Table 2 Information regarding candidate genes and genotyping of tag SNPs

Role	Gene product	Gene symbol	Location	Functional details	Tag SNP	Typing method (restriction enzyme)
Synthetic enzyme	CYP7A1	*CYP7A1*	8q11–q12	Rate–limiting enzyme determining total bile acid pool size	rs8192879	PCR–RFLP (*Hpy*188 I)
					rs11786580	PCR–RFLP (*Fnu*4H I)
					rs6997473	PCR–RFLP (*Mse* I)
					rs3747809	PCR-HRM
					rs8192875	PCR–RFLP (*Mse* I)
					rs1457043	PCR–RFLP (*Hpy*CH4 III)
					rs8192870	PCR-direct DNA sequencing
					rs3808607	PCR–RFLP (*Alw*26 I)
					rs3824260	PCR–RFLP (*Hpy*CH4 IV)
Activators	HNF4α	*HNF4A*	20q13.12	Orphan nuclear receptor activating *CYP7A1* expression as a transcription factor	rs2071197	PCR–RFLP (*Hpy*CH4 IV)
					rs3212180	PCR–RFLP (*Bsr* I)
					rs6017340	PCR–RFLP (*Bso*B I)
					rs6031587	PCR–RFLP (*Ava* II)
					rs11574736	PCR–RFLP (*Taq* I)
					rs6031590	PCR–RFLP (*Bst*U I)
					rs3746575	PCR–RFLP (*Hae* III)
	PGC-1α	*PPARGC1A*	4q15.1	Coactivator enhancing HNF4α activity	rs8192678	PCR–RFLP (*Msp* I)
					rs12374310	PCR–RFLP (*Hsp*I92 II)
					rs4235308	PCR–RFLP (*Hpy*CH4 IV)
Repressors	FXR	*NR1H4*	12q23.1	Bile acid–activated nuclear receptor repressing *CYP7A1* via induction of SHP and FGF19	rs12304867	PCR–RFLP (*Hinf* I)
					rs3789988	PCR–RFLP (*Ban* I)
					rs56163822	PCR–RFLP (*Fok* I)
					rs1327099	PCR-direct DNA sequencing
					rs12424084	PCR–RFLP (*Hsp*92 II)
					rs11110411	PCR-HRM
					rs17030285	PCR–RFLP (*Eco*O109 I)
					rs17030306	PCR–RFLP (*Fok* I)
					rs10860603	PCR–RFLP (*Hpy*CH4 IV)
					rs1030454	PCR–RFLP (*Bst*4C I)
					rs35735	PCR–RFLP (*Eco*91 I)
	SHP	*NR0B2*	1p36.1	Orphan nuclear receptor repressing *CYP7A1* expression	rs7504	PCR–RFLP (*Alw*21 I)
	GPS2	*GPS2*	17p13	Corepressor interacting with SHP	rs2292065	PCR–RFLP (*Pvu* II)
					rs2270981	PCR-HRM
					rs8610	PCR–RFLP (*Hinf* I)
	PXR	*NR1I2*	3q12–q13.3	Bile acid–activated nuclear receptor repressing *CYP7A1* expression	rs3814055	PCR-HRM
					rs2472677	PCR–RFLP (*Hpy*188 I)
					rs7643645	PCR–RFLP (*Bsr*D I)
					rs2472681	PCR–RFLP (*Hpy*188 III)
					rs2472682	PCR–RFLP (*Hsp*92 II)
					rs6785049	PCR–RFLP (*Hph* I)
					rs3814057	PCR–RFLP (*Dde* I)
	FGF19	*FGF19*	11q13	Hormone binding to and activating FGFR4	rs948992	PCR–RFLP (*Bts*C I)
					rs1789364	PCR–RFLP (*Fok* I)
	FGFR4	*FGFR4*	5q35	Receptor repressing *CYP7A1* via its downstream signals	rs351855	PCR–RFLP (*Bcn* I)
	KLB	*KLB*	4p14	Co-receptor working with FGFR4	rs17618244	PCR–RFLP (*Msp* I)
					rs4975017	PCR-HRM
	FOXO1	*FOXO1*	13q14.1	Insulin-activated transcription factor repressing *CYP7A1* expression	rs17592236	PCR–RFLP (*Ava* II)
					rs2755209	PCR-HRM
					rs12865518	PCR-HRM
					rs2995991	PCR–RFLP (*Tsp*R I)
					rs12585434	PCR–RFLP (*Bsl* I)
					rs2721044	PCR–RFLP (*Hinf* I)

PCR polymerase chain reaction, *RFLP* restriction fragment length polymorphism, *HRM* high resolution melting curve analysis

fragments carrying a G or T allele at the rs3808607 SNP site, a 384-bp fragment of the *CYP7A1* promoter region was amplified by PCR from genomic DNA of PBC patients with a G/G or T/T homozygous genotype using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and integrated into the pGL3-Basic plasmids (Promega, Madison, WI, USA) at the *Kpn* I-*Xho* I restriction site using a Rapid DNA Ligation Kit (Roche Diagnostics, Mannheim, Germany). Finally, direct-DNA sequencing was carried out in order to confirm the insertion of the *CYP7A1* promoter region into the reporter gene plasmid vectors.

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10 % fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA). For transient transfection studies, 4.0×10^5 cells were subcultured in each well of a 12-well plate with 1 ml of D-MEM without FBS. When the cells had reached ~60 % confluence, transfection was performed with 1 μg of either pGL3-CYP7A1-G or pGL3-CYP7A1-T using 2.5 μl of X-tremeGENE™ HP transfection reagent (Roche Diagnostics). In addition, 100 ng of pRL-TK (Promega) was added to each transfection fluid as a transfection control for normalization. Forty hours after transfection, HepG2 cells were treated with 0, 25, and 50 μM of CDCA (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured for an additional 24 h and then lysed. Subsequently, luciferase assays were performed using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly and Renilla luciferase intensities were measured by ARVO™ MX 1420 (PerkinElmer, Inc., Waltham, MA, USA). The relative intensity of the Firefly enzyme signal was normalized to that of the Renilla enzyme signal in order to adjust for variations in transfection efficiencies. All experiments were performed in triplicate.

Statistical analysis

Differences in age and the observation period between early- and late-stage PBC patients were evaluated using an unpaired Student's *t* test and Mann–Whitney *U* test, respectively. Likewise, differences in gender and the concomitance of autoimmune diseases were compared by a chi-square test or Fisher's exact test. The unpaired Student's *t* test was used for a comparison of reporter gene expressions. All statistical analyses were performed using the PASW 18 statistical software package (SPSS Japan Inc., Tokyo, Japan).

To determine whether each SNP was in Hardy–Weinberg equilibrium among PBC patients, a chi-square test with Yates' correction was performed using the SNPAlyze® 7.1 standard software package. The frequencies of allele, genotype, haplotype, and diplotype between subgroups of PBC patients were compared by a chi-square test or Fisher's exact test with odds ratio (OR) and 95 % confidence interval (CI) in three different inheritance models—the allele, the minor allele dominant, and the minor allele recessive—using the SNPAlyze® 7.1 standard software package. A P value of less than 0.05 was considered to be statistically significant.

Results

Comparison of PBC patient characteristics

The characteristics were compared between early and late stage PBC patients (Table 1). The mean age and observation period of patients in late stage were significantly older and longer, respectively, than those of patients in early stage ($P < 0.005$ and $P < 0.001$, respectively). These results indicate that some early stage patients might progress to late stage in the future. Meanwhile, there were no significant differences in sex, treatment, and the concordance of autoimmune diseases between the two stages.

Association of genes related to bile acid synthesis with PBC progression

The distributions of alleles and genotypes at 52 tag SNPs in 11 candidate genes were compared between early and late stage PBC patients (data not shown). One tag SNP, rs12304867, in *NR1H4* was not in Hardy–Weinberg equilibrium (data not shown), and was therefore excluded from the association study. Three genes, *CYP7A1*, *HNF4A*, and *PPARGC1A*, showed a significant association with PBC progression (Table 3).

With regard to *CYP7A1*, four tag SNPs (rs1457043, rs8192870, rs3808607, and rs3824260) showed significant differences in allele and/or genotype frequencies in three different inheritance models between early and late stage PBC patients. At rs1457043, the frequencies of a minor A allele in the allele model ($P = 0.025$, OR = 0.662) and its homozygous A/A genotype in the minor allele recessive model ($P = 0.007$, OR = 0.328) were lower in late stage PBC patients as compared to those in early stage patients (Table 3), indicating that the A allele and the A/A genotype of rs1457043 in *CYP7A1* had a protective effect against PBC progression. Conversely, a major G allele and its homozygous G/G genotype or heterozygous G/A genotype of rs1457043 implicated susceptibility to PBC progression. Likewise, the patients possessing either a major homozygous G/G genotype or heterozygous G/T genotype of rs3808607, or a major homozygous A/A genotype or

Table 3 Allele and genotype comparisons in three inheritance models between early and late stage PBC patients in tag SNPs associated with the progression

Gene symbol	Tag SNP (Major > Minor)	Genotype	Number of genotypes (%)		Inheritance model[a]	*P* value	OR	95 % CI
			Early stage	Late stage				
CYP7A1		MAF	0.44	0.34	Allele	0.025	0.662	0.460–0.952
	rs1457043	G/G	74 (32.5)	34 (39.1)				
	(G > A)	G/A	106 (46.5)	46 (52.9)	Dominant	0.268	0.749	0.449–1.250
		A/A	48 (21.1)	7 (8.0)	Recessive	0.007	0.328	0.142–0.757
		MAF	0.16	0.24	Allele	0.022	1.643	1.071–2.518
	rs8192870	C/C	159 (69.7)	49 (56.3)				
	(C > A)	C/A	64 (28.1)	34 (39.1)	Dominant	0.025	1.787	1.074–2.974
		A/A	5 (2.2)	4 (4.6)	Recessive	0.267	2.149	0.564–8.198
		MAF	0.50	0.43	Allele	0.084	0.734	0.516–1.043
	rs3808607	G/G	56 (24.6)	24 (27.6)				
	(G > T)	G/T	115 (50.4)	52 (59.8)	Dominant	0.581	0.855	0.489–1.494
		T/T	57 (25.0)	11 (12.6)	Recessive	0.017	0.434	0.216–0.874
		MAF	0.50	0.43	Allele	0.114	0.753	0.530–1.071
	rs3824260	A/A	60 (26.3)	23 (26.4)				
	(A > G)	A/G	110 (48.2)	54 (62.1)	Dominant	0.983	0.994	0.568–1.740
		G/G	58 (25.4)	10 (11.5)	Recessive	0.007	0.381	0.185–0.785
HNF4A		MAF	0.20	0.29	Allele	0.012	1.663	1.116–2.479
	rs6017340	C/C	145 (63.6)	46 (52.9)				
	(C > T)	C/T	75 (32.9)	31 (35.6)	Dominant	0.082	1.557	0.944–2.567
		T/T	8 (3.5)	10 (11.5)	Recessive	0.012	3.571	1.360–9.377
		MAF	0.43	0.32	Allele	0.012	0.624	0.431–0.903
	rs6031587	C/C	68 (29.8)	39 (44.8)				
	(C > T)	C/T	126 (55.3)	41 (47.1)	Dominant	0.012	0.523	0.314–0.870
		T/T	34 (14.9)	7 (8.0)	Recessive	0.105	0.499	0.213–1.173
PPARGC1A		MAF	0.44	0.53	Allele	0.054	1.411	0.994–2.003
	rs8192678	G/G	73 (32)	15 (17.2)				
	(G > A)	G/A	108 (47.4)	52 (59.8)	Dominant	0.009	2.261	1.214–4.211
		A/A	47 (20.6)	20 (23.0)	Recessive	0.645	1.150	0.635–2.081

