Expression quantitative trait locus analysis

Expression quantitative trait locus analysis (eQTL) was conducted using the web-based tool, Genevar (http://www.sanger.ac.uk/resources/software/genevar) (Yang et al. 2010). We evaluated the correlations between rs2305482 genotypes and the expression of transcripts of *PSMD3* or colony-stimulating factor 3 (*CSF3*) by the Spearman's rank correlation coefficient.

Statistical analysis

In the GWAS and the replication stages, the observed association between a SNP and neutropenia induced by IFN-based therapy was assessed by the Chi square test with a two-by-two contingency table in three genetic models: the allele frequency model, the dominant-effect model and the recessive-effect model. Significance levels after Bonferroni correction for multiple testing were $P = 8.31 \times 10^{-8}$ (0.05/601,578) in the GWAS stage and $P = 2.62 \times 10^{-4}$ (0.05/191) in the replication stage. Categorical variables were compared between groups by the Chi square test, and non-categorical variables by the Student's t test or the Mann–Whitney U test. Multivariate logistic regression analysis with stepwise forward selection was performed with $P < 0.05$ in univariate analysis as the criteria for model inclusion. To evaluate the discriminatory ability of neutrophil counts at baseline to predict neutropenia during IFN-based therapy, receiver operating characteristic curve (ROC) curve analysis was conducted. Changes of serum G-CSF levels from baseline to the period with neutropenia during IFN-based therapy were compared by the repeated measure analysis of variance (ANOVA). Correlations between neutrophil counts and serum G-CSF levels were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was considered significant in all tests.

Results

Genetic variants associated with IFN-induced neutropenia

We conducted two stages of GWAS by changing the terms of neutrophil counts, followed by the replication analysis (Fig. 1). The characteristics of the patients in each group for the GWAS and the replication stage are summarized in Table 1. At the first stage of GWAS (GWAS-1st), we genotyped 416 Japanese CHC patients with minimum neutrophil counts of <750/mm^3 (Case-G1, $n = 114$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 during IFN-based therapy. Here there may still be mixed with undesirable samples that should be removed from the case group. Therefore, we designed and carried out the second stage of GWAS (GWAS-2nd) comparing the patients with more severe neutropenia to the control group: in patients with minimum neutrophil counts of <600/mm^3 (Case-G2, $n = 50$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 using the same samples as used in GWAS-1st. Supplementary Fig. 1 shows a genome-wide view of the single-point association data based on allele frequencies in GWAS-1st and GWAS-2nd. No association between SNPs and IFN-induced neutropenia reached a genome-wide level of significance [Bonferroni criterion $P < 8.31 \times 10^{-8}$ (0.05/601,578)]. Therefore, we selected the candidate SNPs principally

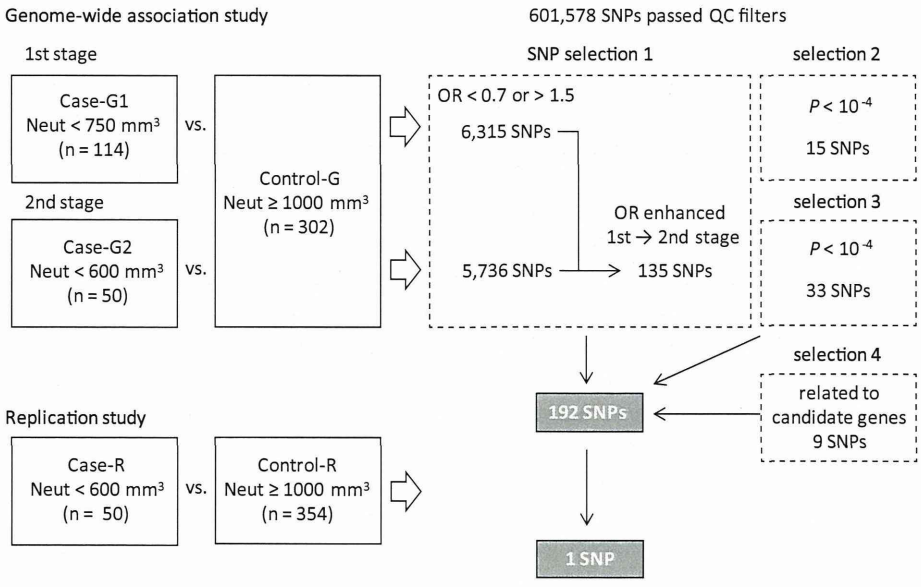

Fig. 1 Outline of the study design. *Neut* neutrophil counts, *SNP* single nucleotide polymorphism, *QC* quality control, *OR* odds ratio

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

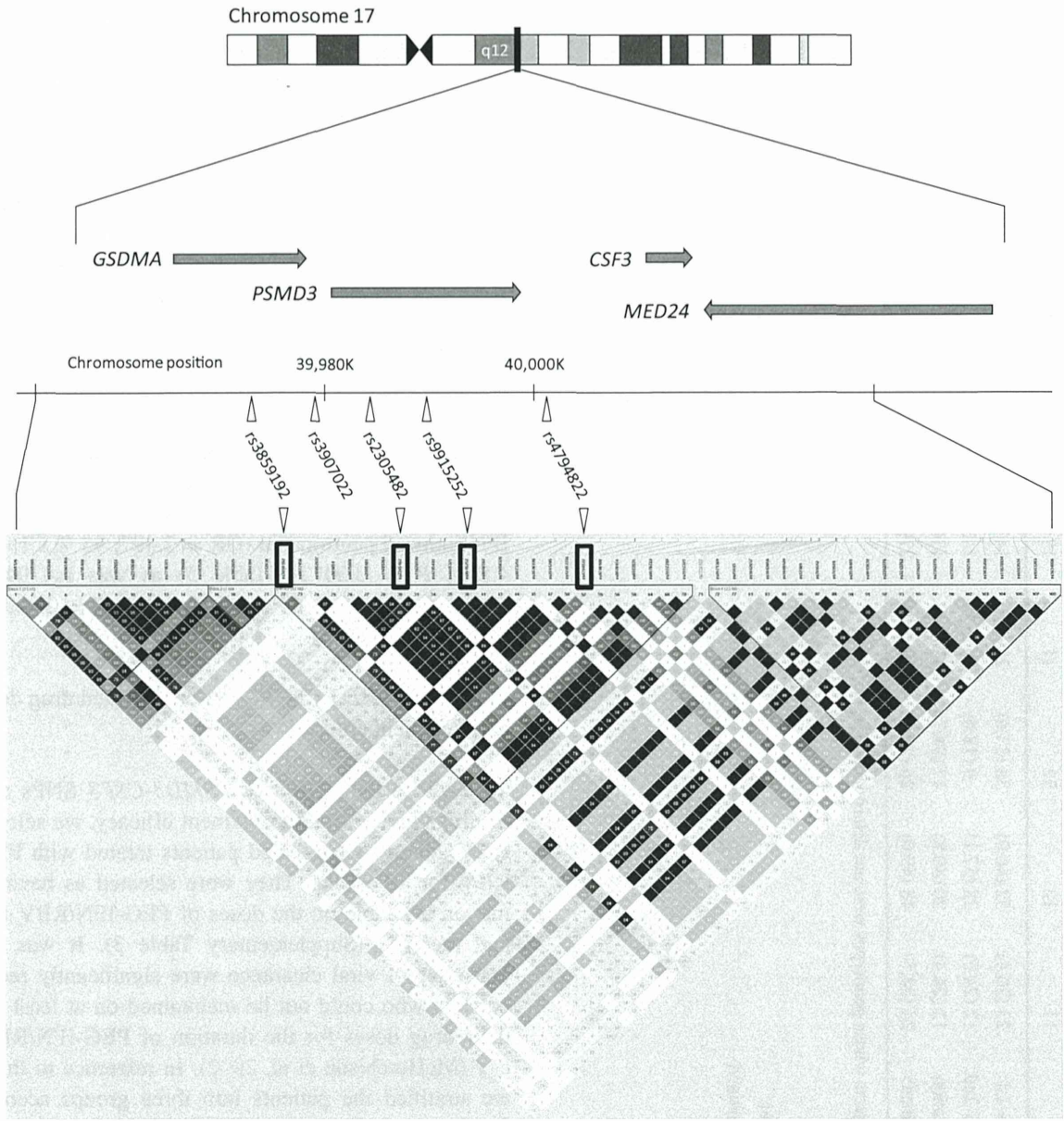

Fig. 2 Position on chromosome and pairwise linkage disequilibrium (r^2) diagrams in the HapMap JPT around the *PSMD3-CSF3* locus

