ONLINE METHODS

Subjects. The Japanese participants in the meta-analysis (4,074 rheumatoid arthritis cases and 16,891 controls) and the replication study (5,277 rheumatoid arthritis cases and 21,684 controls) were obtained through the collaborations of the GARNET consortium (**Supplementary Table 1**)[10,12]. The meta-analysis was conducted on three independent GWAS (from the BioBank Japan Project[18] with 2,414 rheumatoid arthritis cases and 14,245 controls[10], Kyoto University with 1,237 rheumatoid arthritis cases and 2,087 controls[12] and IORRA[19] with 423 rheumatoid arthritis cases and 559 controls). The replication study consisted of two independent cohorts (cohort 1 included 3,830 rheumatoid arthritis cases and 17,920 controls, and cohort 2 included 1,447 rheumatoid arthritis cases and 3,764 controls). We employed a case-control cohort of SLE (891 cases and 3,384 controls)[22] and 1,783 cases with Graves' disease[10]. Details of 5,539 rheumatoid arthritis cases and 20,169 controls included in the meta-analysis in European populations were described elsewhere[15]. All participants provided written informed consent for participation in the study, as approved by the ethical committees of the institutional review boards. Detailed descriptions of the participating subjects are provided (**Supplementary Note**).

Genotyping and quality control in the GWAS. Genotyping platforms and quality control criteria for the GWAS, including cutoff values for sample call rates, SNP call rates, MAF and Hardy-Weinberg *P* values, are given (**Supplementary Table 2**). For the subjects enrolled in each of three GWAS, we excluded closely related subjects with first- or second-degree kinship, which was estimated using PLINK version 1.06 (see URLs). We also excluded the subjects determined to be ancestry outliers from East Asian populations using PCA performed by EIGENSTRAT version 2.0 (see URLs) along with HapMap Phase 2 panels (release 24; **Supplementary Fig. 1**). Genotype imputation was performed on the basis of the HapMap Phase 2 East Asian populations, using MACH version 1.0.16 (see URLs) in a two-step procedure as described elsewhere[25]. We excluded imputed SNPs with MAF < 0.01 or *Rsq* < 0.5 from each of the GWAS. Associations of the SNPs with rheumatoid arthritis were assessed by logistic regression models assuming additive effects of the allele dosages of the SNPs using mach2dat software (see URLs).

Meta-analysis. We included 1,948,139 autosomal SNPs that satisfied quality control criteria in all three GWAS (**Supplementary Table 2**). SNP information was based on a forward strand of the NCBI build 36.3 reference sequence. The meta-analysis was performed using an inverse variance method assuming a fixed-effects model from the study-specific effect sizes (logarithm of odds ratio) and the standard errors of the coded alleles of the SNPs determined with the Java source code implemented by the authors[25]. Genomic control corrections[26] were carried out on test statistics of the GWAS using the study-specific inflation factor (λ_{GC}) and was applied or reapplied to the results of our current meta-analysis (**Supplementary Fig. 2**).

Replication study. We selected a SNP for the replication study from each of the loci that exhibited $P < 5.0 \times 10^{-4}$ in the meta-analysis that had not previously been reported as rheumatoid arthritis susceptibility loci[1–16] (**Supplementary Table 3**). For control subjects, we used genotype data obtained from additional GWAS for non-autoimmune diseases or healthy controls, genotyped using Illumina HumanHap550 BeadChips or HumanHap610-Quad BeadChips, and the cases for rheumatoid arthritis and Graves' disease were genotyped with the TaqMan genotyping system (Applied Biosystems; **Supplementary Table 1**). Selection of the SNP was conducted according to the following criteria: if the SNP with the most significant association in the locus was genotyped in the replication control panel, then that SNP was selected; otherwise, a tag SNP in the replication control panel with the strongest LD was selected (mean $r^2 = 0.89$). For the three SNPs that yielded low call rates (<90%), we alternatively selected proxy SNPs with the second strongest LD. As a result, average genotyping call rates of the SNPs were 99.9% and 99.0% for the controls and cases, respectively. We then evaluated concordance rates between the assayed genotypes by applying these two different methods to samples from 376 subjects who were randomly selected. This procedure yielded high concordance rates of ≥99.9%. Associations of the SNPs were evaluated using logistic regression assuming an additive-effects model of genotypes in R statistical software version 2.11.0 (see URLs). The combined study of the meta-analysis and replication study was performed using an inverse variance method assuming a fixed-effects model[25].

***Cis* eQTL analysis.** For each marker SNP of the newly identified rheumatoid arthritis susceptibility locus, correlations between SNP genotypes and expression levels of genes located 300 kb upstream or downstream of the SNP measured in B-lymphoblastoid cell lines (GSE6536) were evaluated using data from the HapMap Phase 2 east Asian populations[27].

Multi-ancestry analysis of the meta-analyses in Japanese and Europeans. We evaluated the associations of the variants in the validated rheumatoid arthritis susceptibility loci by comparing the results from the current meta-analysis in Japanese with those from a previous meta-analysis in Europeans[15]. We assessed two variants in the *IRF5* locus, where different causal variants were identified in the two populations[24]. For the conditional analysis of the regional associations in the *ARID5B* locus (**Supplementary Fig. 3**), we repeated the meta-analysis at that locus by incorporating genotypes of the referenced SNP(s) as additional covariate(s). For comparison of the odds ratios of the SNPs, we first selected SNPs that were shared between the meta-analyses in Japanese and Europeans. Next, we removed the SNPs located more than 1 Mb away from each of the marker SNPs in the validated rheumatoid arthritis susceptibility loci, except for in the HLA region, where we removed the SNPs located between 24,000,000 bp to 36,000,000 bp on chromosome 6 because of the existence of long-range haplotypes with rheumatoid arthritis susceptibility in this region[28]. LD pruning of the SNPs was conducted for the SNP pairs that were in LD ($r^2 \geq 0.3$) in both HapMap Phase 2 East Asian and Utah residents of Northern and Western European ancestry (CEU) populations (release 24). Correlations of the odds ratios were evaluated using R statistical software version 2.11.0.

