INTRODUCTION

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

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

RESULTS

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

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

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

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

Resequencing and fine-mapping

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

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

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

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

*ORs and 95 % CI are calculated using logistic regression based on the co-dominant model.
†OR of minor allele from 2 × 2 allele frequency table.
‡*P*-values of Pearson's χ^2-test for the allele model.
§Results of Breslow–Day test.
‖Calculated by the Mantel–Haenszel method of combining allele-frequency counts.

Table 2. Genotype distributions of the IL-28B SNPs rs8099917 and rs12979860 in HCV genotype 1b- and 2a-infected patients compared with healthy controls

	rs8099917							rs12979860						
	TT	TG	GG	T (%)	G (%)	*P**	OR† (95 %CI)	CC	CT	TT	C (%)	T (%)	*P**	OR† (95 %CI)
HCV 1b (Set-1, -2, -3)	1180 (75.5)	352 (22.5)	30 (1.9)	86.8	13.2	2.9×10^{-3}	0.60 (0.43–0.84)	1198 (75.1)	367 (23.0)	31 (1.9)	86.6	13.4	1.7×10^{-3}	0.59 (0.42–0.82)
HCV 2a (Set-4)	441 (81.4)	93 (17.2)	8 (1.5)	89.9	10.1	0.41	0.85 (0.58–1.25)	452 (81.6)	92 (16.6)	10 (1.8)	89.9	10.1	0.45	0.86 (0.59–1.23)
Healthy control	236 (83.7)	45 (16.0)	1 (0.4)	91.7	8.3			236 (83.7)	44 (15.6)	2 (0.7)	91.5	8.5		

**P*-value compared with healthy controls under a dominant model for the minor allele.
†OR of subjects infected with HCV having a favourable genotype relative to healthy control subjects.

the LD block defined by the criteria of Gabriel *et al.* (2002) spans about 9 kb (Fig. 1), we carefully resequenced the 42 kb genomic region encompassing IL-28B and IL-28A loci using specific PCR primer sets and identified 71 common SNPs, including 22 novel SNPs with a minor allele frequency greater than 0.05.

Among the 71 common SNPs identified by resequencing, 34 that showed strong linkage disequilibrium (r^2 >0.64) with the landmark SNP (rs8099917) were further genotyped. rs8099917 was found to have the strongest association with treatment outcome, and 14 SNPs exhibited comparable associations, with *P*-values varying by less than one order of magnitude (Table 3 and Fig. 2). Haplotype analysis provided little additional information compared with the single marker analysis (data not shown). These results suggest that the association signal was driven by one of the identified SNPs.

Initial response for SNP rs8099917 in the IL-28B locus

Out of the patients infected with HCV genotype 1b who were treated with PEG-IFN and ribavirin combined therapy (Set-1 and Set-2), 443 patients with sufficient viral load data were analysed for viral kinetics. As shown in Fig. 2(a, b), viral reduction in patients with rs8099917 TT is significantly greater than in those with rs8099917 TG or GG in both weeks 2 and 4. Baseline viral load in patients with rs8099917 TT were slightly but significantly higher than in those with rs8099917 TG or GG (Fig. 2c).

Multivariate logistic analysis

Using the patients infected with HCV genotype 1b who were treated with PEG-IFN, a stepwise forward logistic regression analysis was performed to assess the impact of the IL-28B polymorphism. There were 217 patients with sufficient data for the analysis. We evaluated the following seven factors: age, sex, SNP rs8099917, fibrosis and baseline HCV-RNA level. We found that SNP rs8099917 is an independent factor associated with treatment outcome [OR, 0.27; 95 % confidence interval (CI), 0.12–0.87]. Other independent factors meeting the criteria for inclusion in the model are shown in Table 4.

DISCUSSION

Various viral and host factors determine the outcome of IFN (+ribavirin) therapy (Akuta *et al.*, 2007; Bressler *et al.*, 2003; Casiraghi *et al.*, 2004; Enomoto *et al.*, 1996; Kenny-Walsh & Irish Hepatology Research Group, 1999; Manns *et al.*, 2001; Romero-Gómez *et al.*, 2005). Ethnic differences in the response to IFN therapy and in the rate of spontaneous clearance in chronic hepatitis C suggest the influence of genetic factors (Conjeevaram *et al.*, 2006; Welzel *et al.*, 2009). In the current study, we found that the influence of SNPs in the IL-28 locus was quite high.

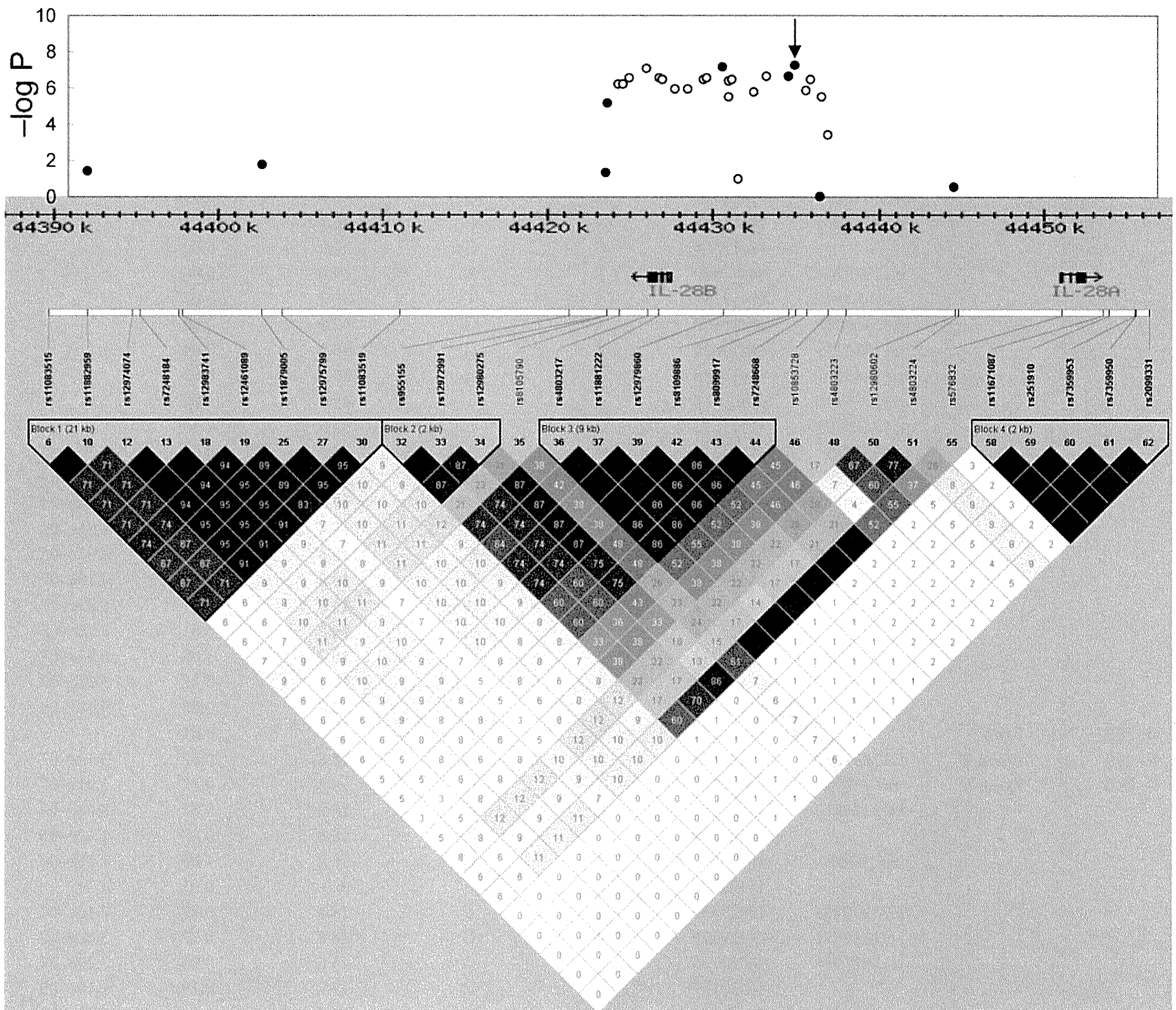

Fig. 1. LD mapping and probabilities for SNPs around the IL-28B locus. The lower panel depicts the haplotype structure around the IL-28B locus. Phase II HapMap JPT genotype data and the HAPLOVIEW program were used to define the LD block. The solid arrow represents the landmark SNP (rs8099917). The block structure is based on the criteria established by Gabriel *et al.* (2002) using pairwise estimates of the disequilibrium coefficient (r^2). Dark grey, regions with high r^2 values; light grey, regions with low r^2 values. The upper panel shows *P*-value plots of case–control association results. *P*-values by the Cochran–Armitage trend test are plotted on a $\log_{10}$ scale. Closed and open dots represent SNP *P*-value in the genome-wide association study (GWAS) and Set-1 fine-mapping stages, respectively.