MAF minor allele frequency, *OR* odds ratio, *CI* confidence interval

[a] Allele, allele model; Dominant, the minor allele dominant model; Recessive, the minor allele recessive model

heterozygous A/G genotype of rs3824260 were at risk for PBC progression. At the remaining tag SNP, rs8192870, the frequencies of a minor A allele in the allele model ($P = 0.022$, OR = 1.643) and its minor homozygous A/A genotype or heterozygous C/A genotype in the minor allele dominant model ($P = 0.025$, OR = 1.787) were increased in late stage patients (Table 3), indicating susceptibility to PBC progression. Taken together, the G allele and G/G or G/A genotype of rs1457043, A allele and A/A or C/A genotype of rs8192870, G/G or G/T genotype of rs3808607, or A/A or A/G genotype of rs3824260 showed a genetic risk factor for PBC progression.

With respect to *HNF4A*, at rs6017340, the frequencies of a minor T allele in the allele model ($P = 0.012$, OR = 1.663) and its homozygous T/T genotype in the minor allele recessive model ($P = 0.012$, OR = 3.571) were higher in late stage PBC patients as compared to those in early stage patients (Table 3), indicating that the T allele and the T/T genotype of rs6017340 in *HNF4A* conferred susceptibility to PBC progression. Whereas, at rs6031587, the frequencies of a minor T allele in the allele model ($P = 0.012$, OR = 0.624) and its homozygous T/T genotype or heterozygous C/T genotype in the minor allele dominant model ($P = 0.012$, OR = 0.523) were decreased in late stage patients (Table 3). Conversely, a major C allele and its homozygous C/C genotype of rs6031587 implicated susceptibility to PBC progression. Thus, the T allele and the T/T genotype of rs6017340 and the C allele and the C/C genotype of rs6031587 were considered to be genetic risk factors for PBC progression.

Table 4 Allele and genotype comparisons in three inheritance models between responders and non-responders to PBC treatment in tag SNPs associated with the progression

Gene symbol	Tag SNP (Major > Minor)	Genotype	Number of genotypes (%)		Inheritance model[a]	*P* value	OR	95 % CI
			Responder	Non-responder				
CYP7A1		MAF	0.44	0.29	Allele	0.011	0.520	0.312–0.867
	rs1457043	G/G	74 (32.5)	20 (48.8)				
	(G > A)	G/A	106 (46.5)	18 (43.9)	Dominant	0.044	0.505	0.258–0.988
		A/A	48 (21.1)	3 (7.3)	Recessive	0.049	0.296	0.088–1.001
		MAF	0.16	0.26	Allele	0.040	1.777	1.020–3.095
	rs8192870	C/C	159 (69.7)	23 (56.1)				
	(C > A)	C/A	64 (28.1)	15 (36.6)	Dominant	0.086	1.803	0.915–3.554
		A/A	5 (2.2)	3 (7.3)	Recessive	0.106	3.521	0.808–15.347
		MAF	0.50	0.38	Allele	0.038	0.603	0.372–0.976
	rs3808607	G/G	56 (24.6)	14 (34.1)				
	(G > T)	G/T	115 (50.4)	23 (56.1)	Dominant	0.198	0.628	0.308–1.280
		T/T	57 (25.0)	4 (9.8)	Recessive	0.041	0.324	0.111–0.950
		MAF	0.50	0.38	Allele	0.050	0.619	0.382–1.002
	rs3824260	A/A	60 (26.3)	14 (34.1)				
	(A > G)	A/G	110 (48.2)	23 (56.1)	Dominant	0.301	0.689	0.339–1.400
		G/G	58 (25.4)	4 (9.8)	Recessive	0.027	0.317	0.108–0.927
HNF4A		MAF	0.20	0.29	Allele	0.058	1.660	0.979–2.815
	rs6017340	C/C	145 (63.6)	22 (53.7)				
	(C > T)	C/T	75 (32.9)	14 (34.1)	Dominant	0.227	1.509	0.772–2.950
		T/T	8 (3.5)	5 (12.2)	Recessive	0.017	3.819	1.184–12.326
		MAF	0.43	0.27	Allele	0.008	0.495	0.294–0.835
	rs6031587	C/C	68 (29.8)	21 (51.2)				
	(C > T)	C/T	126 (55.3)	18 (43.9)	Dominant	0.007	0.405	0.206–0.795
		T/T	34 (14.9)	2 (4.9)	Recessive	0.131	0.293	0.068–1.269
PPARGC1A		MAF	0.44	0.46	Allele	0.117	0.687	0.426–1.101
	rs8192678	G/G	73 (32)	7 (17.1)				
	(G > A)	G/A	108 (47.4)	24 (58.5)	Dominant	0.054	2.288	0.968–5.405
		A/A	47 (20.6)	10 (24.4)	Recessive	0.586	1.242	0.569–2.715

MAF minor allele frequency, *OR* odds ratio, *CI* confidence interval

[a] Allele, allele model; Dominant, the minor allele dominant model; Recessive, the minor allele recessive model

Finally, the number of the patients possessing an A/A genotype or G/A genotype of rs8192678 in *PPARGC1A* was increased in late stage as compared to that in early stage ($P = 0.009$, OR = 2.261; Table 3), indicating that the patients possessing the A/A or G/A genotype at rs8192870 had a genetic risk for PBC progression.

Association of genes related to bile acid synthesis with response to PBC treatment

During the observation period, 41 of 269 patients who were initially diagnosed as early stage progressed to late stage. Since these 41 patients could be considered to be resistant to PBC treatment, we defined these 41 patients as non-responders and the remaining 228 patients as responders to PBC treatment, and investigated whether the SNPs associated with PBC progression were related to response to the treatment. The six SNPs (rs1457043, rs8192870, rs3808607, rs3824260, rs6017340, and rs6031587) of the seven SNPs associated with PBC progression were also significantly associated with response to PBC treatment (Table 4). In regard to all seven SNPs, some parts of patients with the risk genotype for PBC progression showed to be non-responders to the treatment.

Association of CYP7A1 and HNF4A haplotypes with PBC progression

Subsequently, ten haplotypes composed of four tag SNPs in *CYP7A1* and three haplotypes composed of two tag SNPs in *HNF4A*, which displayed significant association with PBC progression in the individual SNP study and

Table 5 *CYP7A1* haplotype comparison in three inheritance models between early and late stage PBC patients

Gene symbol	*rs1457043-rs8192870-rs3808607-rs3824260*	Number of haplotypes (%)		Allele model[a]			Dominant model[b]			Recessive model[b]		
		Early stage	Late stage	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI
CYP7A1	A-C-T-G	192 (42.0)	59 (33.9)	0.060	0.705	0.490–1.016	0.397	0.803	0.483–1.334	0.015	0.366	0.158–0.847
	G-C-G-A	149 (32.9)	57 (32.8)	0.984	0.996	0.687–1.446	0.880	1.039	0.633–1.705	0.848	0.928	0.430–2.000
	G-A-G-A	70 (15.1)	42 (24.1)	0.010	1.755	1.141–2.699	0.013	1.904	1.142–3.174	0.223	2.699	0.660–11.039
	G-C-T-G	32 (7.1)	14 (8.1)	0.657	1.159	0.603–2.223	0.644	1.175	0.593–2.326	–	–	–
	Others	13 (3.0)	2 (1.2)	–	–	–	–	–	–	–	–	–

OR odds ratio, *CI* confidence interval

[a] Each haplotype was compared with other haplotypes combined

[b] Dominant model, the haplotype dominant model; Recessive model, the haplotype recessive model

Table 6 *HNF4A* haplotype comparison in three inheritance models between early and late stage PBC patients

Gene symbol	rs6017340-rs6031587	Number of haplotypes (%)		Allele model[a]			Dominant model[b]			Recessive model[b]		
		Early stage	Late stage	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI
HNF4A	C-T	194 (42.5)	55 (31.6)	0.012	0.624	0.431–0.903	0.012	0.523	0.314–0.870	0.105	0.499	0.213–1.173
	C-C	171 (37.5)	68 (39.1)	0.715	1.069	0.747–1.530	0.767	1.080	0.648–1.801	0.758	1.116	0.554–2.250
	T-C	91 (20.0)	51 (29.3)	0.012	1.663	1.116–2.479	0.082	1.557	0.944–2.567	0.006	3.571	1.360–9.379

OR odds ratio, *CI* confidence interval

[a] Each haplotype was compared with other haplotypes combined

[b] Dominant model, the haplotype dominant model; Recessive model, the haplotype recessive model

were located within the same LD block (supplementary figure), were constructed and identified using the SNPAlyze® 7.1 standard software package. The frequencies of haplotypes and diplotypes of *CYP7A1* and *HNF4A* were compared between early and late stage PBC patients (Tables 5, 6, respectively).

With respect to *CYP7A1* haplotypes, A-C-T-G and G-A-G-A haplotypes were significantly associated with PBC progression (Table 5). The frequency of the A-C-T-G homozygous diplotype (A-C-T-G/A-C-T-G) in the recessive model was decreased in late stage PBC patients as compared to that in early stage patients ($P = 0.015$, OR = 0.366), indicating that the A-C-T-G homozygous diplotype of *CYP7A1* conferred protection against PBC progression. On the other hand, the frequencies of the G-A-G-A haplotype in the allele model ($P = 0.010$, OR = 1.755) and its homozygous or heterozygous diplotype (G-A-G-A/any) in the dominant model ($P = 0.013$, OR = 1.904) were increased in late stage patients, indicating that the G-A-G-A haplotype and its homozygous or heterozygous diplotype of *CYP7A1* conferred susceptibility to PBC progression.