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Predictive Value of Early Viral Dynamics During Peginterferon and Ribavirin Combination Therapy Based on Genetic Polymorphisms Near the *IL28B* Gene in Patients Infected With HCV Genotype 1b

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A study was carried out to determine whether early viral dynamics retain prediction of the outcome of peginterferon (PEG-IFN) and ribavirin combination therapy based on different genetic polymorphisms near the *IL28B* gene, the strongest baseline predictor of response to this therapy. A total of 272 patients infected with hepatitis C virus (HCV) genotype 1b were grouped according to genetic polymorphisms near the *IL28B* gene (rs8099917). The ability of reduced HCV RNA levels at 4 and 12 weeks after starting therapy to predict a sustained virologic response was evaluated based on these genotypes. Among patients with the TT genotype for rs8099917 (associated with a favorable response), the rates of sustained virologic response were higher in patients with a $\geq$3 $\log_{10}$ reduction in serum HCV RNA levels at 4 weeks after starting therapy ($P < 0.0001$). In contrast, among patients with the TG/GG genotype (associated with an unfavorable response), there were no differences in this rate based on the reduction in HCV RNA levels at 4 weeks. Early viral dynamics at 4 weeks after starting therapy retains its predictive value for sustained virologic response in patients with the TT genotype for rs8099917, but not in patients with the TG/GG genotype. Patients who are likely to achieve sustained virologic response despite unfavorable TG/GG genotype cannot be identified based on early viral dynamics during therapy. In contrast, lack of early virologic response at 12 weeks retains a strong predictive value for the failure of sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy. ***J. Med. Virol. 84:61–70, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: chronic hepatitis C; early viral dynamics; genetic polymorphisms near the *IL28B* gene; peginterferon; response-guided therapy; ribavirin

INTRODUCTION

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although this treatment regimen has increased markedly the number of patients with a sustained virologic response, i.e., the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 achieved a sustained virologic response approximately.

Many investigators have examined factors that predict the treatment outcome of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1. In addition to the baseline factors, the response of HCV during combination therapy, i.e., the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti

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et al., 2002; Berg et al., 2003], with the emphasis on "response-guided therapy" [Lee and Ferenci, 2008; Marcellin and Rizzetto, 2008]. Recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been the strongest predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, the predictive value of reduced serum HCV RNA levels at 4 weeks after starting therapy has been clarified further, and a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy has high predictive value that a patient will achieve a sustained virologic response as a final outcome, even in the absence of a rapid virologic response [Toyoda et al., 2011].

In contrast, the lack of an early virologic response, defined as either undetectable serum HCV RNA or HCV RNA levels decreased by >2.0 $\log_{10}$ from the pretreatment level at 12 weeks after starting therapy, has been the most important predictor for the failure of a sustained virologic response in patients infected with HCV genotype 1 reportedly [Fried et al., 2002; Davis et al., 2003]. Therefore, treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment, according to the recommendation in the AASLD guidelines [Ghany et al., 2009].

More recently, several studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 affect the virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Furthermore, genetic polymorphisms near the *IL28B* gene are the strongest baseline predictive factor of the final outcome of combination therapy. An additional report showed the effects of genetic polymorphisms near the *IL28B* gene on HCV viral dynamics during PEG-IFN and ribavirin combination therapy [Thompson et al., 2010].

Although early HCV viral dynamics during therapy was shown originally to have a high predictive value for a sustained virologic response in HCV genotype 1-infected patients before genetic polymorphisms near the *IL28B* gene were linked to a therapeutic response, it is not clear whether early viral dynamics retain their predictive value in light of this additional information. The purpose of the present study was to investigate whether response-guided therapy based on viral dynamics at 4 or 12 weeks after initiating therapy retains its ability to predict the final outcome of PEG-IFN and ribavirin combination therapy after accounting for genetic polymorphisms near the *IL28B* gene.

MATERIALS AND METHODS

Patients and Treatment

Between January 2007 and June 2008, a total of 402 patients with chronic hepatitis C received antiviral combination therapy with PEG-IFN and ribavirin for HCV infection at the Ogaki Municipal Hospital or the Nagoya University Hospital. Among these patients, 272 were infected with HCV genotype 1b and had pretreatment HCV RNA levels >5.0 $\log_{10}$ IU/ml based on a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems, Pleasanton, CA; Lower limit of quantification, 1.7 $\log_{10}$ IU/ml: Lower limit of detection, 1.0 $\log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008]. This study did not include any patients infected with HCV genotype 1a because this genotype is not found in the general Japanese population.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough, Kenilworth, NJ) daily. The PEG-IFN and ribavirin doses were adjusted based on the patient's body weight. Patients weighing $\leq$45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing >45 and $\leq$60 kg were given 80 μg, those weighing >60 and $\leq$75 kg were given 100 μg, those weighing >75 and $\leq$90 kg were given 120 μg, and those weighing >90 kg were given 150 μg. Patients weighing $\leq$60 kg were administered 600 mg of ribavirin per day, those weighing >60 and $\leq$80 kg were given 800 mg per day, and those weighing >80 kg were administered 1000 mg per day. The PEG-IFN and ribavirin doses were modified based on the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of treatment. The treatment duration was extended up to 72 weeks in some patients. In addition, treatment was discontinued before 48 weeks in some patients who had a low likelihood of achieving an eradication of HCV due to the presence of serum HCV RNA at 24 weeks after starting therapy.

A sustained virologic response was defined as undetectable serum HCV RNA at 24 weeks after ending the therapy. A patient was considered to have relapsed when serum HCV RNA was detectable between the end of treatment and 24 weeks after completing treatment, although serum HCV RNA was undetectable during and at the end of therapy. Patients were considered to have non-response if serum HCV RNA was detectable at 24 weeks after initiating therapy (i.e., null response or partial response according to the American guidelines [Ghany et al., 2009]). Patients were considered to have a rapid virologic response if they had undetectable serum HCV RNA at 4 weeks after starting therapy. An early virologic response was defined as the disappearance or decrease in serum HCV RNA levels by at least 2 $\log_{10}$ at 12 weeks after starting therapy. Patients were considered to have a complete early virologic response if serum HCV RNA was undetectable at 12 weeks after starting therapy and a partial early virologic response if the serum

HCV RNA levels had decreased by at least 2 $\log_{10}$ at 12 weeks after initiating therapy. Patients were considered not to have an early virologic response if their HCV RNA levels did not decrease by more than 2 $\log_{10}$ at 12 weeks compared to the pretreatment levels. Patients were considered to have a slow virologic response if the serum HCV RNA became undetectable between 12 and 24 weeks.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Prior to initiating the study, each patient provided written informed consent to use the laboratory data, analyze genetic polymorphisms near the *IL28B* gene, and test stored serum samples.

