Table 1 Clinical characteristics of patients in GWAS and the replication study

	GWAS			Replication study	
	Case-G1 ($n = 114$)	Case-G2 ($n = 50$)	Control-G ($n = 302$)	Case-R ($n = 50$)	Control-R ($n = 354$)
At baseline					
Gender, male/female	48/66	21/29	170/132	24/26	208/146
Age, years	57.9 (8.7)	57.1 (8.3)	57.2 (11.2)	59.1 (10.2)	56.7 (9.6)
Neutrophil count, /mm^3	1,800 (777)	1,662 (897)	2,750 (984)	1,570 (552)	2,724 (985)
Hemoglobin, g/dL	13.6 (1.3)	13.5 (1.3)	14.2 (1.5)	13.6 (1.6)	14.3 (1.5)
Platelet count, $\times 10^9$/L	141 (42)	132 (46)	164 (54)	140 (47)	162 (60)
ALT, IU/L	82.9 (88.6)	70.4 (53.1)	81.5 (77.9)	87.8 (82.7)	85.2 (71.1)
HCV genotype, 1/2/ND	95/18/1	40/10/0	250/51/1	45/5/0	277/77/0
HCV RNA, log IU/mL	5.9 (0.8)	5.9 (1.0)	6.1 (0.8)	6.1 (0.9)	6.1 (0.8)
Liver fibrosis, F0-2/F3-4/ND	62/22/30	25/10/15	168/70/64	21/6/23	229/87/38
rs8099917, TT/TG + GG/ND	74/39/1	35/15/0	189/109/4	31/17/2	278/70/6
Regimen					
PEG-IFN + RBV/IFN + RBV/PEG-IFN/IFN mono	112/0/0/2	48/0/0/2	277/9/9/7	44/4/2/0	351/0/3/0
At week 4					
Neutrophil count, /mm^3	606 (126)	496 (104)	1,551 (501)	501 (89)	1,533 (484)

Data are expressed as number for categorical data or the mean (standard deviation) for non-categorical data

GWAS genome-wide association study, *ALT* alanine transaminase, *ND* not determined, *PEG-IFN* pegylated interferon, *IFN* mono, interferon monotherapy, *RBV* ribavirin

by comparing between GWAS-1st and GWAS-2nd as follows. There were 6,315 and 5,736 SNPs with odds ratios (ORs) <0.7 or >1.5 at GWAS-1st and GWAS-2nd, respectively. Of these, the ORs of 135 SNPs were more notable at GWAS-2nd than at GWAS-1st. In addition to the 135 SNPs, we selected 15 and 33 SNPs with $P < 10^{-4}$ at GWAS-1st and GWAS-2nd, and added 9 SNPs which are located around the candidate genetic regions identified by the GWAS stage and are non-synonymous or related to diseases in previous reports. Consequently, we carried out the replication analysis focusing on this total of 192 SNPs.

In the subsequent replication analysis, we carried out genotyping of the 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients with minimum neutrophil counts of <600/mm^3 (Case-R, $n = 50$) and ≥1,000/mm^3 (Control-R, $n = 354$) at week 2 or 4 during IFN-based therapy (Table 1; Fig. 1). The results in the replication stage combined with GWAS-2nd are shown in Supplementary Table 1. Several SNPs such as rs11743919 and rs2457840 showed strong associations with low P value, however, the MAF of them were <5 %. In general, low frequent SNPs tend to show unsettled associations, especially in statistical analysis with small number of samples. Therefore, we excluded these SNPs from the final candidates. Consequently, we determined the SNP rs2305482, located in the intron of *PSMD3* gene on chromosome 17, as the most promising candidate, which showed a strong association with IFN-induced neutropenia in the combined results of GWAS-2nd and the replication stage (OR = 2.18; 95 % CI = 1.61–2.96, $P = 3.05 \times 10^{-7}$ in the allele frequency model) (Table 2).

Association of SNPs located in *PSMD3-CSF3* with neutropenia

A previous GWAS showed that rs4794822 located between the *PSMD3* and *CSF3* genes was associated with neutrophil counts in Japanese patients including 14 different disease groups (Okada et al. 2010). As shown in Fig. 2, rs4794822 is in strong linkage disequilibrium (LD) with rs2305482 which we identified in the present study. Thus, the pairwise LD (r^2) in the HapMap JPT: Japanese in Tokyo, Japan, is 0.66. Because the SNP rs4794822 is not included in the Affymetrix Genome-Wide Human SNP Array 6.0, we additionally genotyped it together with three other SNPs (rs9915252, rs3859192 and rs3907022) located in the same LD block around the *PSMD3* gene (Fig. 2). The allele frequency of each SNP was compared between patients with minimum neutrophil counts of <600/mm^3 (Case-G2 + R: Case-G2 plus Case-R, $n = 100$) and ≥1,000/mm^3 (Control-G + R: Control-G plus Control-R, $n = 656$) at week 2 or 4 during IFN-based therapy. This showed that, rs4794822 was also strongly associated with neutropenia during IFN-based therapy (OR = 2.24; 95 % CI = 1.63–3.07, $P = 3.63 \times 10^{-7}$ in the allele frequency model) (Table 3).

Table 2 SNP associated with interferon-induced neutropenia

dbSNP rsID	Nearest gene	Risk allele	Allele (1/2)	Stage	Case			Control			OR[a] (95 % CI)	*P* value[b]
					11	12	22	11	12	22		
rs2305482	*PSMD3*	C	C/A	GWAS-1st	23 (20.4)	52 (46.0)	38 (33.6)	26 (8.6)	143 (47.4)	133 (44.0)	1.61 (1.17–2.20)	2.95×10^{-3}
				GWAS-2nd	12 (24.5)	28 (57.1)	9 (18.4)	26 (8.6)	143 (47.4)	133 (44.0)	2.37 (1.54–3.65)	6.47×10^{-5}
				Replication	12 (24.4)	20 (40.8)	17 (34.7)	33 (9.5)	136 (39.1)	179 (51.4)	1.99 (1.30–3.06)	1.46×10^{-3}
				Combined[c]	24 (24.5)	48 (49.0)	26 (26.5)	59 (9.1)	279 (42.9)	312 (48.0)	2.18 (1.61–2.96)	3.05×10^{-7}

Data of allele distribution represent number (%). Data of subjects whose genotypes were not determined were excluded

SNP single nucleotide polymorphism

[a] Odds ratio for the allele frequency model

[b] *P* value by the Chi square test for the allele frequency model

[c] Allele distributions in GWAS-2nd and replication were combined

Predictive factors for IFN-induced neutropenia

The following analyses were carried out for rs2305482 and rs4794822 using the subjects in Case-G2 + R and Control-G + R. Neutrophil counts at baseline correlated with rs2305482 and rs4794822 genotypes (Supplementary Fig. 2), and strongly affected IFN-induced neutropenia as shown by ROC analysis (area under the curve = 0.860) (Supplementary Fig. 3). Furthermore, gender, hemoglobin level, and platelet count at baseline were also significantly associated with IFN-induced neutropenia by univariate analysis (Table 4). Therefore, we analyzed pretreatment predictive factors for IFN-induced neutropenia in logistic regression models that included the following variables: gender, neutrophil count, platelet count, and rs2305482 or rs4794822 genotypes. In addition to neutrophil count, rs2305482 CC was an independent predictive factor for IFN-induced neutropenia (OR = 2.497; 95 % CI = 1.281–4.864, $P = 0.0072$) (Table 5) as was rs4794822 CC (OR = 2.272; 95 % CI = 1.337–3.861, $P = 0.0024$) (Supplementary Table 2).

Impact of *PSMD3-CSF3* SNPs on tolerated drug doses and treatment efficacy

To evaluate the impact of *PSMD3-CSF3* SNPs on doses of drugs given, and on treatment efficacy, we selected 380 HCV genotype 1-infected patients treated with PEG-IFN/RBV for 48 weeks. They were selected as having information available on the doses of PEG-IFN/RBV that they had received (Supplementary Table 3). It was reported that rates of viral clearance were significantly reduced in patients who could not be maintained on at least 80 % of their drug doses for the duration of PEG-IFN/RBV therapy (McHutchison et al. 2002). In reference to this result, we stratified the patients into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients in the <60 % group for PEG-IFN was significantly higher in patients possessing rs2305482 CC than in those with AA/AC ($P = 0.005$), whereas there was no association for RBV (Fig. 3). The same results were found in the analysis of rs4794822 (Supplementary Fig. 4). However, the univariate analysis of pretreatment factors associated with SVR showed that there was no association between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

Candidate SNP-gene association analysis in IFN-induced neutropenia

To investigate whether the SNPs associated with neutropenia affect the expression of nearby genes, we conducted

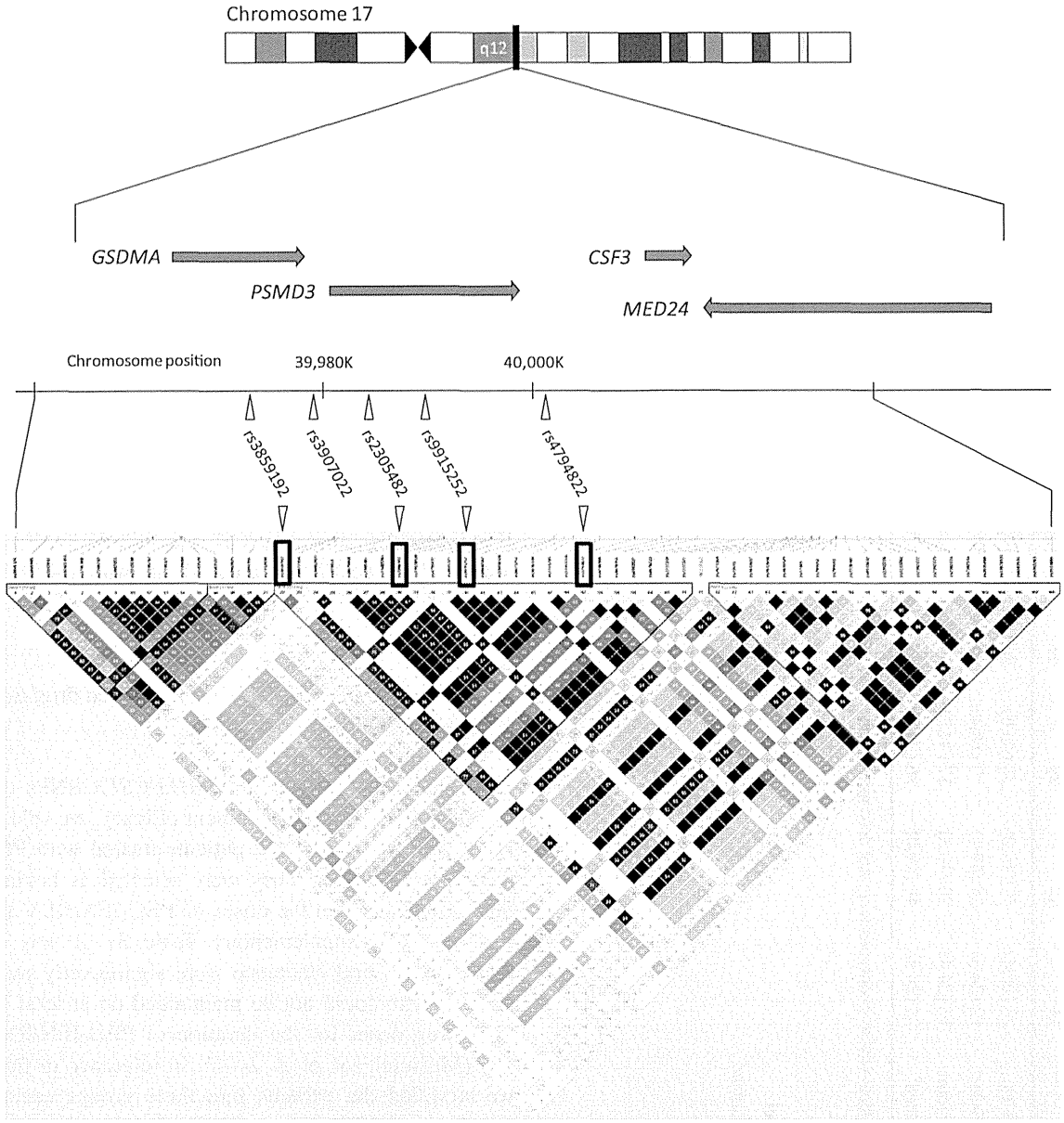

Fig. 2 Position on chromosome and pairwise linkage disequilibrium (r^2) diagrams in the HapMap JPT around the *PSMD3-CSF3* locus

