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). Figure 1a shows a genome-wide view of the single point association data based on allele frequencies using the SNPs that met the following filtering criteria: (i) SNP call rate  $\geq 95\%$ ; (ii) minor allele frequency (MAF)  $\geq 1\%$  for HBV carriers and healthy controls; and (iii) no deviation from Hardy-Weinberg equilibrium (HWE)  $P \geq 0.001$  in healthy controls. We identified significant associations of protective effects against CHB with two SNPs (rs3077 and rs9277542) using the allele frequency model, both of which are located in the 3' UTR of *HLA-DPA1* and in the sixth exon of *HLA-DPB1*, respectively (rs3077,  $P = 1.14 \times 10^{-7}$ , and rs9277542,  $P = 5.32 \times 10^{-8}$ , respectively). The association for rs9277542 reached a genome-wide level of significance in the GWAS panel (Bonferroni criterion  $P < 3.36 \times 10^{-8}$  (0.05/597,789)).

In order to validate the results of GWAS, a total of 32 SNPs, including the associated two SNPs (rs3077 and rs9277542), were selected for replication in two independent sets of HBV carriers and healthy controls (replication-1:256 Japanese HBV carriers and 236 Japanese healthy controls; and replication-2:344 Korean HBV carriers and 151 Korean healthy controls; Table 1). The associations for the original significant SNP (rs9277542) and marginal SNP (rs3077) on GWAS were replicated in both replication sets [replication-1 (Japanese); rs3077,  $P = 2.70 \times 10^{-8}$ , OR = 0.48 and rs9277542,  $P = 3.33 \times 10^{-6}$ , OR = 0.54; replication-2 (Korean); rs3077,  $P = 2.08 \times 10^{-6}$ , OR = 0.47 and rs9277542,  $P = 8.29 \times 10^{-5}$ , OR = 0.54, Table 2]. We conducted meta-analysis to combine these studies using the DerSimonian Laird method (random effects model) to incorporate variation among studies. As shown in Table 2, the odds ratios were quite similar across the three studies (GWAS and two replication studies) and no heterogeneity was observed ( $P_{het} = 0.80$  for rs3077 and 0.40 for rs9277542).  $P_{meta}$  values were  $4.40 \times 10^{-19}$  for rs3077 (OR = 0.46, 95% confidence interval (CI) = 0.39–0.54), and  $1.28 \times 10^{-15}$  for rs9277542 (OR = 0.50, 95% CI = 0.43–0.60). Among the remaining 30 SNPs in the replication study, 27 SNPs were successfully genotyped by the DigiTag2 assay with SNP call rate  $\geq 95\%$  and HWE  $p$ -value  $\geq 0.01$ . Two SNPs (rs9276431 and rs7768538), located in the genetic region including the *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and healthy controls with Mantel-Haenszel  $P$  values of  $2.80 \times 10^{-7}$  (OR = 0.56, 95% CI = 0.45–0.70) and  $1.09 \times 10^{-7}$  (OR = 0.53, 95% CI = 0.42–0.67), respectively, when using additive, two-tailed Cochran Mantel-Haenszel (CMH) fixed-effects model with no evidence of heterogeneity ( $P_{het} = 0.67$  for rs9276431 and 0.70 for rs7768538) (Table S1).

Meta-analysis using the random effects model across 6 independent studies, including 5 additional published data, showed  $P_{meta} = 3.94 \times 10^{-15}$ , OR = 0.55 for rs3077,  $P_{meta} = 1.74 \times 10^{-21}$ , OR = 0.61 for rs9277535 and  $P_{meta} = 1.69 \times 10^{-15}$ , OR = 0.51 for rs9277542, with the SNP rs9277535 being located about 4-kb upstream from rs9277542 and showing strong linkage disequilibrium of  $r^2 = 0.955$  on the HapMap JPT (Table S2). As shown in Table S2, the odds ratio was very similar among the 6 studies, and heterogeneity was negligible with  $P_{het} > 0.01$ .

Moreover, based on GWAS using samples from 94 chronic HBV carriers with LC or HCC and 87 chronic HBV carriers without LC and HCC, we found no significant SNPs associated with CHB progression (Figure S1).