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Relationships between Genetic Variations of *PNPLA3*, *TM6SF2* and Histological Features of Nonalcoholic Fatty Liver Disease in Japan

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Background/Aims: It is important to determine the noninvasive parameters of histological features in nonalcoholic fatty liver disease (NAFLD). The aim of this study was to investigate the value of genetic variations as surrogate markers of histological features. **Methods:** The parameters that affected the histological features of NAFLD were investigated in 211 Japanese patients with biopsy-proven NAFLD. The relationships between genetic variations in *PNPLA3* rs738409 or *TM6SF2* rs58542926 and histological features were analyzed. Furthermore, the impact of genetic variations that affected the pathological criteria for the diagnosis of nonalcoholic steatohepatitis (NASH) (Matteoni classification and NAFLD activity score) was evaluated. **Results:** The fibrosis stage of *PNPLA3* GG was significantly more progressive than that of CG by multiple comparisons. Multivariate analysis identified *PNPLA3* genotypes as predictors of fibrosis of stage 2 or more, but the impact tended to decrease at stage 3 or greater. There were no significant differences among the histological features of the three genotypes of *TM6SF2*. *PNPLA3* genotypes partly affected the definition of NASH by the NAFLD activity score, but *TM6SF2* genotypes did not affect the definition of NASH. **Conclusions:** In Japanese patients with biopsy-proven NAFLD, *PNPLA3* genotypes may partly affect histological features, including stage of fibrosis, but the *TM6SF2* genotype does not affect histological features. **(Gut Liver 2016;10:437-445)**

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Fibrosis stage; Patatin-like phospholipase domain containing 3; Transmembrane 6 superfamily member 2

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently the most common liver disease worldwide across different ethnicities,[1-6] and results in serious health-care issue. NAFLD includes a wide spectrum of liver pathologies ranging from nonalcoholic fatty liver, which is usually benign, to nonalcoholic steatohepatitis (NASH), which may lead to liver cirrhosis, hepatocellular carcinoma, and liver failure without excessive alcohol intake.[7] Vitamin E[8] and Farnesoid X nuclear receptor ligand obeticholic acid[9] practically had improved the histological features. NASH can only be diagnosed by histological components, such as steatosis, lobular inflammation, ballooning, and fibrosis. Although histological diagnosis (such as steatosis, lobular inflammation, ballooning, and fibrosis) is currently the gold standard for diagnosing progressive NASH, liver biopsy has many drawbacks, such as cost, sampling error, and risk of complications.[6] It is important to determine the noninvasive parameters as surrogate markers of histological features.

The severity and progression of NAFLD is influenced by a complex of multiple factors, including environmental factors and genetic variations.[10] Especially, one of the most significant genetic risk factor for NAFLD is a variant located in the *PNPLA3* (patatin-like phospholipase domain-containing 3) gene, the rs738409 encoding an amino acid substitution p.Ile148Met. *PNPLA3* encodes a protein known as adiponutrin, and the rs738409 variant is associated with an increased hepatocyte fat content and the natural course of NAFLD, including the severity of disease.[10-14] Furthermore, the recent report showed that the rs58542926 variant (encoding an amino acid substitution p.Glu167Lys) of *TM6SF2* (transmembrane 6 superfamily member 2), affected hepatocytic triglyceride content (HTGC),

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significantly.[15] The report indicated that the impact of *TM6SF2* to HTGC had been independent of the impact of *PNPLA3* variant, alcohol intake, insulin resistance, or obesity, and that the rs58542926 variant significantly affected the higher levels of serum alanine aminotransferase.[15] However, the impact of *TM6SF2* variant on the pathogenesis and genetic risk of NAFLD is still unclear.[16-18]

The present study included 211 patients with biopsy-proven NAFLD. The aims of the study were to investigate the relationships between genetic variations (*PNPLA3* and *TM6SF2* genotype) and histological features, and to analyze the impact of genetic variations as surrogate markers of histological features.

MATERIALS AND METHODS

1. Patients

A single-center retrospective cohort study was performed based on the patients of biopsy-proven NAFLD. Two hundred eleven Japanese patients were diagnosed with NAFLD by liver biopsy from 1980 to 2015 at Toranomon Hospital. NAFLD was diagnosed based on liver biopsy findings of steatosis in 5% or more of hepatocytes and the exclusion of other liver diseases (such as primary biliary cirrhosis, autoimmune hepatitis, drug induced liver disease, viral hepatitis, hemochromatosis, biliary obstruction, α-1-antitrypsin deficiency-associated liver disease, and Wilson disease). Patients consuming more than 20 g/day alcohol were excluded. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board at Toranomon Hospital. All patients provided written informed consent at the time of liver biopsy.

