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Additive effects of *PNPLA3* and *TM6SF2* on the histological severity of non-alcoholic fatty liver disease

비알코올 지방간의 조직학적 정도에 영향을 미치는 *PNPLA3*와 *TM6SF2*의 부가 효과

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임상의과학과 내과학

주 세 경

A thesis of the Degree of Doctor of Philosophy

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The Department of Clinical Medical Sciences,

Seoul National University

College of Medicine

Sae Kyung Joo

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위 원	이 국 래	(인)
위 원	임 영 석	(인)
위 원	김 지 원	(인)

Abstract

Additive effects of *PNPLA3* and *TM6SF2* on the histological severity of non-alcoholic fatty liver disease

Sae Kyung Joo
Department of Internal Medicine,
Clinical Medical Sciences
The Graduate School
Seoul National University

Introduction: Recent genome-wide association studies have identified that variants in *PNPLA3* and *TM6SF2* are significantly associated with nonalcoholic fatty liver disease (NAFLD) in multiple ethnic groups. However, the data on their genetic impact on NAFLD in Asian populations are limited. Therefore, we investigated the effects of *PNPLA3* rs738409 and *TM6SF2* rs58542926 variants on metabolic phenotypes and their combined effects on the histological severity of NAFLD.

Methods: In a biopsy-proven NAFLD cohort of 525 subjects, *PNPLA3* rs738409 and *TM6SF2* rs58542926 were genotyped. Homeostasis model assessment of insulin resistance (HOMA-IR) and adipose tissue insulin resistance (adipo-IR) were calculated.

Results: The rs738409 and rs58542926 variants were associated with not only non-alcoholic steatohepatitis (NASH) (odds ratio [OR], 2.00; 95% confidence interval [CI], 1.46–2.73 and OR, 1.91; 95% CI, 1.04–3.51) but also with significant fibrosis (≥F2) (odds ratio [OR], 1.53; 95% CI, 1.11–2.11 and OR, 1.88; 95% CI, 1.02–3.46), even after adjustment for metabolic factors. Of both variants, only rs738409 was associated with HOMA-IR and adipo-IR even in healthy controls (P = 0.046 and 0.002, respectively) as well as in the entire study cohort (P = 0.016 and 0.048, respectively). *PNPLA3* and *TM6SF2* risk variants additively increased the risk of NASH and significant fibrosis (OR per risk allele, 2.03; 95% CI, 1.50–2.73 and 1.61; 95% CI, 1.19–2.17). Even in subjects with low insulin resistance, the risk of NASH and significant fibrosis increased as the number of risk alleles increased (P = 0.008 and 0.020, respectively).

Conclusions: *PNPLA3* and *TM6SF2* determine the risk of NASH and significant fibrosis, even after adjustment for insulin resistance, and exert an additive effect on NASH and significant fibrosis.

Keywords: Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, insulin resistance, *PNPLA3*, and *TM6SF2*

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NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NAFL, non-alcoholic fatty live; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein; *PNPLA3*, the patatin-like phospholipase domain-containing-3 gene; *TM6SF2*, the transmembrane 6 superfamily member 2 gene

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the worldwide.[1] The prevalence of NAFLD is rapidly increasing in parallel with the increase in diabetes and obesity.[2] The spectrum of NAFLD is diverse, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can lead to advanced fibrosis (cirrhosis). Currently, biopsy has been regarded as the "gold standard" for the diagnosis and assessment of liver fibrosis.

According to the clinical practice guideline, metabolic syndrome and/or insulin resistance can alert patients with NAFLD to the need for liver biopsy.[3,4] Insulin resistance is one of the major pathophysiological mechanisms in the development of NAFLD[3,4]; therefore, the presence of metabolic syndrome can predict not only the risk of NAFLD[5], but also the presence of steatohepatitis in patients with NAFLD.[6,7]

Currently, several genetic variants have been reported to influence on the risk of NAFLD with metabolic risk factors. The patatin-like phospholipase domain-containing-3 (*PNPLA3*)[8-10] and the transmembrane 6 superfamily member 2 (*TM6SF2*)[11,12] genes are known to increase the risk of NAFLD and the histologic severity of NAFLD. However, there is uncertainty regarding the interaction between genotypes and metabolic risk.[13-15]

In this study, we aimed to determine whether there is an additive effect of genetic variants on the histologic severity of NAFLD. In addition, we

attempted to compare the metabolic profiles across genetic variants.

MATERIALS and METHODS

Subjects

We constructed a prospective cohort from the ongoing Boramae NAFLD registry (NCT 02206841) at the Seoul Metropolitan Government Seoul National University Boramae Medical Center. The details of eligibility criteria and liver biopsy indication were previously reported.[16] Briefly, the inclusion criteria of this study were as follows: (i) ≥18 years old; (ii) bright liver echogenicity observed upon ultrasound scanning; and (iii) unexplained high levels of alanine aminotransferase (ALT) above the upper reference level within the past 6 months.[17] The following conditions were excluded from this study: (i) viral hepatitis, e.g., hepatitis B or C; (ii) autoimmune hepatitis; (iii) drug-induced liver injury or steatosis; (iv) Wilson disease or hemochromatosis; and (v) excessive alcohol intake (male >30 g/day, female >20 g/day),[3] and (vi) diagnosis of malignancy within the past year. Of the eligible study participants, those with clinically suspected NASH or fibrosis[18] underwent liver biopsy. For comparison, control liver tissues were collected from subjects who underwent liver biopsy in a pre-evaluation for donor liver transplantation or in a characterization of solid liver masses that were suspected to be hepatic adenoma or focal nodular hyperplasia based on radiological results without any evidence of hepatic steatosis. This is a single center-based cohort, and the participants were all Asians.

This study was approved by the Institutional Review Board of Boramae

Medical Center (IRB No.16-2014-86) and complied with the 1975 Helsinki Declaration. Informed consent was obtained from all the patients who participated in this study.

Clinical and laboratory assessment

Based on the World Health Organization (WHO) Asia–Pacific criteria[19], Obesity is defined as a body mass index (BMI) ≥25 kg/m^2 and ≥30 kg/m^2 indicated a more severe form of obesity (class II obesity). Metabolic syndrome was defined based on the revised National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) criteria[20], as the presence of at least 3 of the following 5 components: abdominal obesity (waist circumference ≥90 cm for men and ≥80 cm for women[21]), blood pressure ≥130/85 mmHg, triglyceride ≥150 mg/dL, high-density lipoprotein (HDL) cholesterol <40 mg/dL in men and <50 mg/dL in women, and elevated blood glucose levels and fasting blood glucose ≥100 mg/dL. Diabetes mellitus was defined as fasting plasma glucose levels of ≥126 mg/dL, glycosylated hemoglobin (HbA1c) levels of ≥6.5% and/or treatment with anti-diabetic medication at the time of the survey[22]. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg and/or the current use of anti-hypertensive medication. High hsCRP was defined as ≥1 mg/dL[23,24].

Venous blood samples were drawn at the time of biopsy after a 12-hr overnight fasting state, and plasma was separated immediately via centrifugation. The plasma glucose and lipid concentrations were measured

enzymatically using the Hitachi Automatic Analyzer B2400 (Hitachi, Tokyo, Japan). Fasting insulin levels were measured using immune radiometric assays (DIAsource ImmunoAssays, Nivelles, Belgium). Hepatic insulin resistance was indirectly evaluated using the homeostasis model assessment of insulin resistance (HOMA-IR), as described previously[25]. HOMA-IR ≥2.5 was considered to indicate insulin resistance[26,27]. Adipose insulin resistance (Adipo-IR) was calculated as [fasting plasma free fatty acid level (μEq/L) × fasting plasma insulin level (μIU/mL)].[28,29]

Liver histology

Liver biopsy specimens were fixed in 4%-buffered formalin and embedded in paraffin. Two-micrometer-thick sections were stained with hematoxylin-eosin and Masson's trichrome.