Assessments of Serum HCV RNA Levels and Genetic Polymorphisms Near the *IL28B* Gene

After a patient provided informed consent, serum samples were obtained at the patient's regular hospital visits, just prior to initiating treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at −80°C until further use. The HCV RNA levels were measured using a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System).

Genotyping of rs 8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Foster City, California) according to the manufacturer's guidelines. A predesigned and functionally tested probe was used for rs8099917 (C__11710096_10, Applied Biosystems).

Statistical analyses. Quantitative values are reported as the mean ± SD. In between-group differences were analyzed by the chi-square test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predict a sustained virologic response, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$), reduction in peginterferon dose and ribavirin dose, reduction in HCV RNA levels at 4 weeks after starting therapy ($\geq$3 $\log_{10}$ vs. <3 $\log_{10}$), and the type of an early virologic response. All *P*-values are two-tailed, and $P < 0.05$ was considered significant statistically.

RESULTS

The characteristics of the patients examined in this study are shown in Table I. Liver histology was evaluated according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994]. Although some patients had a reduction in their PEG-IFN and ribavirin doses during therapy, respectively, all patients except for those who discontinued the therapy had more than 80% adhesion to both the PEG-IFN and ribavirin regimens. No patients discontinued the therapy because of adverse effects. The treatment duration was extended up to 72 weeks in 51 of 71 patients (71.8%) who exhibited a slow virologic response. As a final outcome, 118 patients (43.4%) achieved a sustained virologic response, 84 patients (30.9%) relapsed, and the remaining 70 patients (25.7%) had no response.

TABLE I. Characteristics of all Study Patients (n = 272)

Age (years)	56.0 ± 10.9
Sex (female/male)	139 (51.1)/133 (48.9)
Body weight (kg)	57.8 ± 10.5
Alanine aminotransferase (IU/L)	64.6 ± 56.4
Aspartate aminotransferase (IU/L)	53.9 ± 42.7
Gamma-glutamyl transpeptidase (IU)	48.5 ± 43.9
Alkaline phosphatase (IU/L)	267.9 ± 101.3
Albumin (g/dl)	4.04 ± 0.37
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4892 ± 1333
Hemoglobin (g/dl)	14.0 ± 1.3
Platelet count ($\times 10^3$/μl)	163 ± 51
Liver histology-activity (A0/A1/A2/A3)*	3 (1.2)/136 (55.3)/ 92 (37.4)/15 (6.1)
Liver histology-fibrosis (F0/F1/F2/F3)*	27 (11.0)/114 (46.3)/ 70 (28.5)/35 (14.2)
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.35 ± 0.79
Reduction in the peginterferon dose	81 (29.8)
Reduction in the ribavirin dose	130 (47.8)
Final outcomes (sustained virologic response /relapse/ no response)	118 (43.4)/ 84 (30.9)/70 (25.7)

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

Reduction in Serum HCV RNA Levels at 4 Weeks after Starting Therapy and Treatment Outcome According to Genetic Polymorphisms Near the *IL28B* Gene

An analysis of genetic polymorphisms at rs8099917 near the *IL28B* gene indicated that 207 patients (76.1%) had a TT genotype, 3 patients had a GG genotype (1.1%), and the remaining 62 patients were TG heterozygote (22.8%). Table II shows the comparison of the background characteristics between patients with the favorable TT genotype and those with the unfavorable TG/GG genotype. As reported previously [Abe et al., 2010], gamma-glutamyl transpeptidase level was higher significantly in patients with the TG/GG genotype. As a final outcome, the rate of a sustained virologic response was higher significantly in patients with the TT genotype. Among 207 patients with the TT genotype, serum HCV RNA became undetectable in 19 patients (9.2%) at 4 weeks after starting therapy (a rapid virologic response). In the remaining 188 patients, the decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.12

TABLE II. Characteristics of Study Patients According to the Genetic Polymorphisms Near the *IL28B* Gene

	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)	*P*-value
Age (years)	56.5 ± 10.4	54.4 ± 12.4	0.4112
Sex (female/male)	107 (51.7)/100 (48.3)	32 (49.2)/33 (50.8)	0.8384
Body weight (kg)	57.8 ± 10.9	57.8 ± 9.4	0.8361
Alanine aminotransferase (IU/L)	65.1 ± 53.3	62.8 ± 65.6	0.2548
Aspartate aminotransferase (IU/L)	53.6 ± 34.8	54.7 ± 62.0	0.3339
Gamma-glutamyl transpeptidase (IU)	44.2 ± 37.1	62.3 ± 59.0	0.0003
Alkaline phosphatase (IU/L)	263.1 ± 90.3	282.8 ± 129.9	0.3875
Albumin (g/dl)	4.04 ± 0.36	4.05 ± 0.43	0.8020
Total bilirubin (mg/dl)	0.79 ± 0.30	0.76 ± 0.32	0.3010
White blood cell count (/μl)	4826 ± 1333	5100 ± 1320	0.1608
Hemoglobin (g/dl)	13.9 ± 1.3	14.1 ± 1.4	0.3339
Platelet count ($\times 10^3/\mu l$)	161 ± 49	169 ± 57	0.3871
Liver histology-activity (A0/A1/A2/A3)*	2 (1.1)/98 (52.4)/ 74 (39.6)/13 (6.9)	1 (1.7)/38 (64.4)/ 18 (30.5)/2 (3.4)	0.3241
Liver histology-fibrosis (F0/F1/F2/F3)*	21 (11.2)/83 (44.4)/ 57 (30.5)/26 (13.9)	6 (10.2)/31 (52.5)/ 13 (22.0)/9 (15.3)	0.6401
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.37 ± 0.85	6.29 ± 0.55	0.0582
Reduction in the peginterferon dose	61 (29.5)	20 (30.8)	0.9644
Reduction in the ribavirin dose	101 (48.8)	29 (44.6)	0.5565
Final outcomes (sustained virologic response /relapse/ no response)	106 (51.2)/ 69 (33.3)/32 (15.5)	12 (18.4)/15 (23.1)/ 38 (58.5)	<0.0001