2. Liver histology

Liver biopsy specimens were obtained using a 14-gauge modified Vim Silverman needle (Tohoku University style; Kakinuma Factory, Tokyo, Japan), a 16-gauge core tissue biopsy needle (Bard Peripheral Vascular Inc., Tempe, AZ, USA) or surgical resection. Tissue was fixed in 10% formalin, and sections were stained with hematoxylin and eosin, Masson trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The specimens were evaluated by three pathologists (M.I., T.F., and T.F.) who were blinded to the clinical findings. An adequate liver biopsy sample was defined as a specimen of length of more than 1.5 cm and/or having more than 11 portal tracts. Specimen with steatosis of <5%, 5%–33%, 34%–66%, and >66% was scored as having steatosis grade of 0, 1, 2, and 3, respectively. Lobular inflammation of no foci, <2 foci, 2–4 foci, and >4 foci per 200× field was scored as 0, 1, 2, and 3, respectively. Hepatocyte ballooning of none, few cells, and many cells was scored as 0, 1, and 2, respectively. NAFLD activity score was the sum of steatosis, lobular inflammation, and hepatocyte ballooning scores (range, 0–8 points; 5–8 points as definition of NASH). Fibrosis stage of none, zone 3 perisinusoidal fibrosis (stage 1), zone 3 perisinusoidal fibrosis with portal fibrosis (stage 2), zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis (stage 3), and cirrhosis (stage 4) was scored as 0, 1, 2, 3, and 4, respectively.[19,20] Patients were also classified into four categories by histology according to the classification by Matteoni *et al*.[21] as follows: type 1, fatty liver alone; type 2, fat accumulation and lobular inflammation; type 3, fat accumulation and ballooning degeneration; type 4, fat accumulation, ballooning degeneration, and either Mallory-Denk body or fibrosis (type 3 or 4 as definition of NASH).

3. Determination of *PNPLA3* and *TM6SF2* genotype

PNPLA3 rs738409 and *TM6SF2* rs58542926 were genotyped by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

4. Clinical parameters

Table 1 summarizes the patients' characteristics at the biopsy of 211 patients, and these factors were investigated to determine the parameters that affected to histological features of NAFLD. Normal range of aspartate aminotransferase (AST) was evaluated as 13 to 33 IU/L. Normal range of alanine aminotransferase (ALT) was evaluated as 8 to 42 IU/L for male, and 6 to 27 IU/L for female. Obesity was defined as a body mass index (BMI) of more than 25.0 kg/m^2. Cardiovascular disease (CVD) risk was investigated by exploring the total cholesterol (TC)/high-density lipoprotein cholesterol (HDL-C) ratio.[22]

5. Statistical analysis

Nonparametric tests (chi-squared test, Fisher exact probability test, and Mann-Whitney U test) were used to compare the characteristics of the groups. Multiple comparisons were examined by the Bonferroni test. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly affected to histological features. The odds ratios (ORs) and 95% confidence intervals were also calculated. All p-values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($p<0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Statistical analyses were performed using the SPSS software version 2 (SPSS Inc., Chicago, IL, USA).

RESULTS

1. Genetic variations and fibrosis stage

Among 211 patients, 140 could be evaluated *PNPLA3* and *TM6SF2* genotype. Table 2 indicated histological features, according to *PNPLA3* and *TM6SF2* genotype.

