

**Figure 2. LD Structure, p Values, and OR Plots in the Association Analysis**

LD maps (A) around *TNFSF15* (chr9: nucleotide position: 116561403–116733452; build 36.3) and (B) around *POU2AF1* (chr11: nucleotide position: 110684600–110802128; build 36.3). The middle panels show estimates of pairwise  $r^2$  for (A) 28 SNPs and (B) 33 SNPs in the high-density mapping with a total of 2,365 samples used. The bottom panels show p values and OR-based chi-square tests for the allelic model for the left panels of 963 samples in the GWAS (●), the right panels of 1,402 samples in the replication study (▲), and the combined analysis (◇).

*POU2AF1* (28 and 33, respectively) for high-density association mapping (Table S5, Figures 2A and 2B). For this follow-up replication analysis, an independent set of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) were genotyped with the DigiTag<sup>2</sup><sup>13</sup> and custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany). The strongest associations identified in the initial GWAS were replicated in the independent set of 1,402 samples (OR = 1.52,  $p = 5.79 \times 10^{-8}$  for rs4979462; OR = 1.29,  $p = 9.32 \times 10^{-4}$  for rs4938534, Table 1). The combined p values were  $2.84 \times 10^{-14}$  (OR = 1.56; 95% CI = 1.39–1.76) for rs4979462 and  $2.38 \times 10^{-8}$  (OR = 1.39; 95% CI = 1.24–1.56) for rs4938534 (Table 1), both of which reached the genome-wide significance level of  $p < 5 \times 10^{-8}$ . In contrast, the other 27 weakly associated SNPs identified in the initial GWAS ( $p$  values  $< 0.0001$ ) were not found to have significant associations with PBC (Table S5). Moreover, no strongly associated SNPs were observed when comparing PBC cases between the early and late stages (Table S5).

A haplotype analysis of the *TNFSF15* and *POU2AF1* regions was conducted with the use of the genotype data from all 2,365 samples (1,274 PBC cases and 1,091 healthy controls). Linkage disequilibrium (LD) blocks were analyzed with Gabriel's algorithm,<sup>14</sup> and five blocks were observed in the *TNFSF15* region and seven blocks in the *POU2AF1* region (Figures 2A and 2B). There were no differences in the LD blocks between PBC cases and healthy controls. The risk haplotypes in each region showed a lower level of association than did the individual SNPs ( $p = 8.26 \times 10^{-14}$  for *TNFSF15* and  $p = 1.00 \times 10^{-4}$  for *POU2AF1*) (Tables S6 and S7).

Next, we focused on data from our initial GWAS in 21 loci that are reportedly associated with susceptibility to PBC in populations of European descent.<sup>4–7</sup> We found that three such loci (*IL7R*, *IKZF3*, and *STAT4*) had p values of less than 0.001 and eight other such loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *NFKB1*, *CD80*, *DENND1B*, and 7p14) showed evidence of marginal associations ( $p < 0.05$ ) in the initial GWAS in 487 Japanese PBC cases and 476 Japanese healthy controls (data not shown). We genotyped three SNPs (rs6890503 for *IL7R*, rs9303277 for *IKZF3*, and rs7574865 for *STAT4*) in an independent set

**Table 1. TNFSF15 SNP rs4979462 and POU2AF1 SNP rs4938534 Associated with Susceptibility to PBC**

dbSNP rsID	Nearest Gene	Risk Allele	Allele (1/2)	Stage	PBC Cases				Healthy Controls				OR <sup>a</sup>	
					11	12	22	RAF	11	12	22	RAF	95% CI	p Value <sup>b</sup>
rs4979462	TNFSF15	T	T/C	GWAS	154 (31.8)	244 (50.4)	86 (17.8)	0.57	98 (20.7)	230 (48.5)	146 (30.8)	0.45	1.63 (1.36–1.95)	1.21 × 10 <sup>-7</sup>
				Replication	253 (32.3)	390 (49.7)	141 (18.0)	0.57	131 (21.6)	305 (50.3)	170 (28.1)	0.47	1.52 (1.30–1.76)	5.79 × 10 <sup>-8</sup>
				Combined	407 (32.1)	634 (50.0)	227 (17.9)	0.57	229 (21.2)	535 (49.5)	316 (29.3)	0.46	1.56 (1.39–1.76)	2.84 × 10 <sup>-14</sup>
rs4938534	POU2AF1	A	G/A	GWAS	114 (23.6)	229 (47.3)	141 (29.1)	0.53	151 (31.8)	247 (52.0)	77 (16.2)	0.42	1.53 (1.28–1.83)	3.51 × 10 <sup>-6</sup>
				Replication	179 (22.8)	391 (49.8)	215 (27.4)	0.52	179 (29.4)	299 (49.2)	130 (21.4)	0.46	1.29 (1.11–1.50)	9.32 × 10 <sup>-4</sup>
				Combined	293 (23.1)	620 (48.9)	356 (28.1)	0.52	330 (30.5)	546 (50.4)	207 (19.1)	0.44	1.39 (1.24–1.56)	2.38 × 10 <sup>-8</sup>

Parentetical numbers indicate the percentage of allele 11, 12, or 22 among total alleles in PBC cases or healthy controls. The following abbreviations are used: PBC, primary biliary cirrhosis; RAF, risk allele frequency; and GWAS, genome-wide association study.

<sup>a</sup>Odds ratio (OR) of minor allele from the two-by-two allele frequency table.

<sup>b</sup>p value of Pearson's chi-square test for the allelic model.

of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) using the DigiTag<sup>2</sup><sup>13</sup> and custom TaqMan SNP genotyping assays. Two SNPs, rs6890853 and rs9303277 located in loci *IL7R* and *IKZF3*, respectively, showed significant associations and the *STAT4* locus (rs7574865) showed suggestive association with PBC in 2,365 Japanese samples (1,274 PBC cases and 1,091 healthy controls) (rs6890853, combined p value = 3.66 × 10<sup>-8</sup>, OR = 1.47 for *IL7R*; rs9303277, combined p value = 3.66 × 10<sup>-9</sup>, OR = 1.44 for *IKZF3*; rs7574865, combined p value = 1.11 × 10<sup>-6</sup>, OR = 1.35 for *STAT4*) (Tables S5 and S8).

Moreover, we genotyped 16 additional associated SNPs, all of which were the same SNPs as identified in previous studies,<sup>4–7</sup> and revealed that six out of 16 SNPs (located on *CXCR5*, *NFKB1*, *CD80*, *DENND1B*, *MAP3K7IP1*, and *TNFAIP2*) were replicated (p < 0.05) in 2,365 Japanese samples (Table S8). The SNP rs2293370, located in the *CD80* locus, showed a significant association and the *NFKB1* locus (rs7665090) showed a suggestive association with PBC in the Japanese population (rs2293370, combined p value = 3.04 × 10<sup>-9</sup>, OR = 1.48 for *CD80*; rs7665090, combined p value = 1.42 × 10<sup>-7</sup>, OR = 1.35 for *NFKB1*). Although further study for determining the primary SNP at each locus is necessary, the remaining ten loci (*RAD51L1*, *PLCL2*, *IL12RB2*, *IRF5*, *SPIB*, *RPS6KA4*, *CLEC16A*, *TNFRSF1A*, *IL12A*, and *MMEL1*) did not show significant association (p < 0.05) with PBC in the Japanese population (Table S8).

In the current GWAS in the Japanese population, we identified two significant susceptibility loci for PBC, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534), which had not been identified in the previous GWAS in populations of European descent. In addition, of the 21 PBC susceptibility loci that have been identified in populations

of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations and two loci (*STAT4* and *NFKB1*) showed suggestive associations with PBC in the Japanese population. Eight other loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *DENND1B*, *MAP3K7IP1*, *TNFAIP2*, and 7p14) also showed marginal associations with PBC in the Japanese population. These results indicate the presence of additional important disease pathways (via *TNFSF15* and *POU2AF1*)—differentiation to T helper 1 (Th1) cells (via *IL7R* and *STAT4*), B cell differentiation (via *IL7R* and *IKZF3*), T cell activation (via *CD80*), and NF-κB signaling—in addition to the previously reported disease pathways in the development of PBC in Japanese populations.

