Variations and early response of Peg-IFN / RBV combination therapy

The impact of RAVs on treatment response to Peg-IFN plus RBV combination therapy was analyzed in 65 patients. The virological response (VR), defined as a greater than 2.0 log reduction of HCV RNA at 12 weeks after therapy, did not differ between patients with or without Y93H RAV among IL28B TT genotype. For comparison, the impacts of the Q80L and S122 variants also were analyzed and these variants also did not affect the VR (Figure 2). The number of patients with IL28B non-TT genotype was too small to analyze the effect of Y93 RAVs on VR.

Discussion

Treatment of HCV has entered the era of DAA-based therapy. Early clinical trials and in vitro studies have shown that treatment with a single DAA can suppress the replication of HCV but the emergence of RAVs is rapid and this may negate the inhibitory effect of the drug. Several previous studies have reported the incidence of RAVs in patients naive to treatment with DAAs. Here, we studied a large number of patients and revealed that naturally occurring RAVs in NS3 and NS5A are not rare in genotype 1b HCV. The frequency of RAVs was 7.9% in NS3 and 20.2% in NS5A. Caution should be used because the presence of these RAVs could attenuate the efficacy of DAA-based therapy.

RAVs for the NS3 protease inhibitors vary for different DAAs and HCV genotypes (4-7, 20-24). RAVs common to first generation protease inhibitors include amino acid positions 36, 54, 155 and 156, while those for second generation protease inhibitors include positions 80,156, and 168 (6,8). The frequency of RAVs in NS3 was higher in the present study than about 1% in a previous report (8,9). The impact of these RAVs on treatment outcome should be evaluated separately for interferon-based therapy and interferon-free combination therapy using DAAs. Bartels et al. reported that the SVR rates of Peg-IFN, RBV and TVR triple therapy were similar in patients with or without TVR-associated RAVs at baseline (9). This observation suggested that RAVs are susceptible to interferon. The result of our study supports this conclusion because the VR to PR therapy did not differ between patients with or without RAVs. On the other hand, several reports have revealed that baseline RAVs significantly attenuated the SVR rate of interferon-free Asunaprevir (NS3 protease inhibitor) and Daclatasvir (NS5A inhibitor) combination therapy. Thus, the presence of RAVs in NS3 could impact the selection of optimal treatment. In cases with NS3 RAVs, interferon-based therapy or interferon-free combination therapy with DAAs other than against NS3 should be preferred for patients with RAVs.

In Japan, an NS5A inhibitor is now becoming a key drug for the interferon-free combination therapy using DAAs. The frequency reported for RAVs in NS5A is higher in

Japan than in Western countries (9,28,29). A strikingly high incidence of the Y93H mutation should influence the outcome of Asunaprevir and Daclatasvir combination therapy, the first interferon-free combination therapy using DAAs approved in Japan, if treatment is given without assessment of baseline RAVs. Previous reports clearly indicated that the rate of SVR decreased to below 50% in patients with baseline RAVs. Because susceptibility to interferon was not attenuated in patients with Y93H RAV, interferon-based therapy, such as with the NS3 inhibitor Simeprevir plus peg-interferon and RBV, may be preferable for these patients. It was shown recently that Daclatasvir plus peg-interferon and RBV combination therapy achieved a high rate of SVR in treatment naïve Japanese patients (30). Taking into account the high prevalence of NS5A RAVs at baseline, as shown here, the result of this report suggested that RAVs in NS5A may not impact the outcomes of interferon-based therapies. Furthermore, newer combination therapies with DAAs are expected to be effective against these naturally occurring RAVs (31-33).

The association between the IL28B genotype and Y93H mutation was an unexpected finding but is in accordance with a recent report of an independent cohort of Japanese patients (29). No other variations in NS3 or NS5A showed an association with IL28B. Prior exposure to interferon therapy was not associated with the presence of Y93H and the precise reason for the association is unclear. Another finding was an association between Y93H and a

high HCV RNA titer. This finding and the high prevalence of Y93H suggest that the Y93H RAV may be replication competent. This is in contrast to RAVs in NS3, where the replication fitness of the variants is reduced compared to the wild type virus, a finding supported clinically by the observation that treatment induced RAVs in NS3 become undetectable after long term follow-up (34-36). However, this result should be considered carefully, since there is no *in vitro* data to support the enhancement of replication by Y93H mutations. Adaptive mutations other than Y93H may be linked to high serum HCV RNA titer. Further studies are necessary to confirm this.

Another factor associated with Y93H was a lower platelet counts. This finding may suggest a more advanced stage of disease in Y93H-infected patients. A prospective observational study is needed to confirm this relationship.