Despite the fact that the total dose of PEG-IFN or IFN that was administered to each patient varied according to HCV genotype and viral titre based on the current standard regimen, the effect of the therapy in all patient cohorts showed significant association with the SNP genotypes. For replication analyses, we also found significant associations in the case of IFN monotherapy (Sets 3 and 4 in Table 1). Although combination therapy with PEG-IFN and ribavirin is currently the recommended treatment for chronic HCV infection, such analysis is still important because patients cannot always receive PEG-IFN and/or ribavirin due to severe side effects or other reasons. Consistent with earlier findings, the heterozygous genotypes show an intermediate effect level of IFN resistance.

Recent studies have demonstrated that genotype frequency of IL-28B polymorphism varied considerably by ethnicity, which may explain much of the difference in response between different population groups and support the prediction that favourable alleles would be very much more

Table 3. Results of resequencing and fine-mapping of the IL-28B locus

Gene	Location	SNP Id	Position	Alleles		Minor allele frequency (%)	r^{2*}	P† (GWAS set)
			Chr 19	Major	Minor			
		rs11083519	44411103	T	A	46.9	0.04	
		rs60635720	44411511	A	G	47.9	0.05	
		rs57593994	44411591	G	C	46.9	0.04	
			44412803	C	G	6.3	0.02	
		rs12976234	44412840	T	A	49.0	0.06	
		rs8107090	44413755	A	T	49.0	0.07	
		rs35408086	44418650	A	G	47.9	0.04	
		rs11883239	44419320	A	G	46.9	0.04	
		rs11883201	44419330	G	A	47.9	0.02	
		rs12979140	44420380	C	T	49.0	0.00	
		rs12460005	44421017	G	C	33.3	0.10	
		rs955155	44421319	G	A	13.5	0.64	4.77e–02
		rs958039	44422141	T	A	17.7	0.66	4.79e–05
		rs35790907	44422595	A	T	17.7	0.66	1.24e–04
		rs12972991	44423587	A	C	17.4	0.69	4.53e–02
		rs12980275	44423623	A	G	18.8	0.73	6.97e–06
			44424552	A	G	18.8	0.87	6.15e–07
			44424592	G	A	19.8	0.81	6.95e–07
			44423744	T	C	19.8	0.81	8.78e–05
		rs8105790	44424341	T	C	16.7	1.00	6.33e–07
			44424963	C	T	19.1	0.87	3.21e–07
		rs4803217	44426060	C	A	18.8	0.87	9.18e–08
IL-28B	Exon 5		44426165	G	A	5.2	0.01	
IL-28B	Intron 2	rs11881222	44426763	A	G	18.8	0.87	3.12e–07
IL-28B	Exon 2	rs8103142	44426946	T	C	18.8	0.87	3.91e–07
		rs28416813	44427484	C	G	18.8	0.87	8.08e–07
			44427759	C	T	18.8	0.87	1.17e–06
		rs8107030	44428559	A	G	18.8	1.00	1.15e–06
			44428927	A	C	6.3	0.01	
		rs73930703	44429353	C	T	18.8	0.87	7.16e–07
		rs11882871	44429450	A	G	18.8	0.87	3.46e–07
		rs12971396	44429706	C	G	15.6	0.93	3.20e–07
			44430157	A	C	17.7	0.79	1.19e–05
		rs12979860	44430627	C	T	17.7	0.94	7.32e–08
		rs4803221	44430969	C	G	15.6	0.93	4.67e–07
			44430995	TT	G	18.8	0.87	3.12e–06
		rs4803222	44431193	G	C	18.8	0.87	4.03e–07
		rs1549928	44431549	T	C	6.3	0.01	1.20e–01
			44432515	C	A	14.1	0.74	1.68e–06
		rs12983038	44432964	G	A	12.8	0.41	
			44433305	C	T	13.8	0.64	2.33e–07
		rs8109886	44434602	C	A	19.8	0.81	2.41e–07
		rs8109889	44434610	C	T	16.7	1.00	9.67e–07
		rs8113007	44434943	A	T	18.8	0.87	8.20e–07
		rs8099917	44435005	T	G	16.7	–	6.62e–08
		rs7248668	44435661	G	A	16.0	1.00	1.61e–06
			44435942	–	GA	16.7	0.81	4.02e–07
		rs10612351	44436647	AC	–	19.8	0.74	3.32e–06
			44436767	C	T	5.2	0.01	
			44436898	C	T	14.6	0.71	3.73e–03
		rs10853728	44436986	C	G	29.2	0.39	4.38e–04
		rs57401101	44437536	–	C	37.5	0.25	
		rs61665163	44438053	–	AAT	13.5	0.00	
		rs4803223	44438059	A	G	8.3	0.19	1.23e–01

Table 3. cont.

Gene	Location	SNP Id	Position	Alleles		Minor allele frequency (%)	r^{2*}	P† (GWAS set)
			Chr 19	Major	Minor			
			44438119	A	G	12.5	0.02	
		rs35811883	44438306	T	C	18.8	0.02	
		rs56116812	44438930	G	A	12.5	0.00	
		rs8101517	44439581	A	C	21.9	0.07	
			44440345	C	T	6.4	0.01	
			44441174	G	A	6.3	0.00	
		rs10424607	44441762	A	C	20.8	0.09	
		rs10407161	44443231	A	C	44.0	0.13	
		rs6508852	44444102	A	G	27.1	0.07	
			44445071	A	C	11.5	0.03	
		rs6508853	44445966	T	C	6.3	0.01	
			44448557	A	G	5.2	0.01	
		rs2596807	44449766	T	C	5.2	0.01	
			44450018	A	G	20.8	0.10	
IL-28A	Intron 3	rs62120533	44451758	T	G	6.3	0.00	
IL-28A	Intron 3		44451814	T	C	6.3	0.00	
IL-28A	Exon 5	rs59746524	44452275	T	C	12.5	0.32	

*Correlation coefficient (r^2) with the landmark SNP (rs8099917).
†Statistical comparisons were performed using the Cochran–Armitage trend test.

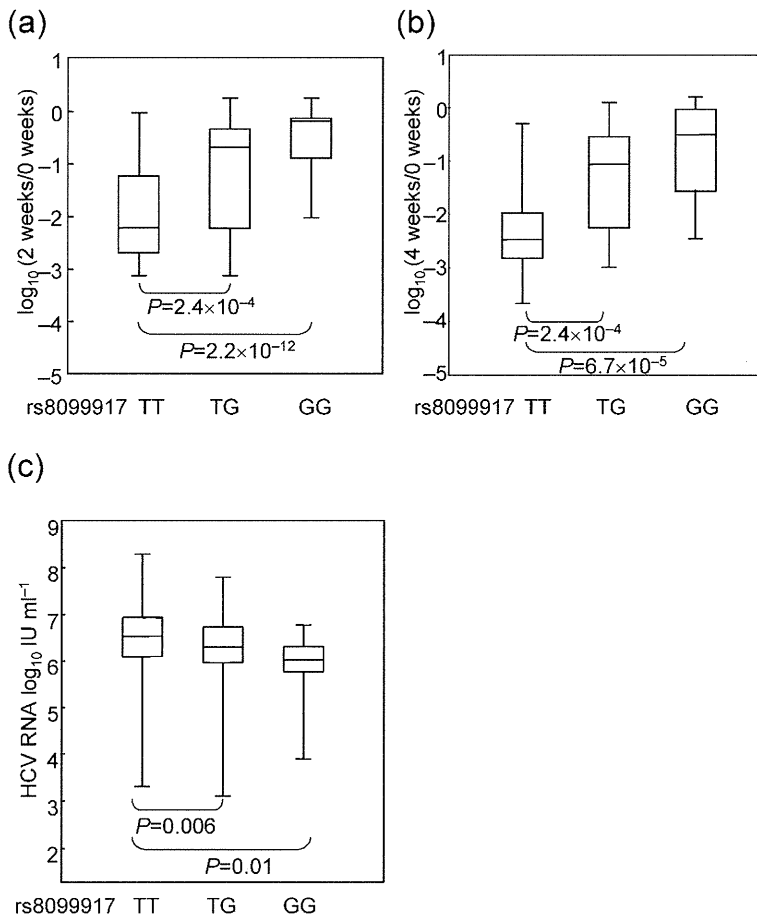

Fig. 2. Association between early response to combination therapy and the top SNP rs8099917 in the IL-28B locus. Viral reduction and basal viral load are shown according to rs8099917 genotype. (a) Viral response at 2 weeks; (b) viral response at 4 weeks; (c) baseline value of viral load. The box indicates the inter-quartile range (25 and 75 %) and the line within the box represents the median. The whiskers represent the range.

