biases may be present, because all the patients had been diagnosed and had received liver biopsies at hepatology centers, which may have caused referral bias. Therefore, validation studies are necessary in the general population.

In conclusion, the measurement of the serum WFA^+-M2BP values using a glycan-based immunoassay provides an accurate and reliable method for assessing the liver fibrosis stage in NAFLD patients. This method appears quite promising as a means for evaluating the natural course of the disease, therapeutic effects, and the suitability of liver biopsies.

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

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ORIGINAL INVESTIGATION

Genome-wide association study identifies a *PSMD3* variant associated with neutropenia in interferon-based therapy for chronic hepatitis C

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Abstract Cytopenia during interferon-based (IFN-based) therapy for chronic hepatitis C (CHC) often necessitates reduction of doses of drugs and premature withdrawal from therapy resulting in poor response to treatment. To identify genetic variants associated with IFN-induced neutropenia, we conducted a genome-wide association study (GWAS) in 416 Japanese CHC patients receiving IFN-based therapy. Based on the results, we selected 192 candidate single nucleotide polymorphisms (SNPs) to carry out a replication analysis in an independent set of 404 subjects. The SNP rs2305482, located in the intron region of the *PSMD3* gene on chromosome 17, showed a strong association when the results of GWAS and the replication stage were combined (OR = 2.18, $P = 3.05 \times 10^{-7}$ in the allele frequency model). Logistic regression analysis showed that rs2305482 CC and neutrophil count at baseline were independent predictive factors for IFN-induced neutropenia (OR = 2.497, $P = 0.0072$ and OR = 0.998, $P < 0.0001$, respectively). Furthermore, rs2305482 genotype was associated with the doses of pegylated interferon (PEG-IFN) that could be tolerated in hepatitis C virus genotype 1-infected patients treated with PEG-IFN plus ribavirin, but not with treatment efficacy. Our results suggest that genetic

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testing for this variant might be useful for establishing personalized drug dosing in order to minimize drug-induced adverse events.

Introduction

Chronic hepatitis C virus (HCV) infection is a significant risk factor for progressive liver fibrosis and hepatocellular carcinoma. Antiviral treatment improves the natural course in chronic hepatitis C (CHC) (George et al. 2009; Yoshida et al. 2004). Newly-developed treatments involving direct-acting antivirals (DAAs), including nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in several clinical trials. Thus, >70 % of patients infected with HCV genotype 1 are reported to achieve sustained virological responses (SVR) (Jacobson et al. 2011; Poordad et al. 2012; Zeuzem et al. 2011). Furthermore, interferon-free (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, IFN-based regimens have been standard-of-care therapies over the last couple of decades.

IFN-based therapies are associated with various adverse effects. Cytopenia is common due to bone marrow suppression cased by IFN or DAA and hemolysis by RBV. This is particularly the case in patients with advanced hepatic fibrosis, but can sometimes also occur in those with mild fibrosis. This then often necessitates dose reduction or premature withdrawal from therapy, resulting in poor response to treatment. For instance, 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). Therefore, pre-treatment prediction of possible adverse effects in order to avoid them and undergo therapy safely is desirable.

Recent genome-wide association studies (GWASs) have identified two important host genetic variants influencing CHC treatment: (1) single nucleotide polymorphisms (SNPs) near the interleukin-28B (*IL28B*) gene, which are strongly associated with response to therapy for chronic HCV genotype 1 infection (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009), and (2) SNPs in the inosine triphosphatase (*ITPA*) gene, which accurately predict RBV-induced anemia in European–American (Fellay et al. 2010) and Japanese population (Ochi et al. 2010). We validated the association between this *ITPA* genetic variant and RBV-induced anemia (Sakamoto et al. 2010), and reported that the *ITPA* genotype affects the tolerated doses of RBV and treatment response in a stratified group (Kurosaki et al. 2011; Matsuura et al. 2014). Additionally, our GWAS showed that *DDRGK1/ITPA* variants are strongly associated with IFN-induced thrombocytopenia as well as anemia during PEG-IFN/RBV therapy (Tanaka et al. 2011). Thompson et al. (2012) also reported that the *ITPA* genetic variant was associated with anemia and thrombocytopenia during PEG-IFN/RBV therapy. However they identified no genetic determinants of IFN-induced neutropenia at the level of genome-wide significance by their GWAS in populations of European Americans, African Americans and Hispanics.

Hence, to identify genetic variants associated with IFN-induced neutropenia, we conducted a GWAS in Japanese CHC patients.

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Materials and methods

Patients

From 2007 to 2012, samples for the GWAS were obtained from 416 CHC patients who were treated at 22 hospitals (liver units with hepatologists) throughout Japan. In the following stage of replication analysis, samples were collected in an independent set of 404 Japanese CHC patients. Most patients were 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 (600–1,000 mg daily according to body weight) for 48 weeks for HCV genotype 1 and 24 weeks for genotype 2. Treatment duration was extended in some patients up to 72 weeks for genotype 1 and 48 weeks for genotype 2 according to physicians' preferences. Other patients were treated with PEG-IFN-α2a or IFN monotherapy, or IFN-α2b plus RBV in standard doses of the regimens. The doses of drugs were reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. Erythropoietin or other growth factors were not given. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study. Written informed consent was obtained from all individual participants in this study and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Inclusion criteria of neutropenia

In the initial stage of GWAS, we defined the inclusion criteria of the case group as minimum neutrophil counts of $<750/mm^3$ at week 2 or 4 during IFN-based therapy, since the dose reduction of IFN is recommended at those levels on the package inserts. Thereafter we did it as minimum neutrophil counts of $<600/mm^3$ at week 2 or 4 in the following GWAS and the replication stages.