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

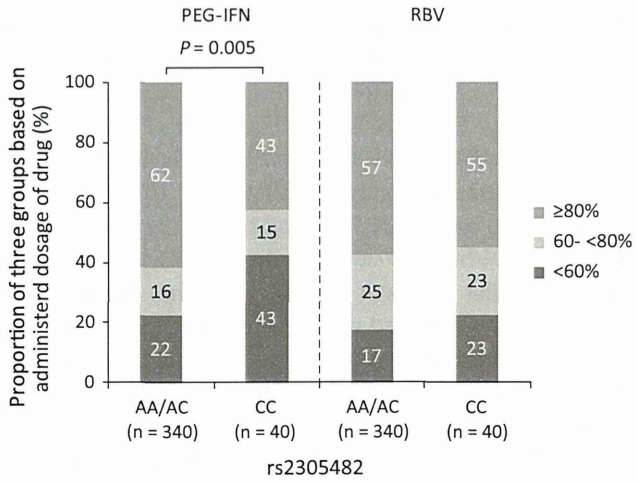

Fig. 3 Administered doses of PEG-IFN and RBV according to rs2305482 genotypes. The patients were stratified into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients receiving <60 % of 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

Liver stiffness measurement for risk assessment of hepatocellular carcinoma

Akihisa Tatsumi,[1] Shinya Maekawa,[1] Mitsuaki Sato,[1] Nobutoshi Komatsu,[1] Mika Miura,[1] Fumitake Amemiya,[2] Yasuhiro Nakayama,[1] Taisuke Inoue,[1] Minoru Sakamoto[1] and Nobuyuki Enomoto[1]

[1]*First Department of Medicine, University of Yamanashi, Chuo, and* [2]*Department of Gastroenterological Medicine, Kofu Municipal Hospital, Kofu, Yamanashi, Japan*

Aim: Liver fibrosis is a risk factor for hepatocellular carcinoma (HCC), but at what fibrotic stage the risk for HCC is increased has been poorly investigated quantitatively. This study aimed to determine the appropriate cut-off value of liver stiffness for HCC concurrence by FibroScan, and its clinical significance in hepatitis B virus (HBV), hepatitis C virus (HCV) and non-B, non-C (NBNC) liver disease.

Methods: Subjects comprised 1002 cases (246 with HCC and 756 without HCC) with chronic liver disease (HBV, 104; HCV, 722; and NBNC, 176).

Results: Liver stiffness was significantly greater in all groups with HCC, and the determined cut-off value for HCC concurrence was more than 12.0 kPa in those with HCV, more than 8.5 kPa in those with HBV and more than 12.0 kPa in those with NBNC. Liver stiffness of more than 12.0 kPa was an independent risk factor for new HCC development in HCV. For HCV, risk factors for HCC concurrence were old age, male sex, low albumin, low platelets and liver stiffness, while for HBV they were old age, low platelets and liver stiffness, and for NBNC they were old age, elevated α-fetoprotein and liver stiffness.

Conclusion: Liver stiffness cut-off values and their association with HCC concurrence were different depending on the etiology. In HCV, liver stiffness of more than 12.0 kPa was an independent risk factor for new HCC development. Collectively, determining the fibrotic cut-off values for HCC concurrence would be important in evaluating HCC risks.

Key words: FibroScan, hepatocellular carcinoma, liver fibrosis

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer in the world and the third most common cause of cancer deaths.[1] HCC, accounting for 90% of primary liver cancer, is a global clinical issue.[2] For improvement in the prognosis of HCC, curative therapy following early detection is important. To this end, it is critical to identify high-risk groups for HCC and perform appropriate surveillance in the clinical practice of chronic liver disease. It has been postulated that hepatitis virus infection, old age, male sex, alanine aminotransferase (ALT) elevation, liver fibrosis, and low albumin (Alb), low platelets (Plt) and α-fetoprotein (AFP) elevation are risk factors for HCC; however, liver fibrosis is the most important risk factor irrespective of its etiology.[3–6]

To date, liver fibrosis has been evaluated by liver biopsy, but it is associated with several problems such as invasiveness, sampling errors, semiquantitation and diagnostic differences among pathologists. With the development of FibroScan (Echosens, Paris, France) using transient elastography, it has become possible to quantitate liver elasticity non-invasively.[7] The diagnostic accuracy of FibroScan for liver fibrosis has been recognized widely for various chronic liver diseases with the exception of some liver conditions such as congestion,

Correspondence: Dr Nobuyuki Enomoto, First Department of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Email: enomoto@yamanashi.ac.jp

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severe inflammation or cholestasis in which liver fibrosis might be overestimated with FibroScan.[8–12] The risk for HCC is evaluable based on liver stiffness measured by FibroScan in cases with hepatitis B virus (HBV) and hepatitis C virus (HCV).[12–19] Nevertheless, in most reports the risk for HCC was only indirectly evaluated based on the value for liver cirrhosis as measured by FibroScan. Liver stiffness related to HCC has not been directly evaluated. Furthermore, the utility of FibroScan in evaluation of the risk for HCC has not been elucidated in non-B, non-C (NBNC) liver disease.

In this study, liver stiffness in patients with chronic liver disease was quantitatively measured and liver stiffness related to HCC occurrence was elucidated separately in cases with HCV, HBV and NBNC liver disease for investigations of its clinical utility.

METHODS

Patients

THE SUBJECTS COMPRISED 1002 patients with chronic liver disease whose liver stiffness was measured by FibroScan consecutively at the University of Yamanashi Hospital between January 2010 and December 2012. Informed consent had been obtained for measurement of liver stiffness before the modality was approved by the national insurance in October 2011. The HCV group (722 cases including 66 sustained virological response [SVR] cases), HBV group (104 cases) and NBNC group (176 cases) were defined as HCV antibody positive, hepatitis B surface antigen (HBsAg) positive, and HBsAg negative and HCV antibody negative cases, respectively. Both HBsAg and HCV antibody positive cases ($n = 3$) and HIV co-infection cases (co-infection with HBV, $n = 1$) were excluded. HCC cases included those with a history of HCC. Among the 1002 cases with chronic liver disease, 246 had HCC and 756 were without HCC. Of those without HCC, 470 hepatitis C cases were followed up by abdominal ultrasonography, contrast computed tomography (CT) or ethoxybenzyl (EOB) contrast magnetic resonance imaging (MRI) every 3–6 months. HCC was diagnosed by contrast ultrasonography, contrast enhancement in the arterial phase and poor enhancement at the equilibrium phase in contrast CT (including CT arteriography and computed tomographic arterial portography) and contrast MRI, and histology by liver tumor biopsy. According to the Declaration of Helsinki, this study was performed after approval was obtained by the ethical committee of the Faculty of Medicine, University of Yamanashi.