an eQTL analysis. The C allele of rs2305482, a risk for neutropenia, was associated with higher expression levels of *PSMD3* in the populations of LWK: Luhya in Webuye, Kenya (rho = 0.30, $P = 0.006$), and MEX: Mexican ancestry in Los Angeles, California (rho = 0.36, $P = 0.015$) (Supplementary Fig. 5a), whereas it was associated with lower expression levels of *CSF3* in CHB: Han Chinese in Beijing, China, in the probe of ILMN_1655639 (rho = −0.48, $P = 5.5 \times 10^{-6}$) (Supplementary Fig. 5b), and in MEX in that of ILMN_1706852 (rho = −0.33, $P = 0.028$) (Supplementary Fig. 5c).

CSF3 encodes a cytokine, known as G-CSF which is produced by different type of cells such as macrophages, monocytes, stromal cells in the bone marrow, fibroblast, and endothelial cells. The eQTL analysis is based on the whole-genome gene expression variations in lymphoblastoid cell lines derived from HapMap individuals. Therefore, it was still necessary to analyze gene expression in G-CSF producing cells, as well as expression at the protein level. Hence, we measured serum G-CSF levels at baseline and week 2 or 4 (at the time of minimum neutrophil counts) in 127 CHC patients receiving IFN-based therapy. There were no differences in serum G-CSF levels at baseline and the time of minimum neutrophil counts as well as in their changes according to rs2305482 or rs4794822 genotypes (Supplementary Fig. 6a, b). In addition, neutrophil counts

Table 3 Association of SNPs located in *PSMD3-CSF3* with interferon-induced neutropenia

dbSNP rsID	Nearest gene	Risk allele	Allele (1/2)	Case-G2 + R[a] ($n = 100$)			Control-G + R[b] ($n = 656$)			OR[c] (95 % CI)	*P* value[d]
				11	12	22	11	12	22		
rs9915252	*PSMD3*	G	G/C	23 (24.0)	47 (49.0)	26 (27.1)	57 (8.9)	276 (43.3)	304 (47.7)	2.13 (1.57–2.89)	9.64×10^{-7}
rs4794822	*PSMD-CSF3*	C	C/T	42 (42.9)	45 (45.9)	11 (11.2)	130 (21.2)	308 (50.2)	176 (28.7)	2.24 (1.63–3.07)	3.63×10^{-7}
rs3907022	*GSDMA-PSMD*	A	A/G	41 (41.8)	45 (45.9)	12 (12.2)	129 (21.3)	306 (50.6)	170 (28.1)	2.11 (1.54–2.89)	2.31×10^{-6}
rs3859192	*GSDMA*	C	C/T	37 (37.8)	44 (44.9)	17 (17.3)	123 (19.9)	313 (50.7)	181 (29.3)	1.82 (1.34–2.48)	1.04×10^{-4}

Data of allele distribution represent number (%). Data of subjects whose genotypes were not determined were excluded

SNP single nucleotide polymorphism

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Odds ratio for the allele frequency model

[d] *P* value by the Chi square test for the allele frequency model

did not correlate with serum G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a), and there was no difference in the changes of serum G-CSF levels from baseline to the time of minimum neutrophil counts between patients with minimum neutrophil counts of $\geq$1,000/mm^3 and <600/mm^3 (Supplementary Fig. 7b).

Discussion

The present GWAS first showed a strong association between genetic variant and IFN-induced neutropenia, namely, with rs2305482 in *PSMD3* on chromosome 17. Although neutrophil counts at baseline were associated with the rs2305482 genotype and the incidence of neutropenia during IFN-based therapy, the logistic regression analysis revealed that the rs2305482 genotype was independently associated with IFN-induced neutropenia.

Intriguingly, the *PSMD3-CSF3* locus was reported to be associated with total white blood cell (WBC) counts based on GWAS of populations with European ancestry (Crosslin et al. 2012; Soranzo et al. 2009) and in Japanese (Kamatani et al. 2010). These findings were replicated in African Americans (Reiner et al. 2011). Moreover, another GWAS by Okada et al. (2010) showed that rs4794822 in *PSMD3-CSF3* was associated with neutrophil counts in 14 different groups of diseases in Japanese patients who were not undergoing chemotherapy. In the present study, rs4794822 as well as rs2305482 was also associated with pretreatment neutrophil counts in CHC patients (Supplementary Fig. 2). However, there have been no reports showing an association between *PSMD3-CSF3* variants and reduction of WBC or neutrophil counts following treatments such as IFN and chemotherapy. The pairwise LD diagram for *PSMD3-CSF3* by HapMap JPT shows that rs4794822 is in strong LD with rs2305482, which we identified here (Fig. 2). In the present study, both rs2305482 and rs4794822 were associated with IFN-induced neutropenia. Collectively, previous reports together with our results imply that the *PSMD3-CSF3* locus is associated with neutropenia in CHC patients under IFN-based therapy as well as with neutrophil counts in healthy individuals and patients without bone marrow suppressive therapy.

In further clinical investigation, the rs2305482 and rs4794822 genotypes were associated with the doses of PEG-IFN that could be given to HCV genotype 1-infected patients treated with PEG-IFN/RBV (Fig. 3; Supplementary Fig. 4). Unfortunately, we could not collect the detailed information about the reason for the reduction of PEG-IFN in this group. However, we highly suppose that these SNPs affected the doses of PEG-IFN through neutropenia in some cases, since neutropenia is one of the major

Table 4 Univariate analysis of pretreatment factors associated with interferon-induced neutropenia

	Case-G2 + R[a] ($n = 100$)	Control-G + R[b] ($n = 656$)	P value[c]
Gender, male/female	45/55	378/278	0.018
Age, years	58.1 (9.3)	56.9 (10.4)	0.262
Neutrophil count, /mm^3	1,614 (735)	2,742 (979)	<0.001
Hemoglobin, g/dL	13.5 (1.5)	14.2 (1.5)	<0.001
Platelet count, $\times 10^9$/L	136 (46)	163 (57)	<0.001
ALT, IU/L	79.1 (69.7)	83.5 (74.3)	0.574
HCV RNA, log IU/ml	6.0 (0.9)	6.1 (0.8)	0.164
Liver fibrosis, F0-2/F3-4/ND	46/16/38	397/157/102	0.674
rs2305482, AA + AC/CC/ND	74/24/2	591/59/6	<0.001
rs4794822, TT + TC/CC/ND	56/42/2	484/130/42	<0.001

Data are expressed as number for categorical data or the mean (standard deviation) for non-categorical data

ALT alanine transaminase, *ND* not determined

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Categorical variables were compared between groups by the Chi square test and non-categorical variables by the Student's t test

Table 5 Logistic regression analysis of pretreatment factors associated with interferon-induced neutropenia

	OR (95 % CI)	P value
Gender, female	1.229 (0.734–2.059)	0.4331
Neutrophil count, /mm^3	0.998 (0.997–0.998)	<0.0001
Platelet count, $\times 10^9$/L	1.005 (0.953–1.059)	0.8604
rs2305482, CC	2.497 (1.281–4.864)	0.0072

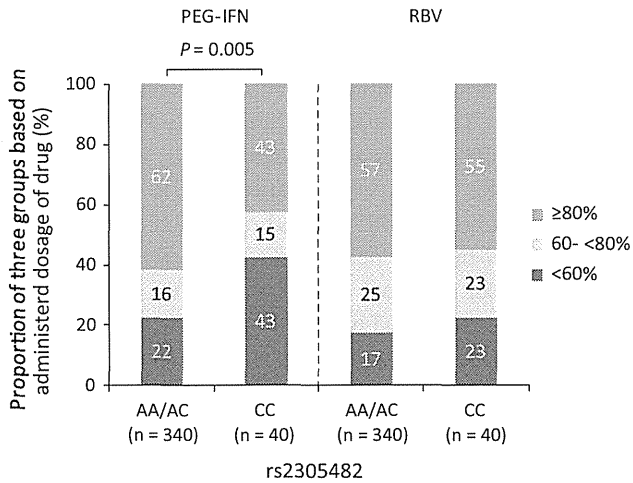

Fig. 3 Administered doses of PEG-IFN and RBV according to rs2305482 genotypes. The patients were stratified into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients receiving <60 % of the PEG-IFN doses was significantly higher in patients with rs2305482 CC than in those with AA/AC ($P = 0.005$, by the Chi square test). *PEG-IFN* pegylated interferon, *RBV* ribavirin

reasons for the dose reduction of PEG-IFN in PEG-IFN/RBV therapy. While, there were no associations between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

PSMD3 encodes the proteasome 26S subunit, non-ATPase 3, a member of the 26S proteasome family, and is involved in the control of cell cycle transition via the ubiquitin–proteasome pathway (Bailly and Reed 1999). *CSF3* encodes G-CSF, which controls the production, differentiation, and function of granulocytes (Nagata et al. 1986). Recombinant G-CSF is widely used to treat patients with severe neutropenia during chemotherapy. Therefore, we hypothesize that *PSMD3-CSF3* variants may influence neutrophil counts through affecting the process of endogenous G-CSF synthesis during IFN-based therapy or other bone marrow suppressive therapies. However, eQTL analysis by Okada et al. (2010) showed that rs4794822 was significantly associated with the expression level of *PSMD3*, rather than that of *CSF3* in the JPT and CHB populations. Our eQTL analysis showed that the risk allele for neutropenia at rs2305482 correlated with higher expression levels of *PSMD3* in LWK and MEX populations (Supplementary Fig. 5a), whereas with lower expression levels of *CSF3* in MEX and especially in CHB populations (Supplementary Fig. 5b, c). However, these results were not replicated in the other probe of *CSF3*. Additionally, we analyzed serum G-CSF levels in CHC patients receiving IFN-based therapy. Although serum G-CSF levels were thought to be increased in response to neutropenia regardless of rs2305482 and rs4794822 genotypes, there was no evidence that they were lower in patients with a risk allele of these SNPs at baseline and during the neutropenic period (Supplementary Fig. 6). Moreover, neutrophil counts did not correlate with serum

G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a). Further functional analyses of these genes and polymorphisms are required to elucidate the reason for the association between *PSMD3-CSF3* and IFN-induced neutropenia as well as neutrophil counts in healthy individuals.