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

$\log_{10}$ to 5.71 $\log_{10}$ (mean, 3.12 $\log_{10}$). The reduction in serum HCV RNA levels was $\geq$3 $\log_{10}$ in 98 patients (47.3%), <3 $\log_{10}$ and $\geq$2 $\log_{10}$ in 52 patients (25.1%), <2 $\log_{10}$ and $\geq$1 $\log_{10}$ in 23 patients (11.1%), and <1 $\log_{10}$ in 15 patients (7.3%). Figure 1A shows the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TT genotype. The rates were higher significantly in patients who achieved a rapid virologic response or had a $\geq$3 $\log_{10}$ decrease in

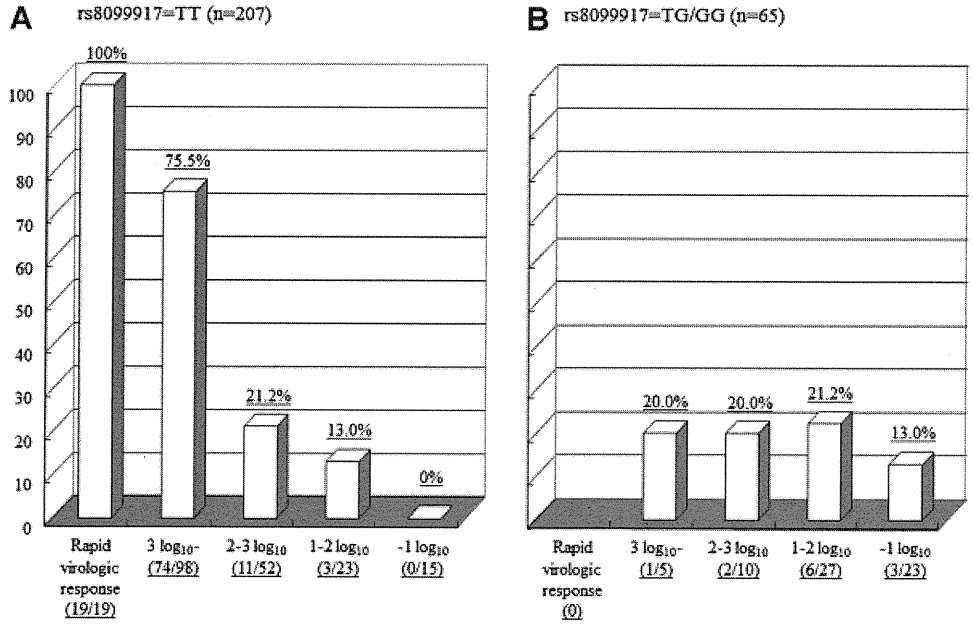

Fig. 1. The rate of sustained virologic responses (%) based on the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

serum HCV RNA levels at 4 weeks compared to those with a <3 $\log_{10}$ decrease in serum HCV RNA levels ($P < 0.0001$). When a 3 $\log_{10}$ decrease in serum HCV RNA levels was defined as the cut-off point, 56.5% of patients were considered to have a ≥3 $\log_{10}$ decrease in serum HCV RNA levels. The sensitivity, specificity, positive predictive value, and negative predictive value for a sustained virologic response were 86.8, 75.2, 78.6, and 84.4%, respectively.

Among the 65 patients who had the TG/GG genotype, no patient achieved a rapid virologic response at 4 weeks after initiating therapy. The decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.11 $\log_{10}$ to 4.75 $\log_{10}$ (mean, 1.66 $\log_{10}$). The reduction in serum HCV RNA levels at 4 weeks after starting the therapy were smaller in patients with the TG/GG genotype than those with the TT genotype (1.66 ± 1.02 $\log_{10}$ in patients with the TG/GG genotype vs. 3.12 ± 1.37 $\log_{10}$ in patients with TT genotype excluding RVR, $P < 0.0001$). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in five patients (7.7%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 10 patients (15.4%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 27 patients (41.5%), and <1 $\log_{10}$ in 23 patients (35.4%). Figure 1B shows the rates of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TG/GG genotype. There were no differences in the rate of a sustained virologic response based on the reduction in HCV RNA levels at 4 weeks after starting therapy; the rate of a sustained virologic response remained at 20% approximately regardless of the reduction in HCV RNA levels in 42 patients with a ≥1 $\log_{10}$ reduction in serum HCV RNA levels.

Association Between an Early Virologic Response at 12 Weeks and Treatment Outcome Based on Genetic Polymorphisms Near the *IL28B* Gene

Figure 2 shows the rate of patients with the TT genotype or TG/GG genotype for rs8099917 who achieved a complete early virologic response, a partial early virologic response, and those who did not achieve early virologic response at 12 weeks after starting therapy based on the reduction in serum HCV RNA level at 4 weeks after initiating therapy. Nearly 75% of patients with the TT genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy achieved a complete early virologic response. In contrast, 80% of patients with the TG/GG genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy showed a partial early virologic response. The majority of patients with the TT or TG/GG genotypes achieved a partial early virologic response when their reduction in HCV RNA levels was <3 $\log_{10}$ and ≥2 $\log_{10}$ or <2 $\log_{10}$ and ≥1 $\log_{10}$.

