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Original Article

Interaction of *SAMM50-rs738491*, *PARVB-rs5764455* and *PNPLA3-rs738409* Increases Susceptibility to Nonalcoholic Steatohepatitis

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Abstract

Background and Aims: Previous studies have reported that the single nucleotide polymorphisms (SNPs) of *SAMM50-rs738491, PARVB-rs5764455* and *PNPLA3-rs738409* are associated with nonalcoholic fatty liver disease (NAFLD). However, no studies have examined the effect of interactions between these three genotypes to affect liver disease severity. We assessed the effect of these three SNPs on nonalcoholic steatohepatitis (NASH) and also examined the gene-gene interactions in a Chinese population with biopsy-confirmed NAFLD. ***Methods:*** We enrolled 415 consecutive adult individuals with biopsy-proven NAFLD. Multivariable logistic regression analysis was undertaken to test associations between NASH and SNPs in *SAMM50-rs738491, PARVB-rs5764455* and *PNPLA3-rs738409*. Gene-gene interactions were analyzed by performing a generalized multifactor dimensionality reduction (GMDR) analysis. ***Results:*** The mean ± standard deviation age of these 415 patients was 41.3±12.5 years, and 75.9% were men. Patients with *SAMM50-rs738491* TT, *PARVB-rs5764455* AA or *PNPLA3-rs738409* GG genotypes had a higher risk of NASH, even after adjustment for age, sex and body mass index. GMDR analysis showed that the combination of all three SNPs was the best model for predicting NASH. Additionally, the odds ratio of the haplotype T-A-G for predicting the risk of NASH was nearly three times higher than that of the haplotype G-C-C. ***Conclusions:*** NAFLD patients carrying the *SAMM50-rs738491* TT, *PARVB-rs5764455* AA or *PNPLA3-rs738409* GG genotypes are at greater risk of NASH. These three SNPs may synergistically interact to increase susceptibility to NASH.

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Abbreviations: BMI, body mass index; CI, confidence interval; CVC, cross-validation consistency; GMDR, generalized multifactor dimensionality reduction; GWAS, genome-wide association studies; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time of Flight; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; OR, odds ratio; PCR, polymerase chain reaction; PERSONS, Prospective Epidemic Research Specifically Of NASH; PKB, protein kinase B; PNPLA3, patatin-like phospholipase domain containing 3; ROS, reactive oxygen species; SAM, sorting and assembly machinery; SD, standard deviation; SNPs, single nucleotide polymorphisms; T2DM, type 2 diabetes.

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Introduction

Nonalcoholic steatohepatitis (NASH), i.e. the histological subtype of nonalcoholic fatty liver disease (NAFLD) with lobular inflammation and hepatocyte injury, frequently progresses to advanced fibrosis, cirrhosis and hepatocellular carcinoma.[1] With rapid growth of the global prevalence of NAFLD, which has occurred over the past two decades, the prevalence of NASH has also increased rapidly worldwide.[2] NAFLD is also associated with an increased risk of developing extra-hepatic diseases, including cardiovascular disease, type 2 diabetes mellitus (T2DM) and some extra-hepatic cancers.[3–7] The latest epidemiological predictive models have estimated that China will undergo the highest

growth of NAFLD and NASH in the near future due to urbanization, which will cause considerable clinical and economic burdens.[8] As the diagnosis of NASH still requires liver biopsy,[9] there is currently an unmet need for accurate noninvasive tests to diagnose and monitor the disease condition.

Recent studies have shown that NAFLD is not only highly prevalent among overweight or obese individuals but it may also occur in lean individuals, in who genetic factors play an important role.[10] Strong evidence indicates that genetic and epigenetic factors may affect the development and progression of NAFLD.[3] In recent years, genome-wide association studies (GWAS) have investigated the genetic background of NAFLD in different ethnic populations.[11–13] The rs738409 polymorphism in the patatin-like phospholipase containing protein-3 (*PNPLA3*) gene has the strongest effect on the entire histopathological spectrum of NAFLD across different countries and ethnicities.[14] However, besides the rs738409 polymorphism in the *PNPLA3* gene, other genetic variants have been implicated in the development and progression of NAFLD. In a GWAS study, Kitamoto *et al*.[13] showed that polymorphisms in the *SAMM50* and *PARVB* genes are also associated with the risk of NAFLD in a Japanese population. However, the contribution of the *SAMM50* polymorphism to the progression of NAFLD from simple steatosis to NASH remains controversial.[13,15]