All biopsy specimens were analyzed by an experienced pathologist who was blinded to the clinical results of the patients. NAFLD was defined as the presence of ≥5% macrovesicular steatosis[30]; and NASH was diagnosed based on an overall pattern of histological hepatic injury consisting of macrovesicular steatosis, inflammation, or hepatocellular ballooning according to Brunt et al.'s criteria[30,31]. Fibrosis was assessed according to a 5-point scale proposed by Brunt and modified by Kleiner et al. as follows: F0, absence of fibrosis; F1, perisinusoidal or periportal fibrosis; F2, perisinusoidal and portal/periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis.[32] Significant fibrosis was defined as ≥F2. We excluded patients with biopsy

lengths that were less than 20 mm, as well as those with biopsies of fewer than eight portal tracts.

Genotyping

Established risk alleles for NAFLD from the previous studies were selected for genotyping; the rs738409 C>G (I148M *PNPLA3*)[8-10], and rs58542926 C>T (E167K, *TM6SF2*)[11,12] single-nucleotide polymorphisms (SNPs) were genotyped in the entire cohort by TaqMan 5'-nuclease assays (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Hardy-Weinberg's equilibrium was confirmed using the chi-square test.

Statistical analysis

Descriptive values are presented as the frequency (percentage) and the median (IQR). Continuous variables were analyzed using the Student t-test or the non-parametric Mann-Whitney U test. Three independent groups were compared using one-way analysis of variance (ANOVA) or the Kruskal-Wallis test. Categorical variables were analyzed using the chi-square test or Fisher's exact test. To investigate the independent determining factors for the presence of significant fibrosis or NASH, a binary logistic regression model adjusted for covariates was generated. The generalized linear model or the linear-by-linear association test was used to identify the trends in metabolic phenotype or histological severity according to genotypes.

The odds of the significant fibrosis or NASH per risk allele was estimated by logistic regression models and adjusted for age and sex. Genetic analyses were performed assuming an additive model (by coding the genotypes as 0, 1, and 2 for wild-type homozygotes, heterozygotes, and alternate allele homozygotes, respectively) or a dominant model for (by coding the genotypes as 0 and 1 for wild-type homozygotes and [heterozygotes + alternate allele homozygotes], respectively) considering minor allele frequency (MAF). Statistical analyses were performed using the IBM SPSS Statistics software package version 20.0 (IBM Inc., Armonk, NY, USA). *P* values less than 0.05 were considered statistically significant.

Results

Clinical characteristics of the study population

Among the 416 subjects (mean age, 52.6 ± 15.5 years), 204 (49%) and 212 (51%) subjects were classified as biopsy-proven NASH and NAFL, respectively (Table 1). We compared their clinical characteristics with those of control subjects (n = 109; mean age, 55.5 ± 13.8 years). As the severity of NAFLD increased, BMI and waist circumference increased along with increasing trends in HOMA-IR, adipo-IR, and serum hsCRP levels ($P < 0.001$). The prevalence of diabetes mellitus, hypertension, metabolic syndrome, and obesity increased as the severity of NAFLD increased (all $P < 0.001$; Table 1).

HOMA-IR, adipo-IR, and serum hsCRP levels were also significantly higher in NASH subjects than in NAFL subjects (all $P < 0.001$; Table 1); however, blood pressure, fasting glucose levels, and lipid profiles were not significantly different between NAFL and NASH groups (Table 1). Subjects with NAFL showed prevalence of diabetes (38.1% vs. 47.0%), hypertension (50.9% vs. 58.3%), obesity (BMI ≥25 kg/m^2; 73.6% vs. 78.3%), metabolic syndrome (70.2% vs. 81.0%) similar to those in patients with NASH (Table 1). Statistically, only class II obesity (BMI ≥30 kg/m^2; odds ratio (OR), 1.96; 95% CI, 1.23–3.13), metabolic syndrome (OR, 1.62; 95% CI 1.01–2.61), insulin resistance (OR, 2.61; 95% CI, 1.63–4.17), and high CRP levels (OR, 2.97; 95% CI, 1.95–4.51) were more frequently observed in NASH than in NAFL after

adjustment for age and sex; however, obesity (BMI ≥25 kg/m^2), hypertension, and diabetes were not significantly different between both groups (Table 2).

Table 1. Characteristics of study participants according to NAFLD status

Total n=525	No NAFLD (n=109)	NAFL (n=212)	NASH (n=204)	P^1	P^2
Age, years	55.5 ± 13.8	51.8 ± 14.7	53.5 ± 16.2	0.111	0.260
Male, N (%)	43 (39.4)	129 (60.8)	89 (43.6)	0.887	<0.001
BMI, kg/m^2	23.8 (22.2, 25.5)	26.9 (24.9, 29.6)	27.7 (25.2, 31.5)	<0.001	0.012
WC, cm	84.0 (78.6, 90.9)	91.0 (85.7, 97.5)	93.7 (87.6, 102.2)	<0.001	0.001
SBP, mmHg	122.7 ± 14.5	129.6 ± 15.5	131.0 ± 17.8	<0.001	0.374
DBP, mmHg	75.0 ± 10.5	80.0 ± 12.2	79.7 ± 12.0	0.001	0.800
Total cholesterol, mg/dL	177.3 ± 40.6	183.8 ± 40.2	183.0 ± 39.5	0.367	0.843
HDL cholesterol, mg/dL	53 (44.5, 63.5)	44 (37.0, 52.0)	44 (37.0, 51.0)	<0.001	0.629
Triglycerides, mg/dL	86.0 (67.5, 131.5)	140.5 (102.3, 199.0)	140.5 (101.8, 189.8)	<0.001	0.913
ALT, IU/L	22.0 (14.0, 34.5)	35.0 (22.0, 53.0)	63.5 (37.3, 111.0)	<0.001	<0.001
AST, IU/L	24.0 (20.0, 34.0)	29.0 (22.0, 38.8)	52.0 (36.3, 75.0)	<0.001	<0.001
GGT, IU/L	26.0 (14.0, 76.0)	32.0 (20.0, 53.8)	57.0 (35.0, 88.3)	<0.001	<0.001
Albumin, g/dL	4.1 ± 0.4	4.2 ± 0.3	4.2 ± 0.3	0.002	0.281
Platelet, x10^9/L	228 ± 63	239 ± 58	222 ± 69	0.008	0.002
Glucose, mg/dL	100.0 (91.5, 112.5)	105.0 (94.3, 118.8)	107.5 (96.0, 127.0)	0.002	0.055
Insulin, μIU/mL	8.0 (6.8, 10.3)	11.6 (8.7, 15.6)	15.5 (10.9, 22.6)	<0.001	<0.001
HOMA-IR	2.03 (1.61, 2.71)	3.07 (2.23, 4.42)	4.23 (2.88, 6.87)	<0.001	<0.001
Adipo-IR	4328 (2892, 6945)	6480 (4591, 10295)	11400 (6978, 17248)	<0.001	<0.001
hsCRP, mg/dL	0.6 (0.3, 1.5)	0.9 (0.5, 1.7)	1.6 (0.8, 3.3)	<0.001	<0.001
Diabetes, N (%)	18 (17.0)	80 (38.1)	94 (47.0)	<0.001	0.068
Hypertension, N (%)	39 (35.8)	108 (50.9)	119 (58.3)	<0.001	0.130
Obesity (BMI ≥ 25), N (%)	34 (31.2)	156 (73.6)	159 (78.3)	<0.001	0.259
Obesity II (BMI ≥ 30), N (%)	6 (5.5)	45 (21.2)	68 (33.5)	<0.001	0.005

Metabolic syndrome, N (%)	40 (36.7)	146 (70.2)	162 (81.0)	<0.001	0.011

The data are expressed as the means ± standard deviations or medians (interquartile ranges)
[1]*P*-values from ANOVA, the *Kruskal-Wallis* test or χ^2 test to compare the subjects with no NAFLD, NAFL, and NASH.
[2]*P*-values from independent *T*-test, the *Mann-Whitney* test or χ^2 test to compare subjects with NAFL and NASH.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein.