In the analysis of *HNF4A* haplotypes, C-T and T-C haplotypes were significantly associated with PBC progression (Table 6). The frequencies of the C-T haplotype in the allele model ($P = 0.012$, OR = 0.624) and its homozygous or heterozygous diplotype (C-T/any) in the dominant model ($P = 0.012$, OR = 0.523) were decreased in late stage patients in comparison to that in early stage patients, indicating that the C-T haplotype and its homozygous or heterozygous diplotype of *HNF4A* conferred protection against PBC progression. Meanwhile, the frequencies of the T-C haplotype in the allele model ($P = 0.012$, OR = 1.663) and its homozygous diplotype (T-C/T-C) in the recessive model ($P = 0.006$, OR = 3.571) were increased in late stage patients, indicating that the T-C haplotype and its homozygous diplotype of *HNF4A* conferred susceptibility to PBC progression.

A functional SNP in CYP7A1 affects its expression in cholestasis

In order to investigate the transcriptional activity of the *CYP7A1* promoter carrying the G allele of rs3808607 as compared to that of another *CYP7A1* promoter carrying the T allele at the same SNP site, a dual luciferase assay was performed using HepG2 cells maintained under normal or cholestatic conditions. Under normal conditions (0 μM CDCA) of bile acid homeostasis, the relative luciferase

intensity of the *CYP7A1* promoter carrying the G allele at rs3808607 was higher than that of the promoter carrying the T allele (Fig. 1). These results supported previously published data [29].

By contrast, under cholestatic conditions (25 and 50 μM CDCA), the *CYP7A1* promoters carrying the G or T allele of rs3808607 tended towards decreased relative luciferase intensities as compared to those obtained under normal conditions in statistical analysis which did not reach statistical significance (Fig. 1). This decrease in transcription activities under cholestatic conditions might reflect diminution of *CYP7A1* expression by the negative feedback regulation mechanism. Interestingly, the relative luciferase intensities obtained with the promoter carrying the G allele were significantly higher than those obtained with the promoter carrying the T allele at both 25 and 50 μM concentrations of CDCA ($P = 0.003$ and $P = 0.007$, respectively).

Discussion

In this study, we demonstrated an association of polymorphisms of the genes *CYP7A1*, *HNF4A*, and *PPARGC1A* with PBC progression. The functions of the progression-associated genes are related to activators of bile acid synthesis in hepatocytes. On the other hand, there were no associations between PBC progression and polymorphisms of other genes that encode repressors of bile acid synthesis via negative feedback regulation. In addition to the association, a reporter gene assay showed that rs3808607, one of the progression-associated SNPs in *CYP7A1*, differently modulated *CYP7A1* promoter activity under normal and cholestatic conditions in vitro. In some PBC patients, the increase of synthetic bile acids may affect the response to UDCA as well as PBC progression. However, the reproducibility of this association in other groups of Japanese PBC patients as well as in other ethnicities remains to be investigated.

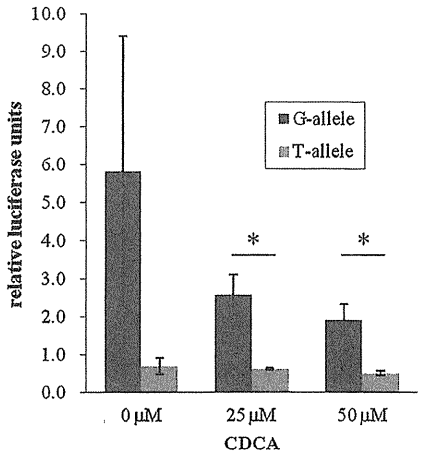

Fig. 1 Transcriptional activities of the *CYP7A1* promoter carrying either the G- or the T-allele at rs3808607 at 0, 25, and 50 μM concentrations of CDCA. Firefly luciferase signals were normalized to those of Renilla luciferase. Data shown represent mean ± standard deviation ($n = 3$). *$P < 0.01$

CYP7A1 is the first and rate-limiting enzyme in the classical bile acid synthetic pathway, and also plays a role in cholesterol catabolism and intestinal lipid absorption. Previous studies have demonstrated associations between genetic variants of *CYP7A1* and clinical phenotypes, including blood cholesterol levels and response to cholesterol-lowering drugs [30]. In addition, *CYP7A1* expression is negatively regulated in the liver of patients with cholestatic liver diseases including PBC [19, 20, 31] via the negative feedback regulation mechanism [15]. Thus, it is reasonable to speculate that the genetic variants of *CYP7A1* that were associated with PBC progression in this study, e.g., the G/G or G/A genotype of rs1457043, A/A or C/A genotype of rs8192870, G/G or G/T genotype of rs3808607, and A/A or A/G genotype of rs3824260, may enhance the expression and activities of CYP7A1, thereby leading to the accumulation of synthetic bile acids in hepatocytes. In particular, with respect to rs3808607 located within the *CYP7A1* promoter region, the transcriptional activity of the *CYP7A1* promoter carrying the G allele, which was the risk allele for PBC progression, was persistently higher under both normal and cholestatic conditions as compared to that of the promoter carrying the T allele. Although the activity of the *CYP7A1* promoter at rs3808607 has already been reported only under normal conditions [29], here we assessed the transcriptional activity of this promoter under not only normal conditions, but also experimental cholestatic conditions. Taken together, the data suggest that the genetic variants of *CYP7A1*, including rs3808607, may accelerate bile acid synthesis, thereby resulting in the accumulation of bile acids in hepatocytes, although it is unknown how much negative feedback regulation contributes to reduction of bile acid synthesis in bile acid homeostasis. The persistent accumulation of bile acids may attribute to the predisposition of PBC progression at any stage.

Another possible mechanism connecting progression-associated genes to PBC progression is the resistance to UDCA treatment. We also demonstrated that progression-associated SNPs of *CYP7A1* and *HNF4A* also showed association with response to PBC treatment, mainly to UDCA. This fact suggests that some parts of progressions we observed in patients who progressed from early to late stages during the observation period may be attributed to UDCA resistance. Under cholestatic conditions, in addition to negative feedback regulation by cholestasis as a

normally pathophysiological mechanism, UDCA also represses *CYP7A1* expression [21]. However, because the dual luciferase assay in this study revealed the higher transcriptional activities of the *CYP7A1* promoter carrying the risk-associated G allele in the experimental cholestatic conditions, UDCA may reduce the repression of *CYP7A1* expression in PBC patients bearing this allele at rs3808607. Furthermore, UDCA may also diminish a decrease in the proportion of potentially toxic hydrophobic bile acids, such as CDCA, to the total of biliary bile acids [32] due to the accumulation of synthetic endogenous bile acids. Thus, overexpression of *CYP7A1* and elevation of the proportion of hydrophobic bile acids in PBC patients with the genetic variants of *CYP7A1* may decrease therapeutic effects of UDCA, thereby resulting in the acceleration of PBC progression.

The *CYP7A1* promoter is activated by interaction of the orphan nuclear receptor HNF4α with PGC-1α, a versatile coactivator that also engages with other nuclear receptors, such as nuclear respiratory factor-1, peroxisome proliferator-activated receptor (PPAR) α and γ [33]. We identified an association between PBC progression and G1444A polymorphism of rs8192678, located in exon 8 of *PPARGC1A*, that leads to an amino acid change (Gly482Ser). A previous report has shown that the amino acid sequences around 482Ser are highly conserved among mammals, and that Gly482 has impaired coactivator activity towards the mitochondrial transcription factor A promoter and the PPAR responsive element in the reporter gene assay [26]. The fact that the frequency of 482Ser was increased in late stage PBC patients supports our hypothesis that the possession of the risk A allele of rs8192678 in *PPARGC1A*, which encodes for 482Ser, may accelerate *CYP7A1* transcriptional activities, resulting in the predisposition to PBC progression.

The mean age and the observation period of patients in the late stage group were higher and longer, respectively, than those in the early stage group. There is a possibility that some patients in the early stage group would progress to late stage in the future. This study, however, is a part of PBC cohort study to be continued. Thus, in the future study, we will get the conclusive results by adjusting age and observation period between the two groups.

In conclusion, we demonstrated the association of *CYP7A1* and its transcriptional activators, i.e., HNF4α with PBC progression. In addition, we demonstrated that one SNP in *CYP7A1* affected the expression of *CYP7A1* in both normal and cholestatic conditions in vitro. Bile acid derivatives are anticipated to exert therapeutic effects on cholestasis, and clinical trials have been conducted using these therapeutic agents to treat cholestatic liver diseases [34]. In addition to these bile acid derivatives, regulation of *CYP7A1* expression is considered to be an attractive therapeutic target. Thus, the genes identified in this study may not only modulate the therapeutic effect of certain drugs, but may also indicate susceptibility to PBC progression. Specifically, the tag SNPs that are associated with PBC progression to late stage have the potential to serve as new genetic biomarkers for PBC progression in Japanese patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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BRIEF ARTICLE

Association of *ITPA* polymorphism with outcomes of peginterferon-α plus ribavirin combination therapy

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Abstract

AIM: To analyzed the association between inosine triphosphatase (*ITPA*) (rs1127354) genotypes and sustained virological response (SVR) rates in peginterferon (Peg-IFN)α + ribavirin (RBV) treatment.

METHODS: Patients who underwent Peg-IFNα + RBV combination therapy were enrolled (n = 120) and they had no history of other IFN-based treatments. Variation in hemoglobin levels during therapy, cumulative reduction of RBV dose, frequency of treatment withdrawal, and SVR rates were investigated in each *ITPA* genotype.

RESULTS: In patients with *ITPA* CC genotype, hemoglobin decline was significantly greater and the percentage of patients in whom total RBV dose was < 60% of standard and/or treatment was withdrawn was significantly higher compared with CA/AA genotype. However, SVR rates were equivalent between CC and CA/AA genotypes, and within a subset of patients with Interleukin 28B (*IL28B*) (rs8099917) TT genotype, SVR rates tended to be higher in patients with *ITPA* CC genotype, although the difference was not significant.

CONCLUSION: *ITPA* CC genotype was a disadvantageous factor for Peg-IFNα + RBV treatment in relation to completion rates and RBV dose. However, CC genotype was not inferior to CA/AA genotype for SVR rates. When full-length treatment is accomplished, it is plausible that more SVR is achieved in patients with *ITPA* CC variant, especially in a background of *IL28B* TT genotype.