Figure 3 shows the rates of a sustained virologic response according to the type of early virologic response in patients with the TT genotype (Fig. 3A) and TG/GG genotype (Fig. 3B). Among patients with the TT genotype, the rate of sustained virologic response was significantly higher in patients with a complete early virologic response than in those with a partial early virologic response ($P < 0.0001$). In contrast, there was no difference in the rate of a sustained virologic response between patients with a complete early virologic response and those with a partial early virologic response ($P = 0.8917$) among patients with

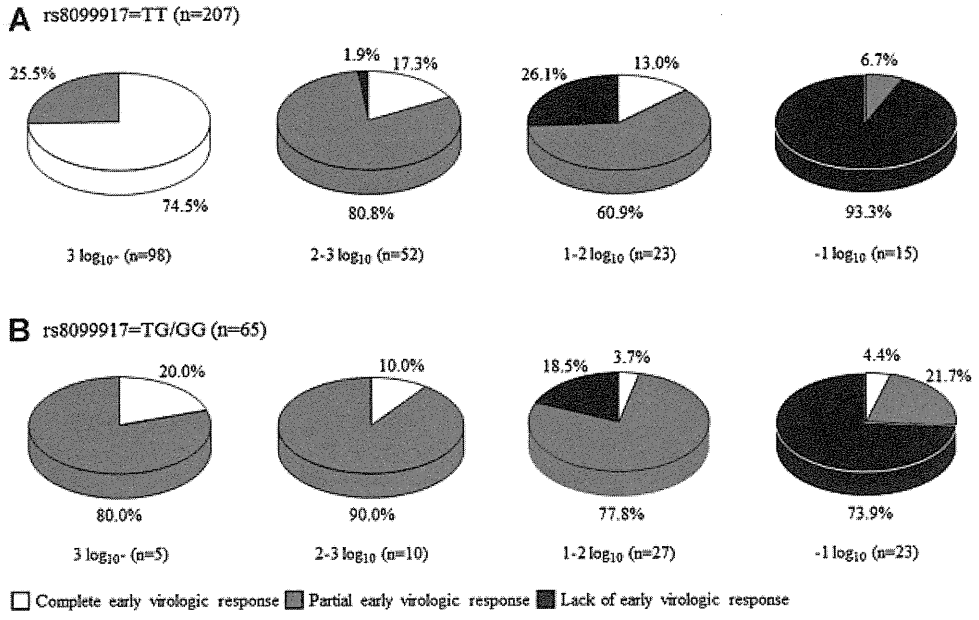

Fig. 2. The association between the virologic responses at 12 weeks after starting therapy and the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

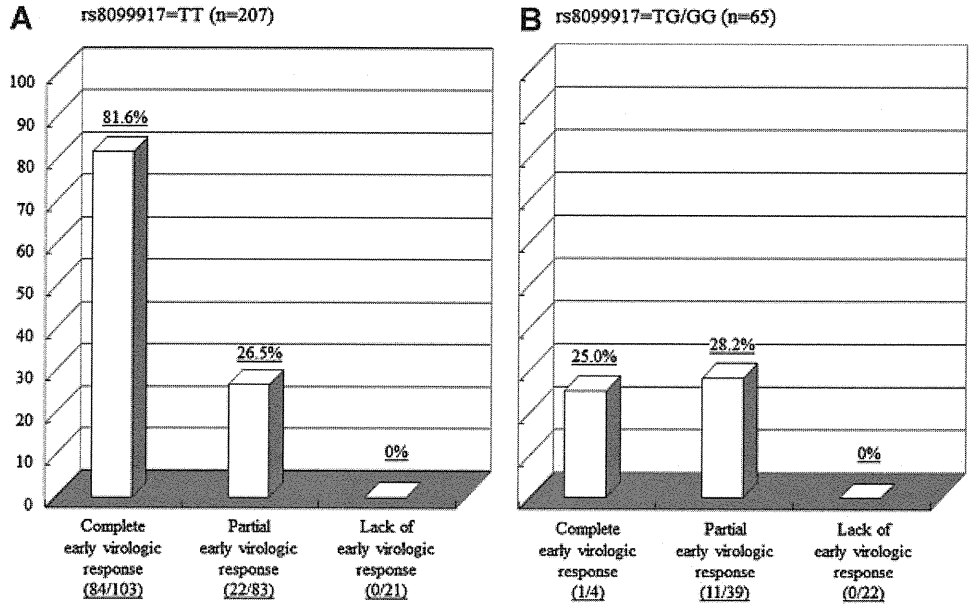

Fig. 3. The rate of sustained virologic responses based on the type of early virologic response. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

the TG/GG genotype. None of the patients with the TT genotype or TG/GG genotype who yielded a lack of an early virologic response reached a sustained virologic response.

Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

Univariate and multivariate analyses were conducted for factors associated with a sustained virologic response based on different genetic polymorphisms near the *IL28B* gene. In patients with the TT genotype, the factors that were associated with a sustained virologic response included serum alkaline phosphatase levels, serum albumin, platelet counts, hepatitis activity grade, liver fibrosis grade, reduction in HCV RNA levels at 4 weeks after starting therapy, and a complete early virologic response based on a univariate analysis (Table IIIA). In a multivariate analysis, the serum albumin levels, reduction in HCV RNA levels 4 weeks after starting therapy, and a complete early virologic response were independent factors that were significantly associated with a sustained virologic response (Table IIIB). A reduction in HCV RNA levels 4 weeks after starting therapy was the strongest factor that affected a sustained virologic response. In patients with the TG/GG genotype, the factors that were associated with a sustained virologic response included patient age, platelet counts, and pretreatment HCV RNA levels based on a univariate analysis (Table IIIA). A reduction in the HCV RNA levels at 4 weeks after starting therapy was not associated with a sustained virologic response. In a multivariate analysis, patient age and pretreatment HCV RNA levels were independent factors that were significantly associated with a sustained virologic response (Table IIIC).

