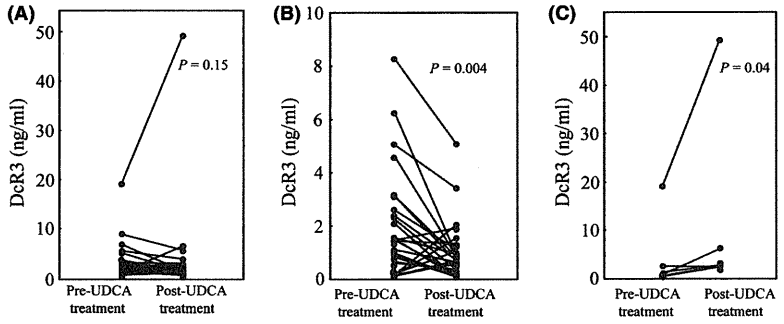

Fig. 7. Effect of UDCA treatment on serum DcR3 levels in PBC patients. In PBC patients who had detectable DcR3 levels by ELISA, serum DcR3 levels before and after UDCA treatment were compared by a two-tailed Wilcoxon's single-rank test. (A) In the group as a whole (n = 39), UDCA treatment tended to lower serum DcR3 levels (pre: 2.1 ± 3.4 ng/ml, post: 2.4 ± 7.9 ng/ml). (B) Serum DcR3 levels were significantly decreased in non-jaundice-stage patients (n = 33) after UDCA treatment (pre: 1.7 ± 1.9 ng/ml, post: 0.90 ± 1.0 ng/ml). (C) Serum DcR3 levels were significantly increased in jaundice-stage patients (n = 6) who progressed to hepatic failure after UDCA treatment (pre: 3.8 ± 7.7 ng/ml, post: 10.7 ± 19.1 ng/ml).

shown that TL1A is localized in macrophages and $CD4^+$ or $CD8^+$ lymphocytes infiltrating the intestinal lamina propria in Crohn's disease, and plasma cells infiltrating the lamina propria in ulcerative colitis (13). TL1A is also localized in macrophages and plasma cells in the synovial tissue in RA (7). In addition, upregulation of TL1A expression has been demonstrated in involved tissues (13, 15). These results indicate that TL1A is involved in the pathogenesis of these chronic inflammatory diseases. In this study, serum TL1A levels tended to correlate with cholangitis but not fibrosis in PBC patients, whereas the TL1A levels correlated with fibrosis in CHC patients. These results may indicate that TL1A is differentially involved in the liver inflammation between PBC and CHC, although there is no apparent difference in TL1A-positive cells as determined by immunohistochemical staining of the liver tissues.

Our immunohistochemical data identified TL1A in intrahepatic small bile ducts as well as infiltrating mononuclear cells, Kupffer cells and blood vessels in the PBC liver. It is noteworthy that the intrahepatic small bile ducts immunohistochemically expressed TL1A; however, the mechanism of TL1A production by these ducts *in vivo* is poorly understood. *In vitro*, TL1A is induced in endothelial cells by TNF-α and IL-1, and in monocytes and dendritic cells by stimulation with TLR ligands (11). Cultured biliary epithelial cells (BECs) constitutively express TL1A (data not shown) and various human TLRs, and produce inflammatory cytokines and chemokines when stimulated with TLR ligands *in vitro* (43, 44). TL1A also induces $CD4^+$ T cells to produce IFN-γ through IL-12 and IL-18 signalling *in vitro* (19), and modulates Th-1 and Th-17 effector responses in animal models of experimental antigen-induced arthritis (16), experimental autoimmune encephalomyelitis (17) and chronic ileitis and colitis (19, 20). Previous reports have shown a significant role for both Th-1 and Th-17 cytokines in the pathogenesis of PBC (45). Although the mechanism of TL1A induction in BEC is as yet poorly understood, we hypothesize that TL1A, which is induced in BEC, blood vessels and mononuclear cells by inflammatory cytokines and TLR signalling, may be involved in the pathogenesis of PBC via modulation in Th-1 and Th-17 effector responses.