Table 4. Predictive factors for treatment outcome in patients with HCV genotype 1b determined by multivariate logistic regression analysis

Variable	*P*	OR	95 %CI
rs8099917	0.0022	0.24*	0.094–0.60
log viral load	0.0100	0.33†	0.140–0.77
Age	0.0180	0.67‡	0.480–0.93
Fibrosis	0.0200	0.40§	0.190–0.86

*Dominant model (GG + GT vs TT).
†Per 1 log increase.
‡Per 10 years increase.
§Severe versus mild.

common in Asian populations than in other ethnic groups (Ge *et al.*, 2009; Thomas *et al.*, 2009). We genotyped rs8099917 and rs12979860 in 282 healthy Japanese volunteers, and the allele frequencies of these SNPs were found to be comparatively high (91 % for both), consistent with the relatively high SVR rates of East Asian populations. Sarrazin *et al.* (2010) reported that genotype 1-infected patients had a lower frequency of the favourable genotype of rs12979860 compared with healthy controls and that genotypes 2/3 patients had an intermediate frequency. This finding was confirmed in this study (Table 2). On the other hand, they reported that the rs12979860 CC genotype, but not the rs8099917 and rs12980275 genotypes was significantly associated with SVR to (PEG-) IFN-α/ribavirin combination therapy in genotypes 2/3-infected patients. In this study, however, rs12979860 and rs8099917 were both strongly associated with SVR. This discrepancy may be due to differences among populations in the degree of linkage disequilibrium between these SNPs, and, in fact, these SNPs were in strong linkage disequilibrium in our healthy control subjects as well.

Of note in our study was that there was a significant difference in the initial viral reduction based on SNP genotype in patients treated with the PEG-IFN and ribavirin combination therapy. This finding holds important implications for the strategy of IFN-based anti-HCV therapy.

The mechanism by which polymorphisms within this locus influence the outcome of therapy has not been established. One of the polymorphisms in strong linkage disequilibrium with the two SNPs is a missense substitution within the IL-28B coding region. Recently, Urban *et al.* (2010) reported that there was no difference in antiviral potency between wild-type IL-28B and amino-acid substituted variant *in vitro* using an HCV replicon system. On the other hand, it has been reported that genetic variation in the IL-28B locus is associated with expression levels of IL-28B (plus IL-28A) IFN in peripheral blood mononuclear cells (Suppiah *et al.*, 2009; Tanaka *et al.*, 2009), although findings by Ge *et al.* (2009) are contradictory. Using a specific assay, Honda *et al.* (2010) and Urban *et al.* (2010) reported that hepatic IL-28B expression was not associated with IL-28B genotype in HCV-infected patients. Post-transcriptional regulation might be affected by the IL-28B variant, which could not be evaluated by the RT-PCR method, although further functional studies are needed to address this issue. IL-28A, IL-28B and IL-29 (IFN-λ II, III and I, respectively) are recently identified IFNs (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003) similar to type I IFNs in terms of biological activity and mechanism of action, but differ structurally and genetically (Maher *et al.*, 2008). The antiviral effects of IFN-λ against hepatitis B virus and HCV have already been reported (Robek *et al.*, 2005). Furthermore, IFNs α and λ act synergistically against HCV (Marcello *et al.*, 2006; Pagliaccetti *et al.*, 2008; Zhu *et al.*, 2005). Whether such synergy actually modulates the effect of IFN therapy against HCV in the liver requires further study. In this regard, recent studies have identified higher pre-treatment hepatic levels of IFN-stimulated genes in NRs than in responders (Chen *et al.*, 2005; Feld *et al.*, 2007; Sarasin-Filipowicz *et al.*, 2008). In addition, association between intrahepatic levels of ISG expression and IL-28B genotype have also been reported (Honda *et al.*, 2010; Urban *et al.*, 2010). On the other hand, the IL-28B variant has recently been reported to be associated with treatment response following liver transplantation in patients infected with HCV (Charlton *et al.*, 2011; Fukuhara *et al.*, 2010). These findings suggest that the IL-28B polymorphism may be associated with innate as well as adaptive immunity.

The OR for the effect of the PEG-IFN and ribavirin combination therapy associated with IL-28B polymorphisms in this study is much lower than the previous report that analysed a similar effect in Japanese patients (Tanaka *et al.*, 2009) and is closer to those of Caucasian, Hispanic and African patients (Ge *et al.*, 2009; Suppiah *et al.*, 2009). Tanaka *et al.*'s non-viral responder (NVR) criteria seem to be very constrained compared with those of other groups including ours. To evaluate possible effects of such limited criteria on ORs for treatment outcome, we divided our NRs into NVRs and transient viral responders (TVRs) by the use of Tanaka and colleagues criteria based on 2 log viral decline at 12 weeks. Among the Set-1 and Set-2 samples, 344 samples were available for this analysis (NVR, 58; TVR, 122; SVR, 164). The ORs in the analyses for week 12 NVR versus SVR and for NVR versus VR (TVR plus SVR) are much greater than those based on our criteria and nearly comparable to those of Tanaka *et al.* (2009) (OR: 20.2, 17.2, respectively). Accordingly, the extreme clinical phenotype of NRs, strongly dictated by early viral response, might account for the strongly associated OR values. Our viral kinetics study also supports this interpretation.

In conclusion, we have shown that polymorphisms located near the IL-28B locus (represented by rs8099917), reported to be associated with the outcome of PEG-IFN and

ribavirin combined therapy, are also associated with that of IFN monotherapy and significantly affect early viral decline. Our resequencing and fine-mapping study identified 15 common genetic variants on the IL-28B locus that are associated with the outcome of IFN therapy for HCV infection. These findings would contribute to better targeting of PEG-IFN plus ribavirin therapy and increase overall treatment efficacy. Genotyping of IL-28B polymorphisms may be useful for predicting treatment outcome as well as estimating the optimal duration of PEG-IFN plus ribavirin combination therapy for viral eradication in HCV patients. Further study is needed to clarify the mechanism and molecular function of the IL-28B polymorphism to establish a more optimal, tailor-made treatment regimen for each patient.

METHODS

Study populations. A total of 2112 patients with chronic HCV infection were included in the study who were treated either with PEG-IFN and ribavirin therapy or with IFN monotherapy at Toranomon Hospital Department of Hepatology, Hiroshima University Hospital, Hiroshima University-Affiliated Hospitals, and the National Taiwan University Hospital. All patients had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, and had not received immunosuppressive therapy before enrollment in the study. Table 5 lists the demographic features of the subjects. Patients were classified into the following two groups based on treatment outcome: SRs and NRs. SRs had no evidence of viraemia at 24 weeks after completion of IFN therapy, whereas NRs were still viraemic at this stage. For IFN monotherapy, patients were treated with 6 million units of IFN intramuscularly every day for 8 weeks, followed by the same dose twice a week for 16 weeks, with a total dose of 528 million units. In the PEG-IFN plus ribavirin combination therapy, subjects received weekly injections of PEG-IFN at 1.5 $\mu g\ kg^{-1}$ body weight and oral administration of ribavirin for 48 weeks (for 24 weeks for Taiwanese patients). The amount of ribavirin was adjusted based on the subject's body weight (600 mg for <60 kg, 800 mg for 60–80 kg and 1000 mg for >80 kg). Only patients with greater than 75 % compliance with prescribed doses of PEG-IFN and ribavirin were included in this study. For patients who underwent IFN-based therapies more than once, only parameters related to the most recent therapy were analysed. In addition, 282 healthy Japanese volunteers were genotyped for rs8099917 and rs12979860. All subjects in the present study received a detailed explanation, and all signed a written informed consent. This study was approved by the Ethical Committee of each participating medical centre and by the Ethical Committee at the SNP Research Center, the Institute of Physical and Chemical Research (RIKEN), Yokohama.

Genotyping. In the association study, we divided the Japanese patients who received PEG-IFN plus ribavirin into two independent groups based on the time of entry into the study (Set-1 and Set-2 in Table 5). For the GWAS stage, 594 Japanese patients with HCV genotype 1b treated with PEG-IFN plus ribavirin (315 SRs and 279 NRs) were initially genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip (Set-1 in Table 5). The other samples were genotyped using the Invader assay, the TaqMan assay, or by direct sequencing as described previously (Ohnishi *et al.*, 2001; Suzuki *et al.*, 2003). The other patients were organized into the following sets: Set-2, 185 genotype 1b Japanese patients treated with PEG-IFN plus ribavirin (67 SRs vs 118 NRs); Set-3, 750 genotype 1b Japanese patients treated with IFN monotherapy (217 SRs vs 533 NRs); Set-4, 513 genotype 2a Japanese patients treated with IFN monotherapy (342 SRs vs 171 NRs); Set-5, 44 genotype 1b Taiwanese patients treated with PEG-IFN plus ribavirin (25 SRs vs 19 NRs); and Set-6, 29 genotype 2a Taiwanese patients treated with PEG-IFN plus ribavirin (25 SRs vs 4 NRs) (Table 5).