SNP genotyping and data cleaning

We conducted two stages of GWAS using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc. Santa Clara, CA) according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to median change. At GWAS, the average overall call rate of patients in the case and the control group reached 98.66 and 98.79 %, respectively. We then applied the following thresholds for SNP quality control (QC) in data cleaning: SNP call rate ≥95 % for all samples, minor allele frequency (MAF) ≥1 % for all samples. A total of 601,578 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups were checked by visual inspection and SNPs with ambiguous genotype calls were excluded. In the replication study, the genotyping of 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients was carried out using the DigiTag2 assay (Nishida et al. 2007). Successfully genotyped SNPs in the replication analysis had a >95 % call rate, and cleared Hardy–Weinberg equilibrium (HWE) $P \geq 0.001$. One SNP could not be genotyped, and hence we obtained data on 191 SNPs including rs9915252. Three SNPs, rs4794822, rs3907022, and rs3859192 located around the proteasome 26S subunits non-ATPase 3 (*PSMD3*) gene and rs8099917 near the *IL28B* gene were genotyped by TaqMan SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol.

Laboratory and histological tests

Blood samples were obtained at baseline and at appropriate periods after the start of therapy and for hematologic tests, blood chemistry, and HCV RNA. Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV RNA level by Roche COBAS Amplicor HCV Monitor test, v.2.0 (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 50 IU/ml or Roche COBAS AmpliPrep/COBAS TaqMan HCV assay (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 15 IU/ml 24 weeks after the completion of therapy. Serum granulocyte colony-stimulating factor (G-CSF) levels were analyzed using Human G-CSF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN).

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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

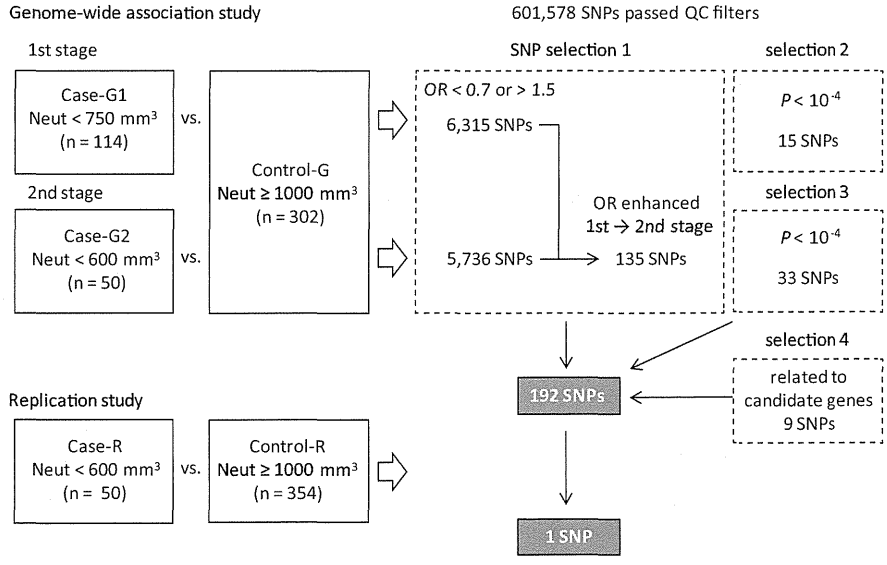

Fig. 1 Outline of the study design. *Neut* neutrophil counts, *SNP* single nucleotide polymorphism, *QC* quality control, *OR* odds ratio