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Key words: Chronic hepatitis C; Interleukin 28B; Inosine triphosphatase; Peginterferon; Ribavirin

Core tip: Inosine triphosphatase (*ITPA*) polymorphism at rs1127354 is significantly associated with hemoglobin decline and reduction of ribavirin (RBV) during peginterferon-α + RBV therapy. However, the effect of the *ITPA* gene single-nucleotide polymorphism on treatment outcome is still unclear. In this study, *ITPA*

CC genotype (rs1127354) was not inferior to CA/AA genotype for sustained virological response rates although CC genotype was a disadvantageous factor for the treatment in relation to completion rates and RBV dose. When full-length treatment is accomplished, the SVR rate tended to be higher in patients with the CC genotype, especially in a subset of patients with the favorable TT genotype (rs8099917) of Interleukin 28B.

Fujino T, Aoyagi Y, Takahashi M, Yada R, Yamamoto N, Ohishi Y, Nishiura A, Kohjima M, Yoshimoto T, Fukuizumi K, Nakashima M, Kato M, Kotoh K, Nakamuta M, Enjoji M. Association of *ITPA* polymorphism with outcomes of peginterferon-α plus ribavirin combination therapy. *World J Gastrointest Pharmacol Ther* 2013; 4(3): 54-60 Available from: URL: http://www.wjgnet.com/2150-5349/full/v4/i3/54.htm DOI: http://dx.doi.org/10.4292/wjgpt.v4.i3.54

INTRODUCTION

Hepatitis C virus (HCV) genotype 1b accounts for around 70% of chronic hepatitis C in Japan[1,2]. A sustained virological response (SVR) in eliminating HCV RNA by peginterferon (Peg-IFN)α + ribavirin (RBV) combination therapy is attained in 40%-50% of individuals with HCV-1b[3-5]. Triple therapy using Peg-IFNα + RBV + telaprevir is anticipated to be effective for SVR in approximately 75% of patients with HCV-1b[6-8]. It is known that polymorphisms located upstream of the Interleukin 28B (*IL28B*) gene, encoding for λ or type Ⅲ interferon (IFN-λ), are major predictors of SVR in the Peg-IFNα-based combination therapies[9-12]. Two single-nucleotide polymorphisms (SNPs), rs8099917 TT genotype and rs12979860 CC genotype, have been independently associated with a higher rate of SVR following Peg-IFNα-based combination therapies in individuals with HCV-1b infection. IFN-λ is believed to upregulate the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway through interaction with a cellular transmembrane receptor, resulting in antiviral activity. In Japanese individuals, strong linkage disequilibrium is recognized between the two *IL28B* SNPs, rs8099917 and rs12979860, and 99% coincidence has been reported[13].

The most important adverse events of Peg-IFNα-based combination therapies include RBV-induced hemolytic anemia, which is severe enough to require dose reduction of RBV in 10%-20% of patients, and which may affect overall efficacy[3]. RBV-induced ATP depletion in red blood cells is believed to be a primary mechanism for RBV-induced hemolytic anemia. A genome-wide association study has shown a strong association between SNPs of the inosine triphosphatase (*ITPA*) gene in chromosome 20 and RBV-induced anemia in patients infected with HCV-1b[14]. Two functional SNPs, a missense variant in exon 2 (rs1127354) and a splicing altering variant in intron 2 (rs7270101), independently reduce the expression of *ITPA*, leading to inosine deficiency and protection against RBV-induced ATP depletion[15-18]. Accordingly, the protective genotypes, rs1127354 CA and AA as well as rs7270101 AC and CC, are associated with decreased *ITPA* activity, which confers protection against RBV-related ATP depletion and hemolytic anemia. The Japanese have the AA genotype exclusively at rs7270101, therefore the CC genotype at rs1127354 is a major predictor of RBV-induced anemia during antiviral combination therapy in Japanese patients infected with HCV-1b[18,19].

However, it is controversial whether *ITPA* (rs1127354) CC genotype, which induces heavier hemoglobin decline, affects therapeutic outcomes. From the standpoint of health economics, it is important to examine the significance of factors predicting viral response to antiviral treatments and therapeutic outcomes. In this study, Japanese patients infected with HCV-1b, who had experienced Peg-IFNα + RBV combination therapy, were retrospectively analyzed. Patients were divided into groups according to genotyping of *ITPA* rs1127354 and *IL28B* rs8099917. Our primary analysis was focused on the quantitative change from baseline in hemoglobin levels and platelet counts, cumulative reduction of RBV dose, frequency of treatment withdrawal, and estimation of treatment outcome.

MATERIALS AND METHODS

Study patients

This retrospective cohort study was performed in 120 patients with chronic HCV-1b infection who were treated with Peg-IFNα + RBV combination therapy at Kyushu Medical Center Hospital between January 2007 and December 2009. The patients met the following inclusion and exclusion criteria. Inclusion criteria were: (1) baseline serum HCV RNA levels > 5.0 log IU/mL; and (2) Japanese patients aged 20-65 years at study entry. Exclusion criteria were: (1) decompensated liver cirrhosis; (2) serum hepatitis B surface antigen; (3) hepatocellular carcinoma or its history; (4) autoimmune hepatitis, alcoholic liver disease, hemochromatosis, or chronic liver disease other than chronic hepatitis C; (5) chronic renal disease or creatinine clearance < 50 mL/min at baseline; (6) hemoglobin < 12 g/dL, neutrophil < 1500/μL or platelets < 100000/μL at baseline; and (7) history of receiving IFN-based treatment. All patients gave consent for analysis of SNPs in *ITPA* and *IL28B* genes. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of Kyushu Medical Center. Written informed consent was obtained from each patient.

Antiviral treatment

Peg-IFNα2b (1.5 μg/kg) or Peg-IFNα2a (180 μg) was injected subcutaneously once weekly. RBV (600-1000 mg/d) was administered after breakfast and dinner. The RBV dose was adjusted by body weight: 600 mg for < 60 kg; 800 mg for 60-80 kg; and 1000 mg for > 80 kg. As a standard combination therapy, Peg-IFNα and RBV were continued for 48 wk. Treatment duration was extended up to

Table 1 Baseline characteristics of patients

Baseline characteristics	*ITPA* polymorphism (rs1127354)		*P* value
	CA/AA (n = 37)	CC (n = 83)	
Age (yr)	61 ± 8	59 ± 11	NS
Gender: male/female	18/19	37/46	NS
HCV RNA (log IU/mL)	6.2 ± 0.6	5.9 ± 0.5	NS
Hemoglobin (g/dL)	13.4 ± 1.5	13.8 ± 1.7	NS
WBC ($\times 10^3/\mu L$)	4.7 ± 1.2	5.0 ± 1.5	NS
Platelet ($\times 10^4/\mu L$)	18.0 ± 6.0	18.0 ± 7.0	NS
AST (IU/L)	56.8 ± 34.9	58.2 ± 42.3	NS
ALT (IU/L)	65.5 ± 40.0	68.4 ± 56.8	NS
GGT (IU/L)	56.1 ± 52.3	55.3 ± 49.4	NS
AFP (ng/mL)	5.3 ± 4.0	24.2 ± 61.8	NS
Staging: $F_{1,2}/F_{3,4}$	19/16	49/27	NS
IL28B: TT/TG + GG	29/8	53/30	NS

ITPA: Inosine triphosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein; NS: Not significant; HCV: Hepatitis C virus; *IL28B*: Interleukin 28B.

72 wk in some patients in whom HCV RNA first became undetectable after week 12 but before week 48. SVR was defined as undetectable serum HCV RNA for 24 wk after treatment completion. Rapid virological response (RVR) and early virological response (EVR) were defined as undetectable serum HCV RNA at 4 wk and 12 wk of Peg-IFNα + RBV treatment, respectively. The RBV dose was reduced by 200 mg in patients receiving 600 or 800 mg (by 400 mg in those receiving 1000 mg) when hemoglobin decreased to < 12 g/dL, and by another 200 mg when it was < 10 g/dL. RBV was withdrawn or stopped temporarily when hemoglobin levels decreased to < 8.5 g/dL. Dose of Peg-IFNα2b (or Peg-IFNα2a) was reduced by 50% when the leukocyte count decreased to < 1500/μL, neutrophil count to < 750/μL, or platelet count to < 80000/μL; Peg-IFNα2b or Peg-IFNα2a was withdrawn when the above measures were decreased to < 1000/μL, < 500/μL or < 50000/μL, respectively.

Laboratory data

Hematological, biochemical, and virological parameters were determined by the clinical laboratory at Kyushu Medical Center. Serum HCV RNA concentrations were determined by the COBAS TaqMan polymerase chain reaction (PCR) HCV test (Roche Diagnostics, Tokyo, Japan). Genotyping for the *IL28B* (rs8099917) and *ITPA* (rs1127354) polymorphisms was performed by TaqMan SNP Genotyping Assays (Applied Biosystems, Branchburg, NJ, United States) that apply a PCR-based restriction fragment length polymorphism assay.

Statistical analysis

Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC, United States). Differences between categorical variables were analyzed using Fisher's exact test or χ^2 test. Mann-Whitney U test was used for continuous variables. Multivariate analysis was used to identify factors independently associated with the achievement of SVR.

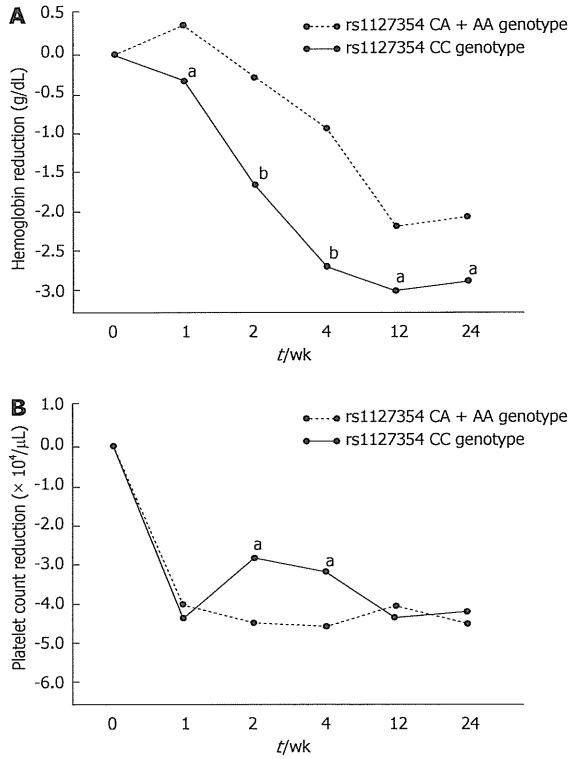

Figure 1 Chronological variation of hemoglobin levels (A) and platelet counts (B) in each inosine triphosphatase genotype at rs1127354. $^aP < 0.05$, $^bP < 0.01$ compared with CA/AA groups.