Table 1. Biopsy Characteristics of 211 Patients Diagnosed with Nonalcoholic Fatty Liver Disease

Characteristic	Value
Demographic data	
No. of patients	211
Gender, male/female	122/89
Age, yr*	52 (20–85)
Body mass index, kg/m^2*	25.9 (18.1–40.4)
Histological findings	
Steatosis, 5%–33%/ 33%–66%/>66%	80/80/47
Lobular inflammation, no foci/<2 foci/2–4 foci/>4 foci per 200× field	25/113/58/11
Ballooning, none/few cells/many cells	28/113/66
Stage, 0/1/2/3/4	30/82/25/56/18
Matteoni classification, type 1/2/3/4	15/11/7/174
NAFLD activity score, ≤2/3,4/≥5	31/81/99
Genetic variation	
PNPLA3 rs738409, CC/CG/GG/not determined	21/60/59/71
TM6SF2 rs58542926, CC/CT/TT/not determined	104/33/2/72
Laboratory data*	
Serum aspartate aminotransferase, IU/L	51 (12–312)
Serum alanine aminotransferase, IU/L	78 (15–338)
γ-Glutamyl transpeptidase, IU/L	68 (11–605)
Serum albumin, g/dL	4.1 (2.8–5.8)
Platelet count, ×10^4/mm^3	21.0 (4.5–38.9)
Fasting plasma glucose, mg/dL	100 (65–273)
Uric acid, mg/dL	5.9 (2.7–10.6)
Total cholesterol, mg/dL	206 (101–370)
Triglycerides, mg/dL	134 (31–610)
High-density lipoprotein cholesterol, mg/dL	45 (14–82)
Low-density lipoprotein cholesterol, mg/dL	125 (28–243)
Total cholesterol/high density lipoprotein cholesterol	4.6 (1.7–10.3)
Serum ferritin, μg/L	231 (10–1,474)
Hyaluronic acid, μg/L	35 (1–814)
High sensitive C-reactive protein, mg/dL	0.097 (0.006–2.240)
Type IV collagen 7S, ng/mL	4.2 (2.0–11.0)

The data displayed represent the number of patients, except those denoted by *, which represent the median (range) values.
NAFLD, nonalcoholic fatty liver disease.

Fig. 1A shows the distribution of *PNPLA3* rs738409 genotype, according to severity of fibrosis stage. *PNPLA3* genotype partly affected to severity of fibrosis stage (stage 0 vs 1–4, p=0.362; stage 0–1 vs 2–4, p<0.001; stage 0–2 vs 3–4, p=0.007; stage 0–3 vs 4, p=0.058). There were significant differences in fibrosis stage among the three genotypes of *PNPLA3* (p=0.001) (Table 2). Especially, fibrosis stage of genotype GG was significantly more progressive than that of genotype CG by multiple comparisons (p=0.001; Bonferroni test) (Table 2). Fig. 1B shows the distribution of *TM6SF2* rs58542926 genotype, according to severity of fibrosis stage. *TM6SF2* genotype did not affect to severity of fibrosis stage (stage 0 vs 1–4, p=0.867; stage 0–1 vs 2–4, p=0.936; stage 0–2 vs 3–4, p=0.955; Stage 0–3 vs 4, p=0.818). There were no significant differences in fibrosis stage among the three genotypes of *TM6SF2* (p=0.990) (Table 2).

2. Factors associated with fibrosis stage, according to severity of fibrosis

Table 3 indicated the factors associated with fibrosis stage, according to severity of fibrosis. Multivariate analysis identified four parameters that independently influenced fibrosis stage 1 or more: AST (≥1.5×ULN; OR, 20.0; p=0.001), high-density lipoprotein cholesterol (<41 mg/dL; OR, 9.17; p=0.012), ferritin (≥191 μg/L; OR, 4.92; p=0.023), and age (≥55 years; OR, 4.35;

Table 2. The Histological Features of Patients Diagnosed with Nonalcoholic Fatty Liver Disease according to *PNPLA3* rs738409 and *TM6SF2* rs58542926 Genotype

	PNPLA3 rs738409			p-value*
	CC	CG	GG	
Steatosis, 5%–33%/>33%–66%/>66%	8/11/2	23/26/10	27/17/14	0.270
Lobular inflammation, no foci/<2 foci/2–4 foci/>4 foci per 200× field	1/14/6/0	7/33/18/1	6/24/20/8	0.074
Ballooning, none/few cells/many cells	3/11/7	8/38/13	5/25/28	0.060
Stage, 0/1/2/3/4	2/12/0/3/4	10/27/7/14/2	5/11/11/24/8†	0.001
Matteoni classification, type 1/2/3/4	1/2/0/18	4/3/3/49	2/2/2/52	0.805
NAFLD activity score, ≤2/3,4/≥5	2/12/7	9/26/25	6/17/36	0.100
	TM6SF2 rs58542926			p-value*
	CC	CT	TT	
Steatosis, 5%–33%/>33%–66%/>66%	42/41/19	15/12/6	1/0/1	0.727
Lobular inflammation, no foci/<2 foci/2–4 foci/>4 foci per 200× field	9/53/35/5	5/17/8/3	0/1/1/0	0.803
Ballooning, none/few cells/many cells	11/57/34	5/15/13	0/2/0	0.574
Stage, 0/1/2/3/4	13/38/12/31/10	4/11/5/9/4	0/1/0/1/0	0.990
Matteoni classification, type 1/2/3/4	5/5/4/88	2/2/1/28	0/0/0/2	0.998
NAFLD activity score, ≤2/3,4/≥5	13/40/51	4/14/15	0/1/1	0.976