Measurement of liver stiffness

FibroScan502 (Echosens) was used for measurement with the M-probe and L-probe. Patients were placed in a supine position with the right hand at the most abducted position for right intercostal scanning. When at least 10 effective measurements were obtained with effective measurement at 60% or higher and interquartile range at less than 30%, such measurements were defined as effective and the median was employed as the result of the measurement.[20]

Analytical methods

In each group of liver diseases (HCV, HBV and NBNC), liver stiffness was compared between patients with and without HCC. Then, the cut-off value of liver stiffness for diagnosis of HCC was determined for later analysis in each group. Patients' backgrounds, laboratory data and liver stiffness in the HCV, HBV and NBNC groups were subjected to univariate, multivariate and subgroup analyses on the relationship with HCC. The 470 HCV patients without HCC at enrollment were followed up with the day of measurement of liver stiffness designated as day 0. Factors related to the development of HCC were examined by univariate and multivariate analyses using values for liver stiffness and blood test results at enrollment.

Statistical analysis

Category data were analyzed by the χ^2-test and Fisher's exact test, while numerical data were examined by Mann–Whitney *U*-test. The cut-off value was set to yield the largest Youden index by receiver–operator curve (ROC) analysis. Multiple logistic analysis was performed for multivariate analysis on factors related to HCC concurrence. The Cox regression hazard model was employed for multivariate analysis of factors related to HCC development. Yearly development of HCC was expressed as per person•year. Cumulative incidence of HCC development was calculated by the Kaplan–Meier curve. *P*-values less than 0.05 were considered significant.

RESULTS

Baseline characteristics

CLINICAL BACKGROUND FACTORS of 1002 patients were compared between patients with and without HCC according to group (Table 1). There were 722 cases in the HCV group, 104 in the HBV group and 176 in the NBNC group. For all groups there was a significant association with older age, low Alb and Plt,

Table 1 Baseline characteristics of patients with and without HCC

Factors	HCV patients ($n = 722$)			HBV patients ($n = 104$)			NBNC patients ($n = 176$)		
	HCC(+) ($n = 167$)	HCC(−) ($n = 555$)	P	HCC(+) ($n = 29$)	HCC(−) ($n = 75$)	P	HCC(+) ($n = 50$)	HCC(−) ($n = 126$)	P
Age (years)	72 (42–89)	61 (20–89)	<0.01	62 (49–76)	52 (19–73)	<0.01	70 (53–88)	63 (19–88)	<0.01
Sex (male/female)	111/56	288/266	<0.01	23/6	47/28	0.11	33/17	69/58	0.16
Alb (g/dL)	3.6 (1.8–5.1)	4.3 (2.1–5.3)	<0.01	4.4 (2.0–5.0)	4.5 (3.5–5.2)	0.04	3.8 (1.9–4.7)	4.1 (2.4–5.5)	<0.01
T-Bil (mg/dL)	0.8 (0.3–4.7)	0.7 (0.2–26.9)	<0.01	0.7 (0.3–1.2)	0.7 (0.2–1.6)	0.45	0.7 (0.1–1.5)	0.7 (0.1–2.3)	0.90
AST (U/L)	48 (13–340)	32 (8–262)	<0.01	28 (16–95)	25 (14–178)	0.06	43 (17–146)	32 (10–291)	0.03
ALT (U/L)	43 (4–557)	32 (2–334)	<0.01	25 (10–134)	21 (9–375)	0.13	29 (10–80)	29 (6–517)	0.99
γ-GT (U/L)	36 (11–918)	28 (9–354)	<0.01	56 (13–267)	21 (8–222)	<0.01	74 (15–628)	55 (7–743)	0.14
Plt (10^9/L)	94 (25–299)	157 (40–343)	<0.01	118 (21–207)	172.5 (58–300)	<0.01	117 (14–264)	168 (30–387)	<0.01
AFP (ng/mL)	12.9 (1.3–54 923)	3.6 (0.8–839)	<0.01	3.8 (1.3–22 421)	2.7 (1.1–70.9)	<0.01	5.8 (1.3–5194)	3.2 (0.8–25.3)	<0.01
Stiffness (kPa)	21.3 (3.9–75.0)	7.8 (3.0–72.0)	<0.01	9.2 (4.7–75.0)	5.6 (2.8–32.4)	<0.01	15.6 (3.3–75.0)	7.4 (2.8–66.4)	<0.01
Hx of IFN Tx (yes/no)	38/129	153/402	0.21	–	–	–	–	–	–
SVR/non-SVR	10/34	56/97	0.09	–	–	–	–	–	–
Tx of NA	–	–	–	16/13	34/41	0.37	–	–	–
HBV-DNA >4 log copies/mL	–	–	–	4/25	16/59	0.38	–	–	–

Values are expressed as the mean (range).

–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV patients, HBs antigen positive patients; HCC, hepatocellular carcinoma; HCV patients, HCV antibody positive patients; Hx, history; IFN, interferon; NA, nucleoside analog; NBNC patients, HBs antigen negative and HCV antibody negative patients; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; Tx, Treatment; γ-GT, γ-glutamyl transpeptidase.

and elevated AFP among those with HCC. The proportion of males was significantly higher among the HCC cases in the HCV group. Stiffness of the liver was significantly greater among the HCC cases in all groups.

Determining cut-off values related to HCC concurrence in each disease group

The cut-off value most related to HCC concurrence was determined by the ROC analysis in each disease group. It was set at more than 12.0 kPa (>12.0 kPa vs ≤12.0 kPa; odds ratio [OR], 14.7; $P < 0.001$) in the HCV group, at more than 8.5 kPa (>8.5 kPa vs ≤8.5 kPa; OR, 8.28; $P < 0.001$) in the HBV group and at more than 12.0 kPa (>12.0 kPa vs ≤12.0 kPa; OR, 4.67; $P < 0.001$) in the NBNC group (Fig. 1).

HCC concurrence-related factors

Hepatocellular carcinoma concurrence-related factors in the HCV group were examined. Univariate analysis revealed that age, sex, Alb, total bilirubin, aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), Plt, AFP and liver stiffness of more than 12.0 kPa were significant factors (Table 2). With the significant factors extracted by univariate analysis, multivariate analysis was performed, and age, sex, Alb, Plt and liver stiffness of more than 12.0 kPa were independent factors (Table 3). Liver stiffness of more than 12.0 kPa was significant with an OR of 4.53 ($P < 0.001$).

Hepatitis C virus patients were categorized into two groups according to liver stiffness of 12.0 kPa or less, and more than 12.0 kPa, and HCC concurrence-related factors were examined in each group. Multivariate analysis extracted age, sex, Alb and AFP in the group with liver stiffness of 12.0 kPa or less as independent factors, and age, Alb and Plt in the group with liver stiffness of more than 12.0 kPa (Table 3).

In the HBV group, HCC concurrence-related factors were examined. Univariate analysis revealed that age, Alb, γ-GT, Plt, AFP and liver stiffness of more than 8.5 kPa were significant factors (Table 2), and multivariate analysis extracted age as an independent factor (OR, 1.12 [range, 1.04–1.21], $P < 0.004$) while low Plt tended to be associated with a high risk for HCC occurrence (OR, 0.99 [range, 0.98–1.00], $P = 0.08$) (data not shown). Subgroup analysis showed that liver stiffness of more than 8.5 kPa was a significant factor for HCC concurrence irrespective of age of more than 60 years or 60 years or less, and Plt less than 150×10^9/L or 150×10^9/L or more (Fig. 2).