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

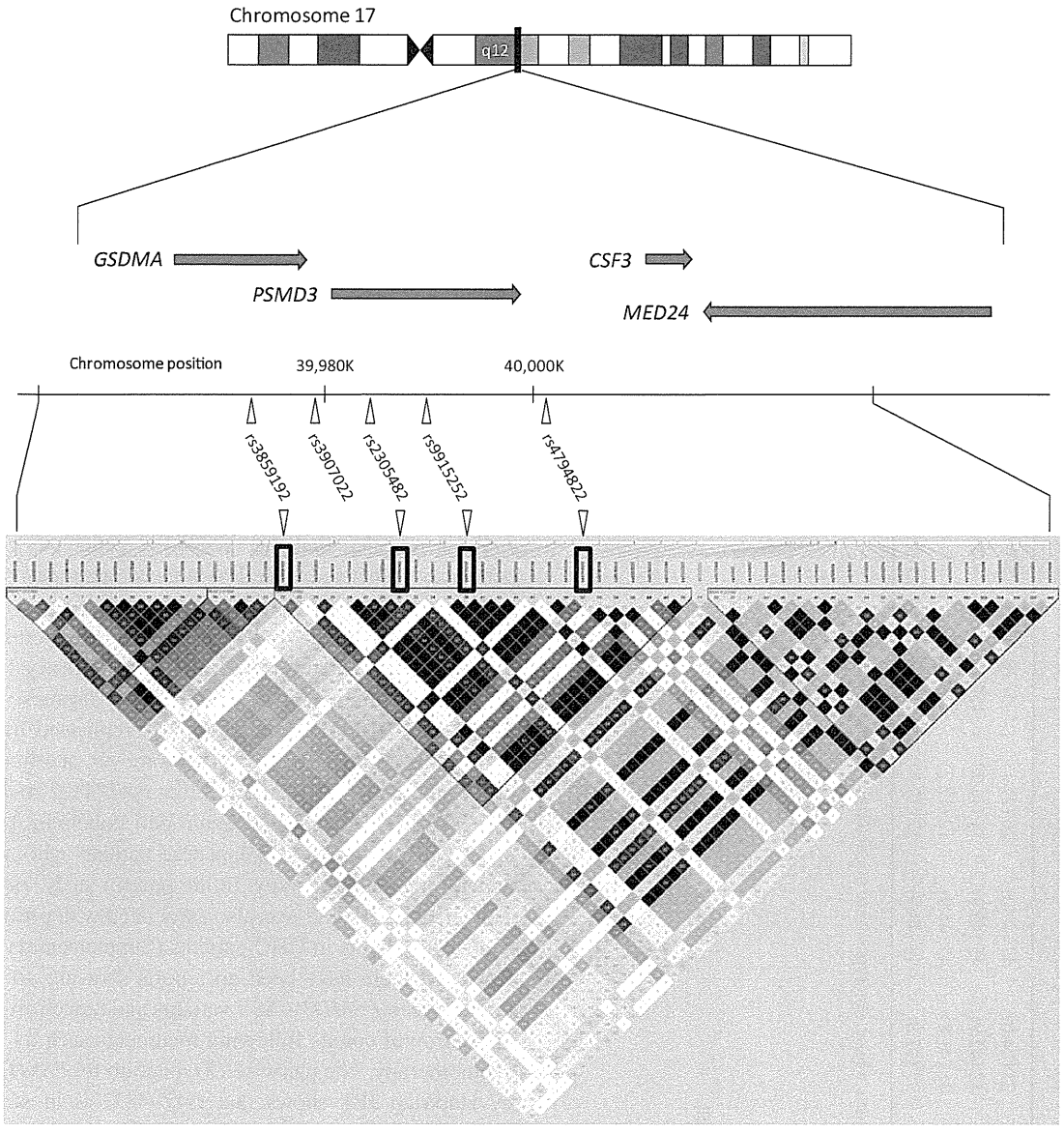

Fig. 2 Position on chromosome and pairwise linkage disequilibrium (r^2) diagrams in the HapMap JPT around the *PSMD3-CSF3* locus

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

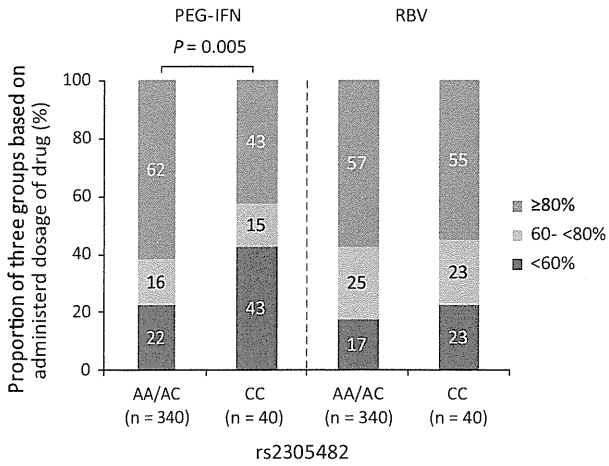

Fig. 3 Administered doses of PEG-IFN and RBV according to rs2305482 genotypes. The patients were stratified into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients receiving <60 % of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

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連 載

各都道府県における肝疾患対策取り組みの現状

岩手県における肝疾患対策取り組みの現状

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▶ はじめに

岩手県では古くから岩手県，岩手医科大学（岩手医大），そして検診業務を担う岩手県予防医学協会（予防医学協会）が連携し，主に慢性ウイルス肝炎対策を推進してきた。一方，急性肝不全対策として岩手医大が中心となり，急性肝障害患者の診療ネットワークを構築し，劇症化予知，早期搬送システムを運営してきた。これらの取り組みについて概説する。

▶ Ⅰ. ウイルス肝炎対策

1. B型肝炎対策を契機とする体制の確立

岩手県では，老人保健法に基づく住民健診が始まる以前の1977年からB型肝炎ウイルス検診を行っている。この取り組みは，岩手県と東京都臨床医学総合研究所の協力体制の下，予防医学協会が中心となって実際の健診業務を担ってきた。予防医学協会は岩手県における予防医学を推進する目的から，任意団体として1970年に設立され，当初，学童の寄生虫検診業務を中心に活動を開始した。その後，農村婦人の貧血検査や循環器健診など，農業協同組合や行政からの委託を受け，岩手県で行われる健診業務の大半を担うように発展してきた団体である。このように，行政，大学医学部，検診施設が一体となった協力体制を構築したことから，県全体の肝炎対策が効率よく推進できたといえる。