To date, to the best of our knowledge, no study has tested the association of *SAMM50* and *PARVB* genes with the presence of NASH in Chinese individuals with biopsy-proven NAFLD. It is important to note that the *PNPLA3, SAMM50* and *PARVB* genes are all located on chromosome 22q13.[13] In addition, the *rs738491*, *rs5764455* and *rs738409* genetic variants are in the same linkage disequilibrium block, and are closely associated with the histological severity of steatosis and NAFLD activity score (NAS).[13] Based on the theory that complex disease traits are affected by the inheritance of different numbers of variants and also gene-gene interactions,[16] we speculated that there is an interaction between these three polymorphisms to affect liver disease severity in NASH. Currently, there are no studies that have tested the effect of interactions among these three genes on disease severity in NAFLD.

Thus, our aim was to evaluate whether *rs738491, rs5764455* and *rs738409* genetic variants are associated with NASH, and to investigate gene-gene interactions and combination effects of these three genetic variants on the susceptibility to NASH in a Chinese population with biopsy-confirmed NAFLD.

Methods

Study population

This study involved analysis of data from the well-characterized Prospective Epidemic Research Specifically Of NASH (known as 'PERSONS').[17,18] Individuals between 18 and 75 years-old with suspected NAFLD (i.e. defined as evidence of hepatic steatosis on imaging techniques, and/or persistently elevated serum liver enzyme levels) were consecutively recruited for liver biopsy examination at the First Affiliated Hospital of Wenzhou Medical University from December 2016 to November 2018. Participants were excluded for the following reasons: (1) excessive alcohol consumption (>140 g/week for men and >70 g/week for women); (2) viral hepatitis (based on serum viral B or C markers) and autoimmune hepatitis (based on serum autoantibodies and histology); (3) chronic use of potentially hepatotoxic drugs; (4) liver cancer (based on imaging and/or pathological data according to the Clinical Practice Guidelines for hepatocellular carcinoma);[19] (5) missing data on genetic polymorphisms or other important laboratory parameters; and (6) fatty liver infiltration <5% on histology. Informed consent was obtained from all participants. The study was approved by the internal review board for ethics of the First Affiliated Hospital of Wenzhou Medical University; the study protocol was registered in the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562).

Clinical and laboratory data

We measured baseline characteristics, including demographics, anthropometry, clinical parameters and comorbidities, in all participants. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured by a standardized method.[18] Hypertension was defined as blood pressure ≥140/90 mmHg and/or use of any antihypertensive drugs. Diabetes was diagnosed by fasting glucose levels ≥7.0 mmol/L and or use of any antihyperglycemic agents. Blood biochemical parameters, including alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glucose and uric acid concentrations, were assessed by an automated analyzer (Abbott AxSYM, Park, IL, USA) using standard laboratory methods.

Liver histology

Detailed methods for liver biopsy examination were described in our previous study.[18] Liver biopsy specimens were reviewed by a single liver pathologist (X.D. Wang), who was blinded to participants' clinical data. Histological parameters of NAFLD were scored based on the NASH Clinical Research Network classification.[20] NASH was diagnosed as the presence of NAS ≥4, with ≥1 point for each of the three individual histologic components of NASH, including steatosis, lobular inflammation, and ballooning.[21–23] Significant liver fibrosis was defined by fibrosis ≥F2 on histology, according to Brunt's diagnostic criteria.[24] Supplementary Figure 1 shows the representative hematoxylin-eosin staining images of liver biopsy in patients with NASH and non-NASH.

Genetic analysis

More details about the genetic analysis have been published elsewhere.[18] Briefly, blood samples were collected from participants and approximately 20 ng of genomic DNA from white blood cells from each participant was extracted for the genetic analysis of polymorphisms in *SAMM50-rs738491, PARVB-rs5764455* and *PNPLA3-rs738409* genes. Genotyping of these three single nucleotide polymorphisms (SNPs) was evaluated by using the MassARRAY System (Agena Bioscience, San Diego, CA, USA). DNA samples were first amplified through locus-specific polymerase chain reaction (PCR), according to Assay Design Suite software (Version 3.1). Detection of rs738491, rs5764455 and rs738409 genotypes was performed by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry.