Table 2. The risk of NASH or significant fibrosis according to obesity, metabolic status

	Odds ratio (95% CI)[1]	*P*-value
Odds ratio for NAFLD in the entire cohort (N = 525)		
BMI ≥ 25 kg/m^2	6.84 (4.27, 10.94)	<0.001
BMI ≥ 30 kg/m^2	6.53 (2.75, 15.50)	<0.001
Metabolic syndrome	6.80 (4.19, 11.03)	<0.001
Diabetes mellitus	4.77 (2.68, 8.49)	<0.001
Insulin resistance (HOMA-IR ≥2.5)	6.62 (4.13, 10.60)	<0.001
High hsCRP level (hsCRP ≥ 1mg/dL)	2.76 (1.76, 4.33)	<0.001
Odds ratio for NASH in NAFLD subjects (N = 416)		
BMI ≥ 25 kg/m^2	1.36 (0.85, 2.18)	0.196
BMI ≥ 30 kg/m^2	1.96 (1.23, 3.13)	0.005
Metabolic syndrome	1.62 (1.01, 2.61)	0.045
Diabetes mellitus	1.45 (0.94, 2.22)	0.092
Insulin resistance (HOMA-IR ≥2.5)	2.61 (1.63, 4.17)	<0.001
High hsCRP level (hsCRP ≥ 1mg/dL)	2.97 (1.95, 4.51)	<0.001
Odds ratio for Significant fibrosis in NAFLD subjects (N = 416)		
BMI ≥ 25 kg/m^2	1.11 (0.68, 1.82)	0.672
BMI ≥ 30 kg/m^2	1.51 (0.92, 2.48)	0.107
Metabolic syndrome	1.19 (0.70, 2.00)	0.526
Diabetes mellitus	2.16 (1.38, 3.40)	0.001
Insulin resistance (HOMA-IR ≥2.5)	2.39 (1.40, 4.07)	0.001
High hsCRP level (hsCRP ≥ 1mg/dL)	2.64 (1.68, 4.17)	<0.001

[1]From age and sex-adjusted logistic analysis

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein.

Approximately one third of NAFLD subjects (141/416, 33.9%) had significant fibrosis. Subjects with significant fibrosis had higher HOMA-IR, adipo-IR, and serum hsCRP levels than those without significant fibrosis ($P < 0.001$ for all comparisons; Table 3). With adjustment for age and sex, diabetes (OR, 2.16; 95% CI, 1.38–3.40), insulin resistance (OR, 2.39; 95% CI, 1.40–4.07), and high CRP levels (OR, 2.64; 95% CI, 1.68–4.17) showed a statistically significant association with significant fibrosis in NAFLD subjects. Metabolic syndrome and class II obesity were not significantly associated with significant fibrosis in NAFLD subjects (Table 2).

Table 3. Characteristics of study participants according to significant fibrosis among NAFLD subjects

	F0-1 (n=275)	F2-4 (n=141)	P^1
Age, years	49.8 ± 15.6	58.0 ± 13.8	<0.001
Male, N (%)	163 (59.3)	55 (39.0)	<0.001
BMI, kg/m^2	27.4 (25.1, 30.4)	27.0 (24.9, 30.9)	0.844
WC, cm	91.9 (86.4, 99.1)	91.9 (86.7, 101.0)	0.396
SBP, mmHg	130.6 ± 15.8	129.6 ± 18.1	0.569
DBP, mmHg	80.6 ± 11.6	78.3 ± 12.7	0.067
Total cholesterol, mg/dL	186.6 ± 37.3	177.1 ± 43.7	0.029
HDL cholesterol, mg/dL	43.5 (37.0, 52.0)	44.0 (35.0, 52.0)	0.525
Triglycerides, mg/dL	142.0 (107.0, 193.0)	139.0 (91.0, 188.5)	0.166
ALT, IU/L	40.0 (25.0, 74.0)	57.0 (31.0, 91.5)	0.001
AST, IU/L	33.0 (24.0, 49.0)	52.0 (36.5, 75.5)	<0.001
GGT, IU/L	37.0 (22.0, 63.0)	56.0 (36.0, 104.0)	<0.001

Albumin, g/dL	4.2 ± 0.3	4.1 ± 0.3	<0.001
Platelet, x10^9/L	244 ± 55	201 ± 71	<0.001
Glucose, mg/dL	104.0 (93.0, 117.0)	112.0 (98.0, 140.0)	<0.001
Insulin, μIU/mL	12.1 (9.2, 16.5)	16.0 (11.0, 23.5)	<0.001
HOMA-IR	3.23 (2.35, 4.47)	4.67 (3.04, 7.90)	<0.001
Adipo-IR	7205 (4899, 11560)	11485 (7107, 17770)	<0.001
hsCRP, mg/dL	1.0 (0.5, 2.2)	1.5 (0.9, 3.5)	<0.001
Diabetes, N (%)	92 (33.9)	82 (59.0)	<0.001
Obesity (BMI ≥ 25), N (%)	211 (76.7)	104 (74.3)	0.582
Obesity (BMI ≥ 30), N (%)	73 (26.5)	40 (28.6)	0.661
Metabolic syndrome, N (%)	198 (73.1)	110 (80.3)	0.109

The data are expressed as the means ± standard deviations or medians (interquartile ranges)

[1]*P*-values from independent *T*-test, the *Mann-Whitney* test or χ^2 test to compare subjects with F0-1 vs. F2-4

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; Adipo-IR, adipose tissue insulin resistance; hsCRP, high sensitivity C-reactive protein.

Association between genetic variants and NASH or significant fibrosis independent of insulin resistance

The genotypic distributions of *PNPLA3* rs738409, and *TM6SF2* rs58542926 were in Hardy–Weinberg equilibrium (P = 0.33, and 0.91, respectively). The minor allele frequencies (MAFs) of the SNPs in the set of all subjects were 0.51 for *PNPLA3* rs738409 (0.38 in subjects with no NAFLD and 0.54 in those with NAFLD; P < 0.001), 0.08 for *TM6SF2* rs58542926 (0.05 for no NAFLD and 0.08 for NAFLD; P = 0.088) (Table 4).

Table 4. Distribution of genotypes and minor allele frequencies according to NAFLD status

	No NAFLD (n=96)	NAFL (n=189)	NASH (n=176)	Minor allele frequency			*P*-value*
				Entire	No NAFLD	NAFLD	
rs738409 *PNPLA3*							
CC	34 (35.8)	62 (32.8)	23 (13.1)	0.51	0.38	0.54	<0.001
CG	50 (52.6)	82 (43.4)	82 (46.6)				
GG	11 (11.6)	45 (23.8)	71 (40.3)				
rs58542926 *TM6SF2*							
CC	87 (90.6)	166 (87.8)	140 (79.5)	0.08	0.05	0.08	0.088
CT	9 (9.4)	23 (12.2)	34 (19.3)				
TT	0	0	2 (1.1)				

*The chi-square test comparing the minor allele frequency between No NAFLD and NAFLD
Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.