NAFLD, nonalcoholic fatty liver disease.
*Histological features were compared among the three genotypes of *PNPLA3* and *TM6SF2*; †p=0.001, compared with the CG genotype by Bonferroni test.

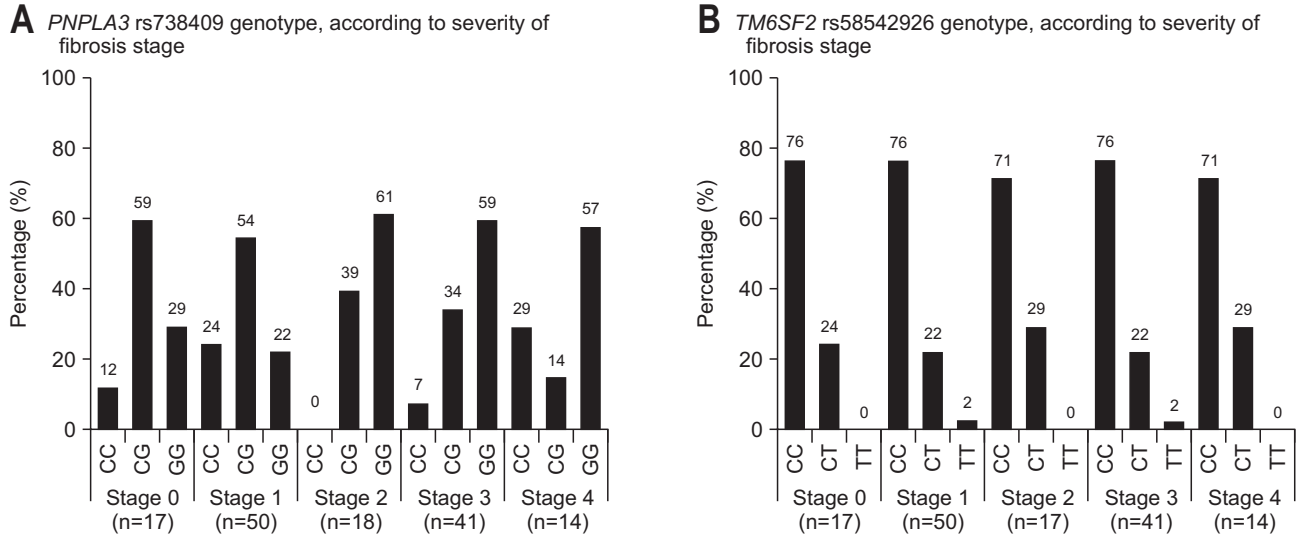

Fig. 1. (A) The distribution of *PNPLA3* rs738409 genotypes, according to severity of fibrosis stage, is shown. The *PNPLA3* genotype partly affected the severity of fibrosis stage (stage 0 vs 1–4, p=0.362; stage 0–1 vs 2–4, p<0.001; stage 0–2 vs 3–4, p=0.007; stage 0–3 vs 4, p=0.058). (B) The distribution of *TM6SF2* rs58542926 genotypes, according to severity of fibrosis stage, is shown. *TM6SF2* genotypes did not affect the severity of fibrosis stage (stage 0 vs 1–4, p=0.867; stage 0–1 vs 2–4, p=0.936; stage 0–2 vs 3–4, p=0.955; stage 0–3 vs 4, p=0.818).