このこともあって，岩手県が国の母子感染対策事業のモデル地区となり，1981年にはHBV母子感染防止の治験に参加し，わが国2例目の症例をはじめとして99例を実施した。これらの検診，母子感染予防事業の推進のために，岩手県と岩手医大の公衆衛生学教室，当時の第一内科（現肝臓内科），盛岡赤十字病院，岩手県立中央病院の協力で，1981年「HBV母子感染予防に関する検討会」が予防医学協会に設置された。この検討会を母体にして1985年，岩手県，岩手医大，岩手県医師会，岩手県赤十字血液センターを主な委員とする「ウイルス肝炎対策専門委員会」（専門委員会）が予防医学協会に設置され，以後30年間にわたって活動を継続している。この委員会は岩手県の諮問機関として，予防医学協会の健診結果，肝炎ウイルスキャリアの追跡結果を解析し，県の肝炎対策のための基本資料を作成している。

以上のように，岩手県ではB型肝炎対策を契機にして，行政，大学医学部，予防医学協会および県の諮問機関が一体となって，早期に全県的な肝炎対策推進体制が確立した。

2. B型肝炎対策の成果

上述の岩手県独自の体制の下，1986年より公費負担によるHBV母子感染防止事業を開始した。その上で専門委員会は，効果を確認する目的で県内の学童のHBs抗原陽性率・抗体陽性率を毎年6,000人以上調査した。その結果，出生年度別にみたHBs抗原陽性率（キャリア率）は1978年出生者の0.98%から1990年出生者の0.02%にまで速やかに低下した[1]（図1）。

3. 国のウイルス肝炎対策と県との連動

国のウイルス肝炎対策として老人保健法に基づくB型，C型肝炎ウイルス検診が節目・節目外検診の形で2002年から5年間実施された。その結果，受診する機会を逸したまま潜在する肝炎ウイルスキャリアが多く存在することが推測された（表1）。この結果を受けて，2008年からは健康増進法に基づく健康増進事業として引き継がれている。岩手県でもこれと連動し，上述の体制のもと，予防医学協会による全県的な検診と専門委員会による解析，報告書の作成が毎年行われている。

ウイルス肝炎治療に関しては，2008年から国の「肝炎治療特別促進事業」が始まり，厚生労働省と都道府県はB型およびC型肝炎のIFN治療に対する医療費の助成を行うようになった。その後，