The number of G alleles at *PNPLA3* rs738409 was positively correlated with the histological grades of steatosis, lobular inflammation, and hepatocellular ballooning ($P < 0.001$, $P = 0.001$, and $P < 0.001$ for trend, respectively) and fibrosis stage ($P < 0.001$ for trend) (Figure 1). In the case of *TM6SF2* rs58542926, we compared the histology between the CC and CT/TT genotypes considering the MAF: only 2 subjects were TT homozygous (Table 4). The presence of the T allele at *TM6SF2* rs58542926 was not significantly associated with steatosis grade (P for trend = 0.377); however, it significantly increased the severity of lobular inflammation (P for trend = 0.041) and fibrosis stage (P for trend = 0.021) (Figure 2).

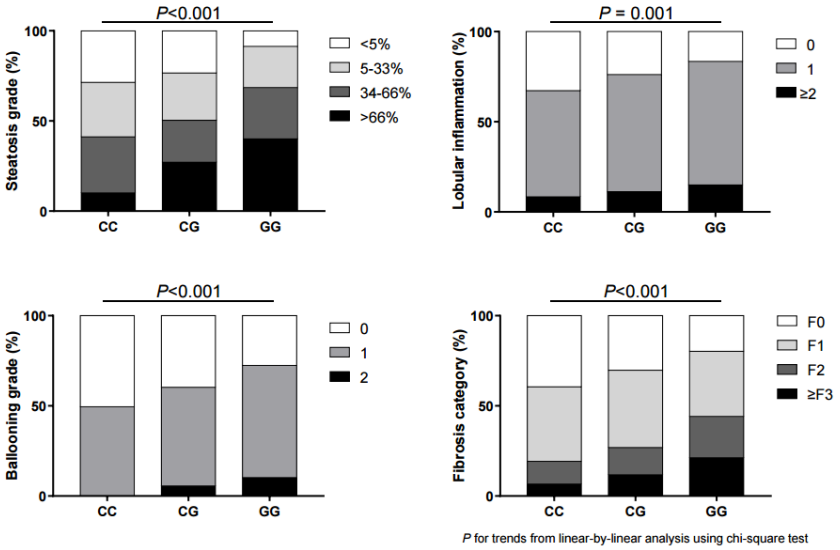

Figure 1. Histologic severity according to *PNPLA3* genotype

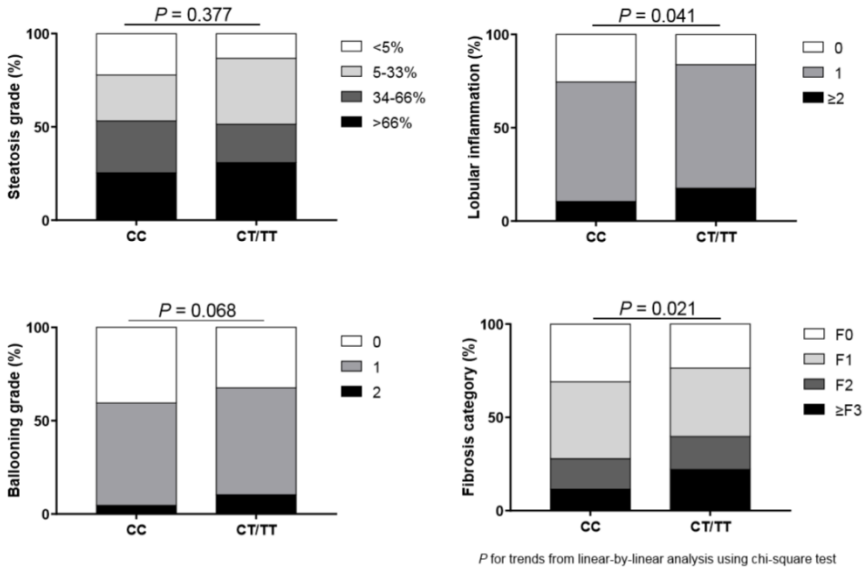

Figure 2. Histologic severity according to *TM6SF2* genotype

Among NAFLD subjects, both *PNPLA3* rs738409 and *TM6SF2* rs58542926 were significantly associated with the risk of NASH or significant fibrosis (Table 5).

An additive model for *PNPLA3* rs738409 and dominant model for *TM6SF2* rs58542926 were assumed considering the MAF and the histological severity of NAFLD according to the genotype. We replicated the associations between NASH and both genetic variants in NAFLD subjects: both *PNPLA3* rs738409 and *TM6SF2* rs58542926 were associated with the risk of NASH in NAFLD subjects, even after adjustment for age and sex (OR for rs734809, 1.97; 95% CI, 1.46–2.66; $P < 0.001$ and OR for rs58542926, 1.86; 95% CI, 1.04–3.32; $P = 0.035$; Model 2 in Table 6).

We found that additional adjustment for metabolic syndrome, obesity, and insulin resistance did not attenuate the significant associations between both variants and NASH or significant fibrosis in NAFLD subjects (Model 5 in Table 6).

Table 5. Risk of NAFLD, NASH, and significant fibrosis according to the genotype

	Odds ratio for NAFLD		Odds ratio for NASH in NAFLD		Odds ratio for ≥F2 in NAFLD	
	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value
rs738409 *PNPLA3*						
Additive	1.88 (1.34, 2.60)	<0.001	2.00 (1.49, 2.69)	<0.001	1.64 (1.21, 2.22)	0.002
Dominant	1.84 (1.13, 2.98)	0.014	3.25 (1.91, 5.54)	<0.001	1.95 (1.12, 3.40)	0.019
Recessive	3.56 (1.83, 6.92)	<0.001	2.16 (1.38, 3.39)	0.001	1.96 (1.24, 3.09)	0.004
rs58542926 *TM6SF2*						
Additive	1.88 (0.91, 3.88)	0.089	1.90 (1.10, 3.29)	0.022	1.73 (1.01, 2.97)	0.045
Dominant	1.86 (0.89, 3.91)	0.099	1.86 (1.05, 3.28)	0.033	1.79 (1.02, 3.15)	0.044
Recessive	–	0.999	–	0.999	1.93 (0.12, 31.1)	0.644

Without adjustment

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis

Table 6. Risk of NAFL, NASH, and significant fibrosis according to the genotype with adjustment for metabolic risk factors

	Odds ratio for NAFLD (95% CI)		Odds ratio for NASH in NAFLD (95% CI)		Odds ratio for ≥F2 in NAFLD (95% CI)	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
rs738409 in *PNPLA3*[1]						
Model 1	1.88 (1.34, 2.60)	< 0.001	2.00 (1.49, 2.69)	< 0.001	1.64 (1.21, 2.22)	0.002
Model 2	1.96 (1.41, 2.71)	< 0.001	1.97 (1.46, 2.66)	< 0.001	1.54 (1.13, 2.11)	0.007
Model 3	2.07 (1.49, 2.69)	< 0.001	1.98 (1.46, 2.69)	< 0.001	1.53 (1.12, 2.10)	0.008
Model 4	1.96 (1.37, 2.80)	< 0.001	1.94 (1.43, 2.80)	< 0.001	1.50 (1.09, 2.06)	0.012
Model 5	1.92 (1.32, 2.80)	0.001	2.00 (1.46, 2.73)	< 0.001	1.53 (1.11, 2.11)	0.009
rs58542926 in *TM6SF2*[2]						
Model 1	1.86 (0.89, 3.91)	0.099	1.86 (1.05, 3.28)	0.033	1.79 (1.02, 3.15)	0.044
Model 2	1.84 (0.87, 3.88)	0.108	1.86 (1.04, 3.32)	0.035	1.90 (1.05, 3.44)	0.033
Model 3	1.91 (0.86, 4.21)	0.111	1.92 (1.06, 3.48)	0.033	1.87 (1.03, 3.42)	0.041
Model 4	1.97 (0.88, 4.41)	0.100	1.89 (1.04, 3.44)	0.038	1.86 (1.02, 3.40)	0.043
Model 5	1.99 (0.87, 4.54)	0.104	1.91 (1.04, 3.51)	0.038	1.88 (1.02, 3.46)	0.042