Also examined were HCC concurrence-related factors in the NBNC group. Univariate analysis revealed that Alb, Plt, AFP and liver stiffness of more than 12.0 kPa

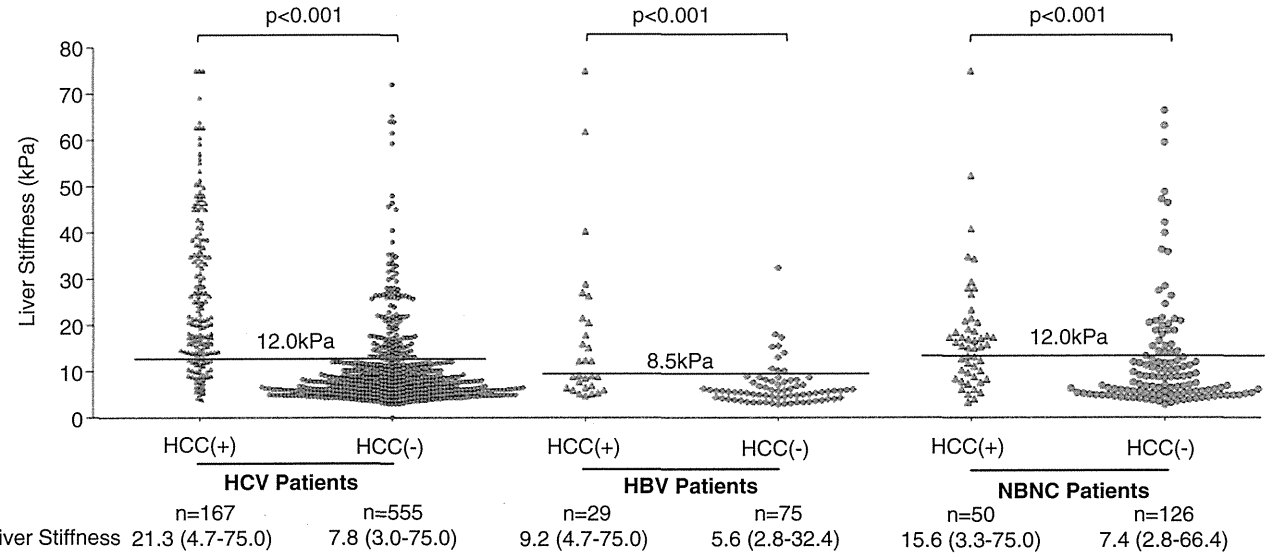


Figure 1 Distribution of liver stiffness categorized by the presence of hepatocellular carcinoma (HCC). Distribution of liver stiffness is shown in cases with liver disease of different etiologies with and without HCC. The cut-off value for liver stiffness was calculated so that sensitivity plus specificity would be the largest. A horizontal line indicating the cut-off value was drawn separately in each etiology group with an insertion of the value. Liver stiffness is shown as the median (range). Liver stiffness scores were significantly higher in cases with HCC concurrence. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B, non-C.

Table 2 Factors related to HCC: univariate analysis

Factors	HCV patients ($n = 722$)			HBV patients ($n = 104$)			NBNC patients ($n = 176$)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Age (years)	1.13	1.11–1.16	<0.001	1.09	1.04–1.14	<0.001	1.07	1.04–1.12	<0.001
Sex (male)	1.84	1.28–2.64	0.001	2.28	0.83–6.29	0.110	1.66	0.84–3.27	0.147
Alb (g/dL)	0.07	0.04–0.11	<0.001	0.20	0.07–0.59	0.003	0.33	0.17–0.63	<0.001
T-Bil (mg/dL)	1.53	1.09–2.14	0.014	1.20	0.24–6.02	0.826	0.80	0.32–2.03	0.639
AST (U/L)	1.01	1.01–1.02	<0.001	1.01	0.99–1.02	0.431	1.00	0.99–1.01	0.554
ALT (U/L)	1.00	0.99–1.01	0.103	0.99	0.99–1.01	0.868	0.99	0.98–1.00	0.281
γ-GT (U/L)	1.00	1.00–1.01	0.005	1.02	1.01–1.03	0.003	1.00	0.99–1.00	0.392
Plt (10^9/L)	0.98	0.97–0.98	<0.001	0.98	0.97–0.99	0.001	0.99	0.98–0.99	<0.001
AFP (ng/mL)	1.01	1.01–1.02	<0.001	1.04	1.00–1.08	0.033	1.14	1.04–1.26	0.007
Stiffness > cut-off value*	14.3	9.27–22.1	<0.001	7.13	2.76–18.4	<0.001	4.67	2.32–9.40	<0.001
Hx of IFN Tx (yes/no)	0.77	0.51–1.15	0.208	–	–	–	–	–	–
SVR patients	0.56	0.28–1.13	0.108	–	–	–	–	–	–
NA Tx	–	–	–	1.48	0.63–3.51	0.369	–	–	–
HBV DNA >4 log copies/mL	–	–	–	0.21	0.05–1.01	0.051	–	–	–

*The cut-off value is 8.5 kPa in HBV patients, and 12.0 kPa in HCV and NBNC patients.
–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV patients, HBs antigen positive patients; HCC, hepatocellular carcinoma; HCV patients, HCV antibody positive patients; Hx, history; IFN, interferon; NA, nucleoside analog; NBNC patients, HBs antigen negative and HCV antibody negative patients; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; Tx, Treatment; γ-GT, γ-glutamyl transpeptidase.

were significant factors (Table 2), and multivariate analysis extracted age and AFP as independent factors (data not shown). In the subgroup aged more than 65 years and AFP of less than 10 ng/mL, liver stiffness of more than 12.0 kPa was a significant HCC concurrence-related factor (Fig. 2).

Risk of HCC development in HCV infection

In the HCV group, the risk of HCC development was evaluated in 470 patients without HCC initially who were followed up. In contrast, evaluation of the risk of development of HCC was not possible in HBV or NBNC cases because no patient in those groups without HCC initially subsequently developed HCC during this limited observation period. These 470 HCV cases were categorized into those with liver stiffness of more than 12.0 kPa and 12.0 kPa or less based on the cut-off value determined at the analysis of HCC concurrence, and Kaplan–Meier curves for HCC occurrence were constructed. Five patients developed HCC over a median

Table 3 Factors related to HCC in HCV patients: multivariate analysis

Factors	All ($n = 722$)			≤12 kPa ($n = 460$)			>12 kPa ($n = 262$)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Age (years)	1.13	1.10–1.17	<0.001*	1.12	1.07–1.19	<0.001*	1.12	1.07–1.16	<0.001*
Sex (male)	3.55	1.98–6.39	<0.001*	43.4	4.88–387	<0.001*			
Alb (g/dL)	0.27	0.14–0.46	<0.001*	0.19	0.06–0.63	0.007*	0.29	0.14–0.61	0.001*
T-Bil (mg/dL)	1.21	0.66–2.22	0.526				1.02	0.52–2.02	0.946
AST (U/L)	1.00	0.99–1.00	0.419						
ALT (IU/L)							0.99	0.99–1.00	0.541
γ-GT (U/L)	1.00	0.99–1.01	0.285						
Plt (10^9/L)	0.99	0.98–0.99	0.008*	0.99	0.98–1.00	0.113	0.99	0.98–0.99	0.036*
AFP (ng/mL)	1.00	0.99–1.01	0.138	1.10	1.01–1.19	0.028*	1.00	0.99–1.01	0.159
Stiffness >12.0 kPa	4.53	2.36–8.69	<0.001*	–	–	–	–	–	–

*Statistically significant.
–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; NA, nucleoside analog; OR, odds ratio; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; γ-GT, γ-glutamyl transpeptidase.