Statistical analysis

Continuous variables were compared by the unpaired Student's *t*-test, one-way analysis of variance or the Mann-Whitney *U* test (if not normally distributed) and expressed as either mean±standard deviation (SD) or medians (25^{th}, 75^{th} percentiles), respectively. Categorical variables were analyzed by the χ^2 test or the Fisher's exact test (as appro-

priate) and expressed as numbers and percentages. Hardy-Weinberg equilibrium for the three aforementioned SNPs in the non-NASH and NASH groups was tested by the χ^2 test. The distribution of genotypes and alleles between the two patient groups were compared by the χ^2 test or the Fisher's exact test. Logistic and linear regression analyses were performed to test the associations between these genetic variants and individual histological features of NASH, after adjustment for age, sex and BMI. The SHEsis online haplotype analysis software (http://analysis.bio-x.cn/) was employed for haplotype analyses.[25,26] The strength of the associations is presented as odds ratio (OR) with 95% confidence interval (CI). Generalized multifactor dimensionality reduction (GMDR) analysis[27] was used to evaluate the gene-gene interactions, which provided cross-validation consistency, training accuracy, testing accuracy, and the sign test (p). The detailed analytical procedure and the definition of each output parameter have been described elsewhere.[28] The effect of interactions of genotypes on the risk of NASH was assessed by logistic regression. Power calculations were completed using the CATS Genetic Power Calculator,[29] with settings of a multiplicative genetic model. Prevalence of NASH was approximately 0.025, as estimated in previous studies.[30] Assuming a minor allele frequency for the rs738491 of 0.5566 (i.e. the frequency found in our population), and an OR of 1.6, the expected power for a one-stage study is 89.2% in our cohort. Using the same assumption for the rs5764455 and rs738409, with minor allele frequency of 0.4590 and 0.4675 separately, the expected power was 91.0% for both SNPs. All statistical analyses were undertaken with SPSS/PC version 25.0 (IBM Corp., Armonk, NY, USA) software. A two-sided level of p <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 591 Chinese individuals with histologically-proven NAFLD were initially included in the study. After exclusion of those with alcoholic fatty liver (n=79), autoimmune liver diseases (n=1), drug-induced hepatitis (n=1), hepatitis B or C virus (n=45) or those with missing records of laboratory parameters (n=50), 415 patients with biopsy-proven NAFLD were identified for the final analysis. Subjects had a mean±SD age of 41.3±12.5 years (range: 18–72 years), and 75.9% were men. Amongst these patients, 246 (59.3%) had definite NASH. Table 1 shows the demographic and clinical characteristics of participants stratified by presence or absence of NASH. Patients with NASH were younger and had higher values of BMI, HOMA-estimated insulin resistance, serum liver enzymes and uric acid compared to the non-NASH group. As also shown in Table 1, NAS also differed significantly between the two patient groups.

Association between genotypes and alleles of rs738491, rs5764455 and rs738409 and NASH

Genotypes of these three SNPs were in Hardy-Weinberg equilibrium for both patient groups ($p_{non\text{-}NASH}$=0.582, 0.542, 0.745; p_{NASH}=0.204, 0.885, 0.171). Table 2 shows that the distribution of genotypes and allele frequencies of *SAMM50-rs738491 PARVB-rs5764455* and *PNPLA3-rs738409* were significantly different between patients with, and without, NASH. In addition, Table 3 shows that genotypes and alleles in each SNP had a strong link with the presence of NASH. TT in *SAMM50-rs738491*, AA in *PARVB-rs5764455* and GG in *PNPLA3-rs738409* were significantly associated with an increased risk of having NASH, even after adjusting for age, sex and BMI (adjusted OR: 2.26, 95% CI: 1.24 to 4.13; adjusted OR: 3.27, 95% CI: 1.73 to 6.18; adjusted OR: 2.89, 95% CI: 1.59 to 5.26, respectively). Besides, Table 3 also shows rs738491-T, rs5764455-A and rs738409-G as the risk alleles significantly associated with NASH (adjusted OR: 1.54, 95% CI: 1.15 to 2.08; adjusted OR: 1.74, 95% CI: 1.29 to 2.35; adjusted OR 1.73, 95% CI: 1.29 to 2.33, respectively).