*TNFSF15* is a newly described member of the TNF superfamily that interacts with death receptor 3 (*DR3* [MIM 603366], also known as *TNFRSF25*) not only to promote effector T cell expansion (i.e., Th1 and Th17 cells) and cytokine production (i.e., interferon-γ [IFN-γ, MIM 147570]) at the site of inflammation, but also to induce apoptosis in cells that overexpress DR3.<sup>15</sup> Interestingly, genetic polymorphisms in *TNFSF15* are associated with susceptibility to CD, UC, ankylosing spondylitis (AS, MIM 106300), and leprosy (MIM 609888)<sup>16–20</sup> (Table S8). Strong association of five SNPs (rs3810936, rs6478108, rs6478109, rs7848647, and rs7869487) in the *TNFSF15* region with CD was first reported for a Japanese population,<sup>16</sup> and the finding was replicated in an independent Japanese population and in European-descent and Korean populations.<sup>21–25</sup> Another SNP within *TNFSF15* (rs4263839) is also associated with susceptibility to CD in populations of European descent.<sup>17,20,26</sup> In addition, the risk alleles of the SNPs were significantly associated with *TNFSF15* mRNA expression in peripheral blood.<sup>27,28</sup> Given that there exists strong LD among SNPs in *TNFSF15*, including those in the promoter region (rs6478109 and

rs7848647) and introns (rs4263839 and rs4979462), it is very probable that the PBC susceptibility haplotype containing rs4979462 also influences *TNFSF15* mRNA expression. Additionally, *TNFSF15* signaling via DR3 synergizes with interleukin-12 (IL-12) and IL-18 to promote IFN- $\gamma$  production.<sup>15</sup> The IL-12 signaling pathway includes *IL12A* and *IL12RB* (MIM 601604), variants of which have been identified as PBC susceptibility loci in previous GWASs of peoples of European ancestry, and has been implicated as a key player in the pathogenesis of PBC.<sup>4–7</sup> *STAT4* is essential for IL-12 signal transduction via the IL-12 receptor (IL12R) for IFN- $\gamma$  production and Th1 polarization.<sup>29</sup> Thus, the evidence that *TNFSF15* and *STAT4* were identified and confirmed as PBC susceptibility loci in the present study might indicate that the IL-12 signaling pathway via IL12R is also operative in PBC pathogenesis in Japanese populations, as it is in populations of European descent.

*POU2AF1* is a B cell-specific transcriptional factor that co-activates octamer-binding transcriptional factors *POU2F1* (MIM 164175) and *POU2F2* (MIM 164176) on B cell-specific promoters; thus, *POU2AF1* is essential for B cell maturation and germinal center formation.<sup>30</sup> The E-twenty six transcription factor *Spi-B* was recently identified as a direct target of the coactivator *POU2AF1*.<sup>31</sup> *Spi-B* is an important mediator of both B cell receptor signaling and early T cell lineage decisions.<sup>32,33</sup> *Spi-B* also induces IL7R-induced CD40 (MIM 109535, MIM 300386) expression.<sup>34</sup> Given that *Spi-B* has been identified as a PBC susceptibility gene in previous GWASs of peoples of European ancestry,<sup>6,7,35</sup> variation of *POU2AF1* might function along with *Spi-B* in this pathway of B cell signaling and differentiation. The lack of *POU2AF1* reportedly prevents the development of autoimmunity in *Aiolos* (also known as *IKZF3*) mutant mice, which have a systemic lupus erythematosus (MIM 152700)-like phenotype, and in MRL-*lpr* mice.<sup>36,37</sup> *IKZF3* and *IL7R* were both replicated and confirmed as PBC susceptibility loci in this study; *IKZF3* functions as a transcription factor that participates in the generation of high-affinity bone marrow plasma cells responsible for long-term immunity, and *IL7R* participates in pre-B cell expansion.<sup>38,39</sup> Collectively, these results strengthen the notion that the B cell signaling pathway is involved in the development of PBC.

In conclusion, *TNFSF15* and *POU2AF1* were identified as significant susceptibility loci for PBC in a Japanese population. Our results provide further evidence for the presence of (1) ethnic differences in genetic susceptibility loci (i.e., *TNFSF15*, *IL12A*, and *IL12RB2*), (2) a new autoimmune pathway (i.e., *TNFSF15* signaling) shared with other autoimmune diseases (CD, UC, and AS), and (3) common pathogenic pathways such as B cell differentiation (i.e., *POU2AF1*, *IKZF3*, and *SPIB*), IL-12 signaling (i.e., *IL12A*, *IL12RB2*, and *STAT4*), and T cell activation (i.e., *CD80*) for the development of PBC in individuals of European descent and Japanese individuals (Table S8). Functional analysis of these genetic loci, as well as the identification

of additional susceptibility loci associated with PBC in eastern Asian populations, should facilitate the analysis of the pathogenesis of PBC worldwide and aid the development of rationale for therapies in the future.

### Supplemental Data

Supplemental Data include two figures, eight tables, and Supplemental Acknowledgments and can be found with this article online at <http://www.cell.com/AJHG/>.

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### Web Resources

The URLs for data presented herein are as follows:

MEXT Integrated GWAS Database, [https://gwas.biosciencedbc.jp/cgi-bin/gwasdb/gwas\\_top.cgi](https://gwas.biosciencedbc.jp/cgi-bin/gwasdb/gwas_top.cgi)

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>

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## Retreatment with Peginterferon $\alpha$ -2a + Ribavirin in Patients Who Failed Previous Peginterferon $\alpha$ -2b + Ribavirin Combination Therapy

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### Key Words

Hepatitis C · Interleukin-28B · Peginterferon · Retreatment · Sustained viral response

### Abstract

**Background/Aims:** Peginterferon (PEG-IFN) + ribavirin (RBV) combination therapy is the current standard of care for chronic hepatitis C. However, more than half of the patients cannot achieve sustained viral response (SVR). In Japan, the clinical benefit of retreatment with PEG-IFN + RBV combination retreatment is still unknown. **Methods:** We collected clinical data in 106 chronic hepatitis C patients who failed to achieve SVR with PEG-IFN $\alpha$ -2b + RBV combination therapy and were retreated with PEG-IFN $\alpha$ -2a + RBV. This retrospective study examined the efficacy of retreatment with PEG-IFN $\alpha$ -2a + RBV by evaluating the time to eradication of hepatitis C virus RNA, early virological response (EVR), and SVR. We compared the results of the previous therapy and retreatment in terms of efficacy and analyzed the factors influencing SVR. **Results:**

The SVR rates in the non-responders and relapsers were 11 and 53%, respectively. EVR and prolonged treatment duration were associated with SVR. We also found that a prior response to PEG-IFN + RBV therapy was more important than the Interleukin-28B genotype for predicting the response to retreatment. **Conclusions:** Retreatment with PEG-IFN $\alpha$ -2a + RBV should be considered for relapsers and partial responders. Our results suggest that prolonged administration is also favorable for EVR cases to attain a higher SVR.

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### Introduction

The development of a combination therapy consisting of peginterferon (PEG-IFN) and ribavirin (RBV) has increased the hepatitis C virus (HCV) RNA response rate to 65–69% at the end of therapy with a sustained HCV RNA response (sustained viral response, SVR) in 54–56% of chronic hepatitis C (CHC) patients. Conversely, this

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indicates that PEG-IFN + RBV therapy does not induce a response in 31–35% of such patients either because HCV RNA is not eliminated during therapy or the therapy is not completed. Furthermore, HCV RNA reappears in 11–13% of patients after the end of therapy [1, 2].

Retreatment with PEG-IFN + RBV for non-responding or relapse patients has been studied in Western countries [3, 4], but no large-scale studies have been performed in Japan. The AASLD guidelines [5] do not recommend retreatment with PEG-IFN + RBV. However, the Japanese guidelines [6] state that ‘after examining cases with no effect in previous treatment, a treatment for SVR or for maintenance should be selected’; thus, retreatment with PEG-IFN + RBV is not completely excluded according to the Japanese guidelines.

A high SVR rate is observed after the addition of telaprevir to PEG-IFN + RBV [7–10]. However, some patients in Japan have not benefited from the launch of telaprevir, because CHC patients in Japan are older than those in Western countries [11, 12] and are often anemic. Thus, we retrospectively analyzed the results of retreatment with PEG-IFN + RBV in Japanese relapse and non-responding patients who previously received therapy with PEG-IFN + RBV. Core amino acid substitutions at position 70 [13] and host genome single-nucleotide polymorphism (SNP) genotyping of rs8099917, an interleukin-28B (IL28B) SNP [14, 15], are related to the efficacy of PEG-IFN + RBV therapy. Therefore, these factors were also examined in the study.

## Materials and Methods

A total of 106 patients received combination therapy with PEG-IFN $\alpha$ -2a + RBV at 12 medical facilities in Japan from 2007 to 2009. We retrospectively evaluated the data of CHC patients who failed to achieve SVR (i.e. non-responders) or became HCV RNA negative on PEG-IFN $\alpha$ -2b + RBV therapy but relapsed again after the end of treatment (i.e. relapsers). The non-responders were divided into two groups according to the maximum decrease in HCV RNA titer during the initial treatment. Retreatments with PEG-IFN $\alpha$ -2a + RBV was performed to examine the relationships between SVR and patient background factors, timing of the HCV RNA response, and treatment duration.