Quality control criteria for the genome-wide study. For the genome-wide survey, we applied the following quality control criteria. Individual samples with genotype call rates less than 98 % and SNPs with call rates less than 99 % were removed, as were non-autosomal SNPs, minor alleles with a frequency less than 0.01, or SNPs deviating from the Hardy–Weinberg equilibrium ($P<1\times10^{-6}$). Related individuals were detected by identity-by-state analysis performed with the PLINK software. One subject from each of the cryptically related pairs of individuals (PI_HAT >0.4) was excluded. We assessed

Table 5. Clinical characteristics of cohorts

SR, Sustained responder; NR, non-responder. PEG + riba, pegylated IFN-α-2b plus ribavirin combination therapy; IFN, IFN-α monotherapy.

Group	Treatment outcome	No. samples	Male (%)	Mean age in years (±SD)	HCV genotype	Therapy	Ethnicity
Set-1	SR	304	197 (64.8)	54.4 (±13.2)	1b	PEG + riba	Japanese
	NR	279	139 (49.8)	59.9 (±10.7)			
Set-2	SR	67	33 (49.3)	57.1 (±13.3)	1b	PEG + riba	Japanese
	NR	118	49 (41.5)	62.7 (±8.5)			
Set-3	SR	217	147 (67.7)	57.2 (±13.8)	1b	IFN	Japanese
	NR	533	311 (58.3)	56.4 (±10.7)			
Set-4	SR	341	203 (59.5)	56.4 (±14.7)	2a	IFN	Japanese
	NR	169	86 (50.9)	54.9 (±12.3)			
Set-5	SR	25	12 (50.0)	50.5 (±9.8)	1b	PEG + riba	Taiwanese
	NR	19	8 (42.1)	51.7 (±7.0)			
Set-6	SR	25	14 (56.0)	49.4 (±12.4)	2a	PEG + riba	Taiwanese
	NR	4	4 (100.0)	48.2 (±13.0)			
Healthy control		282	151 (53.5)	44.1 (±15.0)			Japanese

population stratification using the smartpca program from the EIGENSOFT package (http://genepath.med.harvard.edu/~reich/Software.htm) using genotypes for about 70 000 SNPs informative for the Japanese population according to the method described previously (Yamaguchi-Kabata *et al.*, 2008). Analysis was performed using the Set-1 samples and all four of the HapMap population datasets (CEU/YRI/JPT/CHB). Outliers from JPT/CHB clusters were excluded from the association analysis. Genotype-based associations were tested with the Cochran–Armitage trend test. The genomic control method was applied to evaluate whether the inflation of false-positive rates, indicated by inflation factor λ, was within an acceptable level.

Resequencing. Resequencing around the IL-28B locus was performed by direct sequencing of DNA from 48 unrelated Japanese HCV patients from the enrolled subjects. We used Phase II HapMap JPT genotype data and the HAPLOVIEW program to define an LD block.

HCV RNA level. The HCV RNA level was analysed at three time points: before IFN therapy, at the end of the second week and at the end of the fourth week, using an RT-PCR-based method (the original method, the high range method or the TaqMan RT-PCR test). The measurement ranges of these assays were 0.5–850 kilo (K) IU ml^{-1}, 5–5000 KIU ml^{-1} and 1.2–7.8 log IU, respectively. Set-1 and Set-2 patients with sufficient viral load data falling within the measurable range were included in the viral kinetics analysis.

Liver biopsy. Liver biopsy specimens were evaluated by a pathologist at each institution and were scored for the stage of liver fibrosis according to the classification of Desmet *et al.* (1994). The patients were divided into two categories: mild fibrosis (F0–1) and severe fibrosis (F2–4).

Statistical analysis. Genotype-based associations were tested using the allele model χ^2-test. SNP effects were also evaluated using the dominant, recessive and co-dominant models (using the Cochran–Armitage trend test). Combined analysis was performed following the Mantel–Haenszel method. Viral kinetics were assessed using the non-parametric Kruskal–Wallis test followed by the Steel–Dwass test. Multivariate logistic regression analysis with stepwise forward selection was performed with criteria of $P<0.05$ for inclusion and removal of variables using the StatFlex 5.0 software package (Artec Inc.).

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Research Article

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Predictive value of the *IL28B* polymorphism on the effect of interferon therapy in chronic hepatitis C patients with genotypes 2a and 2b

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Background & Aims: Common *IL28B* locus polymorphisms (SNPs rs8099917 and rs12979860) have been reported to affect peg-interferon plus ribavirin combination therapy (PEG-RBV) for hepatitis C virus (HCV) genotype 1b, but few reports have examined their effect on other two common genotypes, 2a and 2b.
Methods: We analyzed predictive factors for sustained virological response (SVR) in a retrospective study of 719 patients with either genotype 2a (530) or 2b (189). Of these patients, 160 were treated with PEG-RBV and 559 were treated with interferon monotherapy. We evaluated predictive factors including HCV RNA, histological findings, *IL28B* SNP genotypes (rs8099917, rs12979860, and rs12980275), and the effect of treatment regimen and prior treatment history.
Results: HCV RNA viral load, treatment regimen, and rs8099917 genotypes independently contributed to the effect of the therapy. For patients treated with PEG-RBV, rs8099917 and viral load were independent predictive factors for SVR in genotype 2b but not in genotype 2a. Conversely, in patients treated with interferon monotherapy, viral load and rs8099917 were independent predictive factors for SVR in genotype 2a but not in genotype 2b. The favorable rs8099917 genotype is also associated with a steep decline in viral load by the second week of treatment.
Conclusions: Initial viral load and rs8099917 genotype are significant independent predictors of SVR in genotype 2 patients.
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Introduction

Hepatitis C virus (HCV) infection is a major worldwide cause of chronic liver diseases, affecting an estimated 170 million people [1]. Chronic HCV infection may progress to hepatocellular carcinoma (HCC) or liver cirrhosis (LC) [2–6], and in Japan, 60–70% of patients with HCC or LC are HCV carriers [7]. There are two major genotypes (1 and 2) and three sub-genotypes (1b, 2a, and 2b) in Japan as well as in many other countries [8]. Although pathological features of these genotypes are similar [9,10], interferon therapy is more effective against genotype 2 than genotype 1 [11,12]. Compared to the less than 50% of genotype 1 patients who respond to therapy [13–19], more than 80% of genotype 2 patients who received 24-week peg-interferon and ribavirin (PEG-RBV) combination therapy achieved sustained virological response (SVR), defined as absence of HCV RNA six months after the cessation of therapy. Because of this otherwise high success rate, the small subset of genotype 2 patients who fail to respond to therapy should be examined more closely. Although treatment-resistant genotype 2 sub-populations have been reported [20–22], the mechanism underlying variable response to treatment is unclear. Multiple viral (e.g., HCV genotype, amino acid substitutions in the NS5A and core region [22–26]) and host factors (e.g., age [14], body mass index [27], and insulin resistance

Keywords: Interferon therapy; Single nucleotide polymorphism; Ribavirin; Hepatitis C.
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Abbreviations: HCV, hepatitis C virus; IFN, interferon; PEG-IFN, pegylated interferon; RBV, ribavirin; PEG-RBV, pegylated interferon plus ribavirin combination therapy; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NR, non-responder.

Table 1. Baseline characteristics of patients with HCV genotypes 2a and 2b.

	All (n = 719)	2a (n = 530)	2b (n = 189)
Sex (M/F)	403/316	301/229	102/87
Age	57 (49-64)	56 (48-64)	59 (50-66)
Body weight (kg)	59.8 (51-71.4)	60.15 (53.75-71.65)	57.4 (48.5-70)
BMI (kg/m²)	23.2 (20.3-25.7)	24.48 (21.43-26.4)	21.78 (19.89-24.79)
Fibrosis (F0-2/F3-4)	484/101	359/68	125/33
Treatment (IFN/PEG-RBV)	559/160	477/53	82/107
Treatment naïve (Y/N)	689/30	523/7	166/23
HCV RNA (log IU/ml)	5.3 (4.7-5.9)	5 (4.6-5.7)	5.9 (5.5-6.5)
rs8099917 (TT/GT/GG)	572/135/11	425/97/7	147/38/4
rs12979860 (CC/TC/TT)	565/137/11	422/98/7	143/39/4
rs12980275 (AA/GA/GG)	543/158/16	402/116/10	141/42/6
SVR/non-SVR	455/264	340/190	115/74

IFN, interferon monotherapy; PEG-RBV, peg-interferon plus ribavirin combination therapy; SVR, sustained viral responder.

[28]) have been reported to affect the outcome of interferon therapy in genotype 1-infected patients but such factors have not been closely examined in genotype 2 patients.