We also compared the genotype distribution of these three variants in NASH patients, stratified by presence of overweight/obesity (BMI <25 kg/m^2 vs. ≥25 kg/m^2). Table S1 shows that there were no significant differences in distribution of these genetic variants between the two groups of patients. In addition, as shown in Supplementary Table S2, no significant differences were found in the distribution of the three genetic variants in NASH patients stratified by presence of T2DM.

Association between genotypes of rs738491, rs5764455 and rs738409 and clinical features

Due to the fact that TT in *SAMM50-rs738491*, AA in *PARVB-rs5764455* and GG in *PNPLA3-rs738409* increased the susceptibility to NASH, we divided patients into high-risk and low-risk groups of developing NASH based on their genetic polymorphisms (TT *vs.* CC+CT according to rs738491; AA vs. GG+GA according to rs5764455; and GG vs. CC+CG according to rs738409, respectively). Table 4 shows that the proportion of women was greater in the risk genotype group of each of the three SNPs. For *SAMM50-rs738491*, the TT group had higher plasma total cholesterol, LDL-C and HDL-C levels compared to the CC+CT group. As to *PARVB-rs5764455*, the AA group had a higher plasma LDL-C level compared to the GG+GA group. The histological severity of NAFLD (i.e. NAS ≥4) differed significantly between the two genotype groups in each of the three SNPs (Table 4). Furthermore, after adjusting for age, sex and BMI, all three aforementioned SNPs remained statistically associated with NAS ≥4, mostly with higher steatosis grade (Table 5). Fibrosis stage, HOMA-estimated insulin resistance and serum liver enzyme levels did not show any difference between the two patient groups for each of the three SNPs. Supplementary Table S3 shows baseline characteristics of participants, stratified by *SAMM50*-rs738491, *PARVB*-rs5764455 or *PNPLA3*-rs738409 genotypes. Apart from differences in liver histology features (as reflected by NAS ≥4), there were no significant differences in many clinical and biochemical parameters among the different patient groups, except for a slight difference in plasma LDL-C levels.

Analysis of gene-gene interactions

We performed a GMDR analysis to examine the gene-gene interactions. This analysis showed that the *PARVB-rs5764455* was the best single-locus model that predicted the presence of NASH. The best two-locus model was the combination of the *SAMM50-rs738491* and *PNPLA3-rs738409*. The three-locus model had perfect cross-validation consistency (CVC: 10/10) and high testing accuracy (57.23%) (p=0.011), appearing to be the best predictive model (Table 6). All these models were adjusted by age, sex and BMI. When we analyzed the strength of the association between each genetic model and NASH after adjusting for age, sex and BMI, we found that the OR values increased progressively with the increasing number of loci included (adjusted OR: 2.431, 95% CI: 1.384 to 4.269 for a one-locus model; adjusted OR: 2.453, 95% CI: 1.413 to 4.259 for a two-locus model; and adjusted OR: 2.751, 95%

Table 1. Baseline characteristics of participants stratified by nonalcoholic steatohepatitis (NASH) status