For the previous treatment, PEG-IFN $\alpha$ -2b (PegIntron; MSD, Tokyo, Japan) at a dose of 1.5  $\mu\text{g kg}^{-1}$  per week subcutaneously and RBV (Rebetol; MSD) were used. PEG-IFN $\alpha$ -2a (Pegasys; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) + RBV (Copegus; Chugai Pharmaceutical Co., Ltd) was started between 2007 and 2009. In principle, as a starting dose, PEG-IFN was given once weekly at 180  $\mu\text{g}$  PEG-IFN $\alpha$ -2a while RBV was given at 600–1,000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1,000 mg), according to the standard treatment protocol in Japan.

Serum HCV RNA after PEG-IFN $\alpha$ -2b + RBV treatment was assessed by quantification using a Cobas Amplicor HCV monitor test (high range method: detection range, 5–5,000 KIU ml $^{-1}$ , or version 2.0: limit of quantitation, 500 IU ml $^{-1}$ ; Roche Diagnostics Co. Ltd, Tokyo, Japan).

HCV RNA in retreatment was measured using a Cobas TaqMan HCV test (Roche Diagnostics Co. Ltd) at 4-week intervals; the linear dynamic range was 1.2–7.8 log IU ml $^{-1}$ . Samples with undetectable HCV RNA levels were reported as <1.2 log IU ml $^{-1}$  (i.e. no detectable HCV RNA). Patients were judged to have attained SVR status if HCV RNA was not detected for 24 weeks after the end of treatment. Rapid viral response (RVR) was defined when HCV RNA was not detected at week 4; early virological response (EVR) was defined when HCV RNA was not detected at week 12 of treatment.

Univariate analysis was performed using a  $\chi^2$  test and Fisher’s exact test. Multivariate analysis was performed using logistic regression using the stepwise method.

As a rule, dose modification followed the manufacturer’s drug information on the intensity of potential adverse hematologic effects. The PEG-IFN doses were reduced to 50% of the original dose if the neutrophil count fell below 750/mm $^3$  and discontinued if the neutrophil count fell below 500/mm $^3$  or the platelet count (PLT) fell below 50,000/mm $^3$ . RBV was also reduced from 1,000 to 600, 800 to 600, or 600 to 400 mg when hemoglobin (Hb) was below 10 g dl $^{-1}$  and was discontinued when the Hb was below 8.5 g dl $^{-1}$ . Both PEG-IFN and RBV were discontinued if there was a need to discontinue one of the drugs.

The baseline data of the patients are expressed as median values and ranges. In order to analyze the differences between baseline data and the factors associated with SVR, univariate analysis using the Mann-Whitney U test or a  $\chi^2$  test was performed; multivariate analysis was performed using stepwise and multiple logistic-regression models. *p* values <0.05 were considered significant.

This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

## Results

### Characteristics

The median age of the 106 subjects was 60 years. There were 63 non-responders and 43 relapsers who received previous treatment. Non-responders were divided into two groups according to their virological response to the previous treatment: partial responders, maximum HCV RNA decrease of >2 log; null responders, maximum HCV RNA decrease <2 log. HCV RNA genotypes 1 and 2 were detected in 101 and 5 subjects, respectively. The baseline characteristics of the study patients are shown in table 1.

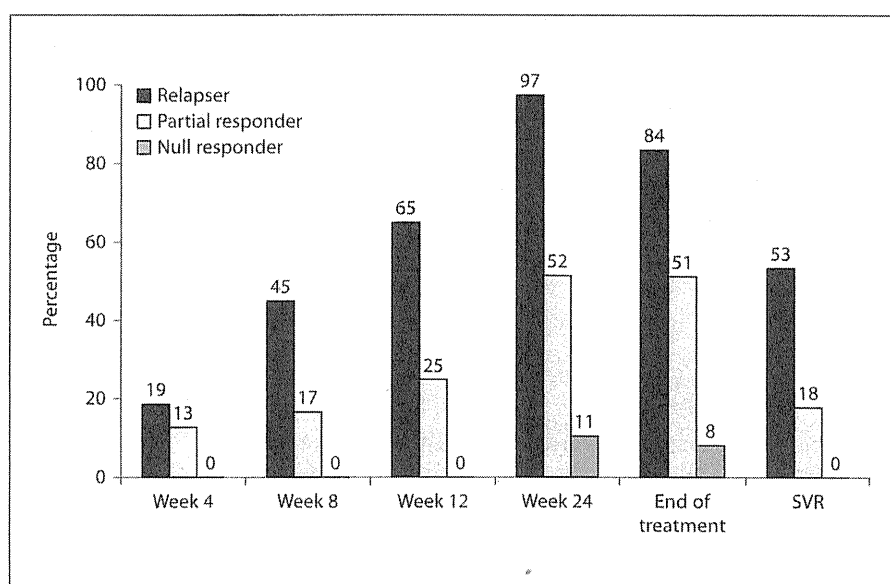
### Efficacy

Retreatment of the 106 subjects with PEG-IFN $\alpha$ -2a + RBV therapy resulted in an SVR rate of 28% (30/106). The

**Table 1.** Baseline patients' characteristics

	Relapser (n = 43)	Partial responder (n = 39)	Null responder (n = 24)
Age, years	64 (44–75)	58 (33–77)	60 (30–69)
Male/female	18/25	19/20	12/12
Body mass index	22.2 (16.6–31.0)	23.4 (18.2–32.3)	22.2 (17.8–29.8)
Genotype 1/2	42/1	36/3	23/1
Viral load, log IU/ml	6.3 (3.7–7.2)	5.7 (1.2–7.2)	6.4 (3.7–7.5)
ALT, IU/ml	39 (15–189)	41 (12–379)	43 (18–275)
$\gamma$ -GTP, IU/ml	24 (9–175)	51 (17–326)	39 (19–366)
WBC count, /mm <sup>3</sup>	4,500 (1,900–8,300)	4,100 (2,000–9,400)	3,495 (1,700–7,100)
Platelet count, $\times 10^4$ /mm <sup>3</sup>	13.8 (7.6–24.9)	14.1 (4.5–28.6)	11.2 (4.5–29.3)
Hemoglobin, g/dl	13.8 (11.9–16.5)	13.3 (9.3–16.5)	12.4 (9.6–15.9)
LDL cholesterol, mg/dl	100 (42–167)	86 (32–128)	74 (15–122)
$\alpha$ -Fetoprotein, ng/ml	4 (1–48)	5.9 (1–30)	7.8 (2–108)
Core aa70, wild/non-wild	13/9	8/7	3/3
IL28B rs8099917 (TT/TG, GG)	21/5	12/7	3/7

Values are medians with ranges in parentheses or numbers.

**Fig. 1.** Virological responses of PEG-IFN $\alpha$ -2a + RBV.

SVR rates in the relapsers, partial responders, and null responders were 53% (23/43), 18% (7/39), and 0% (0/24), respectively. The HCV RNA response rate in the relapsers was 65% at week 12 of the PEG-IFN $\alpha$ -2a + RBV therapy and 84% at the end of treatment (fig. 1). In contrast, the HCV RNA response rates in the non-responders were 15, 37, and 37% at week 12, week 24, and the end of retreatment, respectively. These rates were lower than those of the relapsers.

#### Examination of Previous Relapsers

The durations before an HCV RNA response was observed in the relapsers were  $\leq 12$  weeks in 65% (28/43) and 13–24 weeks in 26% (11/43) of the patients. In the 28 patients who achieved EVR, the SVR rates were 56% (9/16) and 92% (11/12) in the patients for whom retreatment commenced within 48 weeks and who received prolonged therapy, respectively; this difference was significant ( $p = 0.04$ ).



**Table 2.** Factors associated to SVR

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Response of previous treatment, relapsers/non-responders	9.20 (6.3–54)	<0.0001	–	n.s.
$\gamma$ -GTP, <35/ $\geq$ 35 IU/l	2.92 (1.2–7.3)	0.0189	–	n.s.
Platelet counts, $\geq 12 \times 10^4$ / $< 12 \times 10^4$ /mm <sup>3</sup>	2.96 (1.1–7.7)	0.0232	–	n.s.
Hemoglobin, $\geq 13$ / $< 13$ g/dl	3.24 (1.2–8.8)	0.0179	–	n.s.
LDL cholesterol, $\geq 100$ / $< 100$ mg/dl	4.61 (1.5–14)	0.0046	–	n.s.
$\alpha$ -Fetoprotein, $< 6$ / $\geq 6$ ng/ml	4.16 (1.5–11)	0.0112	–	n.s.
Treatment duration, prolonged/standard	3.32 (1.4–8.0)	0.0063	5.16 (1.13–23.6)	0.0343
HCV RNA at week 12, negative/positive	18.5 (6.3–54)	<0.0001	10.3 (1.65–64.7)	0.0022
PEG-IFN adherence, $\geq 80$ / $< 80$ %	4.80 (1.6–14)	0.0025	–	n.s.
Ribavirin adherence, $\geq 60$ / $< 60$ %	3.69 (1.2–12)	0.0214	–	n.s.