In conclusion, RAVs, especially Y93H in the NS5A region, were highly prevalent in DAA-naive patients with genotype 1b hepatitis C in Japan. The presence of RAV Y93H has been reported to attenuate the efficacy of interferon-free combination therapy with DAAs, while the present study revealed that this RAV may be linked to the IL28B TT genotype in Japanese and was susceptible to interferon-based therapy. Thus, the analysis of RAVs in NS3 and NS5A may impact the selection of optimal treatment strategies, where interferon-based therapy with or without combination of DAAs could be an alternative for patients with

RAVs.

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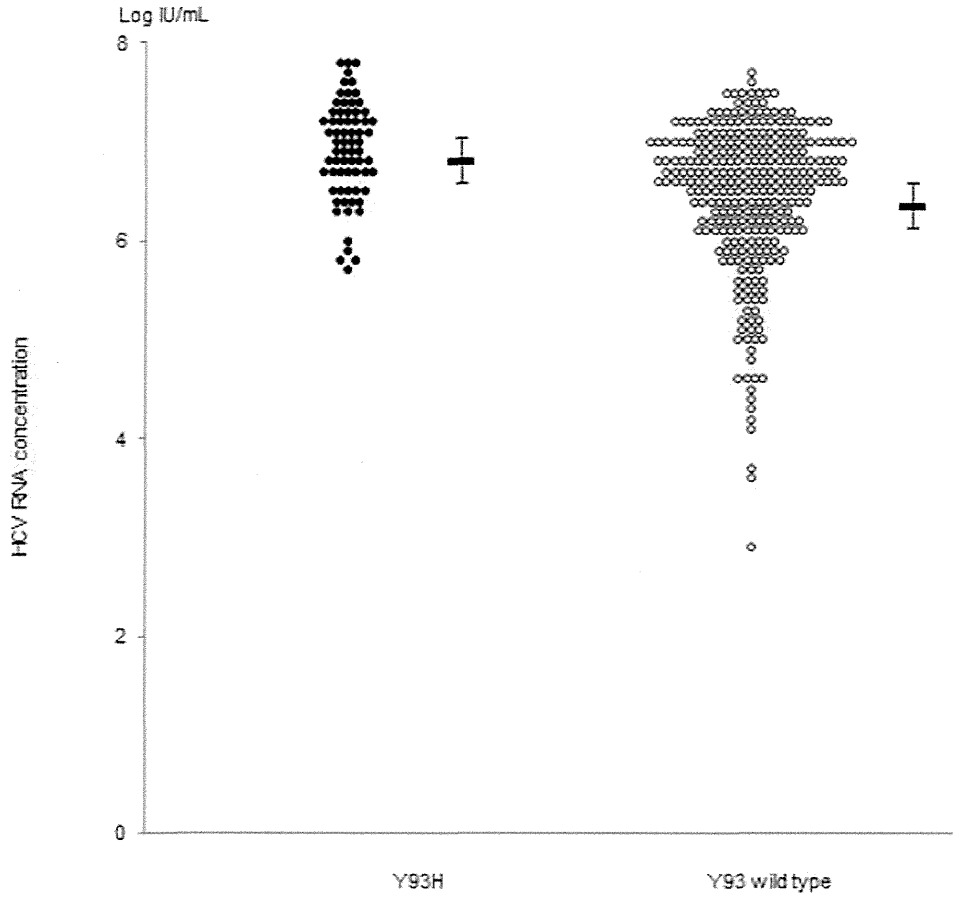

Figure 1. HCV RNA levels according to the presence of Y93H in NS5A

The serum level of HCV RNA was compared between patients with and without Y93H RAV mutations in NS5A. Patients with Y93H had significantly higher HCV RNA levels.