	Non-NASH *n*=169 (40.7%)	**NASH *n*=246 (59.3%)**	***p***
Female sex, *n* (%)	35 (20.7)	65 (26.4)	0.181
Age, years	45 (37–52)	39 (29–48)	<0.0001
BMI, kg/m^2	25.4 (23.4–27.3)	27.36 (25–29.2)	<0.0001
Hypertension, *n* (%)			0.727
Yes	59 (34.9)	90 (36.6)	
No	110 (65.1)	156 (63.4)	
Type 2 diabetes, *n* (%)			0.610
Yes	40 (23.7)	53 (21.5)	
No	129 (76.3)	193 (78.5)	
HOMA-IR	2.73 (1.79–4.44)	3.99 (2.83–6.35)	<0.0001
ALT, U/L	37.0 (23.0–54.0)	68.0 (41.0–124.0)	<0.0001
AST, U/L	27.0 (22.0–36.5)	43.0 (30.0–66.2)	<0.0001
GGT, U/L	42.0 (26.0–69.5)	56.0 (38.0–91.2)	<0.0001
TG, mmol/L	1.81 (1.30–2.59)	1.98 (1.45–2.92)	0.107
TC, mmol/L	4.91±1.12	5.10±1.12	0.094
HDL-C, mmol/L	0.97 (0.85–1.14)	0.98 (0.87–1.14)	0.320
LDL-C, mmol/L	2.95±0.88	3.11±0.91	0.088
UA, mmol/L	364 (310–438)	401 (340.7–481.2)	0.001
Liver histology features			
Steatosis grade			<0.0001
1	156 (92.3)	50 (20.3)	
2	11 (6.5)	82 (33.3)	
3	2 (1.2)	114 (46.3)	
Lobular inflammation grade			<0.0001
0	28 (16.6)	0	
1	138 (81.7)	172 (69.9)	
2	3 (1.8)	69 (28.0)	
3	0	5 (2.0)	
Ballooning grade			<0.0001
0	20 (11.8)	0	
1	127 (75.1)	102 (41.5)	
2	22 (13.0)	144 (58.5)	
Fibrosis grade			0.001
0	80 (47.3)	67 (27.2)	
1	62 (36.7)	117 (47.6)	
2	20 (11.8)	50 (20.3)	
3	6 (3.6)	10 (4.1)	
4	1 (0.6)	2 (0.8)	

Sample size *n*=415. Data are expressed as numbers (percentages) for categorical variables, as mean±SD for normally distributed continuous variables and median (interquartile range) for skewed distributed continuous variables. Differences between the groups were determined using the Student's *t*-test or the Mann-Whitney *U* test for continuous variables, and the Pearson χ^2 test or the Fisher's exact test for categorical variables as appropriate. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglycerides; UA, uric acid.

CI: 1.452 to 5.211 for a three-locus model, respectively). Importantly, the OR of the haplotype T-A-G for predicting the risk of NASH was nearly three times higher compared to that of the haplotype C-G-C. Additionally, the OR of the haplotype T-A-G to C-G-C was nearly twice compared to the haplotype T-G to C-C or A to G, suggesting that the combination of these three risk alleles increases the susceptibility to NASH (Table 7 and Fig. 1).

Table 2. Distribution of genotypes and allele frequencies of genetic variants in *SAMM50-rs738491*, *PARVB-rs5764455* and *PNPLA3-rs738409*, stratified by NASH status

Genotypes and alleles	Non-NASH	NASH	χ^2	*p*
SAMM50-rs738491				
Genotypes			12.62	0.002
CC	43 (25.4)	43 (17.5)		
CT	88 (52.1)	108 (43.9)		
TT	38 (22.5)	95 (38.6)		
Alleles			11.79	0.001
C	174 (51.5)	194 (39.4)		
T	164 (48.5)	298 (60.6)		
PARVB-rs5764455				
Genotypes			16.04	<0.0001
GG	64 (37.9)	57 (23.2)		
GA	83 (49.1)	124 (50.4)		
AA	22 (13.0)	65 (26.4)		
Alleles			15.93	<0.0001
G	211 (62.4)	238 (48.4)		
A	127 (37.6)	254 (51.6)		
PNPLA3-rs738409				
Genotypes			14.75	0.001
CC	65 (38.5)	61 (24.8)		
CG	78 (46.2)	112 (45.5)		
GG	26 (15.4)	73 (29.7)		
Alleles			15.72	<0.0001
C	208 (61.5)	234 (47.6)		
G	130 (38.5)	258 (52.4)		

Data are expressed as number (percentage) and tested by the Pearson χ^2 test or the Fisher's exact test as appropriate. Nash, nonalcoholic steatohepatitis.