In previous reports, *PLBC4*, *DARC*, *CXCL2*, and *CDK5* loci have also been associated with neutrophil or WBC counts in healthy individuals or patients who were not under chemotherapy (Crosslin et al. 2012; Kamatani et al. 2010; Okada et al. 2010; Reiner et al. 2011). However, there were no associations with these loci discernible in our GWAS.

The important limitation of this study is that the association between rs2305482 and IFN-induced neutropenia was not statistically significant in a genome-wide level. Thompson et al. (2012) also identified no genetic determinants of IFN-induced neutropenia during PEG-IFN/RBV therapy at the level of genome-wide significance by their GWAS. Unlike our study design, they analyzed the association between the reduction of neutrophil counts at week 4 and any SNPs. Indeed, we analyzed the association between the reduction of neutrophil counts at week 2 or 4 and rs2305482 or rs4794822, but there was no significant association. Therefore, further independent replication analyses which are designed in the similar way as our study are desirable.

IFN-free therapies are expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. However, combination therapies of DAA and IFN will continue to be used for some time. Our findings contribute to our understanding of the genetic factors influencing IFN-induced neutropenia. Furthermore, these genetic variants may be associated with neutropenia during chemotherapies for various malignant diseases as well as IFN-based therapy for CHC. Therefore, genetic testing of these variants might be useful for establishing personalized doses of such therapies to minimize drug-induced adverse events. Additionally, our results might contribute to the elucidation of the mechanism of drug-induced neutropenia.

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Conflict of interest The following authors are currently conducting research sponsored by the companies: Yasuhito Tanaka, Keisuke Hino, and Yoshito Itoh by Merck Sharp & Dohme, Corp., Chugai Pharmaceutical Co., Ltd., and Bristol-Myers Squibb; Nobuyuki Enomoto, Shuhei Nishiguchi, and Eiji Tanaka by Merck Sharp & Dohme, Corp. and Chugai Pharmaceutical Co., Ltd.; Naoya Sakamoto by Chugai Pharmaceutical Co., Ltd, Bristol-Myers Squibb, Merck Sharp & Dohme, Corp., and Otsuka Pharmaceutical Co., Ltd.; Hiroshi Yatsuhashi by Chugai Pharmaceutical Co., Ltd.; Akihiro Tamori by Merck Sharp & Dohme, Corp.; Satoshi Mochida by Merck Sharp & Dohme, Corp., Chugai Pharmaceutical Co., Ltd., Bristol-Myers Squibb, and Toray Medical Co., Ltd. The other authors have no conflict of interest.

Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

Bailly E, Reed SI (1999) Functional characterization of rpn3 uncovers a distinct 19S proteasomal subunit requirement for ubiquitin-dependent proteolysis of cell cycle regulatory proteins in budding yeast. Mol Cell Biol 19:6872–6890

Crosslin DR, McDavid A, Weston N, Nelson SC, Zheng X, Hart E, de Andrade M, Kullo IJ, McCarty CA, Doheny KF, Pugh E, Kho A, Hayes MG, Pretel S, Saip A, Ritchie MD, Crawford DC, Crane PK, Newton K, Li R, Mirel DB, Crenshaw A, Larson EB, Carlson CS, Jarvik GP (2012) Genetic variants associated with the white blood cell count in 13,923 subjects in the eMERGE Network. Hum Genet 131:639–652. doi:10.1007/s00439-011-1103-9

Fellay J, Thompson AJ, Ge D, Gumbs CE, Urban TJ, Shianna KV, Little LD, Qiu P, Bertelsen AH, Watson M, Warner A, Muir AJ, Brass C, Albrecht J, Sulkowski M, McHutchison JG, Goldstein DB (2010) ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. Nature 464:405–408. doi:10.1038/nature08825

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401. doi:10.1038/nature08309

George SL, Bacon BR, Brunt EM, Mihindukulasuriya KL, Hoffmann J, Di Bisceglie AM (2009) Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. Hepatology 49:729–738. doi:10.1002/hep.22694

Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, Marcellin P, Muir AJ, Ferenci P, Flisiak R, George J, Rizzetto M, Shouval D, Sola R, Terg RA, Yoshida EM, Adda N, Bengtsson L, Sankoh AJ, Kieffer TL, George S, Kauffman RS, Zeuzem S (2011) Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 364:2405–2416. doi:10.1056/NEJMoa1012912

Kamatani Y, Matsuda K, Okada Y, Kubo M, Hosono N, Daigo Y, Nakamura Y, Kamatani N (2010) Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat Genet 42:210–215. doi:10.1038/ng.531

Kurosaki M, Tanaka Y, Tanaka K, Suzuki Y, Hoshioka Y, Tamaki N, Kato T, Yasui Y, Hosokawa T, Ueda K, Tsuchiya K, Kuzuya T,

Nakanishi H, Itakura J, Takahashi Y, Asahina Y, Matsuura K, Sugauchi F, Enomoto N, Nishida N, Tokunaga K, Mizokami M, Izumi N (2011) Relationship between polymorphisms of the inosine triphosphatase gene and anaemia or outcome after treatment with pegylated interferon and ribavirin. Antivir Ther 16:685–694. doi:10.3851/IMP1796

Matsuura K, Tanaka Y, Watanabe T, Fujiwara K, Orito E, Kurosaki M, Izumi N, Sakamoto N, Enomoto N, Yatsuhashi H, Kusakabe A, Shinkai N, Nojiri S, Joh T, Mizokami M (2014) ITPA genetic variants influence efficacy of PEG-IFN/RBV therapy in older patients infected with HCV genotype 1 and favourable IL28B type. J Viral Hepat 21:466–474. doi:10.1111/jvh.12171

McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK (2002) Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology 123:1061–1069

Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H et al (1986) Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature 319:415–418. doi:10.1038/319415a0

Nishida N, Tanabe T, Takasu M, Suyama A, Tokunaga K (2007) Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. Anal Biochem 364:78–85. doi:10.1016/j.ab.2007.02.005

Ochi H, Maekawa T, Abe H, Hayashida Y, Nakano R, Kubo M, Tsunoda T, Hayes CN, Kumada H, Nakamura Y, Chayama K (2010) ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy—a genome-wide study of Japanese HCV virus patients. Gastroenterology 139:1190–1197. doi:10.1053/j.gastro.2010.06.071

Okada Y, Kamatani Y, Takahashi A, Matsuda K, Hosono N, Ohmiya H, Daigo Y, Yamamoto K, Kubo M, Nakamura Y, Kamatani N (2010) Common variations in PSMD3-CSF3 and PLCB4 are associated with neutrophil count. Hum Mol Genet 19:2079–2085. doi:10.1093/hmg/ddq080

Poordad F, Bronowicki JP, Gordon SC, Zeuzem S, Jacobson IM, Sulkowski MS, Poynard T, Morgan TR, Molony C, Pedicone LD, Sings HL, Burroughs MH, Sniukiene V, Boparai N, Goteti VS, Brass CA, Albrecht JK, Bacon BR (2012) Factors that predict response of patients with hepatitis C virus infection to boceprevir. Gastroenterology 143(608–18):e1–e5. doi:10.1053/j.gastro.2012.05.011

Reiner AP, Lettre G, Nalls MA, Ganesh SK, Mathias R, Austin MA, Dean E, Arepalli S, Britton A, Chen Z, Couper D, Curb JD, Eaton CB, Fornage M, Grant SF, Harris TB, Hernandez D, Kamatini N, Keating BJ, Kubo M, LaCroix A, Lange LA, Liu S, Lohman K, Meng Y, Mohler ER 3rd, Musani S, Nakamura Y, O'Donnell CJ, Okada Y, Palmer CD, Papanicolaou GJ, Patel KV, Singleton AB, Takahashi A, Tang H, Taylor HA Jr, Taylor K, Thomson C, Yanek LR, Yang L, Ziv E, Zonderman AB, Folsom AR, Evans MK, Liu Y, Becker DM, Snively BM, Wilson JG (2011) Genome-wide association study of white blood cell count in 16,388 African Americans: the continental origins and genetic epidemiology network (COGENT). PLoS Genet 7:e1002108. doi:10.1371/journal.pgen.1002108

Sakamoto N, Tanaka Y, Nakagawa M, Yatsuhashi H, Nishiguchi S, Enomoto N, Azuma S, Nishimura-Sakurai Y, Kakinuma S, Nishida N, Tokunaga K, Honda M, Ito K, Mizokami M, Watanabe M (2010) ITPA gene variant protects against anemia induced by pegylated interferon-alpha and ribavirin therapy for Japanese patients with chronic hepatitis C. Hepatol Res 40:1063–1071. doi:10.1111/j.1872-034X.2010.00741.x

Soranzo N, Spector TD, Mangino M, Kuhnel B, Rendon A, Teumer A, Willenborg C, Wright B, Chen L, Li M, Salo P, Voight BF, Burns P, Laskowski RA, Xue Y, Menzel S, Altshuler D, Bradley JR, Bumpstead S, Burnett MS, Devaney J, Doring A, Elosua R, Epstein SE, Erber W, Falchi M, Garner SF, Ghori MJ, Goodall AH, Gwilliam R, Hakonarson HH, Hall AS, Hammond N, Hengstenberg C, Illig T, Konig IR, Knouff CW, McPherson R, Melander O, Mooser V, Nauck M, Nieminen MS, O'Donnell CJ, Peltonen L, Potter SC, Prokisch H, Rader DJ, Rice CM, Roberts R, Salomaa V, Sambrook J, Schreiber S, Schunkert H, Schwartz SM, Serbanovic-Canic J, Sinisalo J, Siscovick DS, Stark K, Surakka I, Stephens J, Thompson JR, Volker U, Volzke H, Watkins NA, Wells GA, Wichmann HE, Van Heel DA, Tyler-Smith C, Thein SL, Kathiresan S, Perola M, Reilly MP, Stewart AF, Erdmann J, Samani NJ, Meisinger C, Greinacher A, Deloukas P, Ouwehand WH, Gieger C (2009) A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat Genet 41:1182–1190. doi:10.1038/ng.467

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104. doi:10.1038/ng.447

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109. doi:10.1038/ng.449

Tanaka Y, Kurosaki M, Nishida N, Sugiyama M, Matsuura K, Sakamoto N, Enomoto N, Yatsuhashi H, Nishiguchi S, Hino K, Hige S, Itoh Y, Tanaka E, Mochida S, Honda M, Hiasa Y, Koike A, Sugauchi F, Kaneko S, Izumi N, Tokunaga K, Mizokami M (2011) Genome-wide association study identified ITPA/DDRGK1 variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C. Hum Mol Genet 20:3507–3516. doi:10.1093/hmg/ddr249

Thompson AJ, Clark PJ, Singh A, Ge D, Fellay J, Zhu M, Zhu Q, Urban TJ, Patel K, Tillmann HL, Naggie S, Afdhal NH, Jacobson IM, Esteban R, Poordad F, Lawitz EJ, McCone J, Shiffman ML, Galler GW, King JW, Kwo PY, Shianna KV, Noviello S, Pedicone LD, Brass CA, Albrecht JK, Sulkowski MS, Goldstein DB, McHutchison JG, Muir AJ (2012) Genome-wide association study of interferon-related cytopenia in chronic hepatitis C patients. J Hepatol 56:313–319. doi:10.1016/j.jhep.2011.04.021

Yang TP, Beazley C, Montgomery SB, Dimas AS, Gutierrez-Arcelus M, Stranger BE, Deloukas P, Dermitzakis ET (2010) Genevar: a database and Java application for the analysis and visualization of SNP-gene associations in eQTL studies. Bioinformatics 26:2474–2476. doi:10.1093/bioinformatics/btq452

Yoshida H, Tateishi R, Arakawa Y, Sata M, Fujiyama S, Nishiguchi S, Ishibashi H, Yamada G, Yokosuka O, Shiratori Y, Omata M (2004) Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C. Gut 53:425–430

Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, Focaccia R, Younossi Z, Foster GR, Horban A, Ferenci P, Nevens F, Mullhaupt B, Pockros P, Terg R, Shouval D, van Hoek B, Weiland O, Van Heeswijk R, De Meyer S, Luo D, Boogaerts G, Polo R, Picchio G, Beumont M (2011) Telaprevir for retreatment of HCV infection. N Engl J Med 364:2417–2428. doi:10.1056/NEJMoa1013086

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Original Article

Autoantibody status and histological variables influence biochemical response to treatment and long-term outcomes in Japanese patients with primary biliary cirrhosis

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Aim: The aim of the present study is to evaluate the factors influencing biochemical response to treatment and the value of biochemical response for predicting long-term outcomes in Japanese patients with primary biliary cirrhosis (PBC).