p=0.030). Multivariate analysis identified six parameters that independently influenced fibrosis stage 2 or more: GGT (<219 IU/L; OR, 71.4; p=0.007), *PNPLA3* rs738409 genotype (CG; OR, 18.8; p=0.008; and GG; OR, 38.2; p=0.001), low-density lipoprotein cholesterol (LDL-C) (<86 mg/dL; OR, 28.6; p=0.021), platelet count (<15.0×10^4/mm^3; OR, 21.7; p=0.028), hyaluronic acid (≥51 μg/L; OR, 7.40; p=0.014), and AST (≥1.5×ULN; OR, 5.26; p=0.015). Multivariate analysis identified four parameters that independently influenced fibrosis stage 3 or more: AST (≥1.5×ULN; OR, 14.3; p=0.002), fasting plasma glucose (≥126 mg/dL; OR, 11.9; p=0.011), LDL-C (<86 mg/dL; OR, 11.9; p=0.040), and hyaluronic acid (≥51 μg/L; OR, 8.52; p=0.002).

Table 3. Multivariate Analysis of Factors Associated with Fibrosis Stage, according to the Severity of Fibrosis in 211 Patients Diagnosed with Nonalcoholic Fatty Liver Disease

Factor	Category	OR	95% CI	p-value
Factors associated with stage 1 or more				
Serum aspartate aminotransferase, IU/L	<1.5×ULN	1		
	≥1.5×ULN	20.0	3.48–115	0.001
High-density lipoprotein cholesterol, mg/dL	≥41	1		
	<41	9.17	1.63–52.6	0.012
Serum ferritin, μg/L	<191	1		
	≥191	4.92	1.25–19.4	0.023
Age, yr	<55	1		
	≥55	4.35	1.15–16.4	0.030
Factors associated with stage 2 or more				
γ-Glutamyl transpeptidase, IU/L	≥219	1		
	<219	71.4	3.17–1,000	0.007
PNPLA3 rs738409 genotype	CC	1		
	CG	18.8	2.11–166	0.008
	GG	38.2	4.29–341	0.001
Low-density lipoprotein cholesterol, mg/dL	≥86	1		
	<86	28.6	1.66–500	0.021
Platelet count, $\times 10^4/mm^3$	≥15.0	1		
	<15.0	21.7	1.39–333	0.028
Hyaluronic acid, μg/L	<51	1		
	≥51	7.40	1.51–36.4	0.014
Serum aspartate aminotransferase, IU/L	<1.5×ULN	1		
	≥1.5×ULN	5.26	1.38–20.1	0.015
Factors associated with stage 3 or more				
Serum aspartate aminotransferase, IU/L	<1.5×ULN	1		
	≥1.5×ULN	14.3	2.64–77.6	0.002
Fasting plasma glucose, mg/dL	<126	1		
	≥126	11.9	1.77–80.1	0.011
Low-density lipoprotein cholesterol, mg/dL	≥86	1		
	<86	11.9	1.11–125	0.040
Hyaluronic acid, μg/L	<51	1		
	≥51	8.52	2.19–33.2	0.002
Factors associated with stage 4				
Low-density lipoprotein cholesterol, mg/dL	≥86	1		
	<86	41.7	3.55–500	0.003
Platelet count, $\times 10^4/mm^3$	≥15.0	1		
	<15.0	20.4	3.05–143	0.002

OR, odds ratio; CI, confidence interval; ULN, the upper limit of normal.

Multivariate analysis identified two parameters that independently influenced fibrosis stage 4: LDL-C (<86 mg/dL; OR, 41.7; p=0.003) and platelet count (<$15.0\times10^4/mm^3$; OR, 20.4; p=0.002).

Thus, *PNPLA3* genotype was identified as the predictors of fibrosis stage 2 or more, but the impact tended to decrease on stage 3 or more.

3. Genetic variations and Matteoni classification, NAFLD activity score

Fig. 2A shows the distribution of *PNPLA3* rs738409 genotype, according to type of Matteoni classification. *PNPLA3* genotype