We also showed that serum TL1A levels were significantly decreased in early-stage PBC patients after UDCA treatment. This is consistent with previous reports in which significant decreases in serum TL1A levels were observed in RA patients after anti-TNF monoclonal antibody therapy (14). These results may indicate that serum TL1A serves as a serological marker for disease activity in PBC as well as RA. Although serum ALP and ALT levels decreased significantly to baseline levels in most of the late- and early-stage PBC patients in this study, serum TL1A levels remained significantly higher in late-stage PBC patients as compared with healthy controls, even after UDCA treatment. These results might indicate that UDCA treatment is not sufficient to suppress TL1A-mediated inflammation, particularly in late-stage PBC. Neutralizing anti-TL1A antibodies attenuates inflammation in mouse models of chronic colitis and collagen-induced arthritis (16, 20), suggesting that TL1A might be potentially a new therapeutic target for PBC.

DcR3 binds to TL1A, FasL and LIGHT, and inhibits these ligands-mediated apoptosis or lymphokine production (6). In this study, we demonstrated that serum DcR3 levels were significantly increased at the time of enrolment in both early- and late-stage PBC patients as well as CHC and AIH patients. Under UDCA treatment, serum DcR3 levels were significantly decreased in non-jaundice-stage PBC patients, whereas they were significantly increased in jaundice-stage PBC patients who progressed to hepatic failure. This observation is consistent with previous findings that serum DcR3 is significantly increased in patients with cirrhosis (22) and

autoimmune diseases such as IBD (12, 28), SLE (21) and RA (14). Through downregulation of Th-1 and/or Th-17 immune responses, DcR3 protects against the development and progression of autoimmune diseases in mouse models of conditions such as cyclophosphamide-induced diabetes (31, 32) and experimental autoimmune encephalomyelitis (46). Therefore, it is speculated that the decrease in DcR3 levels during treatment in PBC patients might be because of secondary effect with the decrease in TL1A levels under UDCA treatment. DcR3 is also significantly induced in bacterial infections (29) and is a marker of mortality and multiple-organ dysfunction (47). These reports may indicate that the marked increase in serum DcR3 levels in jaundice-stage PBC patients is caused by bacterial infections and/or multiple-organ dysfunction.

In conclusion, we showed for the first time that serum TL1A and DcR3 levels were increased in PBC patients and that these levels changed in association with disease progression and UDCA treatment, indicating that TL1A and DcR3 might be involved in the pathogenesis of PBC. Further studies are needed to elucidate the exact role of TL1A and DcR3 in the pathogenesis of PBC and to explore its potential as a possible therapeutic target.

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Conflict of interest: None.

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Supporting information

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

Fig. S1. Correlation between TL1A levels and biochemical parameters in sera of PBC patients. The correlations between levels of TL1A and those of ALP (A), ALT (B) or total IgM (C) in sera of PBC patients were analysed by Spearman's rank test; the correlation coefficient and *P*-value are shown.

Fig. S2. Serum TL1A levels in antinuclear antibody-positive and -negative PBC patients. Serum TL1A levels were compared between PBC patients who were positive and negative for anti-gp210 (A) and anticentromere (B) antibodies (anti-gp210 negative vs. positive: $n = 68$, 5.4 ± 5.9 ng/ml vs. $n = 41$, 4.1 ± 3.3 ng/ml; anticentromere negative vs. positive: $n = 79$, 5.0 ± 5.4 ng/ml vs. $n = 30$, 4.4 ± 3.7 ng/ml). Statistical differences were analysed using a two-tailed Mann–Whitney *U*-test.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association of genes involved in bile acid synthesis with the progression of primary biliary cirrhosis in Japanese patients

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Abstract
Background Patients with primary biliary cirrhosis (PBC) exhibit a variety of clinical manifestations and patterns of disease progression. The aim of this study was to identify genetic determinants of PBC progression.
Methods A total of 52 tag single nucleotide polymorphisms (SNPs) of 11 candidate genes involved in regulating bile acid synthesis were analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism, -high resolution melting curve analysis, or -direct DNA sequencing in 315 Japanese patients with PBC.
Results In this study, four tag SNPs of *CYP7A1* (rs1457043, rs8192870, rs3808607, and rs3824260), two tag SNPs of *HNF4A* (rs6017340 and 6031587), and one SNP of *PPARGC1A* (rs8192678) showed a significant association with PBC progression. In addition, a dual luciferase assay revealed that the polymorphism of rs3808607 in *CYP7A1* altered the expression of *CYP7A1* in HepG2. Specifically, the *CYP7A1* promoter carrying the risk G allele for PBC progression induced higher expression of *CYP7A1* under both the normal and cholestatic conditions in vitro as compared to another promoter carrying the non-risk T allele.
Conclusion These results suggested that the genetic variants of *CYP7A1* and its transcriptional activators (*HNF4A* and *PPARGC1A*) may activate bile acid synthesis, resulting in the accumulation of bile acids in hepatocytes and eventually leading to the predisposition to PBC progression. Thus, the regulation of *CYP7A1* expression may represent an attractive therapeutic target for cholestatic liver diseases including PBC.