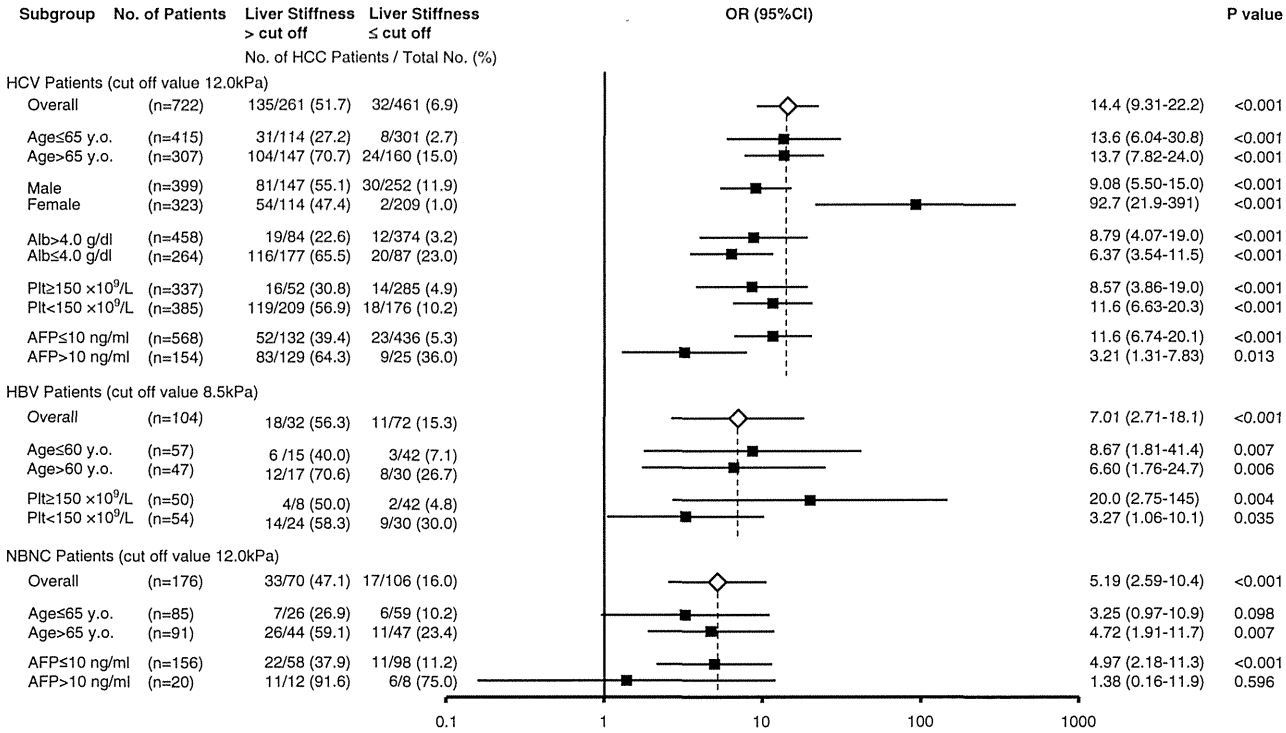


Figure 2 Odds ratio (OR) for the presence of hepatocellular carcinoma (HCC) in specified subgroups associated with liver stiffness over the cut-off value. The OR (95% confidence interval [CI]) for HCC and a *P*-value are shown for each subgroup of hepatitis C virus (HCV) patients with liver stiffness >12.0 kPa, hepatitis B virus (HBV) patients with liver stiffness >8.5 kPa and non-B, non-C (NBNC) liver disease patients with liver stiffness >12.0 kPa. Liver stiffness >12.0 kPa was a HCC concurrence-related factor in all subgroups of HCV patients. In particular, the association was stronger in females than in males. In HBV patients, liver stiffness >8.5 kPa was associated with HCC concurrence irrespective of age >60 years or ≤60 years and platelets (Plt) $\geq 150 \times 10^9$/L or $<150 \times 10^9$/L. In NBNC patients, liver stiffness >12.0 kPa was associated with HCC concurrence in the subcategory of age >65 years and α-fetoprotein (AFP) ≤10 ng/mL.

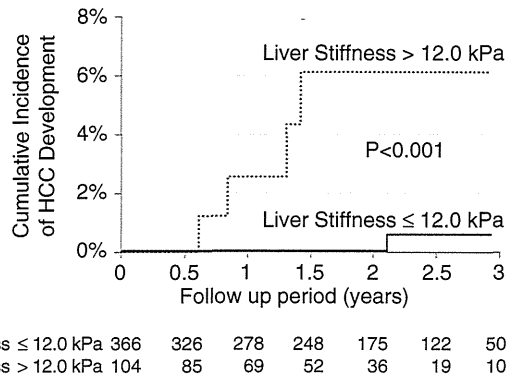
follow-up period of 691 days. The incidence of HCC development was significantly higher among cases with liver stiffness of more than 12.0 kPa than among those with liver stiffness of 12.0 kPa or less ($P < 0.001$, by log–rank test) (Fig. 3).

Factors related to HCC development were examined, and univariate analysis extracted elevated AST, elevated AFP and liver stiffness of more than 12.0 kPa as significant factors, and multivariate analysis revealed that liver stiffness of more than 12.0 kPa was an independent factor. A history of interferon treatment and a SVR were not independent risk factors (Table 4). Cumulative incidence of HCC development was 2.5% in 1 year and 6.1% in 2 years (2.63% per person•year) in patients with liver stiffness of more than 12 kPa. In those with liver stiffness of 12.0 kPa or less, it was 0% in 1 year and 0% in 2 years (0.15% per person•year).

DISCUSSION

WE FOUND THAT stiffness of the liver was significantly greater in those with HCC in the HCV, HBV and NBNC groups than among cases without HCC. In the HCV group, liver stiffness of more than 12.0 kPa was the most appropriate cut-off value for HCC concurrence producing the highest OR and the stiffness significantly correlated with HCC development. Likewise, liver stiffness of more than 8.5 kPa and more than 12.0 kPa were the most appropriate cut-off values associated with HCC concurrence in the HBV group and the NBNC group, respectively.