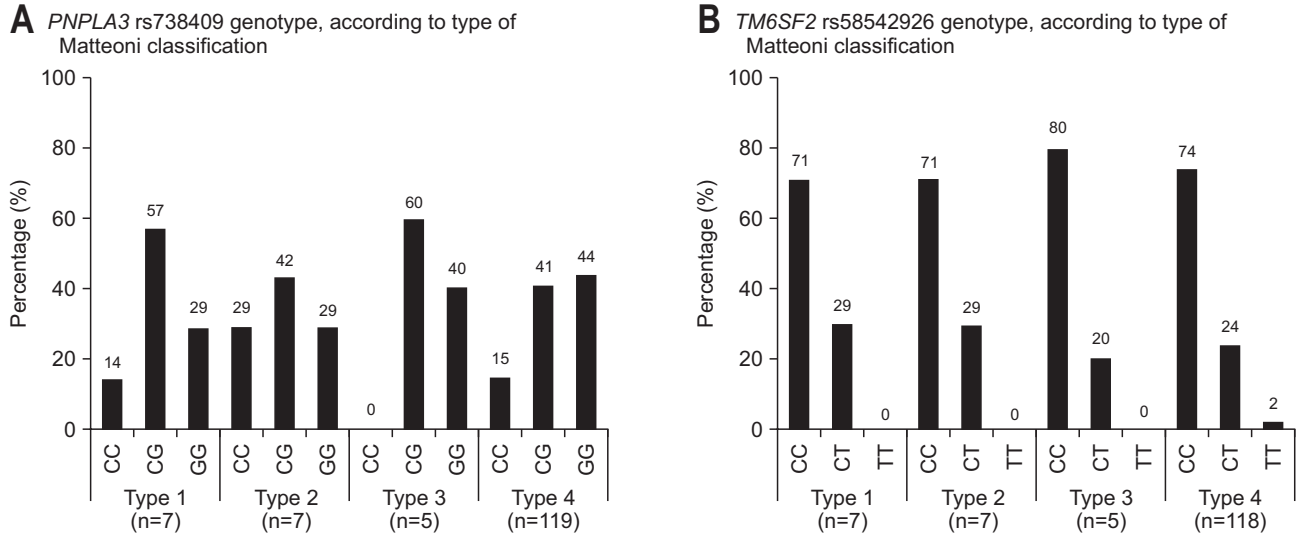

Fig. 2. (A) The distribution of *PNPLA3* rs738409 genotypes, according to type of Matteoni classification, is shown. *PNPLA3* genotypes did not affect the type of Matteoni classification (type 1 vs 2–4, p=0.712; type 1–2 vs 3–4, p=0.533; type 1–3 vs 4, p=0.721). (B) The distribution of *TM6SF2* rs58542926 genotypes, according to type of Matteoni classification, is shown. The *TM6SF2* genotype did not affect the type of Matteoni classification (type 1 vs 2–4, p=0.915; type 1–2 vs 3–4, p=0.828; type 1–3 vs 4, p=0.805).