The OR and 95%CI were also calculated. $P < 0.05$ was considered to be statistically significant.

RESULTS

Association between ITPA deficiency and hemoglobin decline

Baseline characteristics of 120 enrolled patients are shown in Table 1. The study population included 83 patients with major (CC) genotype and 37 patients with minor (CA/AA) genotype of *ITPA* at rs1127354. Within listed items, no significant difference was seen between *ITPA* CC and CA/AA groups. Chronological variation of hemoglobin levels and platelet count during Peg-IFNα + RBV therapy is shown in Figure 1. As reported previously, hemoglobin decline was obvious in patients with *ITPA* CC genotype (rs1127354) and a significant difference was seen at week 1, 2, 4, 12 and 24 (Figure 1A), meaning that *ITPA* deficiency due to CA/CC genotype was associated with slower hemoglobin decline early in treatment. The greatest difference in mean hemoglobin reduction was found at week 4, while platelet reduction was temporally heavier in patients with *ITPA* CA/AA genotype at week 2 and 4 (Figure 1B). Leukocyte and neutrophil counts were equivalent between *ITPA* genotype CC and CA/AA

Table 2 Sustained virological response rates according to total ribavirin dose in each inosine triphosphatase genotype

ITPA genotype (rs1127354)	Patients with > 60% total RBV dose	Patients with < 60% total RBV dose	Total
CA + AA	48.3% (14/29)	12.5% (1/8)	40.5% (15/37)
CC	58.5% (31/53)	20.0% (6/30)	44.6% (37/83)

Each group includes patients in whom treatment was withdrawn. RBV: Ribavirin; *ITPA*: Inosine triphosphatase.

Table 3 Virological response according to classification by inosine triphosphatase and interleukin 28B single-nucleotide polymorphisms *n* (%)

Virological response	*IL28B*: TT		*IL28B*: TG + GG	
	CA + AA (*n* = 29)[1]	CC (*n* = 53)[1]	CA + AA (*n* = 8)[1]	CC (*n* = 30)[1]
RVR	3 (10.3)	10 (18.9)	0 (0.0)	4 (13.3)
RVR + EVR	18 (62.1)	35 (66.0)	1 (12.5)	8 (26.6)
SVR	13 (44.8)	29 (54.7)	2 (25.0)	8 (26.6)

[1]Inosine triphosphatase (*ITPA*). SVR: Sustained virological response; RVR: Rapid virological response; EVR: Early virological response; *IL28B*: Interleukin 28B.

groups during treatment (data not shown).

Treatment outcome in each genotype of ITPA

As a result of hepatocellular carcinoma, therapeutic inefficiency, or adverse events, such as depression, appetite loss, easy fatigability, retinal hemorrhage, and hemolytic anemia, Peg-IFNα + RBV therapy was discontinued in 18 patients with *ITPA* CC genotype (21.7%) and 6 patients with CA/AA genotype (16.2%). Moreover, serious reduction of RBV administration (< 60% of scheduled total dose) was compelled in significantly more patients with CC genotype compared with the CA/AA genotype. The percentage of patients receiving < 60% total RBV dose, including patients with treatment interruption/withdrawal, was significantly higher for the CC genotype (37.3% *vs* 21.6%, $P < 0.05$). To investigate the influence of dose reduction of Peg-IFN on treatment outcome, we also analyzed the dose of Peg-IFN administered for each rs1127354 genotype, and > 70% of the expected total dose was administered to all patients with treatment completion (data not shown). SVR rates were analyzed according to the total RBV dose and *ITPA* genotype (Table 2). In the whole population, SVR rates were higher in *ITPA* genotype CC than CA/AA genotype (44.6% *vs* 40.5%), although the difference was not significant. SVR rates tended to be higher for the CC genotype than the CA/AA genotype in patients with > 60% total RBV dose (58.5% *vs* 48.3%) or < 60% total RBV dose (20.0% *vs* 12.5%), but there were no significant differences between the *ITPA* genotypes.

SVR, RVR and EVR rates were determined for *IL28B* (rs8099917) and *ITPA* (rs1127354) genotypes (Table 3). In a subset of patients with *IL28B* TT genotype, RVR, RVR + EVR and SVR showed higher rates in patients with *ITPA* CC genotype compared with CA/AA genotype, although the difference was not significant. In a subset of patients with *IL28B* TG/GG genotype, SVR rates were equivalent between CC and CA/AA genotypes.

Table 4 Comparison of profile between sustained virological response and non-sustained virological response patients

Factors	SVR (*n* = 54)	non-SVR (*n* = 66)	*P* value
Age (yr)	57 ± 12	61 ± 9	< 0.05
Gender: male/female	21/33	33/33	NS
Body mass index (kg/m^2)	23.5 ± 4.1	22.6 ± 3.3	NS
HCV RNA (log IU/mL)	5.9 ± 0.6	6.1 ± 0.6	< 0.05
Hemoglobin (g/dL)	13.7 ± 1.3	13.8 ± 1.8	NS
WBC (× 10^3/mL)	4.7 ± 1.3	5.1 ± 1.5	NS
Platelet (× 10^4/mL)	20 ± 7	17 ± 6	< 0.05
AST (IU/L)	46.2 ± 25.8	66.7 ± 47.1	NS
ALT (IU/L)	56.1 ± 33.3	75.1 ± 61.1	NS
GGT (IU/L)	39.8 ± 24.1	67.4 ± 61.2	NS
AFP (ng/mL)	8.3 ± 19.8	10.1 ± 24.2	NS
Staging: $F_{1,2}/F_{3,4}$	12/40	28/30	< 0.01
72 wk treatment: +/-	10/44	14/52	NS
Ribavirin dose (%)[1]	90 ± 35	76 ± 41	NS
ITPA: CC/CA + AA	38/16	45/21	NS
IL28B: TT/TG + GG	44/10	38/28	< 0.01

[1]Percentage of ribavirin administration to the scheduled total dose of full-length treatment (48 or 72 wk). SVR: Sustained virological response; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein; ITPA: inosine triphosphatase; NS: Not significant; HCV: Hepatitis C virus; IL28B: Interleukin 28B.

Table 5 Multivariate analysis for predictive factors associated with SVR

Factors	Category	95%CI	*P* value
HCV RNA (log IU/mL)	≥ 6.0: 1.0 < 6.0: 3.94	1.42-10.95	0.008
IL28B (rs8099917)	TG + GG: 1.0 TT: 3.46	1.18-10.10	0.023

HCV: Hepatitis C virus; IL28B: Interleukin 28B; SVR: Sustained virological response.

When background of SVR and non-SVR patients was compared, there was a significant difference in age, HCV RNA concentrations, platelet counts, staging, and *IL28B* SNPs, but not in *ITPA* SNPs (Table 4). Table 5 shows the result of multivariate analysis for predictive factors associated with SVR. The multivariate analysis proved that viral load (HCV RNA < 6.0 log IU/mL) and *IL28B* TT (rs8099917) were independent factors for SVR.

DISCUSSION

It has been shown that the SNP (rs8099917) in the *IL28B* gene is strongly associated with response to IFN-based therapy for chronic HCV-1b infection, and the SNP (rs1127354) in the *ITPA* gene predicts RBV-induced anemia in the Japanese population[19-23]. In this study, patients with *ITPA* (rs1127354) genotype CC showed a higher degree of hemoglobin reduction in response to Peg-IFNα + RBV treatment at week 1, 2, 4, 12 and 24 compared

with those with the CA/AA genotype (Figure 1A). The greatest difference in mean hemoglobin reduction was found at week 4. These findings confirmed the reported evidence that *ITPA* deficiency (rs1127354 CA/AA variants) renders protection against the development of RBV-induced hemoglobin decline in Japanese patients infected with HCV-1b[20-23]. The exact mechanism by which *ITPA* deficiency protects against RBV-induced hemolysis has yet to be resolved. One postulated mechanism for the development of anemia is the accumulation of triphosphorylated RBV in erythrocytes, causing eventual oxidative damage to erythrocyte membranes, and *ITPA* deficiency may confer protection against RBV-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by RBV in the biosynthesis of ATP[24-26].

Thrombocytopenia, which leads to poor treatment efficacy because of the initial or early dose reduction of Peg-IFNα, is one of the critical adverse events caused by IFN-based antiviral therapy. A previous study has reported that the *ITPA* (rs1127354) CA/AA genotype is independently associated with a greater reduction in platelet count as well as protection against the reduction in hemoglobin, whereas patients with the CC genotype have significantly less reduction in mean platelet count[27]. We also evaluated whether genetic variants in the *ITPA* gene were associated with IFN-induced thrombocytopenia. In this study, CC genotype showed lesser trend of reduction at week 2 and 4 compared with CA/AA genotype (Figure 1B). The result may support the association of *ITPA* gene SNP (rs1127354) with platelet decline in response to Peg-IFNα + RBV treatment.

Hemoglobin reduction often necessitates dose reduction of RBV and premature withdrawal from therapy, therefore the *ITPA* (rs1127354) genotype CC may be considered as a disadvantageous factor for Peg-IFNα + RBV treatment. However, although *ITPA* polymorphisms are significantly associated with RBV-induced anemia, their effect on therapeutic outcome is unclear. Some studies have shown no association[14,28-31], and others have reported a possible association with treatment outcomes in chronic hepatitis C patients[21,22]. In the present study, although there was no significant association between *ITPA* polymorphisms and treatment outcome, there was a trend towards higher SVR rates in patients with *ITPA* CC genotype, which seemed to contradict previous studies[21,22,28-31]. The different outcome among the institutes may be due to the difference of inclusion and/or exclusion criteria. In this study, the relationship between *IL28B* and *ITPA* variants were additionally analyzed on treatment outcome. When analyzed in the patients available for treatment outcome, all patients were administered > 70% of the scheduled total Peg-IFNα dose, but the incidence of RBV dose reduction (< 60% of the scheduled dose) and withdrawal was significantly higher in patients with the rs1127354 genotype CC. However, the rate of SVR tended to be higher in patients with the CC genotype, especially in a subset of patients with the favorable TT genotype at rs8099917 of *IL28B*, although the difference was not significant between the CC and CA/AA genotypes (Tables 2 and 3). Independent favorable predictors for SVR identified in multivariate analysis were low viral load (HCV RNA < 6.0 log IU/mL) and TT genotype at rs8099917 of *IL28B*, but not CC genotype at rs1127354 of *ITPA* (Table 5).

There were several limitations to this study. (1) Because of the small sample size which may have contributed to the loss of significance observed or some statistical errors, this study may be ranked at preliminary status; (2) Because of the retrospective nature of the study, enrolled patients may not represent the standard Japanese population infected with HCV; (3) Several other significant SNPs, which have been detected in *ITPA* as well as *IL28B*, may have influenced and distorted the results; and (4) Mutations in other genes and non-genetic factors that may affect response to antiviral therapy against chronic hepatitis C were not determined.