Keywords PBC progression · Bile acid synthesis · CYP7A1 · PGC-1α · HNF4α

Electronic supplementary material The online version of this article (doi:10.1007/s00535-012-0730-9) contains supplementary material, which is available to authorized users.

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Introduction

Primary biliary cirrhosis (PBC) is a chronic and slowly progressive liver disease characterized by immune-mediated destruction of the intrahepatic small bile ducts that leads to cholestasis, fibrosis, cirrhosis, and eventually liver failure. PBC is considered to be an organ-specific autoimmune disease because autoantibodies against mitochondrial and/or nuclear proteins are closely associated with its pathogenesis [1, 2]. Development of PBC is attributed to genetic predispositions and environmental triggers [2, 3]. Previous studies have shown that almost all of the

disease-susceptibility genes are immune-related, e.g., encoding human leukocyte antigens [4–6], cytotoxic T-lymphocyte antigen 4 (CTLA-4) [7–9], interleukin-12 α [5, 10], interleukin-12 receptor β2 [5, 10], and interferon regulatory factor 5-transportin 3 locus [10, 11].

Although the etiology of PBC is due to the dysregulation of immune systems, a majority of PBC patients are treated with ursodeoxycholic acid (UDCA), a secondary bile acid comprising less than 5 % of endogenous bile acids. Clinical manifestations of PBC vary with respect to symptoms, course of progression, and response to treatment [3, 12]. Patients who respond to UDCA have a normal life expectancy, whereas those who do not are at risk for severe disease progression that could lead to cirrhosis and liver failure. Several genes related to PBC progression have been reported, including tumor necrosis factor α [7], solute carrier 4, anion exchanger 2 [7], and *CTLA-4* [13]. By contrast, there is scant information on the genes associated with PBC subphenotypes. To understand the genetic mechanisms underlying these subphenotypes and how they relate to disease progression is an unresolved problem for the clinical management of PBC.

UDCA acts on cholestatic liver diseases through multiple pharmacological actions, including: (1) decreasing the proportion of hydrophobic bile acids, which are toxic to cellular membranes, to the total amount of biliary bile acids; (2) preventing apoptosis of hepatic cells; and (3) positively modulating ductular bile flow by partially altering the expression of genes involved in bile acid homeostasis [14]. The accumulation of endogenous bile acids, such as chenodeoxycholic acid (CDCA), reduces the intrahepatocellular bile acids by repressing both their synthesis and their influx of circulating bile acids from the portal vein and hepatic vessels into hepatocytes (referred to as negative feedback regulation) [15]. At the same time, the accumulation of endogenous bile acids increases their detoxification and efflux to the bile ducts and systemic circulation [15].

From an etiological perspective of bile acid synthesis in hepatocytes, the expression of cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting bile acid synthetic enzyme that plays an important role in determining the size of the bile acid pool, is intricately controlled via multiple mechanisms [16]. Under normal conditions, hepatocyte nuclear factor 4 α (HNF4α), a transcription factor, activates the expression of *CYP7A1* by interaction with peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [17, 18]. However, in the case of cholestasis, this transactivation is inhibited by bile acid-activated nuclear receptors and their downstream signals. Indeed, *CYP7A1* messenger RNA (mRNA) levels are decreased in the liver of patients with advanced PBC [19, 20]. Furthermore, UDCA also represses *Cyp7a1* expression in a rodent model [21]. Therefore, the dysregulation of bile acid synthesis may contribute to severe PBC progression.