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図1　岩手県における出生年度別 HBs 抗原陽性率

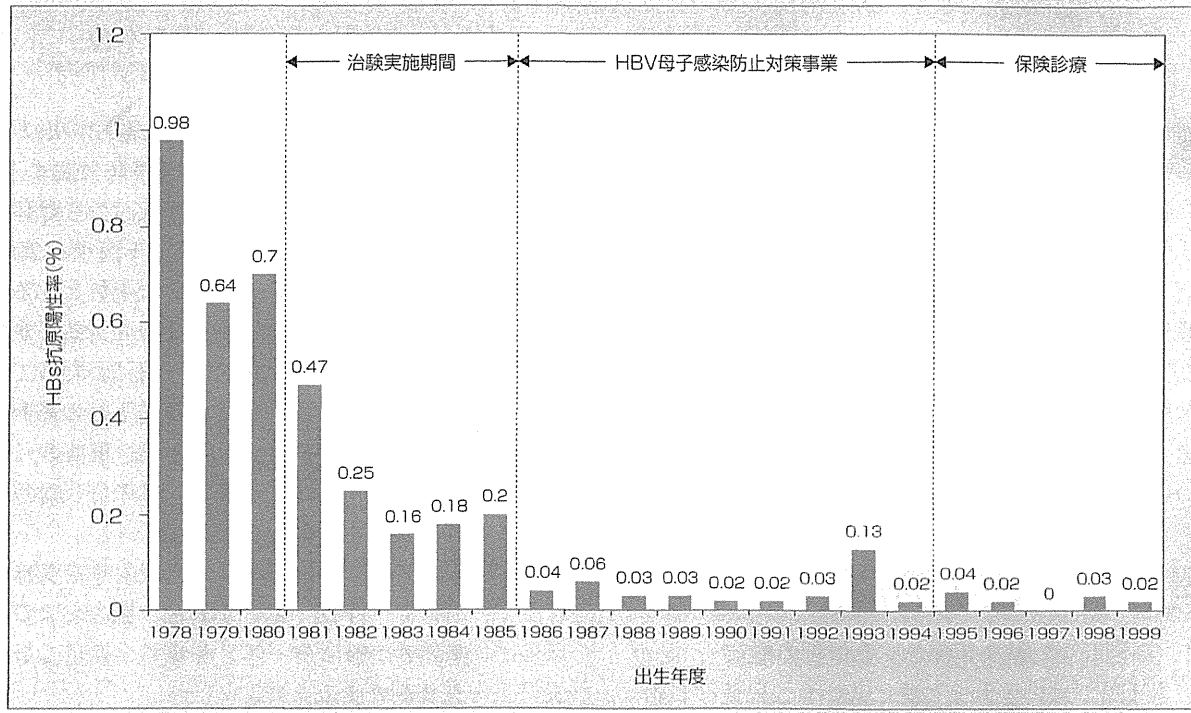


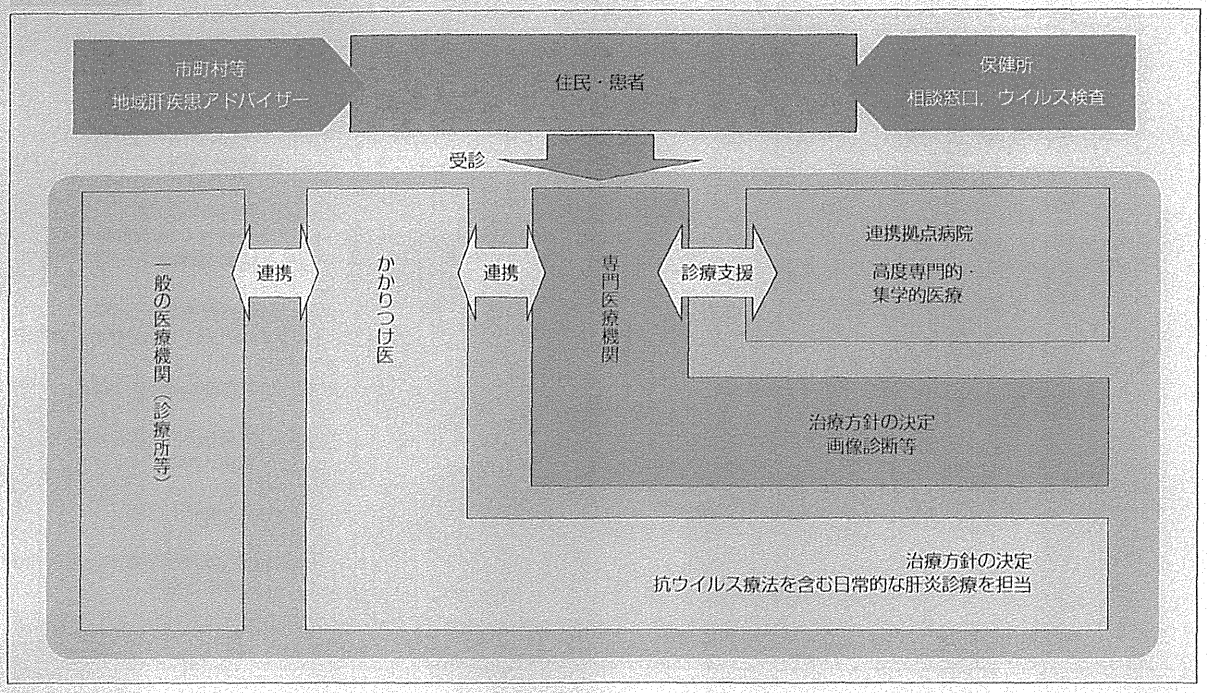
表1　岩手県・全国の節目・節目外検診別の HBV，HCV 陽性率（H14～18年の5年間）

	節目検診		節目外検診		全　体	
	岩手県	全国	岩手県	全国	岩手県	全国
B型	1.3%	1.1%	1.6%	1.1%	1.4%	1.2%
	(1,100/87,159)	(68,148/6,197,199)	(959/61,224)	(31,475/2,400,311)	(2,059/148,383)	(99,623/8,597,510)
C型	0.5%	0.9%	1.0%	1.9%	0.7%	1.2%
	(447/87,752)	(54,743/6,280,111)	(668/64,105)	(45,207/2,354,398)	(1,115/151,857)	(99,950/8,634,509)

厚生労働省 発表資料集計

2010年4月よりB型肝炎の核酸アナログ治療，2014年9月よりC型肝炎のIFN free治療に対しても医療費助成制度が適応となった。助成制度とともに診療体制の整備も行われ，各都道府県において中核医療施設となる「肝疾患診療連携拠点病院」を設置し，患者やその家族などからの相談等に対応する体制がとられている。