Methods: Biochemical response to ursodeoxycholic acid (UDCA) or UDCA plus bezafibrate was defined as good (≤upper limit of normal [ULN]), fair (≤1.5 × ULN) or poor (>1.5 × ULN) at 2 years after initiation of UDCA treatment. Associations

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between various factors (including age, sex, autoantibody status and histological variables at baseline), biochemical response to treatment and long-term outcomes were evaluated in 164 Japanese PBC patients.

Results: Anti-gp210 positivity and a higher bile duct loss score were significant risk factors for worse alkaline phosphatase (ALP) response (odds ratios [OR], 2.78 and 1.85, respectively). Age, anti-gp210 positivity and anticentromere positivity were significant risk factors for worse alanine aminotransferase (ALT) response (OR, 1.05, 4.0 and 2.77, respectively). Anti-gp210 positivity and a higher hepatitis score were significant risk factors for worse immunoglobulin (Ig)M response (OR, 2.10 and 2.06, respectively). Worse ALP and IgM response were significant risk factors for progression to late-stage disease without jaundice (OR, 2.27 and 2.32, respectively). Worse ALT response was a significant risk factor for progression to late-stage disease with persistent jaundice (OR, 11.11).

Conclusion: Biochemical response to treatment at 2 years, which is influenced by autoantibody status and histological variables at baseline, can predict long-term outcomes in Japanese patients with PBC.

Key words: anticentromere antibody, anti-gp210 antibody, bezafibrate, biochemical response to treatment, histological staging and grading for primary biliary cirrhosis, ursodeoxycholic acid

INTRODUCTION

PRIMARY BILIARY CIRRHOSIS (PBC) is a chronic, progressive, cholestatic autoimmune liver disease characterized by intrahepatic bile duct destruction, portal inflammation, cirrhosis and, eventually, hepatic failure.[1-3] It is well known that some patients remain at an early stage of the disease for a long time, while others progress to cirrhosis and hepatic failure requiring liver transplantation. Therefore, in order to prevent disease progression, the prediction of long-term outcome at an early stage of the disease is important.[1-3] For this purpose, risk factors for disease progression have been intensively investigated in the past two decades. Risk factors identified to date include age, sex, baseline serum biochemistry, histological variables on liver biopsy,[4-6] autoantibodies such as anti-gp210 and anticentromere that showed the significant association with the disease activity and prognosis,[7-9] and biochemical response to ursodeoxycholic acid (UDCA) therapy.[10-15] Among these risk factors, biochemical response to UDCA therapy strongly predicts long-term prognosis, although treatment response at 1 year (Paris-II criteria) has not been adopted in clinical practice.[15] This criterion still needs external validation[16] and the relationship between treatment response and other risk factors associated with disease progression remains to be elucidated.

In the present study, we first studied the influence of the known risk factors for disease progression (age, sex, histological variables on liver biopsy, and presence of autoantibodies such as anti-gp210 and anticentromere) on the biochemical response to treatment in a cohort followed by the Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan. We then studied the predictive value of treatment response on long-term outcome using multivariate analysis; we found that a worse alkaline phosphatase (ALP) or immunoglobulin (Ig)M response was a significant risk factor for progression to an advanced stage without jaundice, while a worse alanine aminotransferase (ALT) response was a significant risk factor for progression to an advanced stage with persistent jaundice. In addition to other risk factors such as autoantibody status and histological variables, these findings regarding treatment response help identify PBC patients who may need more intensive treatment to prevent progression to liver cirrhosis and hepatic failure.

METHODS

Patients

THREE HUNDRED AND twenty-eight PBC patients who met at least two of the following internationally accepted criteria for a diagnosis of PBC[1-3] were registered in the present cohort study of the Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan: (i) biochemical evidence of cholestasis based mainly on ALP elevation; (ii) presence of serum antimitochondrial antibodies (AMA); and (iii) histological evidence of nonsuppurative destructive cholangitis and destruction of interlobular bile ducts. The registry consisted of 14 university hospitals from October 2007 to November 2011. After excluding patients with PBC–autoimmune hepatitis (AIH) overlap syndrome, chronic hepatitis virus B and C infection, non-alcoholic steatohepatitis and alcoholic liver disease, a total of 287 PBC patients (median age, 55.5 years [range, 30–83]; female, 88.8%; AMA positivity, 92.3%) were enrolled in the current study.

The diagnosis of PBC–AIH overlap syndrome depended on at least two of the following accepted criteria from Poupon *et al.*:[17] (i) ALT of more than 5 times the upper limit of normal (ULN); (ii) IgG of more than 1.5 times ULN; (iii) anti-smooth muscle antibody positivity; and/or (iv) a liver biopsy specimen showing moderate or severe periportal or periseptal lymphocytic piecemeal necrosis.

Definition of clinical stage

The presence of esophageal varices, liver cirrhosis, ascites or hepatocellular carcinoma was evaluated by gastrointestinal endoscopy, ultrasonography and computed tomography. Clinical stage was defined as follows: stage I (early stage), Scheuer stage 1 or 2 without signs of portal hypertension or liver cirrhosis on liver biopsy; stage II (late stage without jaundice), Scheuer stage 3 or 4 on liver biopsy, or any stage with signs of portal hypertension or liver cirrhosis but no persistent jaundice (total bilirubin <2 mg/dL); and stage III (late stage with persistent jaundice), any liver biopsy stage with persistent or progressive jaundice (total bilirubin, ≥2 mg/dL).[8]

Histological evaluation

A total of 306 needle liver biopsy specimens from 287 PBC patients (Scheuer stage I, $n = 159$; stage II, $n = 92$; stage III, $n = 43$; stage IV, $n = 12$) were re-evaluated according to the new staging and grading system by Nakanuma *et al.* using both hematoxylin–eosin and Orcein staining.[18,19] Orcein staining was used to evaluate the deposition of copper-binding proteins in hepatocytes. The degree of cholangitis, hepatitis, bile duct loss, fibrosis and deposition of Orcein positive granules were graded as 0–3 by two independent observers (Y. N. and M. I.) as follows: cholangitis (0, absent; 1, <1/3 of portal tract; 2, 1/3–2/3 of portal tract; 3, >2/3 of portal tract), hepatitis (0, absent; 1, mild; 2, moderate; 3, severe), bile duct loss (0, absent; 1, 1/3 of portal tract; 2, 1/3–2/3 of portal tract; 3, >2/3 of portal tract), Orcein positive granules (0, absent; 1, 1/3 of portal area; 2, 1/3–2/3 of portal area; 3, >2/3 of portal area), fibrosis (0, none or limited to the portal tract; 1, periportal fibrosis; 2, septal fibrosis; 3, cirrhosis). When there was disagreement between the two independent observers, consensus was reached on further review by the same two observers (Y. N. and M. I.) after thorough discussion by observing the same specimens under the same microscope.

Evaluation of biochemical response

The enrolled patients were retrospectively and prospectively studied for the course of medical treatment and biochemical response in addition to histological re-evaluation of liver biopsies. Among the 287 patients, there were 164 AMA positive PBC patients (median age, 49.5 years [range, 32–78]; female, 89.6%), with data available on the biochemical response to UDCA or UDCA plus bezafibrate at 2 years after initiation of UDCA treatment. The biochemical response was defined as good (≤ULN), fair (≤1.5 × ULN) or poor (>1.5 × ULN) based on ALT, ALP and IgM values at 2 years after initiation of UDCA treatment.[20] In these 164 patients, 160 liver biopsy specimens at the time of initial diagnosis (Scheuer stage I, $n = 96$; stage II, $n = 45$; stage III, $n = 16$; stage IV, $n = 5$; unknown, $n = 2$) were retrievable for analysis of cholangitis, hepatitis, bile duct loss and fibrosis, while 95 specimens were additionally evaluated for Orcein positive granules according to the process described by Nakanuma *et al.*[18,19] The observation period (median, 61.5 months [range, 24–306]) was defined as the time from the date of initial diagnosis until the date of death, liver transplantation, death due to non-liver-related disease or end of follow up, whichever came first. Although the start of follow up was not totally the same as the date of liver biopsy or the date of initiation of UDCA treatment, the liver biopsy was performed in most cases ($n = 134/160 = 83.8\%$) within 1 month before ($n = 119$) or 3 months after ($n = 15$) the initiation of UDCA treatment. In other cases ($n = 26/160 = 16.2\%$), the liver biopsy was performed within 12 months ($n = 12$) and at 48 months ($n = 1$), 68 months ($n = 1$) and 84 months ($n = 1$) before or within 12 months ($n = 3$), 36 months ($n = 3$), 60 months ($n = 4$) and at 78 months ($n = 1$) after the initiation of UDCA treatment.

Enzyme-linked immunosorbent assay (ELISA)

In brief, serial serum samples stored at −20°C at each institution and serum samples obtained at various time points were used to measure autoantibody titers over the observation period. Titers of antibodies to the gp210 C-terminal peptide amino acid 1863–1887 (SPNALPPARKA SPPSGLWSP AYASH) were measured as described elsewhere.[7] Titers for recombinant centromere B proteins and mitochondrial M2 antigens were measured using the CENP-B ELISA kit (MBL, Nagoya, Japan) and the M2 Mesacup-2 kit (MBL), respectively.[8]

Ethics board approval

The present study was approved by the ethics board of the NHO Nagasaki Medical Center and each participating university hospital. All subjects gave informed consent for their serum samples to be used in advancing medical knowledge on the causes of PBC.

Statistical analysis

Data are reported as means ± standard deviation unless otherwise stated. The association between histological scores and autoantibody status was analyzed using an ordinal logistic regression model. Associations among clinical progression, biochemical response to treatment, autoantibody status and histological variables were analyzed using ordinal logistic regression or multivariate logistic regression with stepwise selection. Statistical analysis was performed with SAS statistical software (version 9.2; SAS Institute, Cary, NC, USA). All reported *P*-values are two-sided; $P < 0.05$ was considered statistically significant.

RESULTS

Association between antibody status and histological variables in 287 PBC patients

AS SHOWN IN Table 1, male sex was a significant risk factor for more severe hepatitis (odds ratio [OR], 2.82; 95% confidence interval [CI], 1.44–5.56) and Orcein positive granules (OR, 4.94; 95% CI, 1.88–13.24). Age was a significant risk factor for more severe cholangitis (OR, 1.03; 95% CI, 1.01–1.05) and fibrosis (OR, 1.03; 95% CI, 1.01–1.05). Anti-gp210 positivity was a significant risk factor for more severe hepatitis (OR, 2.33; 95% CI, 1.49–3.66), bile duct loss (OR, 2.82; 95% CI, 1.79–4.47), Orcein positive granules (OR, 4.23; 95% CI, 1.92–9.60) and fibrosis (OR, 2.21; 95% CI, 1.16–4.22).