Model 1, without adjustment
Model 2, with adjustment for age and sex
Model 3, with adjustment for age, sex, and metabolic syndrome
Model 4, with adjustment for BMI ≥ 30 kg/m^2 in addition to model 3
Model 5, with adjustment for HOMA-IR in addition to model 4
[1]Additive model; odds ratio for the number of risk allele (G)
[2]Dominant model; odds ratio for CT/TT genotype

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HOMA-IR, homeostasis model assessment of insulin resistance

In addition, stratified analysis according to insulin resistance showed that even in subjects with HOMA-IR <2.5, the presence of a risk allele (G) increased the risk of NASH in a dose-dependent manner (Figure 3a). As the number of G alleles at *PNPLA3* rs738409 increased, the risk of NASH increased in NAFLD subjects with HOMA-IR <2.5, even after adjustment for age and sex (OR per risk allele, 2.52; 95% CI, 1.26–5.03; Table 7). Among NAFLD subjects, the risk of NASH in subjects with both HOMA-IR ≥2.5 and GG genotype increased by as much as 20 times compared to that in subjects with the CC genotype and HOMA-IR <2.5 (OR, 20.64; 95% CI, 4.47 –95.21; Table 7), implicating that genetic variants affected the histological severity of NAFLD in addition to insulin resistance. The number of risk alleles (G) tended to be positively related with the prevalence of significant fibrosis, even in subjects with HOMA-IR <2.5 (Figure 3b), although the relationship was not statistically significant (OR per risk allele, 2.01; 95% CI, 0.97–4.17; P = 0.062).

In the case of *TM6SF2* rs58542926, similar trends were noticed after stratification according to insulin resistance among NAFLD subjects (Figure 3c-d); however, risk variants at *TM6SF2* rs58542926 were not a significant risk factor for NASH or significant fibrosis in subjects with low insulin resistance (HOMA-IR <2.5) (Table 8).

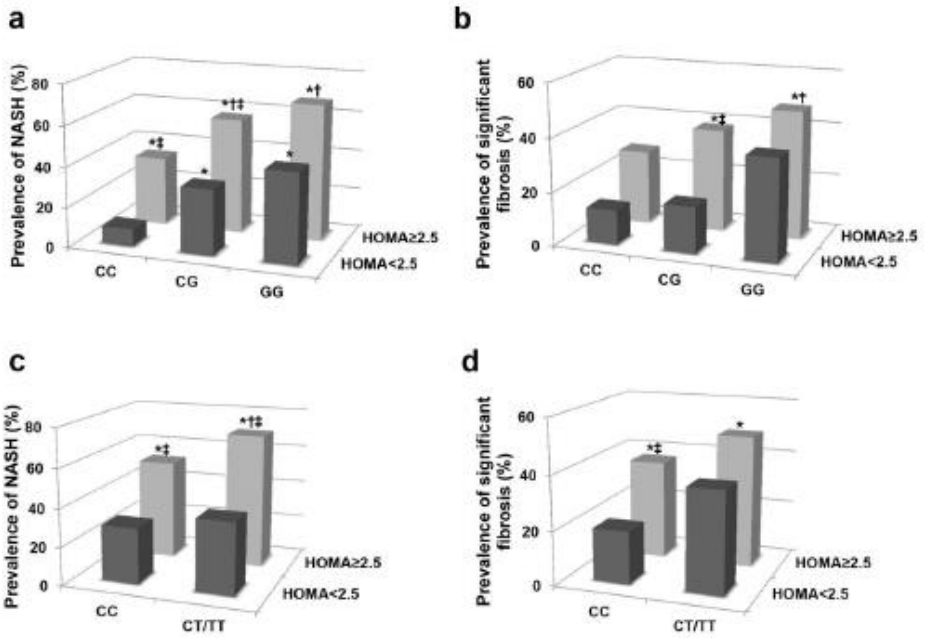

Figure 3. Additive effects of genetic variants and insulin resistance on the risk of NASH and significant fibrosis in subjects with NAFLD

(a–b) Prevalence of NASH and significant fibrosis among NAFLD subjects according to the genotype at *PNPLA3* rs738409 stratified by HOMA-IR is shown (c–d) Prevalence of NASH and significant fibrosis among NAFLD subjects according to the genotype at *TM6SF2* rs58542926 stratified by HOMA-IR is shown. $^{*}P < 0.05$ compared to subjects with HOMA-IR < 2.5 and the CC genotype (with adjustment for age and sex). $^{\dagger}P < 0.05$ compared to subjects with HOMA-IR ≥ 2.5 and the CC genotype (with adjustment for age and sex). $^{\ddagger}P < 0.05$ compared to subjects with the same number of risk alleles and no metabolic risk factors (with adjustment for age and sex).

Table 7. The risk of NASH or significant fibrosis according to *PNPLA3* genotype and metabolic phenotype among NAFLD subjects

		Odds ratio (95% CI)[1]	*P*-value	Odds ratio (95% CI)[1]	*P*-value	Odds ratio (95% CI)[1]	*P*-value
Odds ratio for NASH according to *PNPLA3* genotype & insulin resistance							
HOMA-IR<2.5	CC at rs738409	(reference)	–				
	CG at rs738409	5.25 (1.07, 25.66)	0.041			*Per risk allele*	
	GG at rs738409	8.43 (1.63, 43.54)	0.011			2.52 (1.26, 5.03)	0.009
HOMA-IR≥2.5	CC at rs738409	5.36 (1.13, 25.32)	0.034	(reference)	–		
	CG at rs738409	13.75 (3.06, 61.85)	0.001	2.58 (1.35, 4.93)	0.004	*Per risk allele*	
	GG at rs738409	20.64 (4.47, 95.21)	<0.001	3.87 (1.92, 7.78)	<0.001	1.93 (1.37, 2.74)	<0.001
Odds ratio for significant fibrosis according to *PNPLA3* genotype & insulin resistance							
HOMA-IR<2.5	CC at rs738409	(reference)	–				
	CG at rs738409	1.21 (0.28, 5.24)	0.797			*Per risk allele*	
	GG at rs738409	3.14 (0.73, 13.51)	0.125			2.01 (0.97, 4.17)	0.062
HOMA-IR≥2.5	CC at rs738409	2.14 (0.55, 8.39)	0.275	(reference)	–		
	CG at rs738409	3.96 (1.08, 14.46)	0.038	1.87 (0.93, 3.78)	0.080	*Per risk allele*	
	GG at rs738409	4.98 (1.34, 18.55)	0.017	2.33 (1.12, 4.85)	0.023	1.49 (1.05, 2.13)	0.028

[1]From age and sex-adjusted logistic analysis

Abbreviations: NAFLD, non-alcoholic fatty liver disease; HOMA-IR, homeostasis model assessment of insulin resistance.