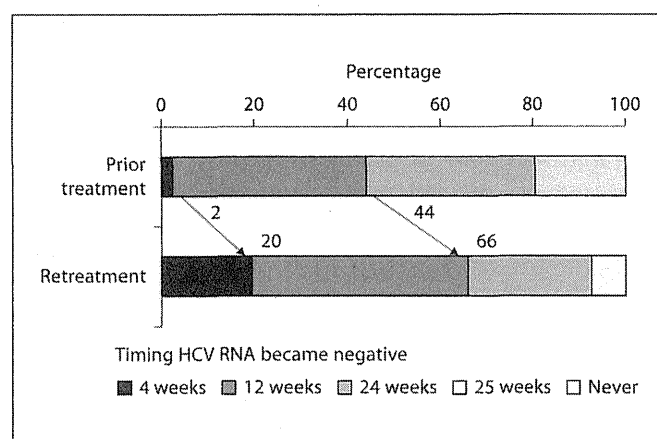
We also compared the RVR and EVR rates between initial treatment and retreatment among the 41 patients whose timing of HCV negativity was available (fig. 2). The rates of RVR and EVR were higher in the retreatment patients (2 and 44% in initial treatment, and 20 and 66% in retreatment, respectively).

#### Examination of Previous Non-Responders

Among the non-responders, an HCV RNA response after retreatment was achieved only in partial responders. Of these patients, 23% (9/39) achieved EVR, 23% (9/39) within 13–24 weeks, and 8% (3/37) after 25 weeks. In the 9 subjects who achieved EVR among partial responders, the SVR rate was 25% (1/4) during the retreatment within 48 weeks but was 60% (3/5) when the retreatment period was prolonged. No SVR was achieved in retreatment subjects with an HCV RNA response that required  $\geq 25$  weeks.

#### Factors Associated with SVR

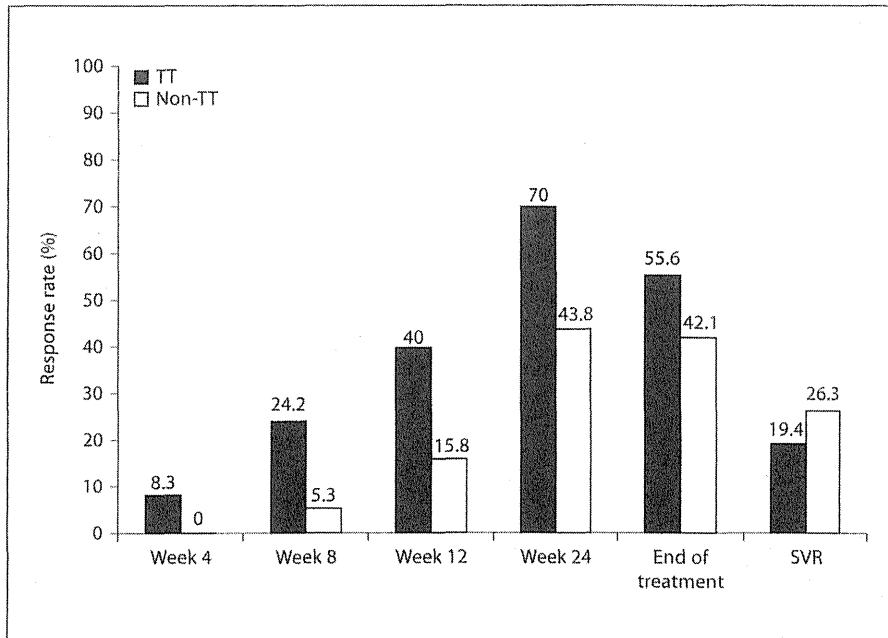
Analysis revealed that the SVR rate was significantly high in the following factors: relapsers; prolonged duration of retreatment; Hb level  $\geq 13$  g dl<sup>-1</sup>; PLT  $\geq 12 \times 10^4$ /mm<sup>3</sup>;  $\alpha$ -fetoprotein (AFP)  $< 6$  ng ml<sup>-1</sup>; low-density lipoprotein cholesterol (LDL-Chol)  $\geq 100$  mg dl<sup>-1</sup>;  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP)  $\geq 35$  IU l<sup>-1</sup>; adherence of PEG-IFN  $\geq 80$ %; adherence of RBV  $\geq 60$ %, and EVR. A multivariate analysis of SVR including the effect of initial treatment, duration of retreatment, Hb, PLT, AFP, LDL-Chol,  $\gamma$ -GTP, PEG-IFN adherence, RBV adherence, and EVR as variables showed that EVR and the period of retreatment were major factors contributing to SVR (table 2).



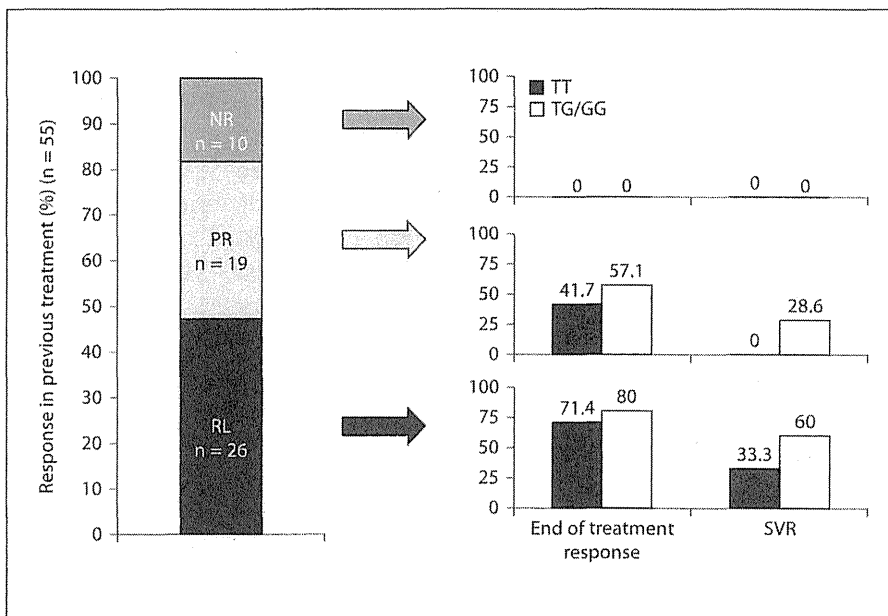
**Fig. 2.** Comparison of time to eradicate HCV RNA. Prior treatment: PEG-IFN $\alpha$ -2b + RBV combination treatment. Retreatment: PEG-IFN $\alpha$ -2a + RBV combination treatment.

#### IL28B SNP Genotype

55 patients agreed to undergo a test for IL28B SNP genotyping. Among them, 36, 18, and 1 had the TT, TG, and GG genotypes, respectively. The patients with TT had significantly lower  $\gamma$ -GTP and AFP levels; moreover, relapse patients were more frequently found to have TT. Among the patients with TT, 40% (14/35) showed an HCV RNA response at week 12, 70% (21/29) at week 24, and 56% (20/36) at the end of treatment. Among the non-TT patients, 16% (3/19) showed an HCV RNA response at week 12, 44% (6/16) at week 24, and 42% (7/19) at the end of treatment (fig. 3).



**Fig. 3.** Virological responses of PEG-IFN $\alpha$ -2a + RBV by IL28B genotype.



**Fig. 4.** Virological responses of PEG-IFN $\alpha$ -2a + RBV by IL28B genotype and response of prior treatment. NR = Null responders; PR = partial responders; RL = relapsers.

When we limited the analysis to relapsers, the EVR rates were 57% (12/21) and 40% (2/5) in the TT and non-TT patients, respectively. At week 24, the HCV RNA response rates were 90% (19/21) and 80% (4/5) in the TT and non-TT patients, respectively (fig. 3, 4).

#### Treatment Discontinuation

33 patients (31.1%) discontinued treatment during the study. 17 patients discontinued treatment due to lack of virological response, 5 due to personal reasons, 3 due to the incidence of hepatocellular carcinoma, and 8 (7.5%) due to adverse events. The reasons for the discontinuation of the 8 patients due to adverse events were anemia

(n = 2), fatigue (n = 2), depressed mental state (n = 1), unidentified fever (n = 1), heart failure (n = 1), and interstitial pneumonia (n = 1).