In conclusion, the SVR rates tended to be higher in patients with the CC genotype than the CA/AA genotype, especially in a subset of patients with *IL28B* (rs8099917) TT genotype, despite a higher rate of RBV dose reduction and treatment withdrawal. Multivariate analysis identified *IL28B* SNP (rs8099917) and HCV RNA as independent predictors of SVR. It is plausible that, in a background of *IL28B* (rs8099917) TT genotype, more SVR is achieved in patients with *ITPA* CC variant when full-length (duration of 48 or 72 wk) treatment is accomplished. These findings indicate that *ITPA* (rs1127354) CC genotype is by no means inferior to the CA/AA genotype for viral response to Peg-IFN + RBV combination therapy.

COMMENTS

Background

A single-nucleotide polymorphism (SNP) at rs1127354 of the inosine triphosphatase (ITPA) gene is associated with hemoglobin decline during peginterferon (Peg-IFN) + ribavirin (RBV) combination therapy in patients with hepatitis C virus infection. However, the effect of the ITPA gene SNP on treatment outcome has not been fully elucidated. Authors analyzed the association between ITPA (rs1127354) genotypes and sustained virological response (SVR) rates in Peg-IFNα + RBV treatment.

Research frontiers

ITPA CC genotype was a disadvantageous factor for Peg-IFNα + RBV treatment in relation to completion rates and RBV dose. However, CC genotype was not inferior to CA/AA genotype for SVR rates. When full-length treatment is accomplished, it is plausible that more SVR is achieved in patients with ITPA CC variant, especially in a background of Interleukin 28B (IL28B) TT genotype.

Innovations and breakthroughs

In patients with ITPA CC genotype, hemoglobin decline was significantly greater and the percentage of patients in whom total RBV dose was < 60% of standard and/or treatment was withdrawn was significantly higher compared with CA/AA genotype. However, SVR rates were equivalent between CC and CA/AA genotypes, and within a subset of patients with IL28B (rs8099917) TT genotype, SVR rates tended to be higher in patients with ITPA CC genotype, although the difference was not significant.

Peer review

The topic is interesting and relevant. The manuscript is well written and concise.

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Research Article

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Clinical milestones for the prediction of severe anemia by chronic hepatitis C patients receiving telaprevir-based triple therapy

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Background & Aims: Anemia is a common adverse effect of telaprevir (TVR) in combination with pegylated interferon (PegIFN)α and ribavirin (RBV) therapy. It occurs at a higher incidence with the TVR relative to PegIFNα and RBV alone. We herein evaluate the baseline and on-treatment predictors of the development of severe anemia by chronic hepatitis C virus (HCV) patients receiving TVR-based triple therapy.

Methods: This prospective, multicenter study consisted of 292 patients (median age: 62 years) infected with HCV genotype 1. All received 12 weeks of TVR in combination with 24 weeks of PegIFNα2b and RBV. The definition of severe anemia during antiviral treatment is hemoglobin (Hb) <85 g/L.

Results: 101 (34.6%) patients developed severe anemia during the treatment period. Multivariable logistic regression analysis of possible pretreatment predictors of the development of severe anemia extracted baseline Hb <135 g/L (Hazard ratio [HR], 2.53; $p = 0.0013$), estimated glomerular filtration rate <80 ml/min/1.73 m^2 (HR, 1.83; $p = 0.0265$), and inosine triphosphatase (ITPA) CC genotype (rs1127354) (HR, 2.91; $p = 0.0024$). For patients with ITPA CC (n = 227), multivariable logistic regression analysis of possible pretreatment and on-treatment predictors of the development of severe anemia extracted Hb level at week 2 (HR, 0.96; $p = 0.0085$) and the initial four weeks of weight-adjusted TVR (HR, 1.05; $p = 0.0281$).

Conclusions: Anemia remains a risk for all patients treated with TVR-based triple therapy. However, ITPA polymorphism (rs1127354) is useful for predicting the development of severe anemia and will be helpful in the management of treatment.

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Keywords: Hepatitis C virus; Anemia; Telaprevir; Inosine triphosohatase; Pegylated interferon; Ribavirin.

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Abbreviations: HCV, hepatitis C virus; PegIFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; TVR, telaprevir; ITPA, inosine triphosphatase; SNP, single nucleotide polymorphism; Hb, hemoglobin; eGFR, estimated glomerular filtration rate; RVR, rapid virological response; HR, hazard ratio; CI, confidence interval; AUROC, area under the receiver operating characteristic curve.

Introduction

Chronic hepatitis C virus (HCV) infection can result in serious health problems such as decompensated cirrhosis and hepatocellular carcinoma [1–3]. The standard of care regimen that includes pegylated interferon (PegIFN)α and ribavirin (RBV) has been the first line for the past decade, however, the rate of sustained virological response (SVR) that can be achieved using this regimen is only 40–52% for patients infected with HCV genotype 1 [4–6].

Of a number of direct-acting antivirals under investigation, nonstructural 3/4A protease inhibitors, including telaprevir (TVR) and boceprevir, have shown promising treatment outcomes in various clinical trials in combination with the current standard of care. The SVR rate is improved to over 70% for HCV genotype 1 patients treated with TVR-based triple therapy [7–9]. Notably, the SVR rate rises to over 80% for prior relapsers [7,10]. However, many adverse effects have been reported, with anemia being one of the most serious. Treatment requires careful management with RBV dose reduction. Because anemia has been shown to occur at a higher incidence with the TVR regimen relative to PegIFNα and RBV alone [7], it is important to understand the characteristics of severe anemia development prior to antiviral treatment.

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A genome-wide association study identified the inosine triphosphatase (*ITPA*) gene single nucleotide polymorphism (SNP) as being significantly associated with RBV-induced anemia [11,12]. Recently, Chayama *et al.* reported in a clinical trial that *ITPA* SNP (rs1127354) is associated with anemia in TVR-based triple therapy and that RBV dose reductions were required significantly earlier for patients with the ITPA CC genotype when compared with the ITPA CA and AA genotypes [13]. However, further improvement of the ability to predict the development of severe anemia will contribute to increasing the likelihood of achieving an SVR by patients whose treatment might otherwise have to be stopped.

The aim of this multicenter, prospective study was to evaluate the baseline and on-treatment predictors of the development of severe anemia (Hb <85 g/L) by chronic hepatitis C patients treated with TVR-based triple therapy.

Patients and methods

Patients

The Kyushu University Liver Disease Study (KULDS) Group consists of the Kyushu University Hospital and its affiliated hospitals in the northern Kyushu area of Japan. This prospective study consisted of 292 Japanese patients with chronic HCV infection aged 20 years or older who received TVR in combination with PegIFNα2b and RBV between December 2011 and October 2012. Exclusion criteria were: (1) positivity for antibody to human immunodeficiency virus or positivity for hepatitis B surface antigen; (2) clinical or biochemical evidence of hepatic decompensation (ascites, bleeding varices, or encephalopathy); (3) baseline hemoglobin (Hb) <120 g/L (female, <60 years), <110 g/L (female, ⩾60 years), <130 g/L (male, <60 years), and <120 g/L (male, ⩾60 years); (4) baseline serum creatinine >1.2 mg/dl (male) and >0.9 mg/dl (female); (5) other causes of liver disease (autoimmune hepatitis, or primary biliary cirrhosis); (6) excessive active alcohol consumption (a daily intake of more than 40 g of ethanol) or drug abuse; (7) suspected hepatocellular carcinoma at entry; or (8) treatment with antiviral or immunosuppressive agents prior to enrollment. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of each participating hospital. Informed consent was obtained from all patients before enrollment.

Clinical and laboratory assessment

Clinical parameters were measured by standard laboratory techniques at a commercial laboratory (SRL Laboratory, Tokyo, Japan). Body mass index was calculated as weight in kilograms/height in square meters. The estimated glomerular filtration rate (eGFR) was calculated based on the Modification of Diet in Renal Disease (MDRD) formula. Aspartate aminotransferase to platelet ratio index (APRI) was calculated, as previously recommended for evaluating severe liver fibrosis [14].

Assessment of liver fibrosis

Liver biopsy for 183 (62.7%) of the studied patients was done by experienced hepatologists. All antiviral treatment was initiated within one month after liver biopsy. The minimum length of the liver biopsy was 15 mm and at least 10 complete portal tracts were necessary for inclusion. For each specimen, the stage of fibrosis was established according to the METAVIR score [15].

Antiviral treatment

All patients received a combination treatment of TVR (Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan), PegIFNα2b (PEG-Intron; MSD, Tokyo, Japan), and RBV (Rebetol; MSD) for 12 weeks, followed by an additional 12 weeks of PegIFNα2b and RBV alone. TVR 750 mg was administrated three times a day at an 8-h interval after each meal (2250 mg/day). PegIFNα2b was injected subcutaneously once weekly at a dose of 1.5 μg/kg. RBV was given orally at a daily dose of 600–1000 mg based on body weight (600 mg for patients weighing <60 kg, 800 mg for those weighing 60–80 kg, and 1000 mg for those weighing >80 kg). The above durations and dosages are those approved by the Japanese Ministry of Health, Labor, and Welfare. In case of a 1.5 to 2.0 times elevation from baseline serum creatinine, the TVR dose was reduced to 1500 mg/day (750 mg twice a day at a 12-h interval after each meal). When serum creatinine was elevated to over 2.0 times the baseline level, TVR was discontinued. If a marked anorexia was developed, the TVR dose could be reduced to 1500 mg/day. If a progressive grade 3 rash developed (severe, involving more than 50% of the body surface, or rash with the appearance of substantial systemic signs of symptoms), TVR was discontinued. However, the patients continued to receive PegIFN and RBV in all of these situations. All treatment was discontinued for patients with less than 2 $\log_{10}$ HCV RNA decrease from baseline to week 12.

Management of anemia

Severe anemia during antiviral treatment was defined as Hb <85 g/L. Complete blood count was checked every week for the first 12 weeks and then at weeks 16, 20, and 24. The management of anemia started with RBV dose reduction. Specifically, the RBV dose was reduced by 200 mg for patients receiving 600 or 800 mg and by 400 mg for those receiving 1000 mg when Hb decreased to <120 g/L and by an extra 200 mg when it lowered to <100 g/L. If Hb decreased to <90 g/L, the TVR dose was reduced to 1500 mg/day. Erythropoietin use was not allowed during treatment, but blood transfusion was allowed when necessary. Discontinuation of TVR-based triple therapy due to severe anemia was primarily based on the discretion of the physicians at each hospital.