Table 8. The risk of NASH or significant fibrosis according to *TM6SF2* genotype and metabolic phenotype among NAFLD subjects

		Odds ratio (95% CI)[1]	*P*-value	Odds ratio (95% CI)[1]	*P*-value
Odds ratio for NASH according to *TM6SF2* genotype & insulin resistance					
HOMA-IR<2.5	CC at rs58542926	(reference)	–		
	CT/TT at rs58542926	1.47 (0.47, 4.57)	0.505		
HOMA-IR≥2.5	CC at rs58542926	2.56 (1.47, 4.43)	0.001	(reference)	–
	CT/TT at rs58542926	5.45 (2.41, 12.34)	<0.001	2.13 (1.05, 4.33)	0.037
Odds ratio for significant fibrosis according to *TM6SF2* genotype & insulin resistance					
HOMA-IR<2.5	CC at rs58542926	(reference)	–		
	CT/TT at rs58542926	2.44 (0.75, 7.99)	0.139		
HOMA-IR≥2.5	CC at rs58542926	2.43 (1.30, 4.56)	0.006	(reference)	–
	CT/TT at rs58542926	4.39 (1.88, 10.23)	0.001	1.82 (0.91, 3.65)	0.092

[1]From age and sex-adjusted logistic analysis

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HOMA-IR, homeostasis model assessment of insulin resistance

Additive effect of *PNPLA3* and *TM6SF2* variants on the risk of NASH and significant fibrosis

Comparing the metabolic profiles across *PNPLA3* rs738409 and *TM6SF2* rs58542926 variants, significant trend toward higher BMI, fasting glucose level, HOMA-IR, and adipo-IR were found as the number of G alleles at *PNPLA3* rs738409 increased (P = 0.021, P = 0.005, P = 0.016, and P = 0.048, respectively; Table 9). However, *TM6SF2* rs58542926 was not significantly associated with these metabolic traits (Table 9). To exclude the indirect effect of the *PNPLA3* variant on these metabolic traits mediated by NAFLD, we retested rs738409 for confirming such association in the control subjects, and verified the positive associations between the number of G alleles at rs732409 and HOMA-IR and adipo-IR even in subjects without NAFLD (P = 0.046 and 0.002, respectively; Figure 4). However, neither BMI nor fasting glucose level were associated with rs738409 in the control subjects (P = 0.132 and 0.250, respectively).

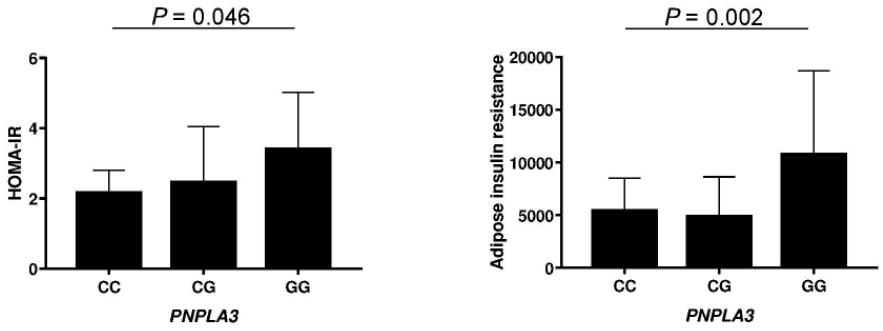

Figure 4. Insulin resistance according to *PNPLA3* genotype in control subjects

Table 9. Comparison of metabolic profiles among genetic variants

	PNPLA3				***TM6SF2***		
	CC (n = 119)	CG (n = 214)	GG (n = 127)	*P* [1]	CC (n = 393)	CT/TT (n = 68)	*P* [1]
BMI, kg/m^2	26.4 (23.6, 29.3)	26.5 (24.1, 29.5)	26.8 (24.9, 30.1)	0.021	26.6 (24.2, 29.4)	26.6 (23.9, 30.1)	0.621
Total cholesterol, mg/dL	181.4 ± 38.1	183.9 ± 40.9	179.2 ± 41.8	0.824	182.9 ± 40.7	177.0 ± 36.3	0.161
HDL cholesterol, mg/dL	46.0 (38.0, 54.0)	45.0 (38.0, 55.0)	45.0 (36.0, 53.0)	0.238	45.0 (37.5, 55.0)	46.0 (38.0, 52.0)	0.605
Triglycerides, mg/dL	130.0 (89.0, 188.0)	129.0 (86.0, 182.3)	131.0 (94.0, 193.0)	0.090	132.0 (90.0, 186.5)	109.0 (84.3, 175.0)	0.880
ALT, IU/L	34.0 (20.0, 54.0)	39.5 (24.0, 66.5)	39.0 (25.0, 79.0)	0.002	37.0 (23.0, 60.5)	43.0 (25.0, 96.8)	0.023
AST, IU/L	29.0 (22.0, 44.0)	34.0 (23.8, 49.0)	40.0 (26.0, 64.0)	0.002	33.0 (24.0, 50.0)	41.0 (24.0, 58.8)	0.289
GGT, IU/L	37.0 (17.0, 77.0)	38.5 (21.0, 64.3)	45.0 (26.0, 84.0)	0.751	40.0 (21.0, 69.0)	43.5 (23.3, 74.0)	0.707
Albumin, g/dL	4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.3	0.994	4.2 ±0.3	4.2 ±0.4	0.775
Platelet, x10^9/L	240 ± 57	231 ± 63	214 ± 71	0.002	229 ± 63	227 ± 70	0.529
Glucose, mg/dL	101.0 (93.0, 113.0)	104.0 (95.0, 121.3)	107.0 (95.0 135.0)	0.005	105.0 (95.0, 122.0)	101.0 (93.3, 116.8)	0.035
Insulin, μIU/mL	11.0 (8.3, 15.2)	11.5 (8.1, 18.2)	12.7 (9.3, 19.3)	0.061	11.4 (8.2, 16.9)	12.4 (9.3, 20.3)	0.014
HOMA-IR	2.74 (1.98, 4.18)	3.05 (2.06, 4.96)	3.85 (2.40, 5.53)	0.016	3.13 (2.14, 4.65)	3.14 (2.25, 5.41)	0.191
Adipose-IR	7157 (4930, 11248)	6686 (4062, 22613)	8706 (5023, 14715)	0.048	7191 (4478, 12140)	7346 (5307, 12913)	0.578
hsCRP, mg/dL	0.8 (0.4, 2.2)	0.9 (0.5, 2.3)	1.3 (0.7, 2.4)	0.080	0.9 (0.5, 2.3)	1.2 (0.6, 2.2)	0.954
Diabetes, N (%)	40 (33.6)	82 (38.3)	50 (39.4)	0.671	151 (38.4)	21 (30.9)	0.755
Obesity (BMI ≥ 25), N (%)	76 (63.9)	138 (64.5)	92 (73.0)	0.060	262 (66.8)	44 (64.7)	0.865
Metabolic syndrome, N (%)	80 (67.2)	140 (66.7)	92 (73.0)	0.436	266 (68.2)	46 (69.7)	0.758

The data are expressed as the means ± standard deviations or medians (interquartile ranges).
[1]From ANOVA or binary logistic regression analysis with adjustment for age and sex

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; Adipo-IR, adipose tissue insulin resistance; hsCRP, high sensitivity C-reactive protein.