岩手県では，このような国の動きとも連動しながら「肝疾患診療連携拠点病院」に岩手医科大学附属病院を指定し，県全体の肝炎治療を統率する役割を改めて委託した。これに併せて二次医療圏で中心的な役割を果たす「肝疾患専門医療機関」を16施設と60余りの地域の「肝炎かかりつけ医」を指定した。「肝炎かかりつけ医」を設けた理由は，専門委員会が行った受診状況の実態調査に基づいている。すなわち，検診によって発見されたキャリアに対して，肝臓専門医が在籍する医療機関のリストを渡して受診誘導しているにもかかわらず，実際にそのような医療機関を受診する割合は約半数にとどまり，残りの半数は地域の診療所などを受診していたことが判明した。このような患者の受診行動の把握から，地域の医療機関同士の情報共有が重要と考えられ，地元医師会や診療所と協力して地域の肝疾患ネットワークづくりを進めることになった（図2）。このように岩手県では，診療拠点病院，専門医療機関，かかりつけ医が協力して，患者の発見・把握に務め，医療費助成制度に則した速やかな医療提供を行うと同時に，岩手県独自の全県的体制を生かして患者の追跡調査を行っている（後述）。

図2　岩手県における肝疾患診療連携イメージ図



4. B型，C型肝炎ウイルス陽性率とキャリアの動向の把握

岩手県においては節目・節目外の肝炎ウイルス検診を行うにあたり，肝炎ウイルスキャリアの追跡調査が行える体制を構築（図3）した。すなわち，HBVおよびHCVキャリアに対して，検診結果の通知時に専門委員会が「受診確認用はがき」を添付し，受診医療機関からのはがきの回収による受診率調査を行った。さらに，受診医療機関に対して毎年受診状況の調査を行い，専門委員会として患者の動向把握に努めている。