## Discussion

We retrospectively examined the therapeutic efficacy and effective predictors after retreatment with PEG-IFN $\alpha$ -2a + RBV combination therapy in 106 CHC patients. These patients showed non-response or relapse after previous PEG-IFN $\alpha$ -2b + RBV combination therapy. SVR was achieved in 53% of the relapsers and 18% of the partial responders. In the relapsers, SVR was more likely to be achieved when HCV RNA became negative within 12 weeks after the commencement of therapy and the therapeutic effect was enhanced with prolonged administration. Among the non-responders, SVR was not achieved in cases without a  $\geq 2$  log decrease in HCV RNA during the previous therapy or in cases with an HCV RNA response after  $\geq 25$  weeks during the retreatment period. Similar to that observed in the relapsers, SVR in the non-responders was achieved after prolonged retreatment in patients who showed an HCV RNA response within 12 weeks.

Patients with genotype 1 who receive PEG-IFN + RBV therapy for the first time undergo response-guided therapy (RGT) [16], in which the therapeutic period is determined according to the timing of the HCV RNA response after the commencement of therapy. In RGT, PEG-IFN + RBV therapy is recommended for 48 weeks for patients

who exhibit an HCV RNA response within 12 weeks after the commencement of therapy (i.e. complete early viral response, cEVR). However, the results of the present study suggest that prolonged administration is favorable for cEVR cases. Moreover, a different RGT is required in patients who receive retreatment and who have previously received PEG-IFN + RBV therapy.

We also examined the status of the IL28B genotype and found that the IL28B non-TT genotype is associated with factors that are unlikely to be related to the response including AFP levels,  $\gamma$ -GTP levels, and mutations in the core regions. However, when limiting the analysis to relapse patients, the rate of HCV RNA negativity did not differ among IL28B genotypes. This suggests that a prior response to PEG-IFN + RBV therapy is more important for predicting the response to retreatment than the IL28B genotype.

As stated earlier, some patients in Japan have not benefited from the launch of telaprevir, because CHC patients in Japan are older than those in Western countries [11, 12] and are often anemic. We believe that retreatment for elderly patients who cannot receive telaprevir-based therapy, especially relapsers and non-responders with a maximum virus decrease of  $\geq 2$  log during the previous therapy, should be considered after PEG-IFN + RBV therapy.

## Disclosure Statement

The authors have no conflicts of interest to disclose.

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# GENETIC POLYMORPHISM-DISEASE ASSOCIATION

## HLA-DP gene polymorphisms and hepatitis B infection in the Japanese population

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The mechanisms underlying the different outcomes of hepatitis B virus (HBV) infection are not fully understood.<sup>1</sup> Kamatani et al<sup>2</sup> identified an association of the single nucleotide polymorphisms (SNPs) human leukocyte antigen (*HLA*)-*DPA1* (rs3077) and *HLA-DPB1* (rs9277535) with chronic HBV infection in a genome-wide association study (GWAS). Additional studies confirmed that rs3077 and rs9277535 were associated with chronic HBV infection in the Han-Chinese population and strengthened the findings from previous GWAS.<sup>3-6</sup> Furthermore, Hu et al<sup>7</sup> reported that SNPs in *HLA-DP* (rs3077 and rs9277535) were associated with both HBV clearance and hepatocellular carcinoma (HCC) development. To investigate the association of these *HLA-DP* variants with the disease progression of HBV infection, we genotyped the 2 SNPs (rs3077 and rs9277535) in different clinical stages of liver disease in Japanese HBV carriers.

### CLINICAL SUMMARY

A total of 241 HBV carriers (positive for hepatitis B surface antigen) who visited the clinics for liver diseases at the Nagasaki University Hospital or Nagasaki Medical Center between 1999 and 2007 were enrolled. As controls, 143 healthy Japanese volunteers (56 men and 87 women aged 16–63 years, with a mean age of 31.3 ± 8.9 years) without any history of liver disease were enrolled. All patients did not have any other types of liver diseases, such as chronic hepatitis C, alcoholic liver disease, autoimmune liver disease, or metabolic liver disease. The study protocol was approved by the Ethics Committees of National Nagasaki Medical Center, and informed consent was obtained from each individual. Of the 241 HBV carriers, 69 were considered to be asymptomatic carriers on the basis of sustained normalization of the serum alanine aminotransferase (ALT) levels together with seropositivity for anti-hepatitis Be antigen throughout the study. On the other hand, 172 of the 241 HBV carriers were considered to have chronic liver disease, such as chronic hepatitis (57), cirrhosis (65), or HCC (50) manifested by elevated ALT levels and by clinical or histologic findings on examination of liver tissue during the follow-up period. Of the 50 patients with HCC, 6 (12%) were found to have chronic hepatitis and 44 (88%) had cirrhosis. All patients were regularly followed with measurements of serum ALT and HBV markers, such as hepatitis B surface antigen, hepatitis Be antigen, anti-hepatitis Be antibody, and HBV-DNA. A total of 79 patients had undergone liver biopsy during the study to assess the degree of liver fibrosis. However, liver biopsy was not performed in patients who had apparent biochemical, endoscopic, and ultrasound features of liver cancer. Tumor markers such as alpha-fetoprotein and des-γ-carboxy-prothrombin were measured with ultrasonography of the liver every 6 months to detect HCC in an early stage. The diagnosis of HCC was made by several imaging modalities in all patients and confirmed histologically by sonography-guided fine-needle tumor biopsy specimens. The genotype of rs3077 (*HLA-DPA1*) and rs9277535 (*HLA-*

*DPB1*) was determined by direct sequencing. The apolipoprotein B mRNA-editing enzyme catalytic peptide 3G (*APOBEC3G* H186R) genotyping was performed on the basis of the report by An et al.<sup>8</sup>

The frequencies of the 2 SNPs of *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) are listed in Table I. There was a significant difference in the frequencies between these 2 SNPs between Japanese HBV carriers and healthy subjects, as described previously.<sup>3</sup> We divided HBV carriers into 2 groups: a nonadvanced group (asymptomatic carriers or chronic hepatitis, n = 115) and an advanced group (liver cirrhosis or HCC, n = 126). The frequencies of CC (rs3077) or GG (rs9277535) genotypes were higher in the advanced group compared with those in the nonadvanced group; however, the difference was not significant (Table I). Next, we stratified the HBV carriers for the presence or absence of the *APOBEC3G* H186R variant and examined the effects of *HLA-DP* polymorphisms on the progression of HBV-related liver disease. Both C and G alleles of rs3077 and rs9277535 significantly increased the risk for advanced liver disease in HBV carriers lacking the H186R variant (Table II).

A 2-stage GWAS identified SNPs including rs3077 and rs9277535 located in *HLA-DPA1* and *HLA-DPB1*, which were associated with a susceptibility to chronic HBV infection.<sup>2</sup> After the first Japanese GWAS, 5 studies replicated the association of these 2 *HLA-DP* SNPs (rs3077 and rs9277535) and chronic HBV infection in the Han-Chinese population.<sup>3-7</sup> Among these studies, an association between HBV-related HCC and rs9277535 or rs3077 was demonstrated.<sup>7</sup> In this study, we examined whether these 2 SNPs (rs3077 and rs9277535) in *HLA-DP* genes were associated with the disease progression and susceptibility to HBV infection in a Japanese population. As demonstrated previously, we reconfirmed that rs3077 and rs9277535 in the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with HBV infection. Although some differences in the frequencies of rs3077 and rs9277535 genotypes between HBV carriers with advanced liver disease (liver cirrhosis and HCC) and those without advanced liver disease were observed, these differences were not statistically significant.