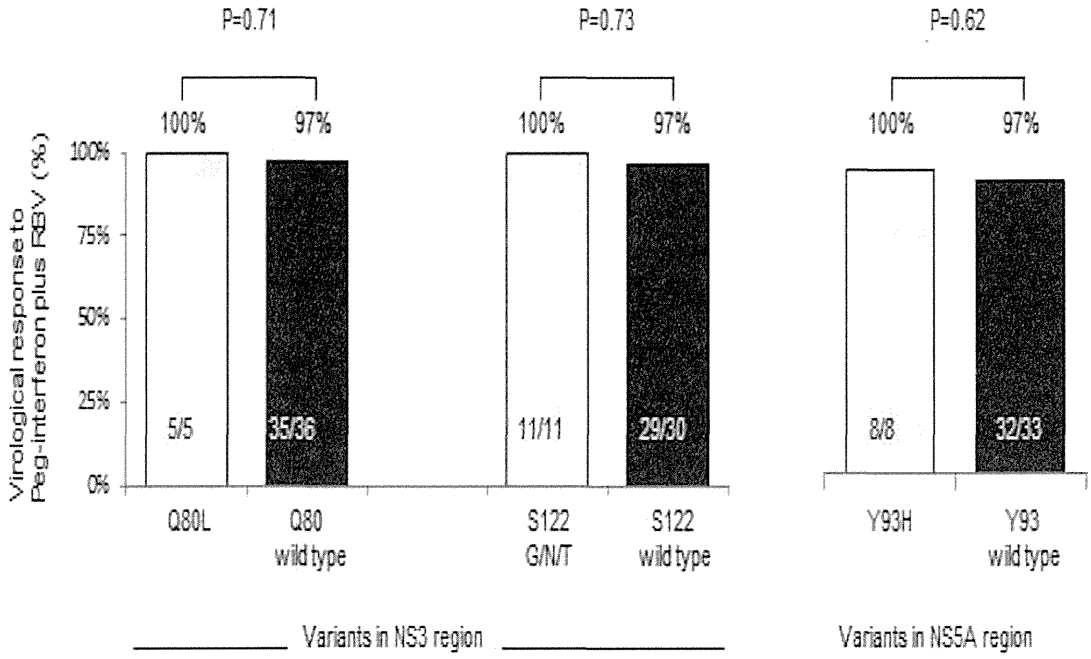

Figure 2. Virological responses to Peg-interferon plus RBV therapy in terms of variations in NS3 and NS5A

The rate of virological response to peg-interferon plus RBV therapy was analyzed in patients with or without frequently observed variations in the NS3 region (Q80L and S122G/N/T) and NS5A region (Y93H), after stratification by IL28B genotype TT.

Table 1. Baseline characteristics

	Total (n=493)	Prior interferon treatment		
		Naïve (n=185)	Experienced (n=308)	P-value
Age	62.9±11.8	59.8±14.1	64.7±9.8	<0.01
Male / Female	175 / 318	64 / 121	111 / 197	0.75
ALT (U/L)	50.1±38.6	55±40.1	47±37.5	0.045
Platelet ($x10^{-9}$/L)	158±58.8	16.6±6.2	15.3±5.7	0.46
Albumin (g/dl)	4.1±0.5	4.1±0.5	4.0±0.5	0.99
AFP (ng/ml)	9.6±18.8	8.9±21.6	10.1±17.1	0.50
HCV RNA (log IU/ml)	6.5±0.8	6.5±0.7	6.5±0.8	0.71
Fibrosis stage (0-1/ 2/ 3-4)	138/ 101/ 125	56/ 34/ 33	82/ 67/ 92	0.16
IL28B (rs8099917) (TT/ TG or GG)	259/ 173	101/ 52	158/ 121	0.06
ISDR mutation (0/ 1-2/ ≥3)	214/ 124/ 30	67/ 46/ 17	147/ 78/ 13	0.03
Core amino acid 70 (Wild type/ Mutant)	189/ 102	89/ 38	100/ 64	0.11

ALT; alanine aminotransferase, AFP; alpha-fetoprotein, IFN; interferon, peg-IFN; pegylated interferon, RBV; ribavirin, ISDR; interferon sensitivity determinant region

Table 2. Incidence of variants in the NS3 region

Position	Total (n=493)	Prior interferon treatment		
		Naïve (n=185)	Experienced (n=308)	P-value
V36I	2 (0.4)	1 (0.5)	1 (0.3)	0.72
V36L	4 (0.8)	4 (2.1)	0 (0)	0.01
T54S*	14 (2.8)	8 (4.3)	6 (1.9)	0.12
V55I	1 (0.2)	0 (0)	1 (0.3)	0.44
N77K	1 (0.2)	0 (0)	1 (0.3)	0.44
N77S	4 (0.8)	2 (1.1)	1 (0.6)	0.61
Q80G	2 (0.4)	2 (1.1)	0 (0)	0.07
Q80K*	11 (2.2)	6 (3.2)	5 (1.6)	0.24
Q80L	77 (13.4)	27 (11.4)	50 (14.6)	0.32
Q80M	1 (0.2)	0 (0)	1 (0.3)	0.44
Q80R*	4 (0.8)	1 (0.2)	3 (0.6)	0.60
Q80stop	1 (0.2)	0 (0)	1 (0.3)	0.43
S122G	93 (18.9)	35 (18.9)	58 (18.8)	0.98
S122I	1 (0.2)	0 (0)	1 (0.3)	0.44
S122N	24 (4.9)	7 (3.8)	17 (5.5)	0.39
S122R	3 (0.6)	2 (1.1)	1 (0.3)	0.30
S122T	36 (7.3)	16 (8.7)	20 (6.5)	0.37
A156S*	1 (0.2)	1 (0.5)	0 (0)	0.20
D168E*	11 (2.2)	6 (3.2)	5 (1.6)	0.24
D168T*	1 (0.2)	0 (0)	1 (0.3)	0.44
V170I	241 (48.9)	100 (54.1)	141 (45.8)	0.14
V170M	5 (1.0)	0 (0)	5 (1.6)	0.08
V170T	1 (0.2)	0 (0)	1 (0.3)	0.44
Any variants	344 (69.8)	133 (71.9)	211 (68.5)	0.43
RAV*	39 (7.9)	19 (10.3)	20 (6.5)	0.13

Data are expressed as the numbers of patients with variations and percentages in parentheses.