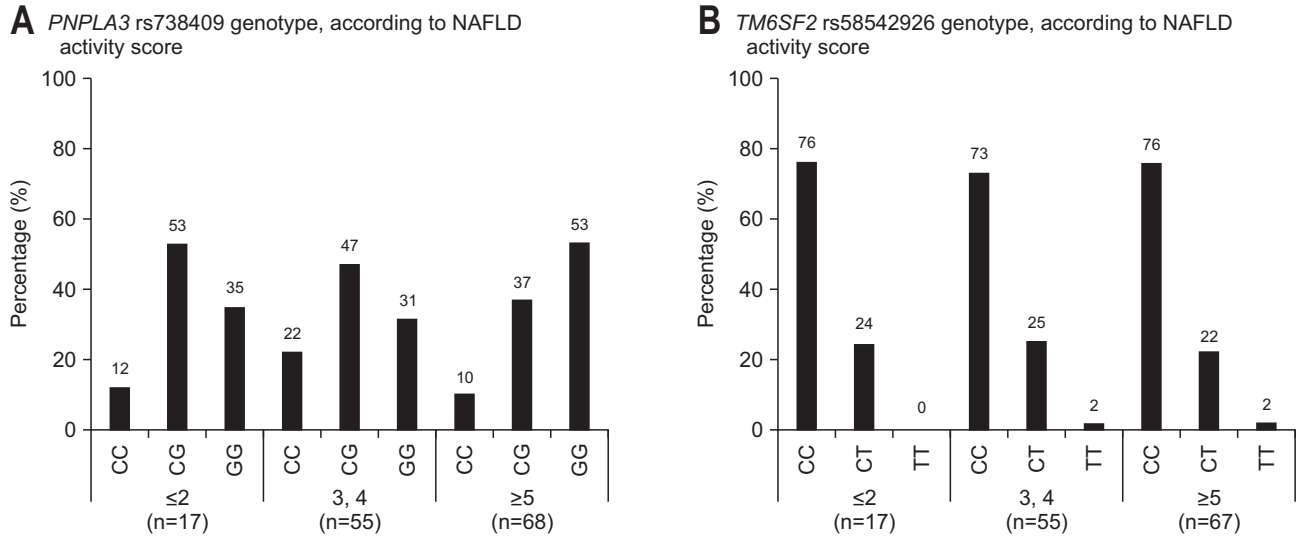

Fig. 3. (A) The distribution of *PNPLA3* rs738409 genotypes, according to nonalcoholic fatty liver disease (NAFLD) activity score, is shown. The *PNPLA3* genotype partly affected the NAFLD activity score (≤2 vs ≥3, p=0.667; ≤4 vs ≥5, p=0.034). (B) The distribution of *TM6SF2* rs58542926 genotypes, according to NAFLD activity score, is shown. The *TM6SF2* genotype did not affect the NAFLD activity score (≤2 vs ≥3, p=0.867; ≤4 vs ≥5, p=0.936).

did not affect to type of Matteoni classification (type 1 vs 2–4, p=0.712; type 1–2 vs 3–4, p=0.533; type 1–3 vs 4, p=0.721). There were no significant differences in type of Matteoni classification among the three genotypes (p=0.805) (Table 2). Fig. 2B shows the distribution of *TM6SF2* rs58542926 genotype, according to type of Matteoni classification. *TM6SF2* genotype did not affect to type of Matteoni classification (type 1 vs 2–4, p=0.915; type 1–2 vs 3–4, p=0.828; type 1–3 vs 4, p=0.805). There were no significant differences in type of Matteoni classification among the three genotypes (p=0.998) (Table 2).

Fig. 3A shows the distribution of *PNPLA3* rs738409 genotype, according to NAFLD activity score. *PNPLA3* genotype partly affected to NAFLD activity score (≤2 vs ≥3, p=0.667; ≤4 vs ≥5, p=0.034). There were no significant differences in NAFLD activity score among the three genotypes (p=0.100) (Table 2). Fig. 3B shows the distribution of *TM6SF2* rs58542926 genotype, according to NAFLD activity score. *TM6SF2* genotype did not affect to NAFLD activity score (≤2 vs ≥3, p=0.867; ≤4 vs ≥5, p=0.936). There were no significant differences in NAFLD activity score among the three genotypes (p=0.976) (Table 2).

DISCUSSION

The impact of *PNPLA3* genotype on the genetic risk and disease progression of NAFLD might appear to be consistent.[11-14] Hotta and coworkers[13] reported that the G-allele of rs738409 was significantly associated with increases in fibrosis stage in the patients with NAFLD, even after adjustment for age, gender, and BMI. Consistent with data previously reported,[12] the present study indicated that fibrosis stage of genotype GG was significantly more progressive than that of genotype CG by multiple comparisons. Interestingly, *PNPLA3* genotype was identified as the predictors of fibrosis stage 2 or more, but was not as the predictors of stage 3 or more. To our knowledge, this is the first report to investigate the different impact of *PNPLA3* genotype, according to severity of fibrosis stage. *PNPLA3* variant might be a strong modifier of the natural course of NAFLD, by modulating hepatocyte fat deposition and the severity of disease.[11-14] The present study might suggest that the impact of *PNPLA3* variant tended to decrease on patients with severe fibrosis stage (such as patients with burned-out NASH, in whom fatty changes and inflammatory cell infiltration resolving in fibrosis has progressed).[6] One limitation of the present study based on NAFLD patients, not including control subjects (steatosis in <5% of hepatocytes), is that it could not be exactly evaluated whether *PNPLA3* variant might affect to the presence of steatosis. Further large-scale studies based on the patients with NAFLD, including burned-out NASH, should be performed to explore the complicated relationships among *PNPLA3* genotype, steatosis, and fibrosis stage.