Discussion

Recent studies have shown that various genes and SNPs play important roles in the development and progression of NAFLD.[3] In addition, it is known that the magnitude of the NAFLD-related SNP effects vary markedly in different ethnic populations.[31] Based on GWAS results, polymorphisms in *SAMM50-rs738491*, *PARVB-rs5764455* and *PNPLA3-rs738409* genes were found to be associated with the development and progression of NAFLD, while the relationship between these SNPs and the presence of NASH was not obvious.[13] With regard to the Han Chinese population, two prior studies have compared genetic variants in the *SAMM50* and *PARVB* genes with the presence of NAFLD as detected by ultrasonography.[15,32] In those two studies, the authors reported that both the *rs738491* T allele in the *SAMM50* gene and the *rs5764455* A allele in the *PARVB* gene were significantly associated with presence of ultrasound-defined NAFLD. Interestingly, in our study that involved patients with biopsy-confirmed NAFLD, we showed for the first time that patients carrying TT in *SAMM50-rs738491*, AA in *PARVB-rs5764455* or GG in *PNPLA3-rs738409* were more likely to have NASH. In addition, we found that the rs738491-T, rs5764455-A and rs738409-G risk alleles remained significantly associated with the risk of NASH, even after adjusting for age, sex and BMI. We also undertook a subgroup analysis comparing these three genetic variants in patients with NASH, stratified by presence of overweight/obesity or T2DM. These data indicate that the three genetic variants may play a role in the development of NASH, regardless of the presence or absence of overweight/obesity and T2DM. However, larger studies are needed to further validate these findings.

From a pathophysiological point of view, previous studies have shown that the *PNPLA3, SAMM50* and *PARVB* genes may be implicated in regulating lipid metabolism,[33] maintaining mitochondrial morphology,[34] and activating Akt/protein kinase B (Akt/PKB) signaling pathways,[35] respectively. Specifically, the PNPLA3 protein is an enzyme with triacylglycerol lipase and acylglycerol O-acyltransferase activity, which promotes remodeling of lipid droplets in hepatocytes.[33] The *PNPLA3-rs738409* C>G genotype reduces this enzymatic activity, leading to the entire histopathological spectrum of NAFLD from simple steatosis to cirrhosis and hepatocellular carcinoma.[3] In our study, the analysis of liver histology features showed that the GG genotype of rs738409 was associated with a higher proportion of NAS ≥4 (especially with a higher steatosis grade), but not with a more atherogenic lipid profile. This finding is also in line with previous data from Speliotes *et al*.[36] showing that the *PNPLA3*-rs738409 variant specifically conferred a higher risk of histologic hepatic fat ac-

Table 3. OR for NASH according to the genotypes and alleles of *SAMM50-rs738491*, *PARVB-rs5764455* or *PNPLA3-rs738409*

Genotypes and alleles	Unadjusted OR (95% CI)	*p*	Adjusted OR (95% CI)[a]	*p*
SAMM50-rs738491				
Genotypes				
CC	1		1	
CT	1.227 (0.739, 2.039)	0.429	1.148 (0.667, 1.975)	0.618
TT	2.500 (1.420, 4.402)	0.002	2.262 (1.240, 4.127)	0.008
Alleles				
C	1		1	
T	1.630 (1.232, 2.156)	0.001	1.544 (1.148, 2.078)	0.004
PARVB-rs5764455				
Genotypes				
GG	1		1	
GA	1.677 (1.067, 2.637)	0.025	1.610 (0.993, 2.611)	0.053
AA	3.317 (1.819, 6.050)	<0.0001	3.265 (1.725, 6.177)	<0.0001
Alleles				
G	1		1	
A	1.773 (1.337, 2.352)	<0.0001	1.744 (1.293, 2.352)	<0.0001
PNPLA3-rs738409				
Genotypes				
CC	1		1	
CG	1.530 (0.972, 2.408)	0.066	1.415 (0.872, 2.297)	0.16
GG	2.992 (1.696, 5.279)	<0.0001	2.894 (1.593, 5.258)	<0.0001
Alleles				
C	1		1	
G	1.764 (1.331, 2.338)	<0.0001	1.732 (1.285, 2.333)	<0.0001

[a]Multivariate logistic regression model adjusted for age, sex and BMI. BMI, body mass index; NASH, nonalcoholic steatohepatitis; OR, odds ratio.

cumulation, but not NAFLD-related metabolic traits. The *SAMM50* gene encodes the protein Sam50 that is part of the sorting and assembly machinery, which is necessary for assembling β-barrel proteins located in the outer membrane of mitochondria.[34] β-barrel proteins play a crucial role in maintaining mitochondrial shape, morphology of mitochondrial cristae, and in assembling the respiratory chain complexes.[34,37] Abnormalities of β-barrel proteins lead to mitochondrial dysfunction, which in turn reduces the removal of reactive oxygen species (ROS).[13] ROS accumulation leads to destruction of organelles oxidizing fatty acids and promotes lipotoxicity, thereby inducing hepatocyte damage.[38] Based on the observation that loss of mitochondrial cristae has been found in the livers of patients with NASH after depletion of the protein Sam50,[39] there is a close link between *SAMM50-rs738491* and proinflammatory factors in hepatocytes.