Recent evidence suggests that *APOBEC3G* inhibits HBV production by interfering with HBV replication through hypermutation of the majority of the HBV genome.<sup>8</sup> Because of the *APOBEC3G* gene's ability to regulate HBV replication, mutations of the gene may cause a deleterious variation that may affect the outcome of HBV infection. Among the SNPs identified in the *APOBEC3G* gene, H186R variant was strongly associated with a decline in CD4<sup>+</sup> T-cell numbers and accelerated progression to acquired immune deficiency syndrome—defining conditions in human immunodeficiency virus-infected individuals.<sup>9,10</sup> Viral disease outcome is influenced by host variability in immune response genes and genes that control viral replication or mutation rate.<sup>11</sup> *APOBEC3G* coding region variant might influence the progression of HBV infection by inducing the replication of HBV.<sup>12</sup> Therefore, genetic diversity of immune response genes, such as *HLA*, and genes that control viral replication, such as *APOBEC3G*, could contribute to the variability in outcome of HBV infection. To minimize the effects

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**Table I.** Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and HBV infection

SNP ID	HBV carrier	Healthy subjects	P value*	OR (95% CI)	Advanced HBV carrier	Nonadvanced HBV carrier	P value*	OR (95% CI)
	n = 241 (%)	n = 143 (%)			n = 115 (%)	n = 126 (%)		
rs3077								
C/C	148 (61.4)	47 (32.9)			77 (67.0)	71 (56.3)		
C/T	79 (32.8)	72 (50.3)			33 (28.7)	46 (36.5)		
T/T	14 (5.8)	24 (16.8)			5 (4.3)	9 (7.1)		
C allele (allele frequencies)	375 (77.8)	166 (58.0)	<0.0001	2.533 (1.843–3.483)	187 (81.3)	188 (74.6)	0.077	1.480 (0.957–2.290)
rs9277535								
G/G	143 (59.3)	45 (31.5)			73 (63.5)	70 (55.6)		
A/G	82 (34.0)	72 (50.3)			36 (31.3)	46 (36.5)		
A/A	16 (6.6)	26 (18.2)			6 (5.2)	10 (7.9)		
G allele (allele frequencies)	368 (76.3)	162 (56.6)	<0.0001	2.471 (1.804–3384)	182 (79.1)	186 (73.8)	0.170	1.345 (0.880–2.056)

Abbreviations: CI, confidence interval; HBV, hepatitis B virus; OR, odds ratio; SNP, single-nucleotide polymorphism.

\*P values were calculated using the chi-square test.

**Table II.** Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and the outcome of HBV infection in HBV carrier without H186R variant

SNP ID	Advanced HBV carrier n = 90 (%)	Nonadvanced HBV carrier n = 108 (%)	P value*	OR (95% CI)
rs3077				
C/C	64 (71.1)	60 (55.6)		
C/T	22 (24.4)	40 (37.0)		
T/T	4 (4.4)	8 (7.4)		
C allele (allele frequencies)	150 (83.3)	160 (74.1)	0.026	1.750 (1.065–2.874)
rs9277535				
G/G	5 (5.6)	10 (9.3)		
A/G	24 (26.7)	39 (36.1)		
A/A	61 (67.8)	59 (54.6)		
G allele (allele frequencies)	146 (81.1)	157 (72.7)	0.049	1.614 (1.000–2.604)

Abbreviations: CI, confidence interval; HBV, hepatitis B virus; OR, odds ratio; SNP, single-nucleotide polymorphism.

\*P values were calculated using the chi-square test.

of viral factors, such as APOBEC3G-mediated HBV editing, and evaluate the effect of *HLA-DP* more precisely, we focused on the subjects without the H186R variant. Because the *APOBEC3G* coding region variant might influence the progression of HBV infection,<sup>11</sup> we investigated the effect of *HLA-DP* polymorphisms on the outcome of HBV infection in HBV carriers lacking the H186R variant.

Our results showed that *HLA-DP* polymorphisms were associated with the progression of HBV infection and that this association was significant in Japanese HBV carriers lacking H186R variants. Our data demonstrated that *HLA-DP* polymorphisms are important in determining the susceptibility and the progression of HBV infection in the Japanese population.

One limitation of our study is the lack of information of HBV genotypes in the patients studied. Another limitation is that the number of HBV carriers (n = 241) is relatively small. Larger studies are needed to confirm the results of our study.

## CONCLUSIONS

We confirmed that rs3077 and rs9277535 SNPs in the *HLA-DP* locus are associated with the susceptibility and progression of HBV infection in the Japanese population. Further functional analyses are warranted to validate the biological plausibility of these SNPs in chronic HBV infection.

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ORIGINAL ARTICLE

## Development of new *IL28B* genotyping method using Invader Plus assay

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### ABSTRACT

*IL28B* polymorphism is associated with the response to pegylated interferon- $\alpha$  with ribavirin (PEG-IFN- $\alpha$ /RBV) treatment in chronic hepatitis C patients. As a genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) in clinical practice, the Invader Plus assay was developed. The accuracy, intra-assay, inter-assay precision, and the limit of detection of the Invader Plus assay were evaluated. Two SNPs (rs8099917 and rs12979860) associated with *IL28B* were genotyped by the Invader Plus and TaqMan assay in 512 Japanese patients. In comparison with direct sequencing, the Invader Plus assay showed 99% accuracy in rs8099917 and 100% accuracy in rs12979860. Intra-assay and inter-assay precision were sufficient to use in clinical practice and the detection limit was 1 ngDNA/assay. Genotyping by rs8099917 showed that 361 (71%), 144 (28%) and seven (1%) of the patients were major homozygous, heterozygous and minor homozygous types, respectively. Five of the 512 cases (1%) had haplotype differences, but none showed differences between the two genotyping methods. For patients with HCV genotype 1, the prevalence of responders in the major homozygous type was 83.3%, and that of non-responders in the minor heterozygous/homozygous type was 72.5%. A convenient *IL28B* genotyping method using the Invader Plus assay could be useful to predict the treatment outcome in clinical practice.

**Key words** interferon, *IL28B*, single nucleotide polymorphism genotyping, invader plus assay.

Hepatitis C virus (HCV) infection results in cirrhosis and hepatocellular carcinoma (HCC) worldwide (1). Pegylated interferon- $\alpha$  with ribavirin (PEG-IFN- $\alpha$ /RBV) is currently the most dominant therapy for chronic HCV infection, but roughly 50% of patients with genotype 1b, the most common in Japan, are not able to achieve a sustained virological response (SVR) determined by the serum HCV-RNA level 24 weeks after treatment (2, 3). In addition, this therapy often leads to side effects, such as flu-like symptoms, depression and anemia(4); therefore, it is valuable to predict the particular response before treat-

ment with PEG-IFN- $\alpha$ /RBV to avoid these side-effects and to avoid ineffective therapy.

Not only viral factors (genotype and viral mutation), but also host factors influence the therapeutic outcome. Age, sex, body mass index and histological grade are considered to determine the individual's treatment regimen and outcome (5, 6, 7). 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- $\alpha$ /RBV

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**List of Abbreviations:** GWAS, genome-wide association study; HCV, hepatitis C virus; NPV, negative predictive value; NVR, null virological response; PEG-IFN- $\alpha$ , pegylated interferon  $\alpha$ ; PPV, positive predictive value; RBV, ribavirin; SVR, sustained virological response; TVR, transient virological response.

**Table 1.** Primers and allele probes used for Invader Plus genotyping of *IL28B* single nucleotide polymorphisms (SNPs)

SNP		(5'-3')
rs8099917	Forward primer	TCA TCC CTC ATC CCA CTT CTG GAA CA
	Reverse primer	CGG GCC ATC TGT TTC CTG CTG
	Major allele probe	agg cca cgg acg AAT TGC TCA CAG AAA GGA A
	Minor allele probe	cgc gcc gag gCA TTG CTC ACA GAA AGG A
	Invader oligo	GCT ACC AAA CTG TAT ACA GCA TGG TTC CAA TTT GGG TGA t
rs12979860	Forward primer	GGA TGG GTA CTG GCA GCG C
	Reverse primer	AGG CGC CTC TCC TAT GTC AGC
	Major allele probe	cgc gcc gag gCG AAC CAG GGT TGA AT
	Minor allele probe	agg cca cgg acg TGA ACC AGG GTT GAA TT
	Invader oligo	CCA GGG AGC TCC CCG AAG GCG a

† Lowercase letters in each probe indicate 5'flap region.

therapy in Japanese (8), European (9), and a multi-ethnic population (10, 11). In particular, the two outstanding SNPs, rs12979860 and rs8099917 (located ~3 kb and 8 kb upstream of *IL28B*, respectively) have been found in strong association with the treatment response (8, 10). The minor allele frequency of rs8099917 was significantly higher in the null virological response (NVR) group compared with the virological response group. By taking advantage of *IL28B* typing, it may be possible to predict a NVR as well as a SVR in order to tailor the most suitable treatment regimens.

In this study, the "Invader Plus genotyping assay *IL28B* SNP" test kit including primers and probe setting was developed and compared with the usual TaqMan probe assay for genotyping *IL28B* SNPs and the pre-treatment prediction of the response to PEG-IFN- $\alpha$ /RBV therapy in HCV infection.

## MATERIALS AND METHODS

### Patients

DNA samples were obtained from 512 Japanese chronic hepatitis C patients, after informed consent, recruited from NHO Nagasaki Medical Center, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, and Kawasaki Medical University Hospital in Japan. Of them, the data of 90 patients were also used in a previous paper (15). All of the subjects had undergone a standard course of PEG-IFN- $\alpha$ /RBV therapy and 316 patients had their virological response status established before this study, of which 272 patients had HCV genotype 1. The NVR, transient virological response (TVR) and SVR were defined 24 weeks after PEG-IFN- $\alpha$ /RBV therapy, as previously described (8). This study classified the response outcome in two categories; responders (including those with SVR

and TVR) and non-responders (including patients with NVR).