FibroScan has been widely used as a non-invasive measurement system for liver fibrosis. The most appropriate cut-off value for diagnosis of liver cirrhosis was 11.8–15.9 kPa with sensitivity ranging 79–87% and



No. at risk

Liver Stiffness ≤ 12.0 kPa	366	326	278	248	175	122	50
Liver Stiffness > 12.0 kPa	104	85	69	52	36	19	10

Figure 3 Cumulative incidence of hepatocellular carcinoma (HCC) development in hepatitis C virus patients. Cumulative incidence of HCC development in cases with liver stiffness >12 kPa and ≤12 kPa is shown. Four and one case developed HCC among cases with liver stiffness >12 kPa and ≤12 kPa, respectively. Liver stiffness >12 kPa was associated with a significantly higher risk of HCC development than liver stiffness ≤12 kPa ($P < 0.001$). No case with liver stiffness ≤12 kPa developed HCC for at least 2 years.

specificity 81–95% in the HCV cases, 11.7 kPa with a sensitivity of 84.6% and specificity of 81.5% in the HBV cases,[17,21–23] and 10.3–17.5 kPa with sensitivity ranging 92–100% and specificity 88–97% in non-alcoholic fatty liver disease cases.[8,11,24] On the other hand, the value for liver stiffness most significantly related to HCC concurrence not to liver cirrhosis in each disease group remains elusive.[16,18,25]

The present analysis revealed that the cut-off value most closely associated with HCC concurrence was 12.0 kPa in the HCV group. Masuzaki *et al.* reported that HCC concurrence was more frequent in the presence of a firmer liver, but presented no appropriate cut-off value.[25] In contrast, Akima *et al.* and Kuo *et al.* reported that 12.5 kPa and 12.0 kPa were, respectively, the most appropriate cut-off values for HCC concurrence. However, their studies included heterogeneous etiologies and the cut-off level was not examined separately according to each etiology.[13,16] On the other hand, these cut-off values were almost comparable with the cut-off of 12.0 kPa in the present study because most cases in these studies were positive for HCV. The cut-off level for liver stiffness at 12.0 kPa, which was most closely associated with HCC concurrence in the present study, was almost comparable to the minimum cut-off level of liver stiffness for diagnosis of liver cirrhosis. In HCV positive cases, HCC concurrence was more frequent in cases with a histological semiquantitative diagnosis of fibrosis at F4 (liver cirrhosis) by liver biopsy.[6,26,27] These clinical observations were consistent with the quantitative results of the present study.

In the HCV group, liver stiffness of more than 12.0 kPa was associated with HCC concurrence independently of other factors associated with HCC concurrence, such as age, sex, Alb and Plt (Table 3). It has been reported that male sex and old age were risk factors for HCC independent of liver fibrosis.[6,28–30] Although it is presumed that low Alb and Plt are indirectly implicated in the advancement to liver cirrhosis, liver stiffness was independent of those factors and may reflect the risk for HCC directly related to fibrosis. Subgroup analysis (Fig. 2) revealed that liver stiffness of more than 12.0 kPa was more closely associated with HCC concurrence in females than in males. It was elucidated that HCC development was more closely associated with advancement of liver fibrosis in females and that measurement of liver stiffness in females was more useful than in males.

Although it is rare, some HCV positive cases develop HCC before clinical advancement to liver cirrhosis, and the clinical characteristics of such cases have been poorly investigated. To investigate HCC concurrence-related factors, we categorized HCV positive cases into two groups according to liver stiffness of more than 12.0 kPa and 12.0 kPa or less (Table 3). In those with mild liver fibrosis with liver stiffness of 12.0 kPa or less, old age, male sex, low Alb and elevated AFP were HCC concurrence-related factors. It was suggested that the risk of developing HCC was increased even in cases with mild liver fibrosis as long as those factors were present. Recently, it was reported that metabolic factors such as diabetes and non-alcoholic steatohepatitis are associated with HCC development independently of liver fibrosis.[31–33] It is necessary to further investigate how metabolic factors influence HCC development in patients with mild liver fibrosis and low values for measurements of liver stiffness.

Furthermore, in the HCV group, 470 cases without HCC were followed up (median, 691 days), and liver stiffness of more than 12.0 kPa was the only independent factor for HCC development (hazard ratio, 12.3; 95% confidence interval, 1.27–132) (Table 4). Curves for cumulative incidence of HCC development revealed that HCC development rates were significantly different between cases with liver stiffness of more than 12.0 kPa and 12.0 kPa or less ($P < 0.001$; log-rank test) and that HCC developed beginning 6 months after measurements in cases with liver stiffness of more than 12.0 kPa, whereas no HCC developed for at least 2 years in cases

Table 4 Factors related to HCC development in HCV patients

Factors	Patients who developed HCC $n = 5$	Patients who did not develop HCC $n = 465$	Univariate			Multivariate		
			HR	95% CI	*P*	HR	95% CI	*P*
Age (years)	60 (51–72)	61 (20–88)	1.01	0.93–1.10	0.837			
Sex (male)	4 (80.0%)	245 (52.7%)	4.49	0.50–40.3	0.180			
Alb (g/dL)	4.6 (3.4–4.8)	4.3 (2.1–5.3)	1.56	0.16–15.7	0.705			
T-Bil (mg/dL)	1.2 (0.5–2.4)	0.6 (0.2–26.9)	1.10	0.86–1.40	0.442			
AST (U/L)	84 (19–131)	32 (8–262)	1.02	1.00–1.03	0.013*	1.01	0.99–1.02	0.358
ALT (U/L)	49 (13–163)	31 (2–334)	1.01	0.99–1.02	0.179			
γ-GT (U/L)	51 (12–130)	28 (9–354)	1.01	0.99–1.02	0.223			
Plt (10^9/L)	98 (82–173)	156 (43–343)	0.98	0.97–1.00	0.128			
AFP (ng/mL)	6.2 (2.1–272.8)	3.5 (0.8–839)	1.00	1.00–1.01	0.025*	1.00	0.99–1.01	0.271
History of IFN	3 (60.0%)	256 (55.1%)	0.62	0.10–3.87	0.609			
SVR patients	1 (20.0%)	124 (26.7%)	0.38	0.04–3.45	0.388			
Stiffness >12.0 kPa	4 (80.0%)	103 (22.2%)	18.9	2.10–171	<0.001*	12.9	1.27–132	0.031*
Follow-up period (days)	477 (223–963)	691 (23–1069)	–	–	–	–	–	–

*Statistically significant.

Values are expressed as the mean (range) or *n* (%).

–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazards ratio; IFN, interferon; NA, nucleoside analog; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; γ-GT, γ-glutamyl transpeptidase.

with liver stiffness 12.0 kPa or less (Fig. 3). According to the HCC surveillance guidelines, an imaging examination every 6 months is recommended in cases with chronic hepatitis C and once in 3–4 months in cases with liver cirrhosis C.[34] In cases with liver stiffness of more than 12.0 kPa, the guidelines can be considered reasonable. In addition, in cases with liver stiffness of 12.0 kPa or less, it was suggested that the surveillance interval may be prolonged, although further accumulation of such cases was necessary.

In the HBV group, the cut-off value at 8.5 kPa most closely correlated with HCC concurrence (OR, 8.28), and both the cut-off value and OR were lower than those in the HCV group, which indicated that there was a weaker association between fibrosis and HCC in the HBV group than in the HCV group. In the HBV group, it was reported that liver stiffness at 8.0 kPa, a cut-off value lower than that in the HCV group, or higher increased the incidence of HCC development.[15] Subgroup analysis (Fig. 2) revealed that liver stiffness of more than 8.5 kPa was a significant factor irrespective of age and Plt. Unfortunately, we could not analyze the HCC developmental risk in cases with HBV because no case without concurrent HCC initially developed HCC during this limited observation period.