Single nucleotide polymorphisms (SNPs) and other genetic factors have been reported to be useful in predicting the outcome of interferon therapy. Polymorphisms in MxA [29,30], interferon alpha-receptor 1 [31], and osteopontin [32] have also been reported to be associated with interferon response. We also identified a MAPKAPK3 SNP [33] that is a predictive factor for interferon mono-therapy. Recently, several groups have reported an association between several SNPs in the *IL28* locus and the effect of PEG-RBV combination therapy for genotype 1b [34–38] but only a few studies have examined the role of these SNPs in the treatment of other genotypes. In this study, we analyzed predictive factors for SVR in genotype 2a and 2b patients treated with PEG-RBV. Because PEG-RBV was only approved for use in Japan in 2005, we also examined predictive factors in patients who were treated with interferon mono-therapy, which is still used in the event of an adverse reaction to ribavirin.

Patients and methods

Patients and study design

We studied 719 Japanese patients with chronic hepatitis C (positive for HCV RNA for more than 6 months) who received interferon therapy with or without ribavirin between 2002 and 2008. Patients were treated at Toranomon Hospital in Tokyo, Hiroshima University Hospital, and hospitals belonging to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html). All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease, and had not received immunosuppressive therapy before enrollment in the study. All patients gave written informed consent to participate in the study in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and according to the process approved by the ethical committees of Hiroshima University and the SNP Research Center at the Institute of Physical and Chemical Research (RIKEN) in Yokohama.

PEG-RBV patients received weekly injections of peg-interferon-alpha-2b at 1.5 g/kg body weight for 24 weeks. Ribavirin was administered orally, and the dosage was determined based on the patient's body weight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Patients receiving interferon mono-therapy were treated daily with 6 million units of IFN intramuscularly for 8 weeks, followed by the same dose three times a week for 16 weeks, for a total of 528 million units. Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA-negative six months after cessation of therapy. Fibrosis stage and activity were diagnosed by pathologists at each hospital according to the criteria of Desmet et al. [39]. Patients were classified as interferon treatment naïve or experienced based on prior interferon treatment but only parameters related to the most recent therapy were used in the analysis.

SNP Genotyping and quality control

We genotyped each patient for three *IL28B* SNPs previously reported to be associated with therapy outcome: rs8099917, rs12979860, and rs12980275. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or the Invader assay, as described previously [40,41]. We were unable to determine genotypes for one of the 796 patients for rs8099917, six of the patients for rs12979860, and two for rs12980275.

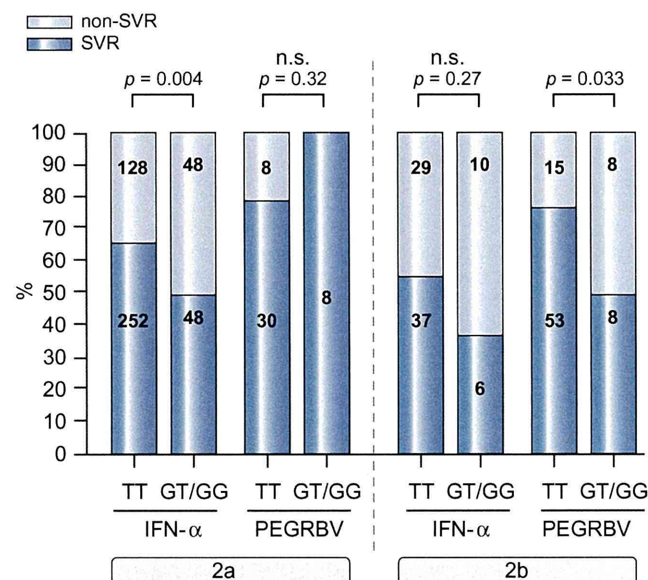

Fig. 1. Effect of interferon therapy on patients with genotype 2a and 2b infection. Sustained viral responders (SVR) and non-responders (non-SVR) were analyzed by IL28B SNP rs8099917 genotype, viral genotype, and treatment type. All patients were interferon-naïve.

Table 2. Predictors for SVR in treatment-naïve patients treated with peg-interferon plus ribavirin combination therapy.

		Simple		Multiple			
Genotype	Variable	n	p	n	OR	(95% CI)	p
2a + 2b	Age	130	0.42				
	Sex	130	0.62				
	Genotype	130	0.21				
	Viral load	127	0.002 **	127	0.19	(0.06-0.55)	0.002 **
	Fibrosis	110	0.25				
	rs8099917	130	0.23				
	rs12980275	129	0.79				
2a	Age	46	0.77				
	Sex	46	0.62				
	Viral load	44	0.16				
	Fibrosis	39	0.75				
	rs8099917	46	0.8				
	rs12980275	45	0.77				
2b	Age	84	0.14				
	Sex	84	0.58				
	Viral load	83	0.01 *	83	0.13	(0.03-0.62)	0.01 *
	Fibrosis	71	0.08				
	rs8099917	84	0.03 *	83	0.23	(0.06-0.80)	0.02 *
	rs12980275	84	0.21				

*p <0.05; **p <0.01; ***p <0.001.

HCV RNA levels

HCV RNA levels, corresponding to initial viral load, were measured using one of several RT-PCR-based methods (the original Amplicor method, the high range method, or the TaqMan RT-PCR test). The measurement ranges of these assays were 0.5–850 KIU/ml, 5–5000 KIU/ml, and 1.2–7.8 log IU, respectively. Saturated samples were diluted with PBS and reanalyzed. All values were reported as log IU/ml.

Statistical analysis

Genotype-based associations were tested using the Cochran–Armitage trend test. Combined analysis was performed using the Mantel–Haenszel method. Simple and multiple regression analyses were used to examine the association between viral and clinical factors using p <0.05 as the criterion for inclusion in the multivariate model. HCV RNA was converted into a binary variable based on the median. Multivariate logistic regression analysis was performed using the Design package in *R* (http://www.r-project.org) with fast backward elimination and validation based on AIC score for model construction.

Results

Clinical characteristics are summarized by genotype in Table 1. The SVR rate was slightly but not significantly higher among patients with genotype 2a (340 out of 530; 64%) compared to genotype 2b patients (115 out of 189; 61%) (p = 0.43). Patients who were treated with PEG-RBV had a slightly but not significantly higher rate of SVR (111 out of 160; 69%) than patients treated with interferon monotherapy (344 out of 559, 61%) (p = 0.08). Because the number of patients treated with interferon monotherapy (559) greatly exceeds the number of patients treated with PEG-RBV (160), patients were analyzed separately by treatment type. Because 30 out of the 719 patients (4%) had received prior interferon treatment, only treatment-naïve patients were included in the analyses mentioned below, followed by a separate analysis of the effect of prior interferon treatment on SVR rate.

IL28B polymorphisms

Minor allele frequencies for rs8099917, rs12979860, and rs12980275 were 0.109, 0.112, and 0.132, respectively. The frequency of the rs8099917 risk allele was lower in SVR patients than non-SVR patients (0.089 vs. 0.14; p = 1.03e-05). The risk allele frequency among all patients was slightly higher than in the HapMap-JPT population (0.109 vs. 0.093; p = 0.01) but lower than in the HapMap-CEU population (0.109 vs. 0.183; p = 1.6e-05). We compared rs8099917 allele and genotype frequencies with 900 healthy Japanese subjects but found no significant differences. 67% of patients (372 out of 552) with the favorable rs8099917 TT genotype achieved SVR, compared to 51% (70 out of 136) of patients with GT or GG genotypes. Fig. 1 shows the joint effects of treatment type, viral genotype, and rs8099917 genotype. In every case results for rs8099917 and rs12979860 are the same, but both factors cannot be included in a multivariate model simultaneously due to multicollinearity, so results for rs8099917 are presented due to the higher genotyping success rate.