Informed consent was obtained from each patient and the study protocol 22 conformed to the ethics guidelines of the Declaration of Helsinki and was approved by 23 the institutional ethics review committee.

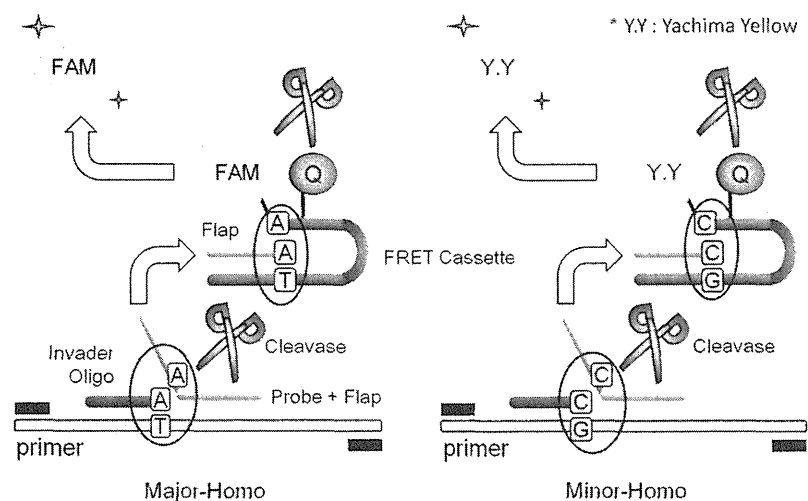
### Samples for assay validation

To assess intra-assay and inter-assay precision and both coefficients of variation (CV%), three different genotype samples (major, minor, and hetero), two positive (major and minor) and one negative control were run in triplicate during three runs (three different days). The limit of detection was evaluated by analyzing three different genotype samples using two different DNA concentrations (1 ng, and 0.3 ng per assay) with the same three controls.

### Invader Plus assay

The Invader Plus assay, which combines polymerase chain reaction (PCR) and the Invader reaction, was performed using the LC480II (Roche Diagnostics, Basel, Switzerland). The enzymes used in Invader Plus are native Taq polymerase (Promega, Fitchburg, WI, USA) and cleavase enzyme (Third Wave Technologies, Madison, WI, USA). Primers and allele probes newly designed for Invader Plus genotyping of *IL28B* SNPs are shown in Table 1 (Third Wave Japan, Tokyo, Japan). The reaction is configured to use PCR primers with a melting temperature ( $T_m$ ) of 72°C and an Invader detection probe with a target-specific  $T_m$  of 63°C. The invader oligonucleotide overlaps the probe by one nucleotide, forming at 63°C an overlap flap substrate for the cleavase enzyme. The first step in Invader Plus is PCR target amplification, in which the reaction is subjected to 18 cycles of a denaturation step (95°C for 15 s) and hybridization and extension steps (70°C for 1 min). At the end of PCR cycling, the reaction mixture is





**Fig. 1. Principle of the Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) (for rs8099917).** First, an Invader Oligo and a probe are annealed to amplified target DNA, overlapping at the SNP position, which clipped out the 5' flap fragment by the cleavase enzyme. Second, the released 5' flap anneals to the fluorescence resonance energy transfer (FRET) cassette and initiates a secondary cleavage reaction that releases the fluorescent dye. The signal is only released when the invasive structure is formed on the target DNA. 'Major allele' is left FAM signal, 'Minor allele' is right Yachima-Yellow (Y.Y) signal, and 'Hetero-type' observed both merged signals, respectively. The fluorescent dye is between major and minor alleles at rs12979860.

incubated at 99°C for 10 min to inactivate the Taq polymerase. Next, the reaction temperature is lowered to 63°C for 15 min to permit hybridization of the probe oligonucleotide and formation of the overlap flap structure (Fig. 1). Data were analyzed by endpoint genotyping software (Roche Diagnostics).

### TaqMan PCR assay

The rs8099917 polymorphism was determined using TaqMan Pre-Designed SNP Genotyping Assays, as recommended by the manufacturer (Life Technologies, Carlsbad, CA, USA). The Custom TaqMan SNP Genotyping Assay Service MGB probe was used to determine the genotype of rs12979860 (Life Technologies). Each genome DNA sample (10 ng) was amplified using the master mix reagent of LightCycler480 Probe Master (Roche Diagnostics). The assays were carried out using the LC480II under the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles: 15 s at 95°C, and 1 min at 60°C. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

### Direct sequencing assay

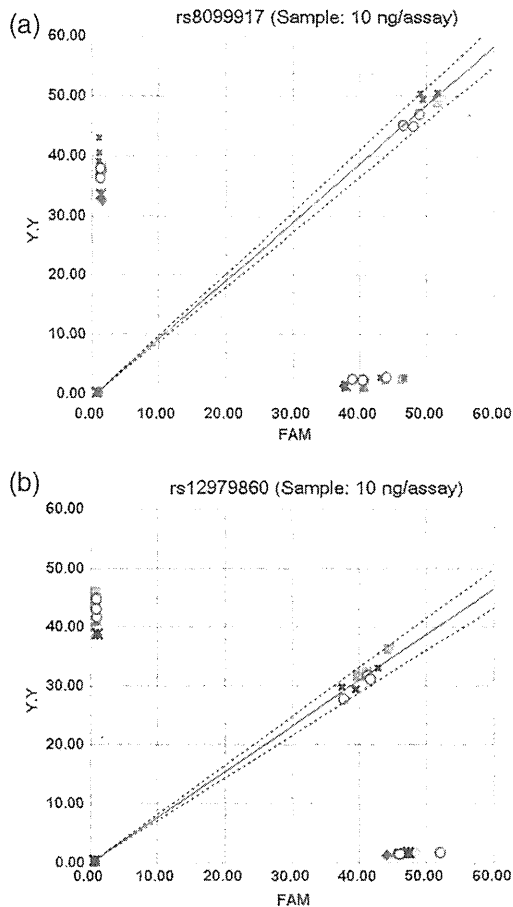
Before proceeding to *IL28B* SNP genotyping by the two methods, 105 DNA samples were genotyped using direct sequencing. To determine the SNP genotype of rs8099917 and rs12979860, the specific primer sets, rs8099917F (5'-AAGTAACACTTGTTTCCTTRTAAAAGATTC-3') and rs8099917R (5'-CGCTATAATTAAGATGTGGGAGAA-TGCAA-3'), rs12979860F (5'-CACGGTCGTGCCTGTC-GTGT-3') and rs12979860R (5'-TGTGCTGTGCCTTCA-CGCTCCGAGCA-3') were used, respectively (Life Technologies). The amplification products were sequenced directly in both forward and reverse directions with Prism

Big Dye (Life Technologies) on an ABI3100 DNA automated sequencer (Life Technologies).

## RESULTS

### Assay validation

In this study, the performance characteristics of the assay were determined before experimental testing. To assess the accuracy of *IL28B* SNP genotyping based on both rs8099917 and rs12979860, the results from the Invader Plus genotyping assay were compared with the results from direct sequencing. Of the 105 DNA samples, 70 samples had the major homozygous allele, 30 samples the minor heterozygous and five samples the minor homozygous allele for both SNPs by direct sequencing. This gave 100% concordant results for rs12979860 between the two assays, although one sample showed different results for rs8099917 with direct sequencing. As a result of direct sequencing, this sample had the major homozygous allele, but the results of the Invader assay and TaqMan assay both showed the minor heterozygous allele. Precision was determined by analyzing six samples (including positive and negative controls) in triplicate during three runs (three different days) with 100% concordant results. Intra-assay coefficient of variation (CV%) for rs8099917 was 0.1–1.6 (%), and for rs12979860 was 0.5–2.5 (%). Then, inter-assay CV% calculated from the angle of the heterozygous allele marker was 1.9 and 2.0 (%), respectively (Fig. 2 and Table 2). To evaluate the limit of detection, 10 samples, including three genotypes, were run in triplicate using three different DNA concentrations with no visible confusion in *IL28B* SNP genotyping or signal intensity, except in the 0.3 ng/assay (Fig. 3).