To the best of our knowledge, no report has demonstrated the association between liver stiffness and HCC concurrence in cases with NBNC liver disease, but when liver stiffness at 12.0 kPa was set as the cut-off value, liver stiffness most closely correlated with HCC concurrence and the cut-off value was almost comparable to that in the HCV group. This result demonstrates that fibrosis also plays an important role in HCC development in NBNC though its contribution is weaker than in HCV. Subgroup analysis revealed that HCC concurrence was more frequent in the group with liver stiffness of more than 12.0 kPa among the elderly aged more than 65 years old and cases with low AFP levels as reported previously,[32] demonstrating that the HCC risk was more greatly dependent on fibrosis in the elderly, while it was high irrespective of fibrosis in cases with elevated AFP in the NBNC group. As for etiologies in the NBNC group, most cases were clinically suspected to have fatty liver-associated diseases. Though information on steatosis-related factors was available only from limited cases in this study, high hemoglobin A1c (HbA1c) value (defined as >6.5) was frequent in NBNC cases (25%) compared to HCV (11%) or HBV cases (17%), and this difference reached statistical significance between HCV and NBNC (data not shown). In addition, high HbA1c value and heavy alcohol intake of more than 70 g/day were more significantly identified in HCC cases compared to non-HCC cases in the NBNC group (data not shown). These observations suggested that fatty liver-associated diseases may be one of the main etiologies in the NBNC group. On the other hand, as with the HBV cases, we could not analyze the HCC developmental risk in cases with NBNC because no case developed HCC during this limited observation period.

In conclusion, evaluation of liver fibrosis based on liver stiffness was useful, in particular, in HCV and NBNC liver disease, because HCC development via advancement of liver fibrosis is a major pathway. Accurate evaluation of liver fibrosis would be important to screen the high risk group for HCC development and analyze causal factors for HCC development other than fibrosis.

REFERENCES

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61** (2): 69–90.
2 El-Serag HB. Hepatocellular carcinoma. *N Engl J Med* 2011; **365** (12): 1118–27.
3 Ikeda K, Saitoh S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998; **28** (6): 930–8.
4 Inoue A, Tsukuma H, Oshima A *et al.* Effectiveness of interferon therapy for reducing the incidence of hepatocellular carcinoma among patients with type C chronic hepatitis. *J Epidemiol* 2000; **10** (4): 234–40.
5 Takano S, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21** (3): 650–5.
6 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999; **131** (3): 174–81.
7 Sandrin L, Fourquet B, Hasquenoph JM *et al.* Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29** (12): 1705–13.
8 Abenavoli L, Beaugrand M. Transient elastography in non-alcoholic fatty liver disease. *Ann Hepatol* 2012; **11** (2): 172–8.
9 Cardoso AC, Carvalho-Filho RJ, Marcellin P. Transient elastography in chronic viral hepatitis: a critical appraisal. *Gut* 2011; **60** (6): 759–64.
10 Castera L. Non-invasive assessment of liver fibrosis in chronic hepatitis C. *Hepatol Int* 2011; **5** (2): 625–34.

11 Yoneda M, Mawatari H, Fujita K *et al.* Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Liver Dis* 2008; **40** (5): 371–8.
12 Kim BK, Fung J, Yuen MF, Kim SU. Clinical application of liver stiffness measurement using transient elastography in chronic liver disease from longitudinal perspectives. *World J Gastroenterol* 2013; **19** (12): 1890–900.
13 Akima T, Tamano M, Hiraishi H. Liver stiffness measured by transient elastography is a predictor of hepatocellular carcinoma development in viral hepatitis. *Hepatol Res* 2011; **41** (10): 965–70.
14 Jung KS, Kim SU. Clinical applications of transient elastography. *Clin Mol Hepatol* 2012; **18** (2): 163–73.
15 Jung KS, Kim SU, Ahn SH *et al.* Risk assessment of hepatitis B virus-related hepatocellular carcinoma development using liver stiffness measurement (FibroScan). *Hepatology* 2011; **53** (3): 885–94.
16 Kuo YH, Lu SN, Hung CH *et al.* Liver stiffness measurement in the risk assessment of hepatocellular carcinoma for patients with chronic hepatitis. *Hepatol Int* 2010; **4** (4): 700–6.
17 Masuzaki R, Tateishi R, Yoshida H *et al.* Comparison of liver biopsy and transient elastography based on clinical relevance. *Can J Gastroenterol* 2008; **22** (9): 753–7.
18 Masuzaki R, Tateishi R, Yoshida H *et al.* Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. *Hepatology* 2009; **49** (6): 1954–61.
19 Wang HM, Hung CH, Lu SN *et al.* Liver stiffness measurement as an alternative to fibrotic stage in risk assessment of hepatocellular carcinoma incidence for chronic hepatitis C patients. *Liver Int* 2013; **33** (5): 756–61.
20 Boursier J, Zarski JP, de Ledinghen V *et al.* Determination of reliability criteria for liver stiffness evaluation by transient elastography. *Hepatology* 2013; **57** (3): 1182–91.
21 Castera L. Liver stiffness and hepatocellular carcinoma: liaisons dangereuses? *Hepatology* 2009; **49** (6): 1793–4.
22 Chon YE, Choi EH, Song KJ *et al.* Performance of transient elastography for the staging of liver fibrosis in patients with chronic hepatitis B: a meta-analysis. *PLoS One* 2012; **7** (9): e44930.
23 Lupsor M, Badea R, Stefanescu H *et al.* Analysis of histopathological changes that influence liver stiffness in chronic hepatitis C. Results from a cohort of 324 patients. *J Gastrointestin Liver Dis* 2008; **17** (2): 155–63.
24 Wong VW, Vergniol J, Wong GL *et al.* Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51** (2): 454–62.
25 Masuzaki R, Tateishi R, Yoshida H *et al.* Risk assessment of hepatocellular carcinoma in chronic hepatitis C patients by transient elastography. *J Clin Gastroenterol* 2008; **42** (7): 839–43.
26 de Oliveria Andrade LJ, D'Oliveira A, Melo RC, De Souza EC, Costa Silva CA, Parana R. Association between hepatitis C and hepatocellular carcinoma. *J Glob Infect Dis* 2009; **1** (1): 33–7.
27 El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. *Hepatology* 2002; **36** (5 Suppl 1): S74–83.
28 Asahina Y, Tsuchiya K, Nishimura T *et al.* alpha-fetoprotein levels after interferon therapy and risk of hepatocarcinogenesis in chronic hepatitis C. *Hepatology* 2013; **58** (4): 1253–62.
29 Asahina Y, Tsuchiya K, Tamaki N *et al.* Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. *Hepatology* 2010; **52** (2): 518–27.
30 Lewis S, Roayaie S, Ward SC, Shyknevsky I, Jibara G, Taouli B. Hepatocellular carcinoma in chronic hepatitis C in the absence of advanced fibrosis or cirrhosis. *AJR Am J Roentgenol* 2013; **200** (6): W610–16.
31 Arase Y, Kobayashi M, Suzuki F *et al.* Effect of type 2 diabetes on risk for malignancies includes hepatocellular carcinoma in chronic hepatitis C. *Hepatology* 2013; **57** (3): 964–73.
32 Reddy SK, Steel JL, Chen HW *et al.* Outcomes of curative treatment for hepatocellular cancer in nonalcoholic steatohepatitis versus hepatitis C and alcoholic liver disease. *Hepatology* 2012; **55** (6): 1809–19.
33 Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51** (5): 1820–32.
34 Kudo M, Izumi N, Kokudo N *et al.* Management of hepatocellular carcinoma in Japan: consensus-based clinical practice guidelines proposed by the Japan Society of Hepatology (JSH) 2010 updated version. *Dig Dis* 2011; **29** (3): 339–64.