Predictive factors for SVR in patients treated with PEG-RBV

Among treatment-naïve patients treated with PEG-RBV, 78% (83 out of 106) of patients with rs8099917 TT achieved SVR compared

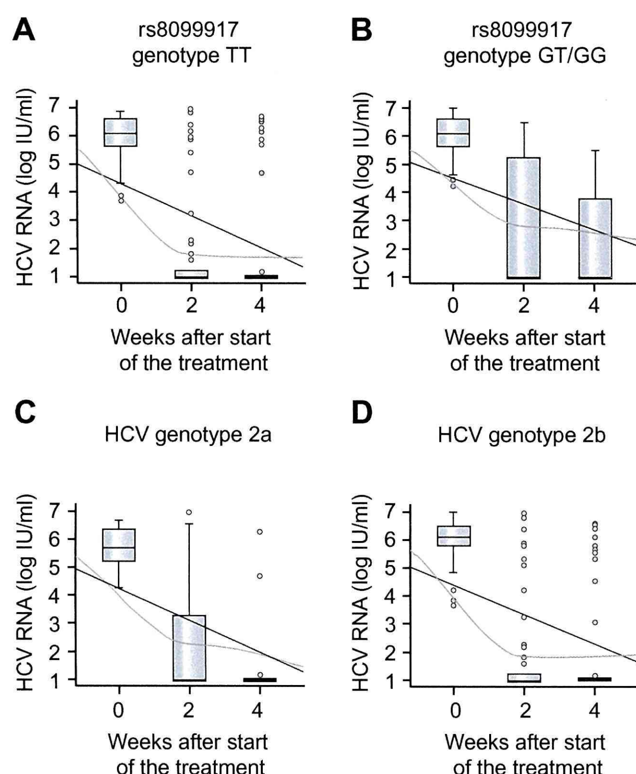

Fig. 2. Effect of rs8099917 genotype and HCV genotype on change in HCV RNA levels. HCV RNA levels at 0, 2, and 4 weeks after the start of peg-interferon plus ribavirin combination therapy in treatment-naïve patients. (A and B) Change in viral load for patients with the protective TT genotype for rs8099917 (A) compared to patients with the GT or GG genotypes (B). (C and D) Change in viral load for patients with HCV genotype 2a (A) versus genotype 2b (B).

to 67% (16 out of 24) of patients with non-TT genotypes ($p = 0.29$). In univariate and multivariate analyses, only viral load was an independent predictive factor for SVR ($p = 0.002$; Table 2), but when we examined genotypes 2a and 2b separately, rs8099917 genotype ($p = 0.02$) and viral load ($p = 0.01$) were both significant independent predictors of SVR for patients with genotype 2b, whereas no significant univariate or multivariate predictors were found for patients with genotype 2a. Notably, however, all 8 patients with genotype 2a with rs8099917 GT/GG achieved SVR (Fig. 1). The same pattern held for patients with rs12979860 TC/TT (9 SVR, 0 non-SVR) and rs12980275 GA/GG (11 SVR, 0 non-SVR) genotypes. Moreover, none of these patients was homozygous for the risk allele at each SNP.

Change in HCV RNA levels for patients treated with PEG-RBV

HCV RNA levels at the start of PEG-RBV therapy and after 2 and 4 weeks of treatment are plotted by rs8099917 genotype and viral genotype in Fig. 2. Under multivariate analysis, rs8099917 genotype was an independent predictive factor for change in HCV RNA level by week 2 ($p = 0.036$) but viral genotype was not significant ($p = 0.15$). For changes in HCV RNA levels by week 4, neither the rs8099917 genotype nor the viral genotype was significant ($p = 0.17$ and $p = 0.22$, respectively).

Predictive factors for SVR in patients treated with interferon monotherapy

Among patients treated with interferon monotherapy, 65% of patients with rs8099917 TT achieved SVR, compared to only 48% of patients with GT or GG genotypes ($p = 0.002$). Viral load and the rs8099917 and rs12980275 genotypes were significant univariate predictors of SVR, and under multivariate analysis viral load and rs8099917 remained as independent predictors (Table 3). When genotypes 2a and 2b were analyzed separately, viral load ($p = 0.001$) and rs8099917 genotype ($p = 0.014$) were independent predictive factors for SVR in patients with genotype 2a but no significant univariate of multivariate terms were found for genotype 2b.

Effect of prior interferon treatment

Thirty out of the 719 patients (4%) had previously received treatment with interferon. Among these patients, only 40% achieved SVR, compared to the 64% SVR rate among treatment-naïve patients. Initial viral load was the only independent predictor of SVR in these patients, whereas in treatment-naïve patients, viral load, rs8099917 genotype, and treatment type (PEG-RBV vs interferon monotherapy) were independent predictors of SVR (Table 4).

Development of resistance to interferon therapy

Over the course of therapy five patients developed resistance to PEG-RBV treatment. In each case the patient showed an initial drop in viremia followed by viral breakthrough. Three out of the five patients were heterozygous (T/G) for the rs8099917 genotype and two out of the five were homozygous for the favorable allele (T/T).

Discussion

As the effect of *IL28B* polymorphism has not been reported separately for genotype 2 and its subtypes so far, we investigated whether the polymorphism influences treatment outcome in patients with HCV genotype 2a and 2b infections. In addition to previously reported effects for genotypes 1 and 4, our results demonstrate that polymorphisms in the *IL28B* locus are also predictive for SVR in genotype 2 (Table 2). We also showed that the favorable *IL28B* SNP genotype is associated with a rapid decrease in HCV RNA levels, which is itself a predictive factor for SVR [42]. Several studies have reported that polymorphisms at the *IL28B* locus affect the outcome of peg-interferon and ribavirin combination therapy in patients with HCV genotype 1b [34–36,38]. In particular, associations with therapy outcome have been reported for two SNPs in strong linkage disequilibrium, rs8099917 (T/G), and rs12979860 (C/T). Only a few studies have examined the effect of the SNP on the treatment outcome for other genotypes. Rallón et al. reported that the rs12979860 genotype is associated with treatment outcome for genotypes 1 and 4 but not genotype 3 in patients with HIV/HCV co-infection [43]. Similarly Rauch et al. reported an association between rs8099917 polymorphism and NVR for genotypes 1 and 4 (difficult-to-treat) but not for genotypes 2 and 3 (easier-to-treat) but the effect due to genotype 2 alone is unclear [38]. In a recent study, Mangia et al. also exam-

Table 3. Predictors for SVR in treatment-naïve patients treated with IFN monotherapy.

		Simple		Multiple			
Genotype	Variable	n	p	n	OR	(95% CI)	p
2a + 2b	Age	559	0.35				
	Sex	559	0.17				
	Genotype	559	0.068				
	Viral load	507	0.0002 ***	506	0.59	(0.45-0.77)	0.0001 ***
	Fibrosis	450	0.61				
	rs8099917	558	0.001 **	506	0.52	(0.33-0.82)	0.005 **
	rs12980275	558	0.009 **				
2a	Age	477	0.19				
	Sex	477	0.2				
	Viral load	425	0.001 **	424	0.6	(0.44-0.81)	0.001 ***
	Fibrosis	382	0.37				
	rs8099917	476	0.003 **	424	0.53	(0.32-0.88)	0.014 *
	rs12980275	476	0.01 **				
2b	Age	82	0.67				
	Sex	82	0.56				
	Viral load	82	0.47				
	Fibrosis	68	0.53				
	rs8099917	82	0.19				
	rs12980275	82	0.44				

$^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

ined genotypes 2 and 3 and found a significant association between rs12979860 genotype and rapid virological response (RVR) at week 4 for genotype 2 [44]. While rs12979860 was not directly associated with SVR in their study, rs12979860 genotype was significantly associated with SVR among those patients who failed to achieve RVR. In this study, we found a significant association between rs8099917 genotype and RVR in multivariate analysis for genotype 2b ($p = 0.028$, data not shown) but not for genotype 2a. When RVR was included as a factor in multivariate logistic regression analysis for genotype 2b, RVR and rs8099917 genotype were both retained in the final model but only RVR was significant (RVR: $p = 4.9e\text{-}05$; rs8099917: $p = 0.0850$; data not shown). When only non-RVR patients were included, no factors were significant; however, there were only six patients who achieved SVR without RVR and only one patient who achieved RVR but then failed to achieve SVR.

Although SVR rate was generally higher for genotype 2a, as reported previously [20,21], we found few differences between genotypes 2a and 2b. However, when analyzed separately, the results suggest an interesting interaction between the *IL28B* genotype, the viral genotype, and treatment type. In particular, we found that rs8099917 was a predictive factor for genotype 2a treated with IFN but not PEG-RBV, and conversely for genotype 2b treated with PEG-RBV but not IFN. This result is likely due to the relatively small sample sizes, but nonetheless all 8 (100%) of the genotype 2a PEG-RBV patients lacking the favorable rs8099917 genotype achieved SVR, compared to less than 50% for IFN therapy or either type of treatment with genotype 2b. In fact, each patient was heterozygous for each of the three *IL28B* SNPs examined. A further complication is that each of the five patients who developed resistance to interferon therapy was infected with genotype 2a, and two of these patients had the favorable rs8099917 TT genotype while the others were heterozygous (GT). More detailed analysis will be required to interpret these results.

Because PEG-RBV therapy was not covered by insurance in Japan until 2005, we also present data comparing the effects of *IL28B* polymorphisms on treatment with the older IFN monotherapy versus the more recent PEG-RBV combination therapy. Although the small sample sizes within each patient group likely underestimate the effect of SNP genotype, we found that rs8099917 influences response to IFN monotherapy in patients with genotype 2a and also influences the response to PEG-RBV therapy in patients with genotype 2b. Although PEG-RBV is currently the standard treatment for chronic hepatitis C infection, interferon monotherapy may still be used in the case of intolerance to ribavirin; therefore, it is important to understand the direct effects of interferon with and without ribavirin. Moreover, even with the advent of protease inhibitors and other antiviral drugs undergoing clinical trials, they are likely to be co-administered with interferon to prevent the otherwise rapid emergence of resistant quasispecies [45].