Next, we counted risk alleles at *PNPLA3* rs738409 (by coding 0, 1, and 2 for CC, CG, and GG genotypes, respectively) and *TM6SF2* rs58542926 (by coding 0, 1, and 2 for CC, CT, and TT genotypes, respectively) for each subject, and investigated whether *PNPLA3* and *TM6SF2* variants exerted an additive effect on the histological severity of NAFLD. Among NAFLD subjects, as the number of risk alleles increased, the prevalence of NASH increased; it was 28.2%, 41.8%, 63.7%, and 69.2% in subjects with 0, 1, 2, and 3 risk alleles, respectively (P for trend < 0.001; Figure 5a). The risk of NASH significantly increased even after adjustment for age and sex (OR per risk allele, 2.04; 95% CI, 1.54–2.71; Model 2 in Table 10). Prevalence of significant fibrosis also increased as the number of risk alleles increased; specifically, it was 22.5%, 28.8%, 43.7%, and 61.5% in subjects with 0, 1, 2, and 3 risk alleles, respectively (age and sex-adjusted OR per risk allele, 1.67; 95% CI, 1.25–2.23; Model 2 in Table 10 and Figure 5b). With additional adjustment for HOMA-IR and hsCRP, the additive effects of *PNPLA3* rs738409 and *TM6SF2* rs58542926 on the risk of NASH and significant fibrosis were maintained (Model 4 in Table 9). After stratification according to insulin resistance, the prevalence of NASH and significant fibrosis significantly increased with increasing number of risk alleles, even in subjects with low insulin resistance (age and sex-adjusted P for trend = 0.008 and 0.020, respectively; Figure 5c-d).

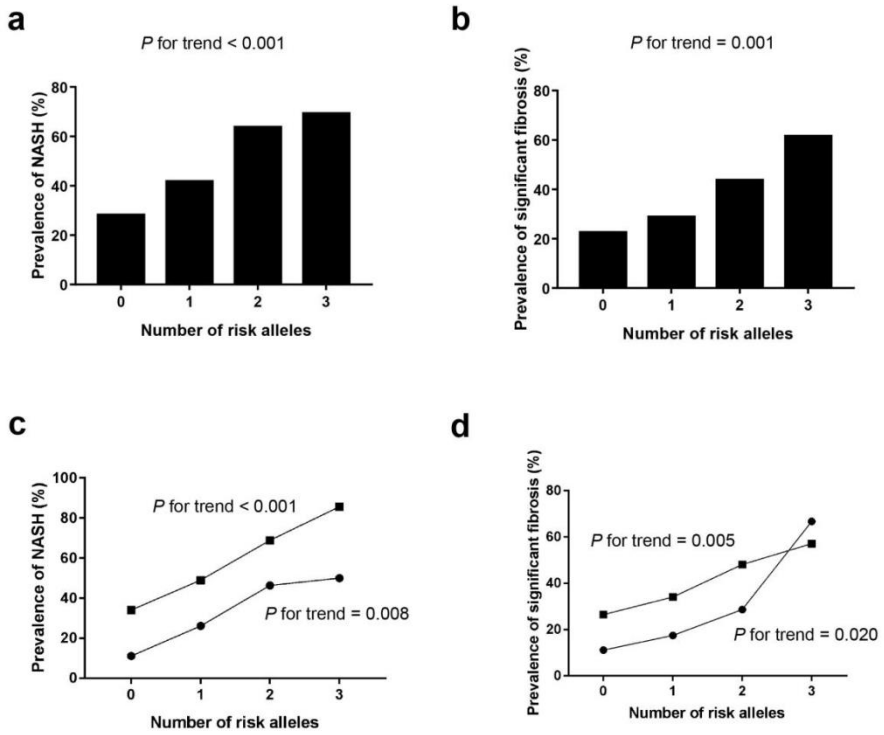

Figure 5. Additive effects of *PNPLA3* and *TM6SF2* genetic variants on the risk of NASH and significant fibrosis in subjects with NAFLD

Prevalence of NASH (a) and significant fibrosis (b) among NAFLD subjects according to the total number of risk alleles is shown. Risk alleles were counted and summed in an additive model at *PNPLA3* rs738409 (by coding 0, 1, and 2 for CC, CG, and GG genotypes, respectively) and in a dominant model at *TM6SF2* rs58542926 (by coding 0, 1, and 2 for CC and CT/ TT genotypes, respectively) for each subjects. As the number of risk alleles increased, the prevalence of NASH (a) and significant fibrosis (b) increased (*P* for trend < 0.001 and *P* for trend = 0.001, respectively), even after adjustment for age and sex. A significantly increasing trend in the prevalence of NASH (c) and significant fibrosis (d) according to the number of risk alleles was observed in both subjects with low insulin resistance (*P* for trend = 0.008 and 0.020, respectively; closed circles) and subjects with insulin resistance (*P* for trend < 0.001 an *P* for trend = 0.005, respectively; closed squares).
Closed circles, subjects with low insulin resistance; closed squares, subjects with insulin resistance.

Table 10. Additive effect of *PNPLA3* rs738409 and *TM6SF2* rs58542926 on the risk of NASH and significant fibrosis in subjects with NAFLD

	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	*P*- value	OR (95% CI)	*P*- value	OR (95% CI)	*P*- value	OR (95% CI)	*P*- value
Odds ratio for NASH in NAFLD subjects								
No risk allele	(reference)		(reference)		(reference)		(reference)	
1 risk allele	1.83 (0.99, 3.38)	0.053	1.85 (0.99, 3.44)	0.052	2.02 (1.07, 3.81)	0.030	2.03 (1.05, 3.90)	0.034
2 risk alleles	4.48 (2.34, 8.36)	<0.001	4.30 (2.28, 8.09)	<0.002	4.45 (2.34, 8.49)	<0.001	4.39 (2.25, 8.54)	<0.001
3 risk alleles	5.74 (1.59, 20.77)	0.008	6.01 (1.63, 22.15)	0.007	8.33 (2.13, 32.60)	0.002	5.89 (1.45, 24.01)	0.013
Per risk gene	2.08 (1.57, 2.75)	<0.001	2.04 (1.54, 2.71)	<.001	2.11 (1.58, 2.81)	<0.001	2.03 (1.50, 2.73)	<0.001
Odds ratio for Significant fibrosis in NAFLD subjects								
No risk allele	(reference)		(reference)		(reference)		(reference)	
1 risk allele	1.39 (0.72, 2.69)	0.332	1.52 (0.77, 3.01)	0.232	1.65 (0.82, 3.32)	0.157	1.62 (0.80, 3.30)	0.180
2 risk alleles	2.67 (1.39, 5.12)	0.003	2.49 (1.27, 4.88)	0.008	2.55 (1.29, 5.07)	0.007	2.39 (1.19, 4.80)	0.014
3 risk alleles	5.50 (1.58, 19.17)	0.007	6.09 (1.67, 22.17)	0.006	7.87 (2.10, 29.46)	0.002	5.82 (1.52, 22.27)	0.010
Per risk gene	1.74 (1.31, 2.31)	<0.001	1.67 (1.25, 2.23)	0.001	1.70 (1.27, 2.29)	<0.001	1.61 (1.19, 2.17)	0.002

The number of the risk allele is the sum of the G allele at rs738409 as an additive model and that of the T allele at rs58542926 as a dominant model.

Model 1, without adjustment
Model 2, with adjustment for age and sex
Model 3, with adjustment for age, sex, and HOMA-IR

Model 4, with adjustment for age, sex, HOMA-IR, and hsCRP

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; CI, confidence interval; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein

Discussion

In this cross-sectional analysis of a prospective cohort consisting of biopsy-proven NAFLD subjects, we replicated the significant associations between NASH and both *PNPLA3* rs738409 and *TM6SF2* rs58542926 genetic variants. Although the associations between NASH and typical metabolic phenotypes, such as metabolic syndrome, obesity, and diabetes, were attenuated among NAFLD subjects because these metabolic phenotypes were frequently noticed even in NAFL subjects, the identification of risk variants in *PNPLA3* and *TM6SF2* as well as insulin resistance was useful for detecting NASH or significant fibrosis among NAFLD subjects. Although the associations between NASH and genetic variants of in *PNPLA3* and *TM6SF2* had been previously established,[8-12] we newly found that these relationships were maintained even after adjustment for metabolic syndrome and insulin resistance.