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ITPA genetic variants influence efficacy of PEG-IFN/RBV therapy in older patients infected with HCV genotype 1 and favourable *IL28B* type

K. Matsuura,[1,2] Y. Tanaka,[1] T. Watanabe,[1] K. Fujiwara,[2] E. Orito,[3] M. Kurosaki,[4] N. Izumi,[4] N. Sakamoto,[5,6] N. Enomoto,[7] H. Yatsuhashi,[8] A. Kusakabe,[3] N. Shinkai,[2] S. Nojiri,[2] T. Joh[2] and M. Mizokami[9] [1]*Department of Virology, Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan;* [2]*Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan;* [3]*Division of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya, Japan;* [4]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan;* [5]*Department of Gastroenterology, Hokkaido University Graduate School of Medicine, Sapporo, Japan;* [6]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan;* [7]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan;* [8]*Clinical Research Center, National Nagasaki Medical Center, Nagasaki, Japan; and* [9]*Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan*

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SUMMARY. Inosine triphosphatase (*ITPA*) genetic variants are strongly associated with ribavirin (RBV)-induced anaemia during pegylated interferon (PEG-IFN) plus RBV therapy. However, the treatment efficacy of *ITPA* genetic variants has not been fully explored. We enrolled 309 individuals infected with hepatitis C virus genotype 1, who were treated with PEG-IFN plus RBV for 48 weeks. The *ITPA* SNP: rs1127354 and *IL28B* SNP: rs8099917 were genotyped. We examined the risk factors for severe anaemia up to week 12 after the start of treatment and treatment efficacy. The incidence of severe anaemia, ≥3 g/dL reduction or <10 g/dL of haemoglobin (Hb) up to week 12, was more frequent in patients with CC at rs1127354 [65% (145/224), 33% (73/224)] than in those with CA/AA [25% (21/85), 6% (8/85)] ($P < 0.0001$). *ITPA* genotype, pretreatment Hb level and age were independent predictive factors for severe anaemia: Hb < 10 g/dL. In *IL28B* favourable type, the sustained virologic response rate was higher in ≥60-year-old patients with CA/AA than in those with CC [71% (22/31) *vs* 40% (26/65), $P = 0.005$], although there was no significant difference in treatment efficacy according to *ITPA* genetic variants in the <60-year-old patients. The proportion of patients administered ≥80% of the dosage of RBV was significantly higher in the patients with CA/AA than in those with CC ($P = 0.025$), resulting in a lower relapse rate. In conclusion, *ITPA* genetic variants were associated with severe RBV-induced anaemia and could influence the efficacy of PEG-IFN plus RBV treatment among elderly patients with *IL28B* favourable type.

Keywords: anaemia, *IL28B*, interferon, *ITPA*, ribavirin.

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide with approximately 170 million people being infected [1]. More than 70% of HCV-infected individuals go on to develop chronic infection, being at significant risk of progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC). Antiviral treatment has been shown to improve liver histology and reduce the incidence of HCC in chronic hepatitis C (CHC) [2, 3]. However, <50% of patients infected with HCV genotype 1 treated with pegylated interferon (PEG-IFN) plus ribavirin (RBV) achieve a sustained viral response (SVR) [4, 5].

Abbreviations: γ-GTP, γ-glutamyl transpeptidase; ALT, Alanine aminotransferase; CHC, chronic hepatitis C; GWAS, genomewide association studies; Hb, haemoglobin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL28B, interleukin 28B; ITPA, inosine triphosphatase; LC, liver cirrhosis; NVR, null viral response; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained viral response; TVR, transient viral response.

Correspondence: Yasuhito Tanaka, M.D., Ph.D., Department of Virology, Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan.
E-mail: ytanaka@med.nagoya-cu.ac.jp

Virus and host factors have been indicated as relevant determinants of treatment outcome. Among patients with HCV genotype 1 infection, factors associated with a lower rate of SVR include baseline high viral load, male gender, older age, African American race, insulin resistance, advanced fibrosis and hepatic steatosis [6]. In addition, the administered dose of PEG-IFN or RBV affects the outcome of treatment, as rates of viral clearance are significantly reduced in patients who cannot be maintained on at least 80% of their PEG-IFN and RBV dosage for the duration of treatment [7].

Anaemia is common with PEG-IFN plus RBV treatment due to the bone marrow suppression by PEF-IFN and haemolysis from RBV. Anaemia is the reason for discontinuation of treatment or dose reduction in drugs in 10–14% of patients treated with PEG-IFN plus RBV [4, 8, 9]. In particular, the incidence of adverse events and laboratory abnormalities is more frequent in older patients, resulting in poor adherence and lower SVR [10–12]. In Japan, HCV-infected patients are relatively old, and some have severe fibrosis [10]. Additionally, the use of erythropoietin-stimulating agents is not allowed in Japan; therefore, anaemia during PEG-IFN plus RBV treatment is an important problem that affects drug adherence and treatment efficacy.

Recent genomewide association studies (GWAS), including our study on HCV infection [13], have identified two important host genetic variants: the single nucleotide polymorphism (SNP) in the interleukin-28B (*IL28B*) gene, which is strongly associated with the response to therapy for chronic genotype 1 HCV infection [13–18], and the SNP in the inosine triphosphatase (*ITPA*) gene, which precisely predicts RBV-induced anaemia in European American [19] and Japanese populations [20]. It has been reported that variants of the *ITPA* gene affected RBV-induced anaemia and the dosage of RBV; however, the effect of the SNP on therapy outcome has not been fully explored. Some reports suggested an association of the polymorphism with SVR [21, 22], and Kurosaki *et al.* reported that the protective variant of *ITPA* was associated with less reduction in RBV and a high SVR rate in patients with the rs8099917 TT genotype [23]. On the other hand, in other reports, those variants were not associated with treatment outcome [19, 24].

In the present study, we aimed to replicate the risk factors in RBV-induced anaemia including *ITPA* genetic variants and the impact of *ITPA* and *IL28B* genetic variants on the effect of PEG-IFN plus RBV treatment in CHC patients.

PATIENTS AND METHODS

Patients and treatment protocol

From April 2007 to April 2010, samples were obtained from 309 patients with chronic HCV genotype 1 infection who were treated at 6 multicentre hospitals, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, Musasino Red Cross Hospital, Tokyo Medical and Dental University Hospital, Yamanashi University Hospital and National Nagasaki Medical Center. All patients had tested positive for HCV RNA for more than 6 months. HBsAg-positive, anti-HIV-positive patients and patients with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis were excluded from this study. Each patient was treated with PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1000 mg daily according to body weight) for 48 weeks. The dose of PEG-IFN or RBV was reduced according to the recommendation on the package inserts or the clinical condition of individual patients. Erythropoietin and other growth factors were not given.

The treatment outcomes were defined as SVR; undetectable HCV RNA levels at 24 weeks after cessation of treatment; transient viral response (TVR); HCV RNA levels became undetectable during treatment, but reappeared after the end of treatment; and null viral response (NVR); HCV RNA levels were persistently detectable.

Written informed consent was obtained from each patient, and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

SNP genotyping

Genetic polymorphisms as SNPs of the *ITPA* (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) or the DigiTag2 assay [25], respectively. In Caucasian patients, two SNPs (rs1127354 and rs7270101) are associated with *ITPA* enzyme activity [19, 24]; however, there are no variants in rs7270101 in the Japanese population [20, 21, 23].

Laboratory tests

Blood samples were obtained before treatment, at weeks 1, 2, 4, 8 and 12 and every 4 weeks up to treatment completion, and they were analysed for haematologic tests, blood chemistry and HCV RNA. Follow-up measurements were obtained at weeks 4, 12 and 24 after the end of treatment. HCV RNA levels were measured throughout the course of therapy using the COBAS TaqMan HCV test (Roche Diagnostics K.K., Tokyo, Japan). The measurement range of this assay was 1.2–7.8 log IU/mL.

Association between ITPA SNP and anaemia during the early periods of treatment

We examined the association between *ITPA* genetic variants and haemoglobin (Hb) levels and the proportion of