その結果，HBVキャリア，HCVキャリアの2002～2006年の医療機関受診率の平均はそれぞれ69.6%，69.7%であった。ところが，キャリアのその後のフォローアップ状況を調べると，定期通院しているキャリアの割合はHBVで39.9%，HCVでは57.7%に留まり，「その後は来院していない」というキャリアはHBVで52.2%，HCVで31.1%に上った。特にHBVでは，「来院せず」と回答した192人中83人（43.2%）は「初診以降来院せず」となっており，十分なフォローアップができていないことが明らかになった。また，HCVキャリアのIFN治療実施率を調べてみると，2006年までの実施率はわずか10%であることも判明した。すなわち，検診によって発見されたキャリアの医療機関への受診率が低いうえに，受診後も単に経過観察になる例が多く，IFN治療などの積極的な治療を受けていないことが明らかにされた。

全期間（2002～2013年）の医療機関受診率を見ると，HBVキャリア，HCVキャリアそれぞれ66.9%，70.3%であり，2002～2006年の調査時と変化ないが，キャリアのその後のフォローアップ状況は，定期通院しているキャリアの割合がHBVで30.4%，HCVで40.1%，「その後は来院していない」というキャリアはHBVで54.9%，HCVで30.5%とやや増加した（図4）。定期通院しているキャリアの割合がHBV，HCVともに減少し，通院を中断したキャリアが，特にHBVで増加した結果となった。一方，HCVキャリアのIFN治療実施率を調べてみると，2013年までの実施率は21.6%とやや上昇しているといった状況であった。これは，蛋白分解酵素阻害薬を加えた，いわゆる3剤併用療法の開発・普及の影響と考えられた。

以上のように，岩手県では，検診による患者の発見から受診状況，診療状況までを一貫して全県的に把握し，一人でも多くの患者に最新の治療を受けてもらえるよう活動している。これには岩手県（行政）と専門委員会（諮問機関），岩手医大（大学医学部），予防医学協会（検診機関），肝疾患

図3 ウイルス肝炎に対する検診・治療体制

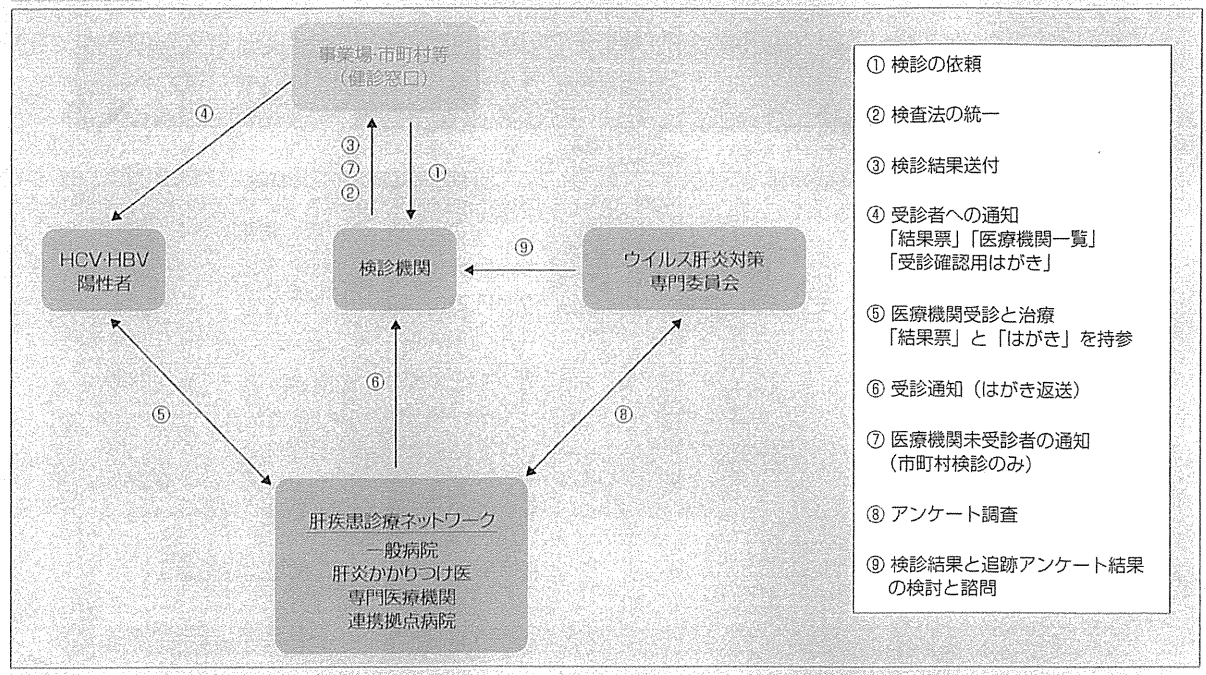
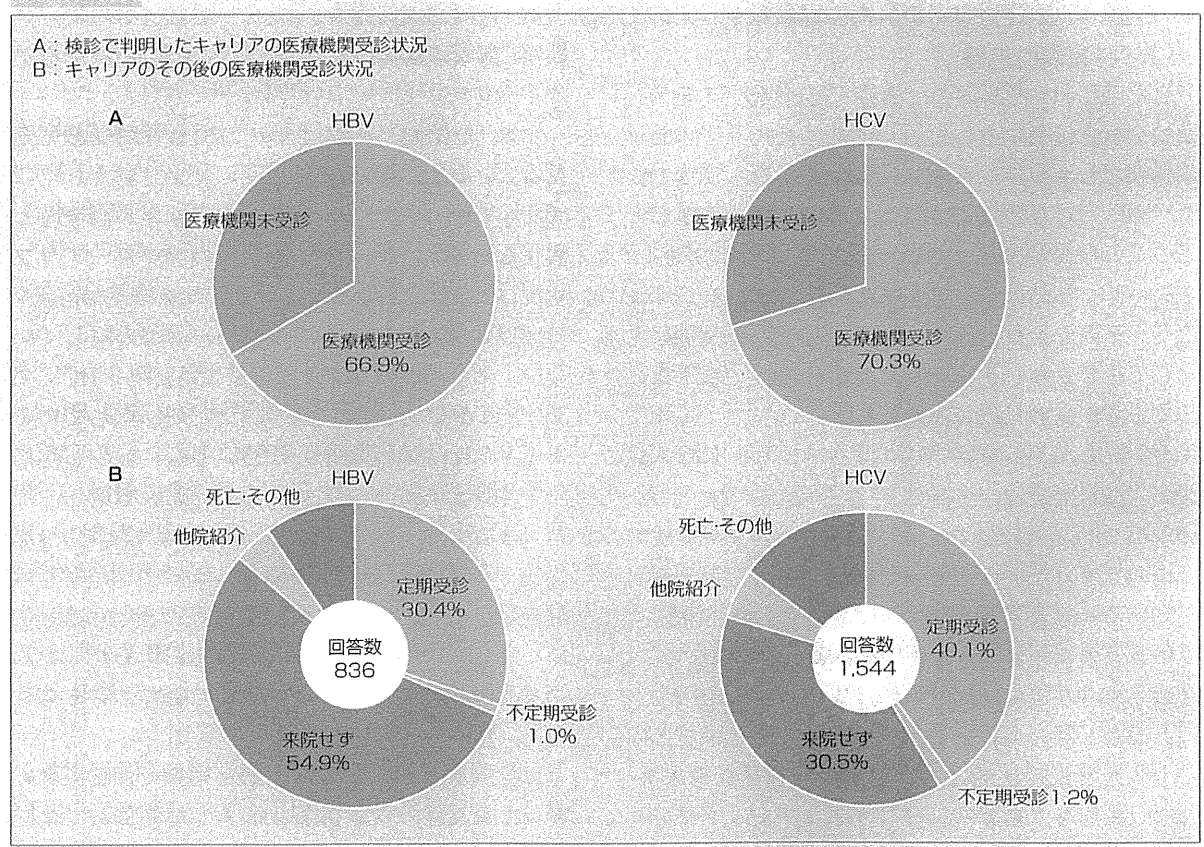