**Fig. 2. Simultaneous and daily repeatability of Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) (three times per day and 3 days of measurement).** Scatter plots of fluorescence data from intra-assay for rs8099917 (a) and for rs12979860 (b). Raw fluorescence data are plotted for each sample and control. The x-axis of rs8099917 is the FAM, corresponding to the major allele, while y-axis is Yachima-Yellow (Y.Y), corresponding to the minor allele. In contrast, the x-axis FAM and y-axis Y.Y of rs12979860 signify minor and major alleles, respectively. The line designates the mean of the angle at the heterozygous allele marker, and the broken line indicates standard deviation +2SD and -2SD. Day 1, control (+); day 1, sample (•); day 2, control (▲); day 2, sample (◆); day 3, control (×); day 3, sample (◊); mean (—); +2SD (---); -2SD (....).

### ***IL28B* typing in PEG-IFN- $\alpha$ /RBV-treated patients**

Of the 512 cases that were analyzed for the rs8099917 genotype, 361 (71%) were shown to have a major homozygous allele, 144 (28%) had a minor heterozygous, and seven (1%) had a minor homozygous allele, which gave concordant results with the TaqMan assay (Table 3). In addition, of the 512 cases that were analyzed for the rs12979860 genotype, 356 (70%) were shown to have a major homozygous allele, 149 (29%) had a minor heterozygous, and seven (1%) had a minor homozygous al-

**Table 2.** Simultaneous and daily repeatability of Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs) (three times per day and 3 days of measurement)

		Coefficient of variation (%)	
		rs8099917	rs12979860
Hetero (10 ng/assay)	Day1	0.1	1.4
	Day2	1.6	2.5
	Day3	1.3	0.5
	Total	1.9	2.0

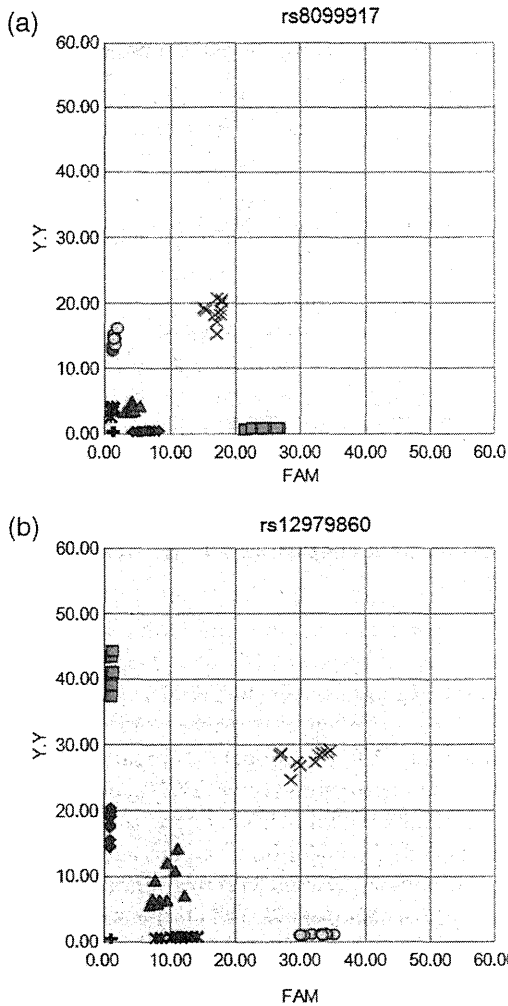
lele, with 100% concordant results with the TaqMan assay (Table 3). Five of the 512 cases (1%) had haplotype differences, but none of the 512 cases showed differences between the two genotyping methods.

### **Prediction of a response to PEG-IFN- $\alpha$ /RBV therapy**

Of the 272 patients with genotype 1 who were evaluated for the treatment response, 169 of 203 patients with the major homozygous allele in rs8099917 exhibited a higher prevalence of a virological response (83.3%), consisting of a SVR and TVR (responder), compared with those with a minor heterozygous (18/66, 27.3%) or homozygous allele (1/3, 33.3%) (Table 4). Therefore, the prevalence of responders with the major homozygous allele was 83.3%, and that of non-responders with the minor heterozygous or homozygous allele was 72.5%. Of the 36 patients with genotype 2a or 2b, patients with a major homozygous allele had 96.7% incidence of virological response and those with a heterozygous allele had 100%. For all cases, 85.2% of the patients with a major homozygous allele were responders to PEG-IFN- $\alpha$ /RBV therapy, and 66.7% of the patients with a minor heterozygous or homozygous allele were non-responders to that therapy.

## **DISCUSSION**

The rate of a NVR after 48 weeks of PEG-IFN- $\alpha$ /RBV therapy among patients infected with HCV genotype 1 is around 20–30%. Previously, there have been no reliable predictors of a NVR or SVR. Some recent studies have focused on the *IL28B* polymorphism as one of the most critical factors with a bearing on the prediction of the treatment response (8–10). In particular, rs8099917 and rs12979860 were significantly associated with the treatment regimen; therefore, it would be useful to examine such genetic markers before IFN-based treatments in clinical practice because it would avoid the unpleasant side effects that commonly accompany the treatment. In addition, such an advanced diagnosis would be economically beneficial, as treatment costs would be reduced.



**Fig. 3. Sensitivity of Invader Plus genotyping assay for *IL28B* single nucleotide polymorphisms (SNPs).** Scatter plots of fluorescence data from intra-assay for rs8099917 (a) and for rs12979860 (b). It was defined that the major allele is "Major", hetero type is "Hetero", the minor allele is "Minor", and Negative control is "Neg", respectively. Major (0.3 ng) (◆); major (1.0 ng) (■); hetero (0.3 ng) (▲); hetero (1.0 ng) (×); minor (0.3 ng) (✱); minor (1.0 ng) (○); + Neg (+).

Some SNPs, such as UGT1A1 polymorphism associated with irinotecan therapy, have already been exploited in clinical practice to avoid severe adverse effects (12, 13). These tailor-made therapies are expected to become more common in clinical practice (14). Similarly, *IL28B* polymorphism detection can be used in tailor-made therapies; thus, it is important to develop a genotyping assay that is convenient, swift, accurate, and inexpensive.

The Invader Plus genotyping assay *IL28B* SNP test kit to genotype *IL28B* SNPs (rs8099917 and rs12979860) from DNA samples was developed and evaluated in this study. This assay contains reagents for DNA amplification, including each SNP. Accuracy was determined by compar-

**Table 3.** Comparisons of the Invader Plus genotyping assay with TaqMan assay for rs8099917 and rs12979860

		Invader Plus			
		Major	Hetero	Minor	total
rs8099917					
TaqMan probe	Major	361	0	0	361
	Hetero	0	144	0	144
	Minor	0	0	7	7
	total	361	144	7	512
rs12979860					
TaqMan probe	Major	356	0	0	356
	Hetero	0	149	0	149
	Minor	0	0	7	7
	total	356	149	7	512

**Table 4.** Effect of the rs8099917 single nucleotide polymorphisms (SNPs) on response to pegylated interferon/ ribavirin (PEG-IFN/RBV) therapy in Japanese patients with hepatitis C virus (HCV) genotype 1

	Major (n = 203) 74.6%	Hetero (n = 66) 24.3%	Minor (n = 3) 1.1%	Total (n = 272)
Mean age (SD)	57.2 (10.1)	54.9 (10.8)	65.7 (6.5)	56.7 (10.3)
Gender (%)				
Females	102 (50.2%)	35 (53.0%)	2 (66.7%)	139 (51.1%)
Males	101 (49.8%)	31 (47.0%)	1 (33.3%)	133 (48.9%)
Responder	169 (83.3%)	18 (27.3%)	1 (33.3%)	188 (69.1%)
Non-responder	34 (16.7%)	48 (72.7%)	2 (66.7%)	84 (30.9%)

ing the Invader assay results with the direct sequencing results. Only one sample (0.95%) showed a discrepant result for rs8099917 by comparing the Invader Plus genotyping assay and direct sequencing, because another rare SNPs existed in the forward primer binding region used for amplification and direct sequencing (15). Intra-assay (0.1–1.6% for rs8099917 and 0.5–2.5% for rs12979860) and inter-assay (1.9 and 2.0% respectively) precision were sufficient for clinical practice and tailor-made therapies. Interestingly, Invader Plus assay is more convenient, swift and inexpensive than direct sequencing. Invader Plus assay is better than TaqMan assay at specificity because no results come up without tagging both probe and Invader oligonucleotide on SNP directly.

For each or both genotypes, influences on pre-treatment prediction with the *IL28B* SNP genotype were evaluated. As a result, especially for patients with genotype 1b, the positive predictive value (PPV) for a NVR was 72.5% and the negative predictive value (NPV) was 83.3%, suggesting that a convenient method using the Invader Plus assay could be useful to predict the treatment outcome in clinical practice.

In conclusion, using the Invader Plus *IL28B* SNP genotyping assay, swift and accurate selection of the optimum treatment strategy for individual patients can be improved by combining with other factors.

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## DISCLOSURE

The authors have no conflict of interest.

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