The impact of *TM6SF2* genotype on NAFLD is controversial.[15-18] Liu and coworkers[16] reported that *TM6SF2* genotype affected necroinflammation score, but they could not replicate this result in their large-scale validation cohort, unfortunately. On the other hand, Liu *et al*.[16] indicated that *TM6SF2* genotype significantly affected liver fibrosis stage in the two explored cohorts by adopting an additive model. On the contrary, Wong and coworkers[17] showed that *TM6SF2* genotype might be not associated either with fibrosis stage or with hepatic fat accumulation in their large-scale study. This is the first report to investigate the impact of *TM6SF2* genotype on NAFLD from Japan. *TM6SF2* genotype was not associated with fibrosis stage, and pathological criteria for the diagnosis of NASH (Matteoni classification and NAFLD activity score). The present study, based on the small number of NAFLD patients, has some limitations. It was a hospital-based study and a retrospective cohort study, which might include the selection bias. All of participants were Japanese, and there is a possibility that the present results might not be applicable for NAFLD patients of the other races or ethnic groups.

Consistent with data previously reported,[15] this preliminary study based on the small number of *TM6SF2* rs58542926 genotype TT (only two patients) indicated that the T-allele was associated with decreases in serum levels of triglycerides in the patients with NAFLD (data not shown). Specifically, serum levels of triglyceride in genotype non CC (median, 112 mg/dL) tended to be lower than those of genotype CC (median, 138 mg/dL) (p=0.056, Mann-Whitney U test). Previous report indicated that the *TM6SF2* variant was significantly associated with HTGC, and the impact of that was independent of the effect mediated by the *PNPLA3* variant.[15] The limitation of present study was that relationships between the *TM6SF2* variant and HTGC could not be investigated. Further study should be performed to investigate the complicated relationships among *TM6SF2* genotype, lipid metabolism, and histological features in NAFLD patients.

Although histological diagnosis is currently the gold standard for diagnosing progressive NASH, liver biopsy has many drawbacks, such as cost, sampling error, and risk of complications.[6] It is important to determine the clinical parameters as surrogate markers of histological features. Furthermore, inter- and intraobserver variability, and pathological diagnosis also presents the serious problems for the histological diagnosis of NASH.[6] To minimize these shortfalls, the impact of genetic variations that affected to pathological criteria for the diagnosis of NASH (Matteoni classification and NAFLD activity score) was evaluated. Kawaguchi and coworkers[14] reported that Matteoni type 4 NAFLD was both a genetically and clinically different subset from the other spectrums of the disease and that the *PNPLA3* gene was strongly associated with the progression of NASH in Japanese population. Consistent with data previously reported,[14] the present study showed that *PNPLA3* genotype partly affected to definition of NASH by NAFLD activity score (5 to 8 points), but *TM6SF2* genotype did not affect to definition of NASH by Matteoni classification and NAFLD activity score. The limitation of the present study, based on the small number of patients, was that there were problems with pathological criteria for the diagnosis of NASH. Specifically, factor of inflammation was not included in the definition of NASH by Matteoni classification, and factor of fibrosis was not included in the definition of NASH by NAFLD activity score.[6] As previously reported,[23-26] further large-scale study should be performed to develop the simple clinical scoring system useful for noninvasive diagnosis of the differentiation between NASH and simple steatosis, the diagnosis of NASH with advanced fibrosis, and the prediction of hepatocarcinogenesis.

In conclusion, genetic variations might partly affect histological changes. *PNPLA3* genotype might partly affect to histological features including fibrosis stage, but *TM6SF2* genotype did not affect to histological features in Japanese patients with biopsy-proven NAFLD. Further comprehensive studies, based on the larger number of patients, should be performed to disclose the molecular mechanisms for the complicated relationships between the impact of *PNPLA3*, *TM6SF2* variant on the genetic risk and pathogenesis of NAFLD.

CONFLICTS OF INTEREST

(1) Norio Akuta has received speakers' bureau from MSD K.K., Mitsubishi Tanabe Pharma, Janssen Pharmaceutical K.K., Bristol-Myers Squibb, and holds a right to get some loyalty from SRL, Inc. (2) Hiromitsu Kumada has received speakers' bureau from MSD K.K., Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, Bristol-Myers Squibb, Janssen Pharmaceutical K.K., GlaxoSmithKline K.K., and holds a right to get some loyalty from SRL., Inc. (3) Fumitaka Suzuki has received speakers' bureau from Bristol-Myers Squibb. (4) Yoshiyuki Suzuki has received speakers' bureau from Bristol-Myers Squibb. (5) Yasuji Arase has received speakers' bureau from MSD K.K. (5) Kenji Ikeda has received speakers' bureau from Dainippon Sumitomo Pharma, Eisai Co., Ltd., Olympus Co. The other authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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