Characteristics of Patients who Achieved a Sustained Virologic Response to the Combination Therapy Despite the Unfavorable TG/GG Genotype Near the *IL28B* Gene

Table IV shows the characteristics of 12 patients who achieved a sustained virologic response despite having the unfavorable TG/GG genotype for rs8099917 near the *IL28B* gene. All but one patient was under 60 years old and had liver fibrosis not more than grade 2 (one patient did not undergo a liver biopsy). Except for one patient, the reduction in the serum HCV RNA levels at 4 weeks after starting therapy was less than 3 $\log_{10}$ and all but one patient showed a partial early virologic response at 12 weeks after starting the therapy. In all 11 patients with a partial early virologic response, the serum HCV RNA was undetectable up to 24 weeks after starting the therapy. All but one patient extended the treatment duration from 48 to 72 weeks (two patients discontinued therapy at 60 weeks during the extended treatment period). When the characteristics of patients who achieved a sustained virologic response were compared between those with the unfavorable TG/GG genotype and those with the favorable TT genotype, patients with the TG/GG genotype were younger (41.8 ± 14.4 years vs. 55.1 ± 10.4 years, $P = 0.0023$) and had lower pretreatment HCV RNA levels (5.91 ± 0.44 $\log_{10}$ IU/ml vs. 6.21 ± 1.05 $\log_{10}$ IU/ml, $P = 0.0199$).

TABLE III. Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

(A) Univariate analyses	*P*-value	
	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)
Age (years)	0.0505	0.0007
Sex (female/male)	0.1830	0.2296
Body weight (kg)	0.6891	0.2456
Alanine aminotransferase (IU/L)	0.7988	0.4032
Aspartate aminotransferase (IU/L)	0.5021	0.1705
Gamma-glutamyl transpeptidase (IU)	0.6340	0.6648
Alkaline phosphatase (IU/L)	0.0315	0.0599
Albumin (g/dl)	0.0002	0.6594
Total bilirubin (mg/dl)	0.2929	0.7130
White blood cell count (/μl)	0.2508	0.5549
Hemoglobin (g/dl)	0.0847	0.2289
Platelet count ($\times 10^3$/μl)	0.0454	0.0411
Liver histology-activity (A0–1/A2–3)	0.0445	0.1117
Liver histology-fibrosis (F0–1/F2–3)	0.0002	0.2283
Pretreatment HCV RNA concentration ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.5279	0.0379
Reduction in the peginterferon dose	0.4316	0.5563
Reduction in the ribavirin dose	0.1823	0.4272
Reduction in HCV RNA levels at 4 weeks after starting the therapy ($\geq$3 $\log_{10}$ vs. < 3 $\log_{10}$)	<0.0001	0.9265
Early virologic response (complete vs. partial)	<0.0001	0.9777
Early virologic response (partial vs. non)	0.8632	0.0686

(B) Multivariate analyses: Patients with TT genotype of rs8099917	*P*-value	Odds ratio (95% confidence interval)
Alkaline phosphatase (IU/L)	0.2617	
Albumin (g/dl)	0.0365	28.287 (1.4107–755.41)
Platelet count ($\times 10^3$/μl)	0.2599	
Liver histology-activity (A0–1/A2–3)	0.6678	
Liver histology-fibrosis (F0–1/F2–3)	0.2307	
Reduction in HCV RNA levels at 4 weeks after starting the therapy ($\geq$3 $\log_{10}$ vs. <3 $\log_{10}$)	<0.0001	16.029 (6.8593–40.406)
Early virologic response (complete vs. partial)	0.0224	0.3685 (0.1557–0.8749)

(C) Multivariate analyses: Patients with TG/GG genotype of rs8099917	*P*-value	Odds ratio (95% confidence interval)
Age (years)	0.0022	0.0034 (0.0000–0.0840)
Platelet count ($\times 10^3$/μl)	0.3344	
Pretreatment HCV RNA concentration ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.0304	0.0548 (0.0020–0.4950)

HCV, hepatitis C virus.

DISCUSSION

Several previous studies reported that patients who achieved a rapid virologic response, in which serum HCV RNA become undetectable at 4 weeks after starting therapy, had a high likelihood of achieving a sustained virologic response [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, several recent studies reported the predictive value of the degree of reduction in serum HCV RNA levels at 4 weeks after starting therapy [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011]. Therefore, the viral dynamics of HCV at 4 as well as 12 weeks after starting therapy is important for response-guided therapy.

Genetic polymorphisms near the *IL28B* gene have emerged as the strongest predictive factor of a sustained virologic response in patients infected with HCV genotype 1 [Hayes et al., 2011; Kurosaki et al., 2011]. In addition, Thompson et al. [2010 reported that genetic polymorphisms near the *IL28B* gene were associated strongly with early viral dynamics during PEG-IFN and ribavirin combination therapy. These findings raised an important issue of whether response-guided therapy, based on the reduction in serum HCV RNA levels at 4 or 12 weeks after starting

TABLE IV. Patients who Achieved a Sustained Virologic Response Despite the TG/GG Genotype for the rs8099917

	Age (years)	Sex	Liver histology	Pretreatment HCV RNA level (log_{10} IU/ml)	HCV RNA reduction at 4 weeks	Response at 12 weeks	HCV RNA became undetectable (weeks)	Treatment duration (weeks)
1.	31	Female	A1/F1	6.13	2.19	partial EVR	20	48
2.	55	Male	A1/F1	5.80	1.77	partial EVR	16	72
3.	57	Female	A1/F1	5.58	3.01	partial EVR	16	72
4.	57	Female	A1/F1	6.21	1.81	partial EVR	20	72
5.	62	Male	N.D.	6.23	1.13	partial EVR	24	72
6.	21	Male	A1/F2	6.04	1.83	partial EVR	24	72
7.	42	Male	A1/F1	6.27	0.57	partial EVR	24	72
8.	29	Female	A1/F2	5.83	1.83	partial EVR	20	60
9.	52	Male	A1/F0	5.91	2.12	complete EVR	12	48
10.	40	Male	A2/F1	5.84	1.34	partial EVR	20	72
11.	27	Male	N.D.	5.63	0.42	partial EVR	24	72
12.	28	Male	A1/F0	6.59	0.76	partial EVR	20	60

N.D., not done; HCV, hepatitis C virus; EVR, early virologic response.

therapy, retains a predictive value when considering genetic polymorphisms near the *IL28B* gene.