Parvin-β, encoded by the *PARVB* gene, takes part in forming the integrin-linked kinase-pinch-parvin complex, which transmits signals from integrin to Akt/PKB.[35] Kinases of Akt/PKB modulate basic cell activation to produce proinflammatory factors.[40] Over-expression of parvin-β not only leads to a concomitant increase in lipogenic gene expression[41] but also promotes apoptosis.[42] Increased hepatic lipogenesis and apoptosis are considered key biological mechanisms occurring with NAFLD progression from simple steatosis to NASH and fibrosis.[43] Polymorphisms of both rs5764455 and rs6006473 in the *PARVB* gene have also been reported to be associated with the development and progression of NAFLD in the Han Chinese population.[32] Unfortunately, we did not examine *PARVB* rs6006473 in this study. However, previous studies have shown that the rs5764455 in the *PARVB* gene showed the strongest association with NASH.[13] Similarly, in our study the rs5764455 in the *PARVB* gene showed the highest OR for NASH amongst all the examined SNPs, comparing the A allele to the G allele.

The progression from simple steatosis to NASH is recognized to have two consecutive steps, which are fat accumulation followed by necroinflammation in the liver.[44] Our study suggests that the *PNPLA3-rs738409* and *SAMM50-rs738491* genetic variants are involved, respectively, in the first and second steps of NAFLD progression, while the *PARVB-rs5764455* variant seems to be involved in both of these steps.

All the findings mentioned above support the existence of an association between these three SNPs and NASH and also suggest possible underlying mechanisms (as schematically shown in Fig. 2). However, it has been shown that the NAFLD-related SNPs identified by GWAS only explain a small part of disease etiology, because the relatedness between complex diseases and multiple genes and/or their interactions are ignored.[45] Therefore, analyses emphasizing gene-gene interactions have been one of the new approaches to gaining a better understanding of the etiology of com-

Table 4. Baseline characteristics of participants stratified by *SAMM50-rs738491*, *PARVB-rs5764455* and *PNPLA3-rs738409* genotypes