Genetic susceptibility might explain the relatively higher prevalence of NAFLD the lower BMI in Asian populations, the prevalence of NAFLD among the non-obese Asian is 15–21%.[33,34] The MAF of *PNPLA3* rs738409 is 0.3–0.5 in the Asian population,[35,36] which is significantly higher than that in the Western population (0.22),[8] suggesting that genetic susceptibility rather than metabolic risk factors might play an important role in the development of NAFLD in the Asian population. It has been confirmed that *PNPLA3* rs738409 C>G confers susceptibility to NAFLD in non-obese individuals in

Asian populations.[13,14] Our study provided robust evidence of the importance of genetic variants in predicting the occurrence as well as the progression of NAFLD in terms of the histological severity.

In terms of genetic determinants of fibrosis in NAFLD, we replicated the significant associations between fibrosis and *PNPLA3* rs738409.[8-10] In the case of *TM6SF2* rs58542926, there have been conflicting data on that: although earlier studies reported that T allele significantly increased the risk of fibrosis in biopsy-proven NAFLD subjects,[37,38] following studies did not.[12,39] Different ethnicities or clinical characteristics of the study subjects might affect the association. Sookoian et al. showed no association between *TM6SF2* rs58542926 and fibrosis in Caucasian NAFLD subjects (n = 226).[12] The mean BMI of that study subjects were > 31 kg/m^2, which was higher than those in our study subjects (NAFL, 26.9 kg/m^2; NASH, 27.7 kg/m^2). Akuta et al. also reported a negative result using Asian NAFLD subjects with similar BMI; however, they analyzed the genetic effect using only 140 NAFLD subjects. In the current study, using 416 biopsy-proven subjects, we found the significant association between rs58542926 C > T and the significant fibrosis even after adjustment for confounders including insulin resistance and obesity. Further large scaled studies are needed to elucidate the effects of *TM6SF2* variants on hepatic fibrosis in NAFLD subjects with diverse ethnic and clinical backgrounds.

We additionally examined the relationships between metabolic traits and genetic variants and we found significant trends towards higher HOMA-IR and adipo-IR as the number of G alleles increased at *PNPLA3* rs738409, even

in subjects without NAFLD. However, *TM6SF2* rs58542926 was not associated with insulin resistance, which suggests that *PNPLA3* and *TM6SF2* might play different roles in the pathogenesis of NAFLD. A limited number of studies have examined the mechanism by which these variants affect the risk of NASH. Previous studies reported that *PNPLA3* rs738409 was not associated with insulin resistance[8] of other metabolic traits such as BMI, lipid levels, and diabetes.[40] Some differences in baseline characteristics of the study subjects may account for this discrepancy between previous and our studies. Previous studies mainly included African-Americans and European-Americans, with mean BMI 27–34 kg/m^2, in a cohort of patients with cardiovascular disease.[8,40] In the current study, the control subjects were of Asian ethnicity and had lower BMI (median, 23.8 kg/m^2) and HOMA-IR (median, 2.03). Further studies are warranted to elucidate the effects of *PNPLA3* variants on systemic insulin resistance in ethnic populations with diverse metabolic profiles. Some evidence suggests that *PNPLA3* is associated with insulin signaling or lipid metabolism.[9,41] Histological data obtained from NAFLD subjects have indicated that the expression levels of insulin receptor, steroid regulatory element binding protein 1c, and peroxisome proliferator-activated receptor-α are decreased in subjects with the GG genotype at rs738409.[9] An in *vitro* study had recently shown that *PNPLA3* promotes the hydrolysis of retinyl palmitate in human hepatic stellate cells in response to insulin,[41] which was markedly reduced in *PNPLA3* 148M as compared to 148I.[41] By contrast, *TM6SF2* rs58542926 C>T may alter microsomal

triglyceride transfer protein expression, resulting in the alteration of the packing and export of triglycerides,[42] rather than insulin signaling.

Given the independent pathways involved in the development of hepatic steatosis by *PNPLA3*[8,9,41] and *TM6SF2* variants,[42] *PNPLA3* and *TM6SF2* might increase the risk of NASH complementarily. We confirmed the additive effects of *PNPLA3* and *TM6SF2* on the histological severity of NAFLD, even after adjustment for insulin resistance and systemic inflammation. Previous studies have also shown an additive effect of both variants on NAFLD risk;[43,44] however, the association was demonstrated using only radiologic imaging studies.[43,44] Our study was based on the histologic severity by liver biopsy and histologic diagnoses of NASH and significant fibrosis were reviewed and established by pathologist who specializes in liver pathology.

The main strength of this study was that we comprehensively investigated the impact of genetic variants along with clinical risk factors on the histological severity of NAFLD using a large prospective biopsy-proven cohort (n = 525), which is exceptional in studies on the Asian population. Additionally, we confirmed the independent associations between genetic variants and the histological severity of NAFLD after adjustment for a variety of clinical confounders, including insulin resistance (HOMA-IR) and inflammatory markers (hsCRP).

One limitation of this study was the inability to infer the causality of the observed relationships owing to the cross-sectional nature of the study. Second, the conclusions might not be generalizable to other ethnic groups.

In conclusion, we replicated the significant associations between histological

severity of NAFLD and *PNPLA3* rs738409 and *TM6SF2* rs58542926 genetic variants. We also found distinct differences in the metabolic profiles among genetic variants and confirmed the additive effects of *PNPLA3* and *TM6SF2* on the risks of NASH and significant fibrosis. Further mechanistic studies are warranted to elucidate the metabolic functions of proteins encoded by mutant genes and large-scale longitudinal cohort studies are needed to develop a novel risk- or outcome-prediction model comprising diverse metabolic phenotypes and genetic variants.

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국문 초록

서론: 최근 다양한 인종의 그룹에서 *PNPLA3* 와 *TM6SF2* 유전자 변이가 비알코올 지방간 환자와 관련되어 있다는 유전체 연구가 발표되었다. 그러나 동양인 비알코올 지방간 환자에서의 유전적 영향에 관한 자료는 부족한 상태이다. 따라서 본 연구는 동양인 비알코올 지방간 환자의 조직학적 정도 및 대사 표현형에 따른 *PNPLA3* 와 *TM6SF2* 유전자 변이의 영향을 알아보고자 하였다.

방법: 조직검사로 비알코올 지방간을 진단 받은 525 명의 환자군에서 *PNPLA3* rs738409 와 *TM6SF2* rs58542926 의 유전자형을 분석하였다.

결과: *PNPLA3* rs738409 와 *TM6SF2* rs58542926 는 지방간염뿐 아니라 F2 이상의 간 섬유화와 상관 관계가 있었으며 이는 대사 위험 인자를 고려하였을 때에도 동일한 결과가 나타났다. 그러나 두 개의 유전체 변이 중 rs738409 만이 HOMA-IR 과 adipo-IR 과 유의한 상관 관계를 보였다. 그 밖에 *PNPLA3* 와 *TM6SF2* 는 지방간염과 F2 이상의 간 섬유화의 위험성에 부가효과를 보였다.

결론: *PNPLA3* rs73840 와 *TM6SF2* rs58542926 는 지방간염과 F2 이상의 간 섬유화와 관련 되어있으며 위험성을 증가 시킨다.

주요어: 비알코올 지방간, 비알코올 지방간염, 인슐린 저항성, *PNPLA3, TM6SF2*

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