Clinical course of the 164 patients who were evaluated for biochemical response

At the time of initial histological diagnosis, the distribution of histological scores was as follows: fibrosis score F0 ($n = 46$), F1 ($n = 66$), F2 ($n = 33$), F3 ($n = 15$) and not done (ND) ($n = 4$); cholangitis score, 0 ($n = 58$), 1 ($n = 87$), 2 ($n = 13$), 3 ($n = 2$) and ND ($n = 4$); hepatitis score 0 ($n = 36$), 1 ($n = 56$), 2 ($n = 45$), 3 ($n = 23$) and ND ($n = 4$); bile duct loss score, 0 ($n = 43$), 1 ($n = 57$), 2 ($n = 30$), 3 ($n = 30$) and ND ($n = 4$); and Orcein positive granules, 0 ($n = 70$), 1 ($n = 14$), 2 ($n = 4$), 3 ($n = 7$) and ND ($n = 69$). Serum titers of antibodies against gp210 were usually measured every 6–12 months in the 164 patients whose biochemical response was evaluated. AMA and anticentromere antibodies were measured annually. In these 164 patients, AMA, anti-gp210 and anticentromere antibodies were positive at the time of initial diagnosis in 164 (100%), 50 (30.5%) and 52 (31.7%) patients, respectively. While AMA and anticentromere antibodies did not seroconvert from positive to negative or vice versa, anti-gp210 antibodies seroconverted from positive to negative in 10 patients and vice versa in 10 patients.

In this cohort of 164 patients, 139 were at clinical stage I (early stage), 21 were at stage II (late stage without jaundice) and four were at stage III (late stage with persistent jaundice) at the time of initial diagnosis. During the observation period (median, 61.5 months [range, 24–306]), 115 patients (70.1%) were treated with UDCA alone (300–900 mg/day), 34 patients (20.7%) were treated with UDCA plus bezafibrate

Table 1 Association between autoantibody status and histological variables

Variable	†OR (95% CI) for higher histological score				
	Cholangitis	Hepatitis	Bile duct loss	Orcein positive granules	Fibrosis
Male sex	1.33 (0.68–2.65)	**2.82 (1.44–5.56)**	1.84 (0.99–3.41)	**4.94 (1.88–13.24)**	1.33 (0.68–2.65)
Age	**1.03 (1.01–1.05)**	1.01 (0.99–1.03)	0.99 (0.97–1.01)	0.99 (0.96–1.02)	**1.03 (1.01–1.05)**
Anti-gp210 positivity	0.94 (0.58–1.52)	**2.33 (1.49–3.66)**	**2.82 (1.79–4.47)**	**4.23 (1.92–9.60)**	**2.21 (1.16–4.22)**
Anticentromere positivity	0.95 (0.57–1.59)	0.97 (0.60–1.56)	1.19 (0.72–1.94)	2.01 (0.83–4.86)	0.74 (0.40–1.37)

†Ordinal logistic regression.
Bolding indicates statistical significance.
CI, confidence interval; OR, odds ratio.

(200–400 mg/day) and 15 patients (9.1%) were treated with low-dose prednisolone (≤5 mg/day) in addition to UDCA with or without bezafibrate. The daily dose of UDCA was escalated from 300 mg to 600 mg or from 600 mg to 900 mg within 3–6 months of treatment when the biochemical response of ALP was inadequate. Administration of bezafibrate (200–400 mg/day) was started at 3 months to 16 years (median, 30 months) after the initiation of UDCA in some patients when the biochemical response of ALP was inadequate.

When biochemical response was evaluated 2 years after the initiation of UDCA treatment, 125 (76.2%) were being treated with UDCA alone, 23 (14.0%) with UDCA plus bezafibrate and 14 (8.5%) with UDCA with or without bezafibrate plus low-dose prednisolone. In addition, there was no significant difference in the treatment among PBC patients with three different clinical stages at the end of observation (χ^2, 4.3105; $P = 0.3656$).

The number of good, fair, and poor responders with respect to ALT, ALP and IgM were as follows. ALT response was graded as good in 124 patients, fair in 16 and poor in 24. ALP response was considered good in 107 patients, fair in 32 and poor in 25. IgM response was classified as good in 88 patients, fair in 42, poor in 28 and ND in six. There were 19 out of 139 patients at clinical stage I who progressed to clinical stage II, and three progressed from stage I to stage III. In addition, four out of 21 patients at clinical stage II progressed to clinical stage III. Consequently, there were 117 patients at clinical stage I, 36 at stage II and 11 at stage III at the end of the observation period.

Association between autoantibody status or histological score and biochemical response

We first studied the association between autoantibody status or histological score and biochemical response to treatment using ordinal logistic regression (Table 2). Anti-gp210 positivity was a significant risk factor for worse ALT, ALP and IgM response (ALT response, OR, 4.00; 95% CI, 1.58–10.10; ALP response, OR, 2.78; 95% CI, 1.30–5.91; and IgM response, OR, 2.10; 95% CI, 1.02–4.31), while anticentromere positivity was a significant risk factor for worse ALT response (OR, 2.77; 95% CI, 1.03–7.46) (Table 2a). In addition, higher score of bile duct loss was a significant risk factor for worse ALP response (OR, 1.85; 95% CI, 1.21–2.82) and worse ALT response (OR, 1.550; 95% CI, 0.950–2.525). Higher score of hepatitis was a significant risk factor for worse IgM response (OR, 2.06; 95% CI, 1.24–3.43) (Table 2b). In contrast, higher score of cholangitis was a protective factor against worse ALP response (OR, 0.551;

Table 2 Factors influencing biochemical response to treatment

a

Autoantibody status as a risk factor for a worse biochemical response, OR (95% CI)†

	ALT response	ALP response	IgM response
Male sex	3.289 (0.863–12.500)	1.285 (0.374–4.405)	1.553 (0.462–5.076)
Age	**0.944 (0.902–0.988)**	0.999 (0.963–1.035)	0.988 (0.956–1.021)
Anti-gp210 positivity	**4.000 (1.582–10.101)**	**2.785 (1.307–5.917)**	**2.105 (1.026–4.310)**
Anticentromere positive	**2.770 (1.033–7.462)**	1.047 (0.460–2.381)	1.623 (0.779–3.378)

b

Higher histological score as a risk factor for a worse biochemical response, OR (95% CI)†

	ALT response	ALP response	IgM response
Male sex	2.369 (0.679–8.264)	1.135 (0.323–3.984)	1.283 (0.374–4.424)
Age	**0.955 (0.914–0.998)**	1.007 (0.971–1.045)	0.986 (0.953–1.019)
Hepatitis score	1.050 (0.553–1.996)	1.277 (0.746–2.183)	**2.066 (1.24–3.436)**
Bile duct loss score	**1.550 (0.950–2.525)**	**1.855 (1.215–2.825)**	0.837 (0.563–1.243)
Cholangitis score	0.841 (0.390–1.815)	**0.551 (0.283–1.074)**	1.037 (0.577–1.862)
Fibrosis score	0.808 (0.341–1.915)	0.594 (0.286–1.231)	0.671 (0.342–1.315)

†Ordinal logistic regression.
Bolding indicates statistical significance.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

95% CI, 0.283–1.074). Fibrosis score was not associated with any biochemical response (Table 2b). Age was a significant protective factor against worse ALT response (Table 2).

Association between PBC progression and biochemical response, histological scores and autoantibody status

The risk factors for the progression to clinical stage II or III were analyzed by stepwise regression using all 164 patients at the end of observation. Worse ALT response was a significant risk factor for progression to clinical stage III (OR, 11.11; 95% CI, 4.16–50.0). Worse ALP and IgM responses were significant risk factors for progression to clinical stage II (ALP response, OR, 2.27; 95% CI, 1.28–4.16; and IgM response, OR, 2.32; 95% CI, 1.33–4.16) (Table 3a). In addition, higher scores for hepatitis, bile duct loss and fibrosis were significant risk factors for progression to clinical stage II (hepatitis, OR, 2.26; 95% CI, 1.22–4.39; bile duct loss, OR, 1.90; 95% CI, 1.14–3.29; and fibrosis, OR, 2.20; 95% CI, 1.14–4.44) (Table 3b). Anti-gp210 positivity was a significant risk factor for progression to clinical stage II (OR, 3.54; 95% CI, 1.49–8.82) and to stage III (OR, 29.88; 95% CI, 5.01–579.39), while anticentromere positivity was a significant risk factor for progression to clinical stage II

Table 3 Risk factors for progression to clinical stage II or III

a

A worse biochemical response as a risk factor for clinical progression

Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.07 (1.03–1.13)	–
ALT response	–	11.11 (4.16–50.00)
ALP response	2.27 (1.28–4.16)	–
IgM response	2.32 (1.33–4.16)	–

b

Higher histological score as a risk factor for clinical progression

Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.07 (1.03–1.13)	–
Cholangitis score	–	–
Hepatitis score	2.26 (1.22–4.39)	–
Bile duct loss score	1.90 (1.14–3.29)	–
Fibrosis score	2.20 (1.14–4.44)	–

c

Autoantibody status as a risk factor for clinical progression

Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.05 (1.01–1.10)	0.90 (0.83–0.96)
Anti-gp210 positivity	3.54 (1.49–8.82)	29.88 (5.01–579.39)
Anticentromere positivity	3.73 (1.58–9.27)	–

†Stepwise logistic regression.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

Table 4 Multivariate analysis of risk factors for progression to clinical stage II or III

a		
Autoantibody status, higher histological score and a worse biochemical response as risk factors for clinical progression		
Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.08 (1.01–1.15)	–
Anti-gp210 positivity	4.83 (1.26–21.98)	–
Anticentromere positivity	23.59 (5.17–155.21)	–
Cholangitis score	–	–
Hepatitis score	4.18 (1.79–11.33)	–
Bile duct loss score	–	–
Fibrosis score	3.83 (1.66–10.25)	–
ALT response	–	10.86 (3.98–55.55)
ALP response	4.23 (1.85–11.36)	–
IgM response	–	–

b		
Autoantibody status and a worse biochemical response as risk factors for clinical progression		
Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.08 (1.03–1.15)	–
Anti-gp210 positivity	5.49 (1.89–17.89)	13.94 (2.04–284.87)
Anticentromere positivity	5.44 (1.87–17.74)	–
ALT response	–	10.10 (3.43–55.55)
ALP response	2.43 (1.26–5.00)	–
IgM response	2.22 (1.19–4.34)	–

†Stepwise logistic regression.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

(OR, 3.73; 95% CI, 1.58–9.27) (Table 3c). Age was a significant risk factor for progression to clinical stage II (OR, 1.05–1.07) (Table 3).

Multivariate analysis of risk factors for PBC progression

When risk factors such as age, sex, autoantibody status, histological score and biochemical response were analyzed at the same time using multivariate logistic regression with stepwise selection, anti-gp210 positivity, anticentromere positivity, higher hepatitis and fibrosis scores, and worse ALP response remained significant risk factors for progression to clinical stage II (Table 4a), and worse ALT response (OR, 10.86; 95% CI, 3.98–55.5) remained a significant risk factor for progression to clinical stage III (OR, 10.86; 95% CI, 3.98–55.5) (Table 4a). When variables involving histological scores were excluded from the multivariate analysis, both anti-gp210 positivity and worse ALT response remained significant risk factors (OR, 13.94; 95% CI, 2.04–284.87 and OR, 10.10; 95% CI, 3.43–55.55, respectively) for progression to clinical stage III (Table 4b). Anti-gp210 positivity, anticentromere positivity, worse ALP response and worse IgM response remained significant risk factors for progression to stage II (Table 4b).