HCV RNA level and HCV genotype

Clinical follow-up of HCV viremia was done by real-time reverse transcriptase PCR assay (COBAS® TaqMan® HCV assay) (Roche Diagnostics, Tokyo, Japan), with a lower limit of quantitation of 15 IU/ml and an outer limit of quantitation of 6.9×10^7 IU/ml (1.2 to 7.8 log IU/ml referred to $\log_{10}$ IU/ml) [16]. HCV RNA levels were measured at baseline, regularly during treatment, at early discontinuation, and at follow-up visits after the end of treatment. Virological response was categorized as follows: rapid virological response (RVR) was an undetectable HCV RNA at week 4 and SVR was an undetectable HCV RNA at week 24 after the end of treatment. HCV genotype determination was by sequence determination in the 5′-non-structural region of the HCV genome followed by phylogenetic analysis [17].

Genetic testing

Human genomic DNA was extracted from peripheral blood. Genotyping of the *ITPA* (rs1127354) genes was performed using the ABI TaqMan allelic discrimination kit (7500 Real Time PCR System; Applied Biosystems, Carlsbad, CA, USA). Heterozygotes (CA) or homozygotes (AA) of the minor allele (A) are described as having the *ITPA* minor allele, whereas homozygotes for the major allele (CC) are described as having the *ITPA* major allele [12].

Statistical analysis

Statistical analyses were conducted using SPSS statistics 19.0 (IBM SPSS Inc, Chicago, IL, USA). Baseline continuous data are expressed as median (first-third quartiles) or mean (±standard deviation), and categorical variables are reported as frequencies and percentages. Univariate analyses were done using the Chi-square, Fisher's exact, or Mann-Whitney U tests as appropriate. Variables with p <0.05 in univariate analysis were evaluated using multivariate logistic regression to identify variables significantly associated with the development of severe anemia. The results are expressed as hazard ratios (HR) and their 95% confidence interval (CI). The significance of trends in values was determined with the Cochran-Armitage trend test.

Area under the receiver operating characteristic curve (AUROC) analysis was done to evaluate the relationship between the Hb level and development of severe anemia. The cut-off values were selected from the receiver operating characteristic (ROC) curve to maximize the total sensitivity and specificity. A p value less than 0.05 was regarded as statistically significant in all analyses.

Results

Patient characteristics and the development of severe anemia

The baseline characteristics of the 292 studied patients as classified by the development of severe anemia are shown in Table 1.

Table 1. Baseline risk factors for the development of severe anemia by chronic hepatitis C patients treated with telaprevir-based triple therapy.

Characteristic	All patients n = 292	Severe anemia n = 101	Non-severe anemia n = 191	*p* value*
Age (yr)	62 (54-66)	64 (57-68)	60 (53-65)	<0.0001
Men, n (%)	135 (46.2)	33 (32.7)	102 (53.4)	0.0007
Body mass index (kg/m^2)	23.3 (21.6-25.6)	22.9 (20.9-25.1)	23.4 (21.8-25.7)	0.0408
Alanine aminotransferase (IU/L)	50 (33-93)	50 (30-93)	50 (34-93)	0.3499
Serum albumin (g/L)	40 (37-42)	39 (36-42)	40 (38-43)	0.0054
Estimated glomerular filtration rate (ml/min/1.73 m^2)	80 (72-92)	76 (70-90)	83 (74-94)	0.0024
α-fetoprotein (ng/ml)	5.4 (3.5-11.0)	5.4 (3.3-10.2)	5.5 (3.6-11.7)	0.1753
Hemoglobin (g/L)	136 (127-147)	132 (124-140)	141 (133-153)	<0.0001
Platelet count (x10^9/L)	154 (120-190)	150 (118-200)	156 (121-189)	0.9279
HCV RNA level ($\log_{10}$ IU/ml)	6.5 (6.0-6.9)	6.4 (6.1-6.8)	6.5 (6.0-6.9)	0.5359
APRI <2.0/≥2.0, n (%)	234/58 (80.1/19.9)	77/24 (32.9/41.4)	157/34 (67.1/58.6)	0.2292
Stage of fibrosis				
F0-2/F3-4, n (%)	117/66 (63.9/36.1)	43/28 (36.8/42.4)	74/38 (63.2/57.6)	0.4505
Not determined, n (%)	109	30	79	
ITPA SNPs (rs1127354) CC/CA or AA, n (%)	227/65 (77.7/22.3)	90/11 (39.6/16.9)	137/54 (60.4/83.1)	0.0004
Treatment-naïve/experienced, n (%)	90/202 (30.8/69.2)	32/69 (35.6/34.2)	58/133 (64.4/65.8)	0.8169

Data are expressed as number (%) or median (first-third quartiles).
All demographic and clinical data are those at the start of antiviral treatment.
Severe anemia is diagnosed by hemoglobin level <85 g/L during antiviral treatment.
HCV, hepatitis C virus; ITPA, inosine triphosphatase; SNP, single nucleotide polymorphism; APRI, aspartate aminotransferase to platelet ratio index.
*Comparison between severe anemia and non-severe anemia.

HCV genotype 1b was detected in 290 (99.3%) patients and genotype 1a in the other two. Severe anemia (Hb <85 g/L) was developed during the treatment period by 101 of the 292 (34.6%) patients. The percentages of patients experiencing on-treatment severe anemia are shown in Fig. 1. No patient experienced severe anemia before week 2, and only two patients developed severe anemia after week 12. The onset of severe anemia was most frequently seen from weeks 8 to 12. The allele of the *ITPA* SNP (rs1127354) was determined for each patient. The percentages of patients with the *ITPA* CC, CA, and AA genotypes were 77.7% (n = 227), 19.2% (n = 56), and 3.1% (n = 9), respectively. There were no significant differences in sex (male 45.4% and 49.2%), age (median 62 and 59 years), BMI (23.2 and 23.7 kg/m^2), eGFR (80 and 83 ml/min/1.73 m^2), baseline Hb level (137 and 139 g/L), or initial four-week RBV dosage (9.7 and 10.5 mg/kg) between the *ITPA* CC and CA/AA genotypes.

Baseline factors associated with the development of severe anemia

Univariate analysis extracted older age (p <0.0001), female sex (p = 0.0007), lower BMI (p = 0.0408), lower serum albumin (p = 0.0054), lower eGFR (p = 0.0024), lower baseline Hb level (p <0.0001), and *ITPA* CC (p = 0.0004) as significantly associated with the development of severe anemia during treatment (Table 1). Prior therapeutic experience was not associated with the development of severe anemia (p = 0.8169). In multivariable logistic regression analysis of possible pretreatment predictors of the development of severe anemia, significant independent pretreatment predictors were baseline Hb <135 g/L (HR, 2.53; 95% CI, 1.43–4.51; p = 0.0013), eGFR <80 ml/min/1.73 m^2 (HR, 1.83; 95% CI, 1.07–3.16; p = 0.0265), and *ITPA* CC (HR, 2.91; 95% CI, 1.44–6.32; p = 0.0024). No independent predictive relationship was found between age, sex, BMI, or serum albumin. The rates of severe anemia development stratified by *ITPA* genotype (CC and CA/AA), eGFR level (≥80 and <80 ml/min/1.73 m^2), and baseline Hb level (≥135 and <135 g/L) are shown in Fig. 2.

Hb levels during antiviral treatment stratified by *ITPA* SNPs are shown in Fig. 3A. Hb levels from week 2 to week 24, except at week 12, for patients with *ITPA* CC were significantly lower than those of patients with *ITPA* CA/AA. Similarly, Hb decrement and the decrease ratio throughout the initial 12 weeks stratified by *ITPA* SNPs are shown in Fig. 3B and C, respectively. Hb decrement and the decrease ratio from week 2 to week 8 for patients with *ITPA* CC were significantly lower than for patients with *ITPA* CA or AA.

Baseline factors associated with Hb decrease by over 50 g/L

Hb decline over 50 g/L during the treatment period was found for 128 of the 292 (43.8%) patients. Univariate analysis extracted younger age (p = 0.0011), male sex (p = 0.0011), lower eGFR (p = 0.0161), higher baseline Hb level (p <0.0001), and *ITPA* CC (p = 0.0009) as significantly associated with the decline of Hb to ≥50 g/L. In multivariable logistic regression analysis, baseline Hb ≥135 g/L (HR, 2.73; 95% CI, 1.55–4.86; p = 0.0005), eGFR <80 ml/min/1.73 m^2 (HR, 1.74; 95% CI, 1.04–2.93; p = 0.0355), and *ITPA* CC (HR, 3.36; 95% CI, 1.78–6.63; p = 0.0001) were independently associated with an Hb decline of over 50 g/L.

Relationship between pretreatment or on-treatment variables and the development of severe anemia stratified by ITPA SNPs

Table 2 shows the development of severe anemia according to the *ITPA* SNPs. Univariate analysis of patients with *ITPA* CC (n = 227) extracted older age (p = 0.0004), female sex (p = 0.0011), lower serum albumin (p = 0.0083), lower eGFR (p = 0.0041), lower baseline Hb level (p <0.0001), lower Hb level

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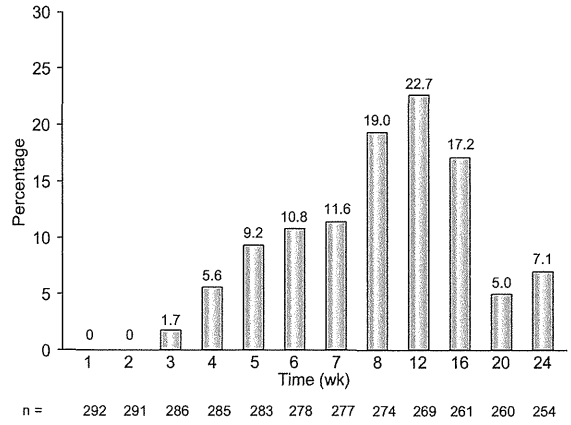

Fig. 1. The percentage of patients experiencing on-treatment severe anemia (hemoglobin <85 g/L). No patient experienced severe anemia before week 2, and the onset of severe anemia was most frequently observed from weeks 8 to 12.