In summary, we showed that the *IL28B* SNP genotype is an important predictive factor for SVR and early viral dynamics in patients with HCV genotypes 2a and 2b.

Conflict of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Table 4. Comparison of predictive factors for SVR based on prior treatment with interferon.

		Simple			Multiple			
	Variable	n	p		n	OR	(95% CI)	p	
All	Age	719	0.70						
	Sex	719	0.28						
	Genotype	719	0.42						
	Viral load	663	6.00E-02	***	662	0.63	(0.51-0.79)	4.30E-05	***
	Fibrosis	585	0.83						
	rs8099917	718	0.002	**	662	0.57	(0.38-0.85)	0.0055	**
	rs12980275	717	0.03	*					
	Treatment	719	0.054						
Naïve	Age	689	0.58						
	Sex	689	0.18						
	Genotype	689	0.62						
	Viral load	634	0.0011	**	633	0.53	(0.41-0.69)	2.00E-06	***
	Fibrosis	560	0.95						
	rs8099917	688	0.00059	***	633	0.5	(0.33-0.77)	0.0015	**
	rs12980275	687	0.013	*					
	Treatment	689	0.0013	**	633	3.01	(1.82-4.99)	1.80E-05	***
Experienced	Age	30	0.91						
	Sex	30	0.75						
	Genotype	30	0.14						
	Viral load	29	0.032	*	29	0.21	(0.05-0.87)	0.032	*
	Fibrosis	25	0.53						
	rs8099917	30	0.12						
	rs12980275	30	0.1						
	Treatment	30	N/A						

*p <0.05; **p <0.01; ***p <0.001.

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Research Article

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IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy

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Background & Aims: Common genetic variation within the IL28 locus has been found to influence the effect of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. Expression of *IL28* in peripheral blood cells has been reported to be higher in patients with *IL28* SNP genotypes associated with favorable response.
Methods: We analyzed 52 liver and 114 blood samples obtained from patients with HCV genotype 1b. We used reverse transcription-real time polymerase chain reaction to analyze expression levels of *IL28* and several interferon stimulated genes (ISGs), including *MxA*, double stranded RNA dependent protein kinase (*PKR*), 2′–5′ oligo-nucleotide synthetase (*OAS1*), *ISG15*, and *SOCS1*.
Results: Interestingly, expression of *IL28* was significantly lower in patients with the response-favorable rs8099917 TT genotype compared to those with TG or GG genotypes (p <0.005). In hepatic cells, expression of *MxA*, *PKR*, *OAS1*, and *ISG15* were also significantly lower in rs8099917 TT patients (p <0.001, p = 0.005, p = 0.001, p <0.001, respectively), whereas in peripheral blood mononuclear cells ISG expression levels did not differ significantly. Among patients treated with peg-interferon plus ribavirin therapy, liver mRNA levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly or marginally lower in responders who became negative for HCV RNA (p = 0.001, 0.004, 0.014, 0.051, and 0.015, respectively).
Conclusions: Expression levels of ISGs are differentially regulated in the liver and peripheral blood. The mechanism underlying the expression levels of IL28 and ISGs and the correlation with the effect of the therapy should be further investigated.

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Introduction

Chronic hepatitis C virus infection often results in the development of chronic hepatitis, which leads to cirrhosis and hepatocellular carcinoma [1,2]. Currently, patients with chronic HCV infection are treated with a combination of pegylated interferon and ribavirin [3,4]. The eradication rate of the virus has been reported to be about 50% in patients treated with the standard 48 week therapy [4–6]. Although the eradication rate of the virus has been slightly improved by extending the treatment period to 72 weeks, there are many patients who fail to eradicate the virus [7]. Furthermore, many patients fail to complete the therapy because of severe side effects.

Many predictive factors have been reported so far that affect response to combination therapy. Viral factors, such as substitutions at core amino acids 70 and 91 [8,9], or within the interferon sensitivity determining region (ISDR) [10,11] or the interferon and ribavirin response determining region (IRRDR) [12] have been reported.

Among host factors, many single nucleotide polymorphisms (SNPs) associated with outcome of therapy have been identified. They include SNPs in interferon-alpha pathway genes [13] and interferon induced genes [14], within the promoters of the *MxA* [15] and osteopontin [16] genes, and within an intron of *MAPK APK3* [17].

Recently, Ge *et al.* [18] identified SNPs located 5′ to the *IL28B* gene that affect response to combination therapy. Furthermore, two other research groups also independently reported that these SNPs are associated with the effectiveness of combination therapy [19,20]. More recently, Thomas *et al.* reported that the SNP allele related to favorable therapy response is also associated with spontaneous clearance of HCV [21]. They reported that the allele related to HCV clearance is the major allele in the majority of Asian and European countries.

Keywords: IL28; Liver biopsy; ISG15; MxA; Single nucleotide polymorphism.
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Abbreviations: HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; IRRDR, interferon and ribavirin response determining region; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NVR, non-viral responder; OAS1, 2′–5′ oligoadenylate synthetase 1 ; PKR, double stranded RNA dependent protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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IL28A, *IL28B*, and *IL29* gene products belong to the interferon lambda family [22,23]. These cytokines are interferons functionally, but have been reported to be structurally related to the IL-10 family [24]. IL29 has been reported to reduce the replication levels of the HCV replicon [25] as well as hepatitis B virus [26]. IL29 has also been reported to reduce the replication of HCV cooperatively with interferon alpha and gamma [27]. These observations suggest that higher expression levels of interferon lambda should be observed in the liver and should correspond with a favorable response to therapy. However, no report has analyzed the expression levels of these cytokines and levels of ISG expression in the liver. In this study, we investigated mRNA expression levels of *IL28*, IL28 receptor, and several ISGs using biopsy samples obtained from patients with chronic hepatitis C and analyzed the relationship between the *IL28* genotype and the effect of combination therapy.

Materials and methods

Patients

We analyzed liver specimens from 52 patients who underwent liver biopsies at Hiroshima University Hospital between December 2002 and November 2008 and who were treated with a peg-interferon plus ribavirin combination for chronic hepatitis C genotype 1b at the same or other hospitals. Clinical characteristics of patients are shown in Table 1. Patients received weekly injections of peg-interferon-alpha-2b for 48 weeks with the dosage adjusted by body weight (60 μg for 35–45 kg, 80 μg for 46–60 kg, 100 μg for 61–75 kg, 120 μg for 76–90 kg, and 150 μg for 91–120 kg). Ribavirin was administered orally with the dosage based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels were reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. The response to therapy categories are defined as follows: sustained viral responders (SVR) were negative for HCV RNA 24 weeks after cessation of therapy; relapsers were negative for HCV RNA only transiently during and after the therapy; and non-viral responders (NVR) never became negative for HCV RNA. Liver biopsy specimens, which were obtained in routine clinical practice in an amount beyond what was needed for pathological diagnosis, were kept frozen at −80 °C until analysis. Liver samples obtained by surgical operation from patients who received resection for hepatocellular carcinoma were also kept frozen. Fibrosis stage and activity were diagnosed according to the criteria of Desmet *et al.* [28].

Although we attempted to analyze blood samples from the same patients who provided liver specimens, more than half of these patients were not treated at Hiroshima University Hospital. Accordingly, we collected blood samples from 114 genotype 1b patients who visited Hiroshima University Hospital from November 2009 to March 2010 to analyze ISG mRNA levels. We excluded patients who were under treatment with therapies including interferon or immunosuppressants. Patients who had eliminated HCV with therapy were also excluded. Clinical characteristics of patients who contributed blood samples for ISG analysis are shown Table 1.

All patients provided written informed consent to participate in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the ethical committee of Hiroshima University and RIKEN.

Genotyping

We genotyped SNPs rs8099917 and rs12979860 from 52 patients using either the Invader assay or the Taqman assay. In the Invader assay, allele-specific oligonucleotide pairs and invasive probes were designed and supplied by Third Wave Technologies (WI). FRET probes were labeled with FAM or VIC corresponding to alleles. The 10 μl reaction volume consisted of 0.5 μl of signal buffer, 0.5 μl of FRET probes, 0.5 μl of structure-specific cleavage enzyme, 1 μl of allele-specific probe mix, and 2 μl of PCR product diluted 1:10. Samples were incubated at 95 °C for 5 min and then at 63 °C for 15 min in an ABI PRISM 7700 (Applied Biosystems), and then fluorescence data were collected. Signal intensity was calculated as the ratio of FAM or VIC to ROX, an internal reference. Genotypes were determined visually in the dye components view of the SDS software.

In the TaqMan assay, we carried out PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, CA), 1 ng DNA, 0.2 μM of each primer, and 40 nM of probe provided by Applied Biosystems in 3-μl reactions. Each 384-well plate contained 376 samples of an unknown genotype and 8 no-DNA control samples. Thermal cycle conditions were 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 92 °C for 15 s, and 58 °C for 1 min. Thermal cycling was done on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems), and then fluorescence data were collected and the genotypes were determined using the SDS software [29,30].