DISCUSSION

IN THE PRESENT study, we formally studied the relationship among four variables (autoantibody status, histological lesions, biochemical response to UDCA or UDCA plus bezafibrate treatment, and disease

progression) in a cohort of Japanese PBC patients that were not previously studied.[7,8] We clearly showed that biochemical response to treatment at 2 years after initiation of UDCA treatment, which is significantly influenced by baseline autoantibody status and histological variables, is a useful biomarker for predicting long-term outcomes in Japanese patients with PBC.

Many studies have shown that UDCA contributes to improved liver enzyme levels, delayed histological progression and prolonged survival in patients with PBC, particularly those who are diagnosed at an early stage.[21] In order to evaluate the effect of UDCA treatment on long-term outcomes in patients with PBC in a clinical setting, prognostic criteria such as biochemical response to UDCA have recently been introduced into clinical practice.[10–15] These include the Barcelona,[10] Paris-I,[11] Rotterdam,[12] Toronto[13] and Ehime criteria,[14] which show that a good biochemical response at 1 to 2 years after initiation of UDCA treatment is associated with good prognosis, similar to estimates for matched controls. In addition, the Paris-II criterion, which incorporates ALP and AST levels ($<1.5 \times$ ULN) after 1 year of UDCA treatment, can predict the absence of adverse outcomes including liver-related death, liver transplantation, referral to a transplant unit, complications of cirrhosis or histological evidence of cirrhosis in patients with histological or clinical early stage PBC.[15] However, the timing for assessing response to treatment with UDCA remains under debate.[16] Furthermore, progression to liver cirrhosis and hepatic failure with jaundice, which are respectively defined as clinical stage II and III in the present study, were not separately analyzed in these studies, thus limiting the ability to predict the most unfavorable outcomes of PBC.[7,8]

Bezafibrate in combination with UDCA was recently found to be effective for normalizing serum levels of liver enzymes, especially ALP and IgM.[22–25] Although UDCA is currently the only drug approved for the treatment of PBC, bezafibrate has been widely used in Japanese patients with an inadequate response to UDCA treatment alone since the first report of a favorable effect with bezafibrate was observed in Japanese PBC patients.[22] Bezafibrate is effective in approximately 80% of PBC patients in whom liver enzymes do not normalize on UDCA monotherapy within 6 months.[24] However, the long-term effects of bezafibrate are not yet clearly established, and criteria for interpreting the biochemical response to bezafibrate have not been defined to date.

In the present study, bezafibrate treatment was initiated at 3–24 months after the initiation of UDCA treatment in approximately 22.5% of patients in whom ALP levels did not normalize. In contrast, serum levels of ALP, IgM or both continued to decrease in the second to third year of UDCA treatment in some patients (data not shown). Therefore, we arbitrarily defined biological response to UDCA or UDCA plus bezafibrate with guidance from previous studies[13,15] as good (<ULN), fair ($\leq 1.5 \times$ ULN) or poor ($>1.5 \times$ ULN) based on ALT, ALP or IgM levels at 2 years after starting UDCA.[20]

Although the present study includes both retrospective and prospective data, the analysis clearly showed that: (i) a worse ALP or IgM response to treatment is significantly associated with progression to clinical stage II (Table 3a); and (ii) a worse ALT response to treatment is associated with progression to clinical stage III (Table 3a). In addition, the present study showed for the first time that (iii) anti-gp210 positivity and more severe bile duct loss are significantly associated with worse ALP response (Table 2); (iv) anti-gp210 and anticentromere positivity are significantly associated with worse ALT response (Table 2); and (v) anti-gp210 positivity and more severe hepatitis are significantly associated with worse IgM response (Table 2). Although the mechanisms underlying these association remain to be elucidated, the present results strongly imply the involvement of different pathophysiological mechanisms in PBC progression, namely, bile duct damage or destruction as represented by the ALP response, hepatocyte damage or destruction as represented by the ALT response, and immunological alteration as represented by the IgM response.[18,19] Based on this hypothesis, more severe bile duct damage (i.e. worse ALP response) is not enough for progression to clinical stage III, but more severe hepatocyte damage (i.e. worse ALT response) is necessary for the progression to clinical stage III. Because anti-gp210 positivity represents both more severe bile duct and hepatocyte damage,[7,8] our findings in the present study seem to reasonably suggest that anti-gp210 positivity contributes to the progression to either clinical stage II or III.

In order to find more feasible surrogate markers for predicting the long-term outcome of PBC in the clinical setting, we performed multivariate analysis with various combinations of risk factors including age, sex, autoantibody status, histological variables and biochemical response to treatment. Multivariate analysis including all of these factors revealed that worse ALT response is the strongest risk factor for progression to clinical stage III, whereas anti-gp210 positivity no longer remained a significant predictor for progression to clinical stage III. Anticentromere positivity became the strongest risk

factor for progression to clinical stage II (Table 4a). When histological variables were excluded from the multivariate analysis, anti-gp210 positivity and worse ALT response became the strongest risk factors for progression to clinical stage III, and anti-gp210 and anticentromere positivity became the strongest risk factors for progression to stage II (Table 4b). Worse ALP and IgM responses were also significant risk factors for progression to clinical stage II. Thus, histological variables dramatically affected the significance of autoantibody status for PBC progression to clinical stage II or III. However, because the number of PBC patients who undergo liver biopsy is declining markedly in recent clinical practice, we contend that the combination of autoantibody status and biochemical response to treatment is a feasible surrogate marker for identifying patients at risk for a poor long-term outcome during the initial phase of follow up. In order to establish the most appropriate criteria for identifying patients who will progress to clinical stage II or III, further studies are needed in larger numbers of PBC patients with clinical stage II and III in the prospective study.

In the present study, anti-gp210 antibodies disappeared in 10 out of 164 patients (6.0%) after the initiation of UDCA treatment. All of these patients were at clinical stage I and showed good ALP and ALT responses. These patients had less severe hepatitis and bile duct loss compared to clinical stage I patients who were persistently positive for anti-gp210 antibodies with good ALP and ALT responses (data not shown). These results may support the hypothesis that anti-gp210 antibody production is closely associated with bile duct and hepatocellular pathology specific to the pathogenesis of PBC that is reversible by treatment with UDCA or UDCA plus bezafibrate during the early stage of the disease.[7] On the other hand, anticentromere antibodies were persistently positive even after treatment with UDCA or UDCA plus bezafibrate. They were not significantly associated with any histological variables examined in the present study (Table 1). In addition, six out of 27 patients positive for anticentromere antibodies (22.2%) with good ALT and ALP responses to UDCA treatment progressed from clinical stage I to II during the observation period. These results suggest that the presence of a currently unknown mechanism is involved in disease progression among patients positive for anticentromere antibodies, beyond a more severe ductular reaction that may lead to the progression of fibrosis.[8]

In conclusion, we demonstrated comprehensively for the first time the association among risk factors for the progression of PBC. These risk factors include autoantibody status (i.e. anti-gp210 and anticentromere antibodies), histological lesions and biochemical response to treatment with UDCA or UDCA plus bezafibrate.

In order to better characterize practical and robust surrogate markers for predicting long-term outcomes and to better understand the mechanisms underlying disease progression in PBC, a prospective cohort study of biochemical response and genetic approaches including genome-wide association study is currently underway.[26–32]

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REFERENCES

1 Poupon R. Primary biliary cirrhosis: a 2010 update. *J Hepatol* 2010; **52**: 745–58.
2 Lindor KD, Gershwin ME, Poupon R, Kaplan M, Bergasa N, Heathcote EJ. AASLD practice guidelines: primary biliary cirrhosis. *Hepatology* 2009; **50**: 291–308.
3 European Association for the Study of the liver. EASL clinical practice guidelines: management of cholestatic liver diseases. *J Hepatol* 2009; **51**: 237–67.
4 Degott C, Zafrani ES, Callard P, Balkau B, Poupon RE, Poupon R. Histopathological study of primary biliary cirrhosis and the effect of ursodeoxycholic acid treatment on histology progression. *Hepatology* 1999; **29**: 1007–12.
5 Corpechot C, Carrat F, Poupon R, Poupon RE. Primary biliary cirrhosis: incidence and predictive factors of cirrhosis development in ursodiol-treated patients. *Gastroenterology* 2002; **122**: 652–8.
6 Poupon RE, Lindor KD, Pares A, Chazouilleres O, Poupon R, Heathcote EJ. Combined analysis of the effect of