In order to dissect the mechanisms contributing to individual differences in PBC progression, we investigated whether polymorphisms of candidate target genes involved in bile acid synthesis and its regulatory pathways are associated with PBC progression in Japanese patients. In this study, we focused on the rate-limiting enzyme CYP7A1 in the bile acid synthesis and its regulation pathways, which play an important role in bile acid homeostasis. Then, 11 candidate genes were selected as follows: (1) the bile acid synthetic enzyme, CYP7A1 (encoded by *CYP7A1*); (2) activators of *CYP7A1* expression, e.g., HNF4α (encoded by *HNF4A*) and PGC-1α (encoded by *PPARGC1A*); and (3) repressors of *CYP7A1* expression, e.g., farnesoid X receptor (FXR; encoded by *NR1H4*), short heterodimer partner (SHP; encoded by *NR0B2*), G protein pathway suppressor 2 (GPS2; encoded by *GPS2*), pregnane X receptor (PXR; encoded by *NR1I2*), fibroblast growth factor 19 (FGF19; encoded by *FGF19*), fibroblast growth factor receptor 4 (FGFR4; encoded by *FGFR4*), Klotho β (encoded by *KLB*), and forkhead box O1 (FOXO1; encoded by *FOXO1*).

Methods

Subjects

The cohort study consisted of 315 unrelated Japanese patients with PBC. The patients were registered in the PBC cohort study of the National Hospital Organization Study Group for Liver Disease in Japan (NHOSLJ) from August 1982 to September 2008. The time of entry was defined as the date of the initial PBC diagnosis. The study protocol was approved by the Ethics Committee dealing with the Human Genome and Gene Analysis at Nagasaki University and National Hospital Organization Nagasaki Medical Center, and written informed consent was obtained from each patient.

The patients were diagnosed with PBC if they met at least two of the following internationally accepted criteria [22]: biochemical evidence of cholestasis based upon alkaline phosphatase elevation, the presence of serum antimitochondrial antibodies, and histological evidence of nonsuppurative destructive cholangitis and destruction of the interlobular bile ducts. A liver biopsy was performed in 233 out of 315 patients at the initial diagnosis. Patients with acute or autoimmune hepatitis (alanine aminotransferase >200 IU/L, aspartate aminotransferase >200 IU/L), a maintenance dose of prednisolone >5 mg/body weight for concomitant autoimmune hepatitis, persistent hepatitis

virus B or C infection, alcoholic liver disease, and other chronic liver diseases were excluded from this study.

During the observation periods, 304 (96.5 %) patients received the following treatments: 300–900 mg/day UDCA alone ($n = 202$), 200–400 mg/day bezafibrate alone ($n = 4$), ≤5 mg/day maintenance prednisolone alone ($n = 4$), UDCA + bezafibrate ($n = 65$), UDCA + maintenance prednisolone ($n = 16$), UDCA and/or bezafibrate + maintenance prednisolone ($n = 12$), or UDCA + fenofibrate ($n = 1$).

Classification of clinical stages of PBC

PBC patients were classified into the following two groups based on liver biopsy results and/or clinical manifestations: early stage included the findings of Scheuer's stage 1 or 2 [23] in liver biopsy or an unknown histological stage without any signs indicating portal hypertension or liver cirrhosis; late stage included the findings of Scheuer's stage 3 or 4 in liver biopsy or any histological stage with signs indicating portal hypertension, liver cirrhosis, or persistent jaundice (total bilirubin >2 mg/dL). At the initial diagnosis, 269 and 46 patients were in early and late stages, respectively. During the observation period, 41 out of 269 patients in early stage progressed to late stage. The characteristics of the two subgroups are shown in Table 1.

The observation period was defined as the time from initial diagnosis until the date of latest observation as of May 2010 (86.7 %), the date of death from liver-associated diseases (2.2 %) or non-liver-associated diseases (1.0 %), liver transplantation (2.5 %), or end of follow-up (7.6 %), whichever came first.

Selection of tag single nucleotide polymorphisms in candidate genes

All of the single nucleotide polymorphisms (SNPs) in the candidate genes that we selected for this study were obtained from Japanese data in Tokyo (JPT: Rel 24/phaseII Nov08, on NCBI B36 assembly, dbSNP b126), available on the International HapMap website (http://www.hapmap.org). Candidate tag SNPs were selected from all SNPs in each chromosomal region including 2-kb upstream with priority in minor alleles with a frequency of more than 10 % in the International HapMap data. Subsequently, genotyped tag SNPs among the candidate tag SNPs were determined based on linkage disequilibrium (LD) tagging using the Haploview 4.2 software program [24] or the iHap software program [25]. However, genotyped SNPs of two genes, *PPARGC1A* and *KLB*, were selected based on well known functional SNPs with regard to gene product activity or protein stability [26, 27]. Information on the candidate genes and genotyping of tag SNPs is shown in Table 2.