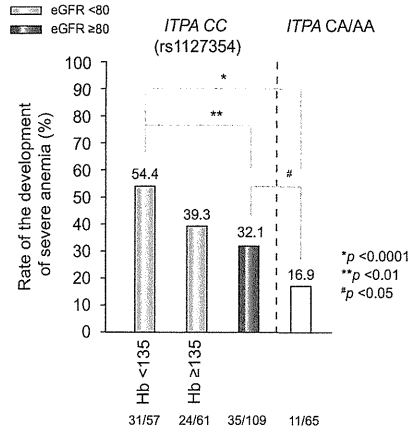

Fig. 2. The percentages of patients who developed severe anemia stratified by *ITPA* SNPs (rs1127354), baseline estimated glomerular filtration rate (eGFR), and hemoglobin (Hb) level. The percentages of *ITPA* CC patients with severe anemia were significantly increased with lower eGFR and Hb levels. Severe anemia was developed by only 16.9% of the *ITPA* CA/AA patients (the Cochran-Armitage trend test: p <0.0001).

at week 2 (p <0.0001), and higher initial four week weight-adjusted dosage of TVR (p = 0.0455) as significantly associated with the development of severe anemia. In multivariable logistic regression analysis of possible pretreatment and on-treatment predictors of the development of severe anemia, significant independent predictors were Hb level at week 2 (HR, 0.96; 95% CI, 0.93–0.98; p = 0.0085) and the initial four weeks of weight-adjusted TVR (HR, 1.05; 95% CI, 1.01–1-10; p = 0.0281). The percentages of *ITPA* CC patients experiencing on-treatment severe anemia stratified by the initial four weeks of TVR (25 mg/kg/day) are shown in Fig. 4A. The rates of severe anemia of the TVR ⩾25 mg/kg/day group were significantly higher than those of the TVR <25 mg/kg/day group at weeks 7, 8, 12, 16, and 24.

In contrast, univariate analysis of patients with *ITPA* CA/AA (n = 65) extracted only lower baseline Hb level (p = 0.0022) and lower Hb level at week 2 (p = 0.0081) as significantly associated with the development of severe anemia. No predictive relationship was found between age, sex, eGFR, or the initial weight-adjusted dosages of RBV or TVR and the development of severe anemia. The percentages of *ITPA* CA/AA patients experiencing on-treatment severe anemia are shown in Fig. 4B. Severe anemia was developed only between weeks 7 and 16, unlike patients with *ITPA* CC.

ROC curve analysis of the Hb level at week 2 and the development of severe anemia by patients with ITPA CC

The adequacy of the multivariate model was confirmed by a ROC curve analysis. This analysis was performed to determine the optimal threshold values for the Hb level at week 2 for predicting the development of severe anemia by the 227 patients with *ITPA* CC. The corresponding AUROC was 0.70 (p <0.0001) for *ITPA* CC and the cut-off value for the Hb level at week 2 was 116 g/L (sensitivity 79.0%, specificity 57.0%).

ROC curve analyses of the Hb level at baseline and week 2 and the development of severe anemia by patients with ITPA CA/AA

ROC curve analyses were performed to determine the optimal threshold values for the Hb levels at baseline and week 2 for predicting the development of severe anemia by the 65 patients with *ITPA* CA/AA. The corresponding AUROCs were 0.75 (p = 0.0089) for Hb level at baseline (cut-off values 122 g/L; sensitivity 98.0%, specificity 55.0%) and 0.68 (p = 0.0538) for Hb level at week 2, which indicates that the Hb level at baseline is more effective than that at week 2 for predicting the development of severe anemia by patients with *ITPA* CA/AA.

Treatment efficacy

The overall rates of RVR and SVR were 75.0% (219 of 292) and 82.2% (240 of 292), respectively. The RVR and SVR rates of patients with *ITPA* CC were 74.9% (170 of 227) and 81.5% (185 of 227), respectively. For patients with *ITPA* CC, there was no significant difference in the initial four weeks of weight-adjusted TVR between the RVR (28.7 [24.4–33.1] mg/kg/day) and non-RVR groups (28.7 [23.9–31.7] mg/kg/day) (p = 0.2467). However, the SVR rates for the initial four weeks of the TVR ⩾25 group (83.5%, 137 of 164) were higher than those of the TVR <25 (mg/kg/day) group (76.2%, 48 of 63), but did not reach significance (p = 0.2106).

Premature discontinuation of treatment or blood transfusion due to anemia

Of the 292 patients, 38 (13.0%) had TVR-based triple therapy discontinued during the treatment period. Of these 38 patients, 8 (21.1%) had treatment discontinued because of severe anemia between weeks 8 and 16. Of these 8 patients, 5 (62.5%) were women, 6 (66.7%) were aged over 60 years, and 6 (66.7%) were *ITPA* CC. On the other hand, 23 (7.9%) received blood transfusion without treatment discontinuation. Of these 23 patients, 13 (56.5%) were women, 16 (69.6%) were aged over 60 years, and 18 (78.3%) were *ITPA* CC.

Discussion

This prospective, multicenter study was carried out to evaluate the baseline and on-treatment predictors of the development of

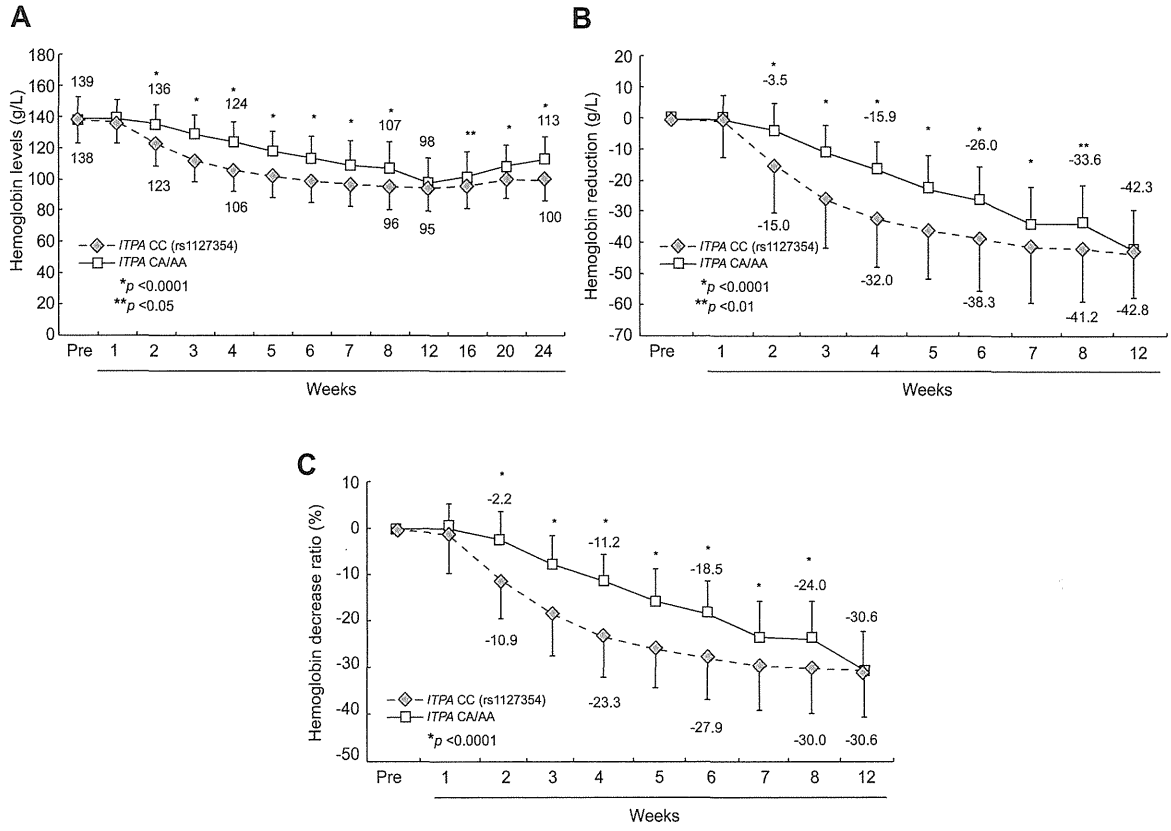

Fig. 3. Mean hemoglobin (Hb) levels, Hb decrement, and Hb decrease ratio during antiviral treatment stratified by *ITPA* SNPs. (A) Hb levels from week 2 to week 24, except at week 12, for patients with *ITPA* CC were significantly lower than those of patients with *ITPA* CA/AA. (B) Hb decrement from week 2 to week 8 for patients with *ITPA* CC was significantly lower than for those with *ITPA* CA/AA. (C) The Hb decrease ratio from week 2 to week 8 for patients with *ITPA* CC was also significantly lower than for those with *ITPA* CA/AA.

severe anemia (Hb <85 g/L) by patients treated with TVR in combination with PegIFNα2b and RBV. Several pretreatment factors, including lower baseline Hb level, lower eGFR, and *ITPA* CC (rs1127354) genotype, were independently associated with the development of severe anemia. Moreover, analysis of patients with the *ITPA* CC genotype that included baseline and on-treatment parameters found the Hb level at week 2 and the initial four week, weight adjusted dosage of TVR to be independent, significant predictors of the development of severe anemia. These findings will help increase the rate of successful completion of treatment by allowing doctors to take steps to predict severe anemia according to the *ITPA* polymorphism.

The availability of protease inhibitors has profoundly changed the management of chronic hepatitis C by achieving higher rates of SVR. However, adverse events are experienced by almost all patients. The most frequently reported adverse effects associated with TVR have been hematological disorders (anemia, thrombocytopenia and leukocytopenia), skin disorders (pruritus and rash), gastrointestinal disorders (nausea and diarrhea), general fatigue, and elevated serum levels of uric acid, bilirubin, and creatinine [7–9,18,19]. Moderate and severe anemia has been shown to develop more frequently in TVR-based triple therapy than in PegIFNα2b and RBV alone [7] and by Japanese more often than by Americans/Europeans [18,19] because Japanese patients with chronic hepatitis C average more than 10 years older than those in Western countries [20–22]. However, these findings were based on clinical trials, thus this is the first study to show the predictors of development of severe anemia as evaluated in clinical practice.

Severe anemia leads to hypoxia in organs, and this condition may be a sign of hyperdynamic circulation, tachycardia, and left heart strain, which have the potential risk of heart failure. Our results showed that the development of severe anemia began at week 3, with a peak frequency at week 12. Therefore, it is important to adjust the dosage as needed in order for patients to be able to complete the overall treatment duration.

We investigated the baseline characteristics associated with both the development of severe anemia and Hb decline by over 50 g/L. *ITPA* CC genotype and lower eGFR level were extracted as the independent risk factors in common. In the analysis of Hb decline, a higher baseline Hb level was extracted as an independent factor of higher Hb decline during treatment. This finding can be interpreted in relation to the more adequate RBV adherence of the patients with high baseline Hb levels. The above show the importance of TVR-based triple therapy strategy adjustment according to the *ITPA* SNPs (rs1127354). Of the patients with *ITPA* CC, 39.6% developed severe anemia during treatment. Their Hb levels after week 2 were significantly lower than those