We calculated linkage disequilibrium using the LD method in the genetics library in the R 2.11 statistics package (http://www.r-project.org) and found high linkage disequilibrium between rs8099917 and rs12979860 ($r^2 = 0.99$ and $D' = 1$).

Table 1. Characteristics of the two cohorts of patients analyzed for ISG expression levels. All patients were infected with HCV genotype 1b.

	Treated patients	WBC patients
Characteristic	(n = 52)	(n = 114)
Age [median (range)]	56 (31-77)	63 (30-88)
Sex (Male/Female)	29/23	63/51
ALT [median (range)] IU/L	47 (13-246)	62 (15-259)
γ-GTP [median (range)] IU/L	47 (15-708)	53 (10-469)
Fibrosis (F1/F2/F3/F4)	20/18/4/10	23/28/17/11
Activity (A1/A2/A3)	13/30/9	14/48/14
Virus titer [median (range)] kIU/L	850 (15-6500)	850 (0.5-8200)
Core 70[a] (Wild/Mutant/ND)	27/19/6	40/21/53
Core 91[a] (Wild/Mutant/ND)	24/22/6	34/27/53
ISDR [b] substitutions (0/1/>2/ND)	14/16/12/10	37/12/9/56
rs8099917 allele (TT/TG/GG)	30/17/5	88/33/1
Outcome of therapy (SVR/relapser/NVR)[c]	25/19/8	not applicable

Outcome of therapy	TT	TG	GG
SVR	20	5	0
Relapser	4	8	2
NVR	1	4	3
Total	42	24	6

[a]Hepatitis C virus core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants. ND, not determined.
[b]Interferon sensitivity determining region: the number of substitutions relative to the ISDR of the reference sequence [31].
[c]SVR, sustained viral responder; NVR, non-viral responder.

Quantitative analysis of mRNA of ISGs

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of each RNA sample was reverse transcribed with ReverseTra Ace (TOYOBO Co. Ltd., Japan) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the mRNA for *IL28*, *MxA*, 2′–5′ oligoadenylate synthetase1 (*OAS1*), double stranded RNA dependent protein kinase (*PKR*), *ISG15*, and *SOCS1* with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). As it was difficult to measure *IL28A* and *IL28B* mRNA separately, we measured *IL28A* plus *IL28B* mRNA and expressed *IL28* mRNA. Amplification and detection were performed using an ABI PRISM 7300 (Applied Biosystems). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of MxA protein in peripheral mononuclear cells

The MxA protein level in whole blood sample was measured using an ELISA system (MxA ELISA Kit, Kyowa Medex, Tokyo, Japan). Briefly, lysing solution was added to blood samples and the lysate was applied to ELISA plates coated with

a MAb (KM1135, Kyowa Medex, Tokyo, Japan). After 2 h of incubation, the plates were washed, and a different peroxidase-labeled MAb (KM1124, Kyowa Medex) was added. After 1 h of incubation and washing, substrate was added. Chemiluminescence was detected using Multiskan MS (Labsystems Version 8.0, Helsinki, Finland). The sensitivity of MxA in this ELISA system was 3.2 ng/ml.

Analysis of amino acid sequences in the core and ISDR region

PCR amplification and nucleotide and amino acid sequence analysis of core and ISDR were performed as reported previously [31] with a slight modification. Briefly, HCV RNA was extracted from 100 µl serum samples by SepaGene RV-R (Sanko Junyaku Co., Tokyo, Japan) and dissolved in 20 µl of H_2O. The RNA was then reverse-transcribed with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The resultant cDNA was then amplified by nested PCR. PCR was performed in 25 µl of the reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer, and 1.25 U of LA Taq (Takara Bio Inc., Otsu, Japan) with a buffer supplied by the manufacturer. One microliter of 10 × -diluted products from the first PCR was used as a template for the second PCR. The PCR primer sequences are listed in Table 2. The PCR protocol involved initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing of primers for 1 min at 57 °C and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 7 min. The amplified DNA fragments were separated onto a 2% agarose gel and purified with the QIAquick gel extraction Kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).

The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype 1b HCV-J (GenBank Accession No.: D90208) [32]. Amino acids at positions 70 and 91 of the core region identical to the reference sequence (arginine and leucine, respectively), were considered as wild type. The number of amino acid substitutions in the ISDR was determined as in Enomoto *et al.* [11,12].

Statistical analysis

Statistical analysis was performed using R version 2.11 or PASW Statistics 18 (SPSS Inc., IL). Categorical data were analyzed using Fisher exact tests, and continuous data were analyzed using the non-parametric Mann–Whitney *U* test. Given the large number of possible predictors, we used multiple logistic regression with variable selection to identify a model with the most important predictors for virological response. To identify independent predictive factors, variables that were significant at the 0.05 level in univariate non-parametric tests were considered as candidate factors for multiple logistic regression analysis. Multicollinearity among predictor variables were examined using hierarchical clustering based on Spearman rank. The model was reduced using forward/backward stepwise selection using the stepAIC function in R, and then boostrap validation was performed using the rms library (formerly called the Design library). Partial residual plot and leverage plots were examined to identify outliers and assess model assumptions. The rms calibrate function was used to calculate R^2 shrinkage, and log odds were corrected for over-optimism using penalized maximum likelihood [33].

Table 2. Primers used in this study.

HCV core protein	
outer forward	5'-GCC ATA GTG GTC TGC GGA AC-3'
outer reverse	5'-GGA GCA GTC CTT CGT GAC ATG-3'
inner forward	5'-GCT AGC CGA GTA GTG TT-3'
inner reverse	5'-GGA GCA GTC CTT CGT GAC ATG-3'
HCV NS5A ISDR[a]	
outer forward	5'-TTC CAC TAC GTG ACG GGC AT-3'
outer reverse	5'-CCC GTC CAT GTG TAG GAC AT-3'
inner forward	5'-GGG TCACAG CTC CCA TGT GAG CC-3'
inner reverse	5'-GAG GGT TGT AAT CCG GGC GTG C-3'

[a]Interferon sensitivity determining region.

Results

IL28B SNP genotype and mRNA expression levels of ISGs in liver samples

We genotyped two SNPs (rs8099917 and rs12979860) in the *IL28B* locus, which have been reported to affect the outcome of the therapy, and compared them with mRNA expression levels in ISGs. Because of linkage disequilibrium, the results are the same for both SNPs, and thus only results for rs8099917 are presented. Other SNPs in this locus for the association with therapy outcome were several orders of magnitude less significant (data not shown). Expression levels of *IL28* mRNA in blood cells have been reported to be significantly higher in the patients homozygous for the response favorable allele (rs8099917 TT or rs12979860 CC) in peripheral blood [19,20]. However, our results showed that expression levels of *IL28* mRNA in the liver were significantly lower in rs8099917 TT patients (Table 3). Furthermore, hepatic mRNA levels of each of the major anti-viral ISGs, i.e., *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly lower in rs8099917 TT patients (Table 3). In contrast, expression levels of *SOCS1*, which functions as a repressor of interferon signaling, did not differ significantly between the two groups of patients (Table 3).

IL28B SNP genotype and mRNA expression levels of ISGs in peripheral blood

We examined mRNA expression levels in blood cells. In contrast to liver expression levels, mRNA expression levels of *IL28* and other ISGs were not statistically different between the two groups of patients (Table 3). *IL28B* mRNA levels, as well as four of the five ISGs, were only slightly higher in rs8099917 TT patients (Table 3).

MxA protein levels in peripheral mononuclear cells

We examined the levels of MxA protein in the peripheral mononuclear cells of 43 patients with genotype 1b chronic hepatitis C who were treated with combination therapy. In this case, consistent with previous reports [19,20], the protein levels of MxA were marginally higher in patients homozygous for the major allele (Fig. 3). Furthermore, MxA protein levels in these patients were significantly higher two days after the beginning of therapy (Fig. 1).

IL28 locus genotypes and the effect of combination therapy

Fifty-two patients with chronic hepatitis C genotype 1b were treated with combination therapy. Numbers of SVR, relapser, and NVR patients were 25 (48%), 19 (37%) and 8 (15%), respectively. Responses to therapy by rs8099917 genotype are noted in Table 1. SVR was most frequent in rs8099917 TT patients.

Effect of the combination therapy and mRNA expression levels

As shown in Fig. 2, when patients were divided into VR (SVR + relapser) and NVR categories, expression levels of *IL28*, *MxA*, *PKR*, *OAS1*, and *ISG15* were significantly higher in NVR patients (Fig. 2). There was no significant difference in *SOCS1* mRNA expression between the two groups of patients. Similarly, when patients were classified as SVR and non-SVR (relapser and