図4 岩手県における肝炎ウイルスキャリア追跡調査結果（2002～2013年）

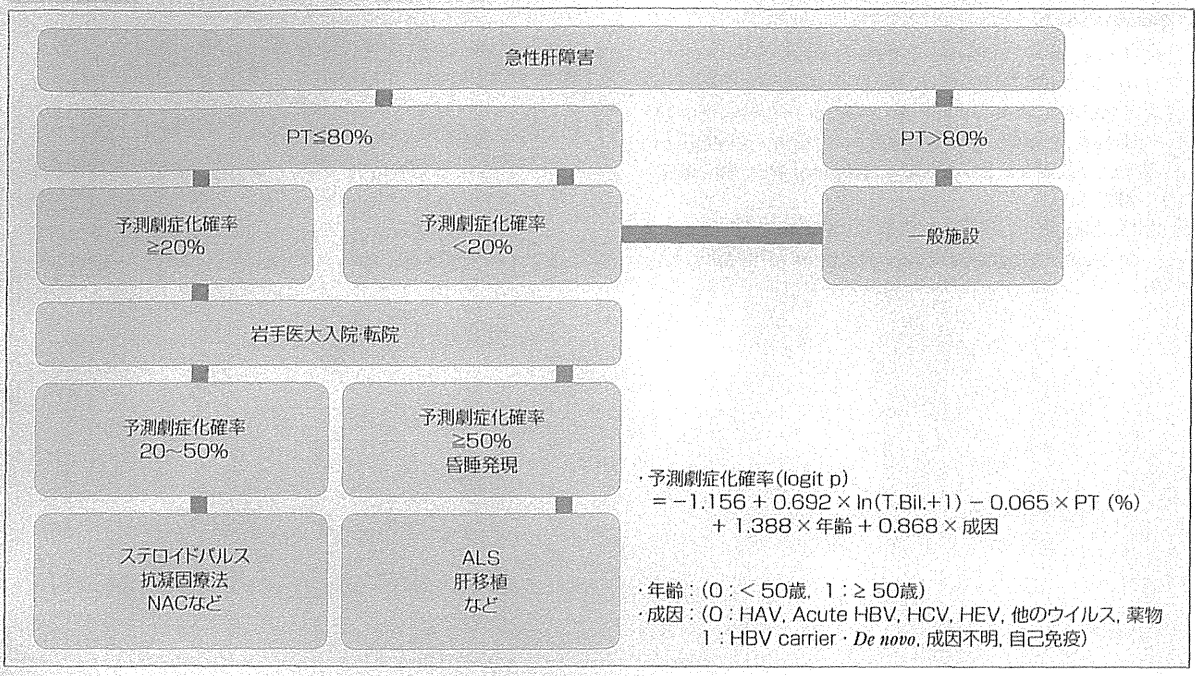


診療ネットワーク（医療機関）が長年にわたり一致協力した体制を構築してきたことが寄与していると思われる。

▶ Ⅱ. 急性肝障害搬送システム

上述の慢性肝疾患対策の主体は，無症状の患者

図5　岩手医大を中心とした急性肝障害の搬送システム



を発見し，肝硬変・肝癌への進行を阻止することにあるが，急性肝疾患対策は，劇症肝炎などの急性肝不全の診療体制の構築が重要である。

昏睡型急性肝不全は急性肝障害の1～2%にみられる稀な病態であるが，内科的救命割合が約30%と極めて予後不良である。肝不全に至らなければ急性肝障害の多くは自己終息的な経過をとり，特殊な治療を要することはほとんどないため，一般の施設で管理可能である。これに対し，昏睡発現すなわち劇症化した場合は，人工肝補助などの特殊治療を要するうえ，肝移植を必要とすることも多いため，移植可能な専門施設で管理することが望ましい。したがって，急性肝障害の診療にあたっては，地域の肝不全専門施設と一般施設が役割を分担して，重症度に応じた治療を行う体制を構築すべきである。このような共同の管理体制を可能にするためには，急性肝障害の正確かつ客観的な重症度判定により，昏睡発現（劇症化）が懸念される場合はただちに専門施設へ搬送するという体制が必要となる。

北東北では，岩手医大を中心として，40余りの病院と地域医療ネットワーク（ネットワーク）を構築し，岩手医大が開発した劇症化予知式を基にした急性肝障害の共同管理体制を取っている[2]（図5）。このシステムは2004年から開始され，11年間に急性肝不全約230例を含む500例以上の急性肝障害例が登録され，約150例が岩手医大への搬送・加療を受けている。そのうち昏睡発現例は27例（肝炎による急性肝不全の7.5%）で，7例が脳死あるいは生体肝移植を受けている。

そのために，中心となる専門施設として岩手医大では，劇症化予防治療，人工肝補助，生体肝移植，脳死肝移植など，ほぼすべての治療選択肢を準備している。また，一般病院との連絡を迅速に行うために，専用の登録用FAX用紙を事前に各施設に配布し，これによる登録，重症度判定，搬送の要否の判定とその通知，治療法アドバイス等を行っている。

この共同管理体制により，重症急性肝障害の早期搬送，予防治療が迅速に行われ，劇症化率の低下に繋がっていると推定される[3]。また，地域全体の急性肝障害の発生状況，成因・予後調査などが可能になっている。

▶ おわりに

岩手県では，慢性肝疾患，急性肝疾患ともに，全県的な体制で対策が取られており，その仕組み，成果を紹介した。

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Cancer Medicine

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ORIGINAL RESEARCH

Sorafenib for the treatment of advanced hepatocellular carcinoma with extrahepatic metastasis: a prospective multicenter cohort study

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Keywords
Extrahepatic metastasis, hepatic reserve, hepatocellular carcinoma, long-term treatment, sorafenib

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Abstract

Sorafenib, an oral multikinase inhibitor, is approved for advanced hepatocellular carcinoma (HCC) treatment. However, its therapeutic effect in advanced HCC patients with extrahepatic metastasis remains uncertain. This study aimed to prospectively assess the efficacy, safety, and survival risk factors and evaluate the prognostic impact of sorafenib treatment in advanced HCC patients with or without extrahepatic metastasis. Between May 2009 and March 2014, 312 consecutive advanced HCC patients who received sorafenib were enrolled in this study. We evaluated their characteristics and compared the clinical outcomes of those with and without extrahepatic metastasis. Of the enrolled patients, 245 (81%) received sorafenib treatment for more than 1 month, with a median duration of 3.6 months. Eighteen patients demonstrated partial response to sorafenib therapy, 127 had stable disease, and 134 had progressive disease at the first radiologic assessment. The median survival time (MST) and progression-free survival (PFS) were 10.3 and 3.6 months, respectively. Multivariate analysis identified gender, Child-Pugh class, baseline serum des-gamma-carboxy prothrombin level, and treatment duration as independent risk factors for survival. Extrahepatic metastasis was detected in 178 patients. However, the MST, PFS, and therapeutic effect were comparable between patients with and without extrahepatic metastasis. The independent risk factors for decreased overall survival in patients with extrahepatic metastasis were similar to those affecting all patients. Our results indicated that sorafenib could be administered for hepatic reserve and as long-term treatment for advanced HCC patients regardless of their extrahepatic metastasis status.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [1]. Recent advances in imaging have increased the early detection rate of HCC. Curative therapies, such as hepatic resection, liver transplantation, and radiofrequency ablation, are possible in early-stage HCC and thus improve patient survival rates [2, 3]. Otherwise, transarterial chemoembolization is an important locoregional treatment for patients with unresectable HCC [4]. However, long-term survival remains limited due to high rates of recurrence, even after such curative therapies [5]. In particular, the development of advanced HCC with macroscopic vascular invasion or