treatment with ursodeoxycholic acid on histologic progression in primary biliary cirrhosis. *J Hepatol* 2003; 39: 12–6.
7 Nakamura M, Shimizu-Yoshida Y, Takii Y *et al.* Antibody titer to gp210-C terminal peptide as a clinical parameter for monitoring primary biliary cirrhosis. *J Hepatol* 2005; 42: 386–92.
8 Nakamura M, Kondo H, Mori T *et al.* Anti-gp210 and anti-centromere antibodies are different risk factors for the progression of primary biliary cirrhosis. *Hepatology* 2007; 45: 118–27.
9 Czaia A. Autoantibodies as prognostic markers in autoimmune liver disease. *Dig Dis Sci* 2010; 55: 2144–61.
10 Pares A, Caballeria , Rodes J. Excellent long-term survival in patients with primary biliary cirrhosis and biochemical response to ursodeoxycholic acid. *Gastroenterolgy* 2006; 130: 715–20.
11 Corpechot C, Abenavoli L, Rabahi N *et al.* Biochemical response to ursodeoxycholic acid and long-term prognosis in primary biliary cirrhosis. *Hepatology* 2008; 48: 871–7.
12 Kuiper EM, Hansen BE, de Vries RA *et al.* Improved prognosis of patients with primary biliary cirrhosis that have a biochemical response to ursodeoxycholic acid. *Gastroenterology* 2009; 136: 1281–7.
13 Kumagi T, Guindi M, Fischer SE *et al.* Baseline ductopenia and treatment response predict long-term histological progression in primary biliary cirrhosis. *Am J Gastroenterol* 2010; 105: 2186–94.
14 Azemoto N, Kumagi T, Abe M *et al.* Biochemical response to ursodeoxycholic acid predicts long-term outcome in Japanese patients with primary biliary cirrhosis. *Hepatol Res* 2011; 41: 310–7.
15 Corpechot C, Chazouilleres O, Poupon R. Early primary biliary cirrhosis: biochemical response to treatment and prediction of long-term outcome. *J Hepatol* 2011; 55: 1361–7.
16 Papastergiou V, Tsochatzis EA, Rodriquez-Peralvarez M *et al.* Biochemical criteria at 1 year are not robust indicators of response to ursodeoxycholic acid in early primary biliary cirrhosis: results from 29-year cohort study. *Aliment Pharmacol Ther* 2013 [Epub ahead of print].
17 Poupon R. Autoimmune overlapping syndromes. *Clin Liver Dis* 2003; 7: 865–78.
18 Hiramatsu K, Aoyama H, Zen Y, Aishima S, Kitagawa S, Nakanuma Y. Proposal of a new staging and grading system of the liver for primary biliary cirrhosis. *Histopathology* 2006; 49: 466–78.
19 Nakanuma Y, Zen Y, Harada K *et al.* Application of a new histological staging and grading system for primary biliary cirrhosis to liver biopsy specimens: interobserver agreement. *Pathol Int* 2010; 60: 167–74.
20 The Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan, Working Subgroup (English version) for Clinical Practice Guidelines for Primary BiliaryCirrhosis. Guidelines for the management of primary biliary cirrhosis. *Hepatol Res* 2014; 44 (Suppl 1): 71–90.
21 Corpechot C, Carrat F, Bahr A, Chretien Y, Poupon RE, Poupon R. The effect of ursodeoxycholic acid therapy on the natural course of primary biliary cirrhosis. *Gastroenterology* 2005; 128: 297–303.
22 Kurihara T, Maeda A, Shigemoto M, Yamashita K, Hashimoto E. Investigation into the efficacy of bezafibrate against primary biliary cirrhosis, with histological references from cases receiving long-term monotherapy (letter). *Am J Gastroenterol* 2002; 97: 212–14.
23 Iwasaki S, Ohira H, Nishiguchi S *et al.* The efficacy of ursodeoxycholic acid and bezafibrate combination therapy for primary biliary cirrhosis: a prospective, multicenter study. *Hepatol Res* 2008; 38: 557–64.
24 Takeuchi Y, Ikeda F, Fujioka S *et al.* Additive improvement induced by bezafibrate in patients with primary biliary cirrhosis showing refractory response to ursodeoxycholic acid. *J Gastroenterol Hepatol* 2011; 26: 1395–401.
25 Lens S, Leoz M, Nazal L, Bruguera M, Pares A. Bezafibrate normalizes alkaline phosphate in primary biliary cirrhosis patients with incomplete response to ursodeoxycholic acid. *Liver Int* 2014; 34: 197–203.
26 Nakamura M, Yasunami M, Kondo H *et al.* Analysis of HLA-DRB1 polymorphisms in Japanese patients with primary biliary cirrhosis (PBC): the HLA-DRB1 polymorphism determines the relative risk of antinuclear antibodies for disease progression in PBC. *Hepatol Res* 2010; 40: 494–504.
27 Juran BD, Lazaridis KN. Update on the genetics and genomics of PBC. *J Autoimmun* 2010; 35: 181–7.
28 Aiba Y, Nakamura M, Joshita S *et al.* Genetic polymorphisms in CTLA4 and SLC4A2 are differentially associated with pathogenesis of primary biliary cirrhosis in Japanese patients. *J Gastroenterol* 2011; 46: 1203–12.
29 Poupon R, Ping C, Chretien Y *et al.* Genetic factors of susceptibility and of severity in primary biliary cirrhosis. *J Hepatol* 2008; 49: 1038–45.
30 Inamine T, Higa S, Noguchi F *et al.* Association of genes involved in bile acid synthesis with the progression of primary biliary cirrhosis in Japanese patients. *J Gastroenterol* 2013; 48: 1160–70.
31 Ohishi Y, Nakamuta M, Ishikawa N *et al.* Genetic polymorphisms of OCT-1 confer susceptibility to severe progression of primary biliary cirrhosis in Japanese patients. *J Gastroenterol* 2014; 49: 332–42.
32 Nakamura M, Nishida N, Kawashima M *et al.* Genome-wide association study identifies TNFSF15 and POU2AF1 as susceptibility loci for primary biliary cirrhosis in the Japanese population. *Am J Hum Genet* 2012; 91: 721–8.

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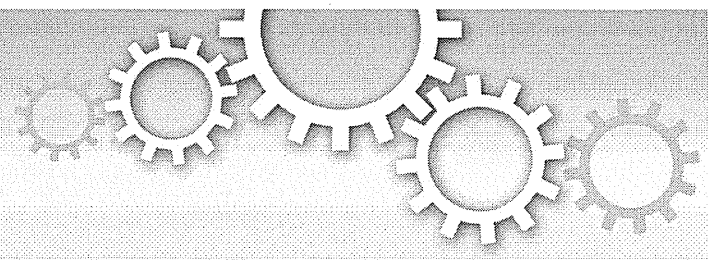

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Small tRNA-derived RNAs are increased and more abundant than microRNAs in chronic hepatitis B and C

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Persistent infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) account for the majority of cases of hepatic cirrhosis and hepatocellular carcinoma (HCC) worldwide. Small, non-coding RNAs play important roles in virus-host interactions. We used high throughput sequencing to conduct an unbiased profiling of small (14-40 nts) RNAs in liver from Japanese subjects with advanced hepatitis B or C and hepatocellular carcinoma (HCC). Small RNAs derived from tRNAs, specifically 30–35 nucleotide-long 5′ tRNA-halves (5′ tRHs), were abundant in non-malignant liver and significantly increased in humans and chimpanzees with chronic viral hepatitis. 5′ tRH abundance exceeded microRNA abundance in most infected non-cancerous tissues. In contrast, in matched cancer tissue, 5′ tRH abundance was reduced, and relative abundance of individual 5′ tRHs was altered. In hepatitis B-associated HCC, 5′ tRH abundance correlated with expression of the tRNA-cleaving ribonuclease, angiogenin. These results demonstrate that tRHs are the most abundant small RNAs in chronically infected liver and that their abundance is altered in liver cancer.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are phylogenetically unrelated non-cytopathic viruses that infect the liver[1]. While HBV is a DNA virus, and HCV is a positive-strand RNA virus, both have the capacity to persist for years in some infected individuals. Hundreds of millions of people worldwide are chronic carriers of HBV or HCV, 30–50% of whom have chronic liver disease[2]. Together, these viral infections are responsible for ~60% of liver cirrhosis and ~80% of hepatocellular carcinoma (HCC), a leading cause of cancer-related deaths worldwide. Numerous studies suggest that microRNAs (miRNAs), small 21–23 nt non-coding RNAs are important in the pathogenesis of these infections, modulating viral replication as well as host responses and possibly influencing the risk of carcinogenesis[3]. For example the HBV X protein represses expression of miR-148a, potentially enhancing tumorigenesis[4]. In contrast, HCV infection is associated with higher expression of miR-21, which targets key components of Toll-like receptor signaling pathways, possibly facilitating viral evasion of innate immune responses[5]. miR-122 stabilizes HCV RNA and promotes its replication[6,7], and the importance of this interaction is reflected in the clinical development of an anti-miR-122 antagomir (miravirsen) as an antiviral therapeutic[8].

Somewhat larger, 30–35 nt RNAs derived from the 5′ half of tRNA (5′ tRHs) represent a second major class of small non-coding RNA[9]. Increased expression of 5′ tRHs has been associated with viral and rickettsial infections in animals[10,11], and may serve to prevent apoptosis and promote cell survival[12]. However, they have not been studied previously in the context of viral hepatitis. To our knowledge, only one study has described unbiased profiling of small RNAs in the liver during chronic viral hepatitis[13], but the analysis was restricted to miRNAs. We sequenced small (14–40 nts) RNAs in liver biopsies from subjects with chronic hepatitis and HCC, examining both non-tumor and matched cancer tissue, and found a surprisingly high proportion of reads representing 5′ tRHs[9]. Our results document their presence in human tissue, demonstrate that they are the most highly abundant

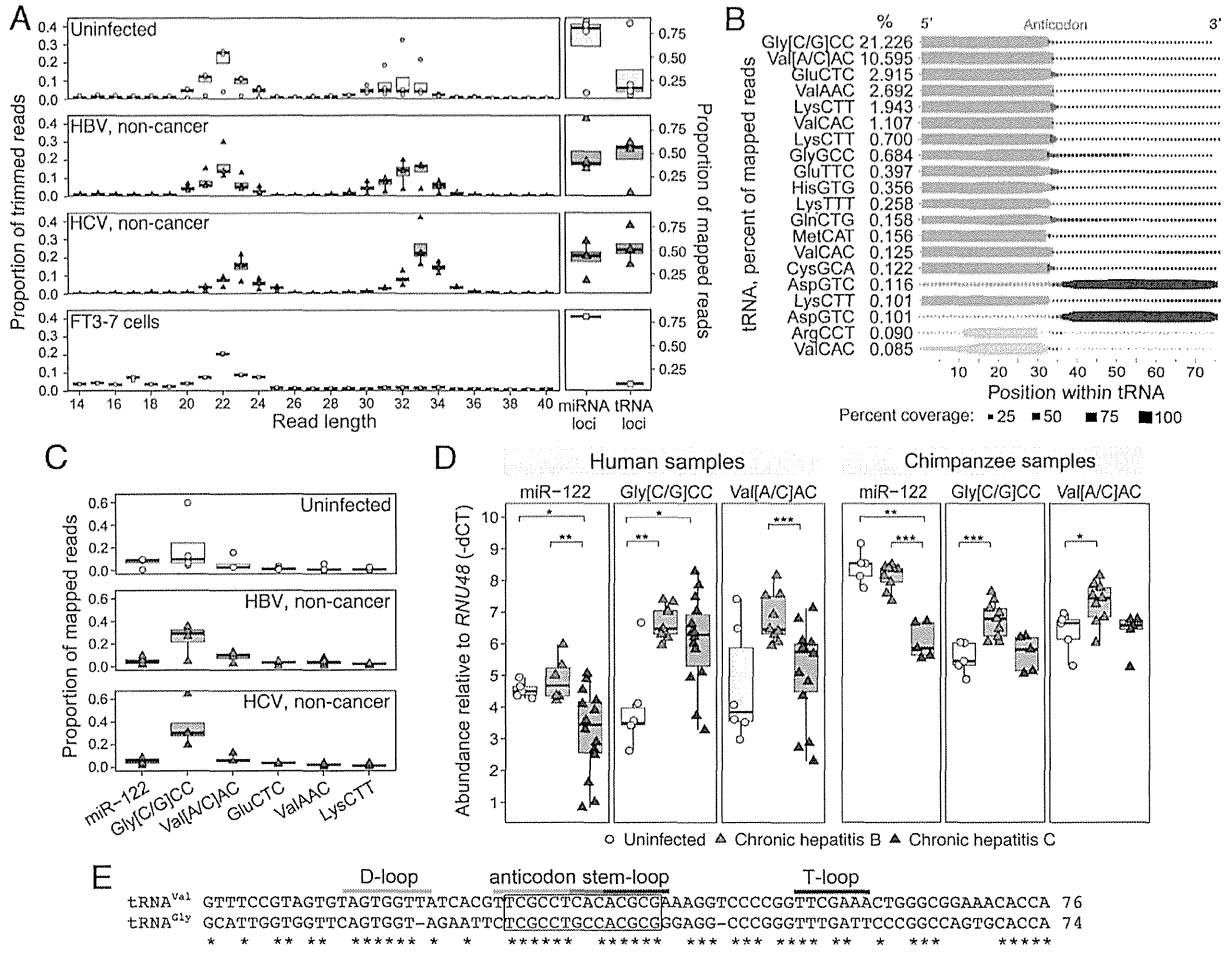

Figure 1 | tRH abundance in HBV- and HCV-infected liver. (a) (left) Read length distribution of 14–40 nt RNAs in non-malignant liver from uninfected, HBV-, or HCV-infected subjects (n=4 each), and FT3-7 cells (n=3 replicates). (right) Proportion of reads mapping to miRNA versus tRNA loci. Boxes represent median ± 1.5 * interquartile range. (b) tRNA coverage plot from the average of the 20 non-cancer samples. Dot size represents percent of reads mapping at each base position within each tRNA (top 20 by average abundance). The anticodon is red, with 5′ bases green and 3′ bases blue. Gray: bases of RNAs that are non-tRHs. See Supplemental Figure 1. (c) Proportion of mapped reads aligning to miR-122 versus the five most abundant tRNA-derived RNAs. (d) (left) Expression levels (RT-qPCR) of miR-122, 5′ tRH^{Gly} ("Gly[C/G]CC") and 5′ tRH^{Val} ("Val[A/C]AC") in uninfected (n=5–6), HBV-infected (n=6–9) and HCV-infected (n=14) human liver. Numbers of samples differ due to limited RNA. (right) Similar results from uninfected (n=5), HBV-infected (n=9), and HCV-infected C (n=5) chimpanzees. *RNU48* was used as a normalizer. *P < 0.05; **P < 0.01; ***P < 0.005 by Mann-Whitney U-test. (e) ClustalW[43] multiple sequence alignment of representative tRNA^{Gly} and tRNA^{Val} genes from which 5′ tRH^{Gly} and 5′ tRH^{Val} could originate (see Supplemental Figure 3). tRNAs regions are highlighted according to the color scheme in panel (b). The box identifies a unique conserved sequence motif described in the text. "Mapped reads" represents all reads aligning to miRNAs or tRNAs (see Methods).

small RNAs in virus-infected liver, and show that their abundance is altered in various disease states including hepatocellular carcinoma.