In the present study, the predictive value of the decrease in serum HCV RNA levels was evaluated at 4 and 12 weeks after starting therapy in Japanese patients infected with HCV genotype 1b based on genetic polymorphisms near the *IL28B* gene. Consistent with previous reports, patients with the TG/GG genotype for rs8099917 had a smaller reduction in serum HCV RNA levels at 4 weeks after starting treatment ($P < 0.0001$), which indicates an unfavorable response to the combination therapy. Patients with the TT genotype for rs8099917, which is associated with a favorable response to the combination therapy, exhibited a significant difference in the rate of a sustained virologic response based on the reduction in serum HCV RNA levels at 4 weeks after initiating the therapy. Patients with a rapid virologic response or with a ≥ 3 log_{10} reduction in HCV RNA levels had a higher likelihood of achieving a sustained virologic response.

In contrast, these factors did not have any predictive value in patients with the TG/GG genotype. Only 18.5% of patients achieved a sustained virologic response (12 of 65 patients), and it was difficult to identify these patients based on the reduction in HCV RNA levels at 4 weeks or the type of an early virologic response at 12 weeks after starting therapy. Patients who achieved a sustained virologic response, despite the TG/GG genotype for rs8099917, were identified among those with a <2 log_{10} and ≥ 1 log_{10} or even <1 log_{10} reduction in HCV RNA levels at 4 weeks after starting therapy. Interestingly and paradoxically, the possibility of a sustained virologic response can be expected in patients with a <1 log_{10} reduction in HCV RNA levels at 4 weeks after starting therapy only when they have the unfavorable TG/GG genotype.

In the evaluation at 12 weeks after starting therapy, patients with the TT genotype who achieved a complete early virologic response had a higher rate of a sustained virologic response significantly than patients who achieved a partial early virologic response, whereas this difference was not found in patients with the TG/GG genotype. No patients who failed to achieve an early virologic response achieved a sustained virologic response regardless of the genetic polymorphisms near the *IL28B* gene. Thus, the lack of an early virologic response retained a strong predictive value for the failure of achieving a sustained virologic response. This result supports the recommendation in the AASLD guidelines, in which treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment.

The characteristics of patients who achieved a sustained virologic response despite the unfavorable TG/GG genotype were younger in age and lower pretreatment HCV RNA levels. Most patients with the TG/GG genotype who achieved a sustained virologic response showed a partial early virologic response and extended the treatment duration. It was difficult to identify these patients according to viral dynamics at 4 or 12 weeks after starting therapy.

There are several limitations in this study. Some patients with a slow virologic response did not have their treatment period extended from 48 to 72 weeks. This is because the effectiveness of a 72-week combination therapy regimen in patients with HCV genotype 1 with a slow virologic response [Berg et al., 2006; Pearlman et al., 2007] had not been established in Japan in the earlier part of this study. This fact might have influenced the treatment outcome especially in patients with the unfavorable TG/GG genotype. Another limitation is a smaller sample size of patients with the TG/GG genotype in comparison to that of patients with the TT genotype. This sample size could have caused the lack of statistical significance in the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy or according to the type of an early virologic response in patients with the TG/GG genotype. In addition, the data were based on Japanese patients infected with HCV genotype 1b. Therefore, these results should be confirmed in other ethnicities and patients infected with HCV genotype 1a.

In conclusion, among patients infected with HCV genotype 1b with the TT genotype for rs8099917, a rapid virologic response or a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, or a complete early virologic response indicate strongly that these patients will achieve a sustained virologic response as a final outcome for PEG-IFN and ribavirin combination therapy. Early viral dynamics retain the predictive value in this patient subpopulation. A reduction in HCV RNA levels at 4 weeks after starting therapy or the type of an early virologic response does not predict the likelihood that patients with the TG/GG genotype will achieve a sustained virologic response. In contrast, the lack of an early virologic response retains a strong predictive value for the failure to achieve a sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy.

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Genetic Polymorphisms of the Human PNPLA3 Gene are Strongly Associated with Severity of Non-Alcoholic Fatty liver Disease in Japanese

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) includes a broad range of liver pathologies from simple steatosis to cirrhosis and fibrosis, in which a subtype accompanying hepatocyte degeneration and fibrosis is classified as nonalcoholic steatohepatitis (NASH). NASH accounts for approximately 10-30% of NAFLD and causes a higher frequency of liver-related death, and its progression of NASH has been considered to be complex involving multiple genetic factors interacting with the environment and lifestyle.

Plincipal Findings: To identify genetic factors related to NAFLD in the Japanese, we performed a genome-wide association study recruiting 529 histologically diagnosed NAFLD patients and 932 population controls. A significant association was observed for a cluster of SNPs in *PNPLA3* on chromosome 22q13 with the strongest *p*-value of 1.4×10^{-10} (OR=1.66, 95%CI: 1.43-1.94) for rs738409. Rs738409 also showed the strongest association ($p=3.6 \times 10^{-6}$) with the histological classifications proposed by Matteoni and colleagues based on the degree of inflammation, ballooning degeneration, fibrosis and Mallory-Denk body. In addition, there were marked differences in rs738409 genotype distributions between type4 subgroup corresponding to NASH and the other three subgroups ($p=4.8 \times 10^{-6}$, OR=1.96, 95%CI: 1.47-2.62). Moreover, a subgroup analysis of NAFLD patients against controls showed a significant association of rs738409 with type4 ($p=1.7 \times 10^{-16}$, OR=2.18, 95%CI:

1.81-2.63) whereas no association was obtained for type1 to type3 (p=0.41). Rs738409 also showed strong associations with three clinical traits related to the prognosis of NAFLD, namely, levels of hyaluronic acid (p=4.6 x 10^{-4}), HbA1c (p=0.0011) and iron deposition in the liver (p=5.6 x 10^{-4}).

Conclusions: With these results we clearly demonstrated that Matteoni type4 NAFLD is both a genetically and clinically different subset from the other spectrums of the disease and that the *PNPLA3* gene is strongly associated with the progression of NASH in Japanese population.

Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a broad range of pathologies from fatty liver (simple steatosis), steatonecrosis, and steatohepatitis to cirrhosis [1–3]. NAFLD often accompanies other lifestyle-related pathologies of metabolic syndrome such as diabetes mellitus, hypertension and dyslipidemia, and the number of NAFLD patients is increasing worldwide along with the escalation in the incidence of metabolic syndrome [4]. Prevalence of NAFLD is considered as approximately 8% in Japanese and 6-35% in Europeans [4,5]. The majority of NAFLD shows simple steatosis with a good prognosis, but approximately 10-30% of NAFLD histologically diagnosed as nonalcoholic steatohepatitis (NASH) shows hepatocyte degeneration (ballooning hepatocyte), necrosis, inflammation and fibrosis, with a higher frequency of liver-related death both in Japanese and European populations [6,7]. Insulin resistance and oxidative stress are considered to be key players in the progression of NASH [8,9]. However, the progression of NASH has been considered to be complex involving multiple genetic factors interacting with the environment and lifestyle, because only a portion of NAFLD patients develops NASH.

The first Genome-wide association (GWA) study searching for such genetic factors identified the *PNPLA3* gene as a major genetic determinant for the predisposition to NAFLD in Hispanic, African American and European American populations according to liver fat

contents [10], which was subsequently confirmed in Europeans and Asians according to liver biopsy. Association of *PNPLA3* with not only fatty liver and TG content, but also inflammation and fibrosis were shown in the subsequent studies, so *PNPLA3* may be widely associated with the development of NAFLD [11–13]. More recently, another GWA study reported the association of four additional genes with NAFLD in Europeans [14]. Also, a candidate gene-based approach revealed the association between NAFLD and the apolipoprotein C3 gene in Indians [15]. However, the precise role of such genes in the development of NASH still remains to be elucidated. In addition, no GWA study has been reported for Asian populations to date although the genetic components and their relative contribution may be different between ethnicities.

The Japan NASH Study Group was founded in 2008 aiming at the identification of genetic determinants predisposing to NASH in the Japanese population. Here we report the first GWA study of NAFLD in the Japanese using DNA samples of patients with liver histology-based diagnoses recruited through this multi-institutional research network.

Results

Genome-wide association analysis of NAFLD in Japanese

We conducted a GWA study using DNA samples of 543 patients with NAFLD and 942 controls. After quality controls of genotyping results (see materials and methods for details), a total of 529 patients consisting of four NAFLD subgroups according to Matteoni's classification [2] (type1; 100, type2; 73, type3; 29, type4; 327) and 932 controls were subjected to statistical analyses. This index pathologically classifies NAFLD according to the degree of inflammation, hepatocyte degeneration, and the existence of fibrosis and Mallory-Denk body in the liver. Genome scan results of 932 DNA samples collected for other genetic studies were used as general Japanese population controls [16]. After standard quality control procedure as described in materials and methods, genotype distributions of 484,751 autosomal SNP markers were compared between the NAFLD cases and control subjects by exact trend test. A slight inflation of *p*-values was observed by genomic control method (λ=1.04) (Supplementary figure 1).

We identified six SNP markers located at chromosome 22q13 showing genome-wide significance ($p<1.04 \times 10^{-7}$) (Figure 1). Among them, four SNPs, namely, rs2896019, rs926633, rs2076211 and rs1010023, located in the *PNPLA3* gene and in strong linkage disequilibrium (LD) ($r^2>0.93$), returned *p*-values smaller than 1×10^{-9} ($p=1.5 \times 10^{-10}$, 7.5 x

10^{-10}, 1.4 x 10^{-9} and 1.5 x 10^{-9}, respectively) (Table 1). Rs738407 and rs3810662 also located in *PNPLA3* showed significant but weaker associations (p=1.0 x 10^{-7} and 1.0 x 10^{-7}, respectively) than the above four SNP markers. Rs738491, rs2073082, rs3761472, rs2235776, rs2143571 and rs6006473 were in the neighboring *SAMM50* gene which is outside of the linkage disequilibrium (LD) block where the top SNP markers were distributed (Figure 2). These markers were in moderate LD with each other (r^2>0.42) and showed p-values between 3.9 x 10^{-6} and 6.4 x 10^{-7} but did not reach genome-wide significance (Supplementary table 1). Rs738409, the SNP which showed the strongest association with NAFLD in the first GWA study [10], was not included in the SNP array used in our study. This SNP was therefore genotyped using Taqman technology in the same case and control samples that were used for genome scan. Rs738409 showed the strongest association with the disease (p=1.4 x 10^{-10}, OR=1.66, 95%CI: 1.43-1.94) among all the SNP markers examined in this study. The association remained after the correction for population stratification with EIGENSTRAT [17] (p=2.3 x 10^{-11}). Although a peak consisting of a cluster of SNPs was observed at the *HLA* locus on chromosome 6 (minimal p-value of 4.10 x 10^{-7} for rs9262639 located at the 3' of *C6orf15* gene), the association disappeared when EIGENSTRAT was applied (p>1.6 x 10^{-3}). We consider this as a result of population stratification between the cases and controls.

Impact of *PNPLA3* polymorphisms to the pathogenicity of NAFLD

We next examined whether or not the seven SNPs in the *PNPLA3* gene were associated with the pathogenic status of NAFLD. The genotype distributions of these SNPs were compared by Jonckheere-Terpstra test among the four subgroups of NAFLD patients categorized by Matteoni's classification (type1 to type4). There was a significant increase in the frequency of the risk allele from Matteoni type1 to type4 for all of the seven SNPs (p-values ranging from 3.6×10^{-6} to 0.0017) (Table 2). Among them, rs738409 again showed the strongest association ($p=3.6 \times 10^{-6}$) as seen in the simple case/control analysis (Table 2). On the other hand, there was no significant association between control and Matteoni type1 ($p=0.76$).

In order to clarify how rs738409 influences the pathogenicity of NAFLD, we performed pairwise comparisons of genotype distributions in the four subgroups of NAFLD patients. There were marked differences in genotype distributions between type4 subgroup and the other three subgroups by multivariable logistic regression adjusted for age, sex and body mass index (BMI) ($p=2.0 \times 10^{-5}$, OR=2.18, 95%CI: 1.52-3.18 between type1 and type4; $p=1.4 \times 10^{-3}$, OR=1.81, 95%CI: 1.26-2.62 between type2 and type4; $p=0.027$, OR=1.85, 95%CI: 1.07-3.19 between type3 and type4) (Figure 3). On the other hand, no significant