RAV*: resistance-associated variants

Table 3. Incidence of variants in the NS5A region

Position	Total (n=410)	Prior interferon treatment		
		Naïve (n=136)	Experienced (n=274)	P value
L23I	3 (0.7)	0 (0)	3 (1.1)	0.22
L28M	26 (6.3)	6 (4.4)	20 (7.3)	0.26
R30H	1 (0.2)	1 (0.7)	0 (0)	0.16
R30L	1 (0.2)	1 (0.7)	0 (0)	0.16
R30Q	44 (10.7)	14 (10.3)	30 (10.9)	0.84
R30S	1 (0.2)	0 (0)	1 (0.4)	0.48
L31I*	3 (0.7)	2 (1.5)	1 (0.4)	0.22
L31M*	6 (1.5)	3 (2.2)	3 (1.1)	0.42
F37I	8 (2.0)	2 (1.5)	6 (2.2)	0.62
F37L	206 (50.2)	61 (44.9)	145 (52.9)	0.12
F37V	3 (0.7)	1 (0.7)	2 (0.7)	1.00
Q54C	1 (0.2)	0 (0)	1 (0.4)	0.48
Q54E	1 (0.2)	0 (0)	1 (0.4)	0.48
Q54H	154 (37.6)	53 (39.0)	101 (36.9)	0.68
Q54L	9 (2.2)	3 (2.2)	6 (2.2)	0.99
Q54N	7 (1.7)	2 (1.5)	5 (1.8)	0.79
Q54V	2 (0.5)	1 (0.7)	1 (0.4)	0.61
Q54Y	12 (2.9)	4 (2.9)	8 (2.9)	0.99
P58A	4 (1.0)	2 (1.5)	2 (0.7)	0.47
P58L	6 (1.5)	1 (0.7)	5 (1.8)	0.39
P58Q	2 (0.5)	0 (0)	2 (0.7)	0.32
P58R	2 (0.5)	0 (0)	2 (0.7)	0.32
P58S	21 (5.1)	9 (6.6)	12 (4.3)	0.49
P58T	2 (0.5)	2 (1.5)	0 (0)	0.04
P58V	1 (0.2)	1 (0.7)	0 (0)	0.16
Y93H*	78 (19.0)	21 (15.4)	57 (20.8)	0.19
Y93N	1 (0.2)	0 (0)	1 (0.4)	0.48
Y93P	1 (0.2)	0 (0)	1 (0.4)	0.48

Y93S	2 (0.5)	0 (0)	2 (0.7)	0.32
Y93T	1 (0.2)	0 (0)	1 (0.4)	0.48
Any variants	344 (83.9)	109 (80.1)	235 (85.8)	0.15
RAV*	83 (20.2)	24 (17.6)	59 (21.5)	0.36

Data are expressed with number of patients with variations and percentage in parenthesis.

RAV,*: resistance-associated variants

Table 4. Characteristics associated with Y93H of NS5A region

	Y93		Univariate	Multivariate	
	Wild type (n=332)	H (n=78)	P-value	OR (95%CI)	P-value
Age (years)	62.4±11.8	65.9±10.6	0.02		
Male / Female	116 / 216	26 / 52	0.79		
ALT (U/L)	49.5±40.2	49.6±34.0	0.98		
Platelet ($x10^9$/L)	162±60	141±50	<0.01	2.43 (1.34-4.41)	0.003
Albumin (g/dl)	4.1±0.4	4.0±0.5	0.13		
AFP (ng/ml)	8.9±19.2	11.6±20.0	0.34		
HCV RNA (logIU/ml)	6.5±0.8	6.9±0.5	<0.01	3.42 (1.62-7.2)	0.001
Prior therapy (Naïve/experienced)	115/ 217	21/ 57	0.19		
Fibrosis stage (0-2/ 3-4)	170/ 84	44/ 24	0.73		
IL28B (rs8099917) (TT/ TG or GG)	159/ 141	59/ 14	<0.01	3.44 (1.69-7.01)	0.001
ISDR mutation (0/ ≥1)	141/ 106	43/ 21	0.14		
Core amino acid 70 (Wild type/ Mutant)	115/ 75	33/ 14	0.22		

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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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