Results

tRNA-half abundance is significantly increased in chronic viral hepatitis. We employed high-throughput sequencing to characterize the small RNA transcriptome in liver tissue from Japanese adults with advanced hepatitis B or hepatitis C and concomitant HCC (see Supplemental Table 1 for patient information; see Supplemental Tables 2–4 for summary statistics on RNA, qRT-PCR, and sequencing). Initial studies focused on non-malignant tissue from 4 subjects with hepatitis B (mean age 53 ± 4 yrs s.e.m.), 4 with hepatitis C (63 ± 2 yrs), and 4 uninfected individuals undergoing resection of metastatic tumors (60 ± 10 yrs)[14]. A large proportion of the sequencing reads were 19–25 nts in length (median 38%, range 10–73%), as expected for miRNAs[15] (Figure 1a, left). However, we detected an equal or greater abundance of 30–35 nt reads in HBV- and HCV-infected liver (median 54%, range 14–80%). These larger RNAs were less abundant in uninfected tissue (median 21%, range 14–84%) and in human hepatoma (FT3-7) cells (median 9%, range 8.7–9.3%).

Most (~65%) of the 30–35 nt reads in infected samples aligned perfectly to the region 5′ of the anticodon triplet in annotated tRNA genes[16] (Figure 1b, Supplemental Figure 1, Supplemental Table 5 and 6). We refer to these as "5′ tRNA-halves" (5′ tRHs)[9]. Many of the remaining 30–35 nt reads also aligned to the 5′ end of tRNAs, particularly tRNA^{Gly}, but with one or more nucleotide deletions. Also present were 3′ tRHs (~36–39 nts) mapping to the region 3′ of the anticodon, including the 3′ terminal CCA (Figure 1b, Supplemental Figure 1). Additionally, we identified shorter reads derived from 3′ or 5′ tRNA termini, referred to previously as "tRNA fragments"[9] (tRFs), or the region immediately 5′ or 3′ of the anticodon loop, but these

were much less frequent. In 6 of 8 infected livers, more reads mapped to tRNA loci[16] than to known miRNAs[17] (see Methods), while the opposite was true in 3 of 4 uninfected tissues as well as FT3-7 cells (Figure 1a, right).

There are 625 annotated tRNA genes in the human genome (hg19) encoding 458 unique tRNA sequences. We identified reads mapping to 348 of these 458 sequences. Notably, in 11 of the 12 subjects, the same five 5′ tRHs comprised >80% of tRNA-derived reads (Supplemental Figure 2a). The two most abundant 5′ tRHs were Gly[C/G]CC ("5′ tRH^{Gly}"), which could be derived from any of 10 $tRNA^{Gly}$ genes with identical 5′ sequence, and Val[A/C]AC ("5′ tRH^{Val}"), which could originate from any of 15 $tRNA^{Val}$ genes (Figure 1b and C, Supplemental Figure 1 and 3, and Supplemental Table 5)[16]. 5′ tRH^{Gly} accounted for 54 ± 9% (s.d.) and 5′ tRH^{Val} 17 ± 9% of all tRNA-derived RNA reads (Supplemental Figure 2a). Remarkably, 5′ tRH^{Gly} abundance exceeded that of miR-122, one of the most abundant liver miRNAs[13], in 7 of 8 virus-infected tissues.

We used real-time reverse transcription quantitative PCR (RT-qPCR) to validate these results and compare 5′ tRH^{Gly}, 5′ tRH^{Val} and miR-122 abundance in liver tissue from 22 additional subjects (Supplemental Table 1–3)[14]. These analyses confirmed that 5′ tRH^{Gly} abundance was increased in HBV- and HCV-infected liver compared with uninfected tissues ($P<0.01$ and $P<0.05$, respectively) (Figure 1d, left). A similar trend was observed for 5′ tRH^{Val} (HBV $P=0.07$; HCV $P=0.7$). 5′ tRH^{Gly} and 5′ tRH^{Val} were more abundant than miR-122 in HBV- and HCV-infected liver (5′ tRH^{Gly}, $P<0.005$ for both HBV and HCV; 5′ tRH^{Val}, $P<0.005$ for HBV and $P<0.01$ for HCV) (Figure 1d left). Notably, 5′ tRH^{Val} abundance was higher in HBV- than in HCV-infected tissues ($P<0.005$).

Chimpanzees (*Pan troglodytes*) recapitulate many aspects of HBV and HCV infections in humans[18,19], and are free of potential confounding variables (e.g., alcohol intake, smoking) that are difficult to control in human cohorts. Similar to humans, we found that intrahepatic 5′ tRH^{Gly} and 5′ tRH^{Val} abundance was increased in archived liver tissue from chimpanzees chronically infected with HBV compared to uninfected animals ($P<0.005$ and $P<0.05$, respectively) (Figure 1d, right, and Supplemental Table 7). However, 5′ tRH abundance was not increased in chronically HCV-infected chimpanzee liver.

In human tissues, the relative abundance of specific tRNA-derived RNAs correlated with codon usage (codon frequency in DNA sequence) (Spearman's $rho=0.32$, $P=0.01$) and the number of possible tRNA genes from which each could originate ($rho=0.41$, $P=0.001$) (Supplemental Figure 2b). However, tRNAs representing potential sources of the five most abundant tRHs were not the most highly ranked by gene number or codon usage, suggesting that additional factors likely determine tRH biogenesis (Supplemental Figure 4). Interestingly, those tRNAs from which 5′ tRH^{Gly} and 5′ tRH^{Val} are potentially derived share a unique sequence motif in the anticodon stem-loop region (Figure 1e) not present in other tRNAs (Supplemental Figure 3).

tRNA-half abundance is altered in viral hepatitis associated cancer. In HCC tissue from HBV-infected subjects, RT-qPCR analysis showed that 5′ tRH^{Gly} and 5′ tRH^{Val} abundance was significantly reduced ($P<0.005$ for both) (Figure 2a). Similar reductions were evident in HCV-associated cancer tissue, but significant only for 5′ tRH^{Val} ($P<0.05$). We then sequenced small RNAs in cancer tissue from 4 HBV- and 4 HCV-infected subjects. The proportion of reads mapping to tRNA genes was reduced in 4 of 7 samples for which a paired analysis with non-malignant liver was possible, and relatively unchanged in the other 3 (Figure 2b). Although tRNA-derived RNA expression profiles were similar across non-malignant tissues from different subjects, there was substantial variation when compared to cancer tissues (Figure 2c). This suggests that the relative abundance of specific tRNA-derived RNAs is altered in HCC. Notably, the relative abundance of 5′ tRH^{Gly} was reduced by ~50–60% in both HBV- and HCV-associated cancer (Figure 2d).

tRNA-half abundance correlates with angiogenin levels in HBV-associated cancer. Angiogenin (encoded by the gene *ANG*) is best known for its role in angiogenesis, but several studies suggest its RNase activity contributes to tRH biogenesis[20,21]. Consistent with this, analysis of previous microarray data obtained from these tissues[14] revealed that *ANG* mRNA was reduced in both HBV- and HCV-associated cancer compared to non-malignant tissue ($P<0.01$ and $P<0.005$, respectively) or uninfected liver ($P<0.005$ and $P=0.01$) (Figure 3a). Analysis of data from The Cancer Genome Atlas (https://tcga-data.nci.nih.gov/tcga/) also indicates that *ANG* expression is reduced in HCC compared to non-malignant tissue, although the difference is significant only for HBV-associated cancer (HBV $P<0.005$, HCV $P=0.12$) (Supplemental Figure 5). *ANG* mRNA abundance correlated strongly with 5′ tRH expression in the HBV-infected subjects we studied (5′ tRH^{Gly}: Spearman's $rho=0.67$, $P<0.01$; 5′ tRH^{Val}: $rho=0.74$, $P<0.005$) (Figure 3b). Quantitative immunoblot analyses (Supplemental Figure 6) confirmed a correlation between ANG protein abundance and 5′ tRH expression in HBV-associated cancer (5′ tRH^{Gly}: $rho=0.83$, $P<0.005$; 5′ tRH^{Val}: $rho=0.87$, $P<0.005$) (Figure 3c). ANG was expressed within the cytoplasm of hepatocytes (Figure 3d), and although its expression varied substantially in different tumors (Figure 3e), reductions in ANG expression likely explain the reduced tRH abundance we observed in most HBV-associated cancers. Unfortunately, however, the available tissue sections from these subjects were insufficient to power a formal analysis of the correlation between cytoplasmic versus nuclear expression of ANG and tRH abundance. ANG expression correlated poorly with tRH abundance in HCV-infected livers, suggesting that other factors determine tRH biogenesis.

Discussion

Recent advances in high-throughput sequencing technology have unveiled the complexity and diversity of functional small RNAs. We found that small RNAs derived from tRNAs, specifically 5′ tRNA-halves[9] (5′ tRHs, ~30–35 nts), are abundant in liver, significantly increased during chronic viral infection, and altered in abundance in liver cancer associated with these infections. We do not believe that these tRNA-halves are products of stochastic endonuclease cleavage of tRNAs for several reasons. First, the same tRNA-halves were found to be increased in chronic viral hepatitis across all individuals. Second, each tRNA-half family exhibited a uniform length distribution (e.g., 5′ tRH^{Gly} was represented primarily by reads of length 32–34 in every individual). Third, tRNA-halves were preferentially induced in chronic HBV infection (as compared to chronic HCV infection) in both human and chimpanzee tissue, indicating biological specificity. Finally, tRH abundance was correlated with disease state (cancer versus non-cancer), indicating reproducible sensitivity to the cellular environment.

Several models of disease have been shown to exhibit an increase in tRH abundance, including cultured human airway cells infected with respiratory syncytial virus[22], mice infected with spotted-fever group rickettsia[23], and rats treated with cisplatin[24]. While their function is not well understood, previous work in cell culture suggests that some tRHs promote cell survival, are anti-apoptotic[12], reduce translation[25], and promote the formation of stress granules[26]. Preliminary studies in our laboratory do not support a role for 5′ tRH^{Gly} or 5′ tRH^{Val} in the regulation of global protein translation in human hepatoma cells (Supplemental Figure 7–8); however, more detailed investigation is required to uncover the potential functions of tRHs. It has also been suggested that tRHs may alter the immune response due to their enrichment in mouse lymphoid organs[27], high