SNP genotyping

Genomic DNA was extracted from whole blood samples using a NucleoSpin® Blood L Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. A total of 52 tag SNPs were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), -high resolution melting curve analysis (HRM), and -direct DNA sequencing (Table 2). The genotyping procedures of PCR–RFLP, -HRM, and -direct DNA sequencing were previously described [28].

Haplotype structures of CYP7A1 and HNF4A

Haplotype structures of *CYP7A1* and *HNF4A*, which were comprised of the tag SNPs associated with PBC progression in individual SNP study and were in LD in each gene, and diplotype structures were estimated based on the expectation–maximization algorithm using the SNPAlyze® 7.1 standard software package (Dynacom Inc., Chiba, Japan).

Dual luciferase reporter assay

Luciferase reporter gene plasmids regulated by the *CYP7A1* promoter were constructed based on the methods of De Castro-Orós et al. [29]. To obtain *CYP7A1* promoter

Table 1 Characteristics of PBC patients in each stage

	Patients	Patients		*P* value
		Early stage	Late stage	
Total number	315	228	87	
Age, mean ± SD (years)	64.1 ± 11.5	62.9 ± 11.4	67.4 ± 10.5	<0.005
Male/female (% of male)	45/270 (14.3)	28/200 (12.3)	17/70 (19.5)	0.100
Observation period, mean ± SD (months)	71.3 ± 63.9	63.1 ± 59.6	93.0 ± 70.1	<0.001
Receiving treatment (%)	96.5	95.6	98.9	0.301
Concomitance of autoimmune diseases (%)	29.5	27.6	34.5	0.233

SD standard deviation

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.

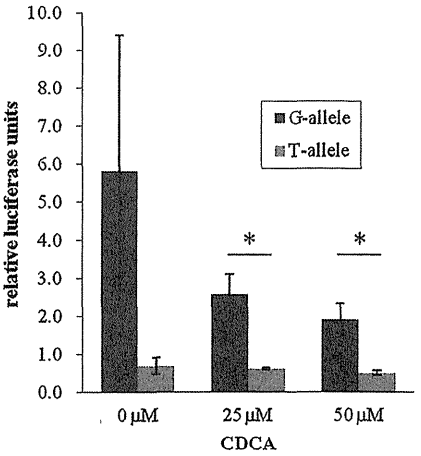

Fig. 1 Transcriptional activities of the *CYP7A1* promoter carrying either the G- or the T-allele at rs3808607 at 0, 25, and 50 μM concentrations of CDCA. Firefly luciferase signals were normalized to those of Renilla luciferase. Data shown represent mean ± standard deviation ($n = 3$). *$P < 0.01$

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.

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.

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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.

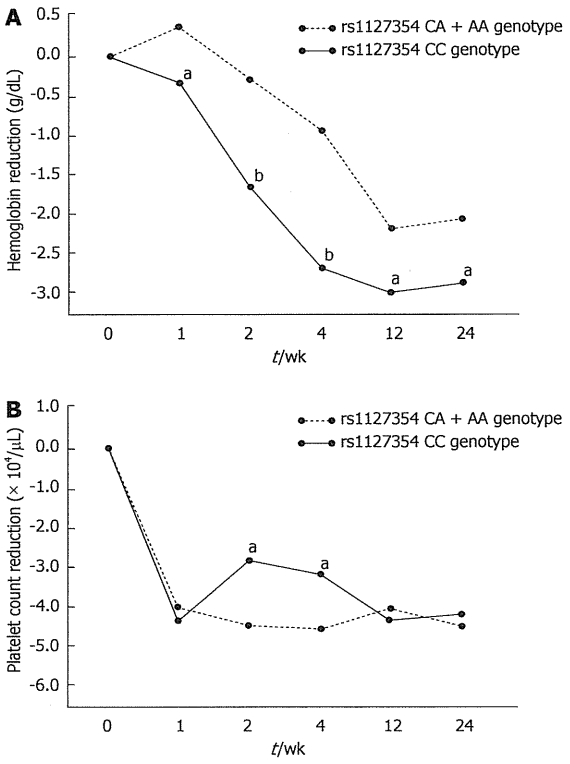

Figure 1 Chronological variation of hemoglobin levels (A) and platelet counts (B) in each inosine triphosphatase genotype at rs1127354. $^aP < 0.05$, $^bP < 0.01$ compared with CA/AA groups.

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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