	SAMM50-rs738491			***PARVB-rs5764455***			***PNPLA3-rs738409***		
	CC+CT	**TT**	***p***	**GG+GA**	**AA**	***p***	**CC+CG**	**GG**	***p***
n (%)	282 (67.95)	133 (32.05)		328 (79.04)	87 (20.96)		316 (76.14)	99 (23.86)	
Female sex (%)	58 (20.6)	42 (31.6)	0.014	70 (21.30)	30 (34.50)	0.011	70 (22.2)	30 (30.3)	0.098
Age, years	43 (32–50)	41 (32–49)	0.655	42 (32–50)	42 (31–52)	0.841	42 (31–50)	42 (33–48)	0.977
BMI, kg/m^2	26.57 (24.22–28.68)	26.95 (24.63–28.80)	0.339	26.72 (24.22–28.67)	26.53 (24.65–28.73)	0.711	26.69 (24.22–28.83)	26.63 (24.62–28.67)	0.893
Hypertension			0.957			0.344			0.913
Yes	101 (35.8)	48 (36.1)		114 (34.8)	35 (40.2)		113 (35.8)	36 (36.4)	
No	181 (64.2)	85 (63.9)		214 (65.2)	52 (59.8)		203 (64.2)	63 (63.6)	
Type 2 diabetes			0.337			0.470			0.959
Yes	67 (23.8)	26 (19.5)		76 (23.2)	17 (19.5)		71 (22.5)	22 (22.2)	
No	215 (76.2)	107 (80.5)		252 (76.8)	70 (80.5)		245 (77.5)	77 (77.8)	
HOMA-IR	3.61 (2.43–5.60)	3.27 (2.19–5.15)	0.165	3.61 (2.42–5.44)	3.20 (2.11–5.29)	0.246	3.45 (2.35–5.43)	3.54 (2.22–5.42)	0.504
ALT, U/L	49 (29.7–88)	55 (36–99)	0.168	49 (30–88.7)	60 (37–101)	0.100	49.5 (30–89.7)	56 (36–91)	0.204
AST, U/L	33.5 (25–52)	36 (26–60)	0.202	32.5 (25–52)	38 (26–61)	0.051	33 (25.5–52)	37 (26–59)	0.165
GGT, U/L	51 (33–84.2)	52 (32–80.5)	0.750	52 (33–84.7)	51 (31–77)	0.383	51 (33–83)	51 (33–80)	0.709
TG, mmol/L	1.96 (1.33–2.92)	1.82 (1.44–2.54)	0.303	1.96 (1.40–2.91)	1.82 (1.33–2.50)	0.111	1.95 (1.35–2.92)	1.87 (1.45–2.41)	0.282
TC, mmol/L	4.94±1.12	5.20±1.12	0.027	4.97±1.11	5.22±1.15	0.059	4.99±1.12	5.13±1.12	0.263
HDL-C, mmol/L	0.96 (0.85–1.12)	1.01 (0.89–1.16)	0.031	0.96 (0.85–1.13)	1.03 (0.89–1.15)	0.070	0.96 (0.85–1.13)	1.02 (0.89–1.19)	0.107
LDL-C, mmol/L	2.96±0.89	3.23±0.89	0.004	2.98±0.87	3.30±0.96	0.003	3.00±0.89	3.18±0.92	0.074
UA, mmol/L	389 (325–470.2)	380 (327–452)	0.741	389 (325–466)	378 (334–456)	0.788	389.5 (325.5–469)	377 (325–441)	0.370
Liver histology features									
NAS ≥4			0.001			0.002			0.001
Yes	154 (54.6)	95 (71.4)		184 (56.1)	65 (74.7)		176 (55.7)	73 (73.7)	
No	128 (45.4)	38 (28.6)		144 (43.9)	22 (25.3)		140 (44.3)	26 (26.3)	
Fibrosis stage			0.893			0.847			0.730
F≥2	61 (21.6)	28 (21.1)		71 (21.6)	18 (20.7)		69 (21.8)	20 (20.2)	
F<2	221 (78.4)	105 (78.9)		399 (78.4)	68 (79.3)		247 (78.2)	79 (79.8)	

Sample size *n*=415. Data are expressed as number (percentage) for categorical variables, as mean±SD for normally distributed continuous variables and median (interquartile range) for skewed distributed continuous variables. Differences between the groups were determined using the Student's *t*-test or the Mann-Whitney *U* test for continuous variables, as well as the Pearson χ2 test or the Fisher's exact test for categorical variables. ALB, albumin; ALP, alkaline phosphatase.

Table 5. Associations between SNPs and individual histologic features of NAFLD

	Steatosis grade[a]		Hepatocyte ballooning[a]		Lobular inflammation[a]		NAS ≥4[b]		Significant fibrosis (F≥2)[b]	
	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*
SAMM50-rs738491										
CC+CT	Ref.		Ref.		Ref.		Ref.		Ref.	
TT	0.896 (0.202)	<0.0001	−0.113 (0.211)	0.591	0.327 (0.241)	0.175	0.676 (0.241)	0.005	−0.115 (0.262)	0.662
PARVB-rs5764455										
GG+GA	Ref.		Ref.		Ref.		Ref.		Ref.	
AA	0.952 (0.233)	<0.0001	−0.064 (0.242)	0.792	0.152 (0.275)	0.581	0.859 (0.289)	0.003	−0.144 (0.302)	0.634
PNPLA3-rs738409										
CC+CG	Ref.		Ref.		Ref.		Ref.		Ref.	
GG	0.920 (0.220)	<0.0001	−0.139 (0.230)	0.545	0.344 (0.261)	0.188	0.818 (0.268)	0.002	-0.144 (0.289)	0.618

Data are expressed as beta coefficient and standers error (SE). [a]Multivariate linear regression model adjusted for age, sex and BMI. [b]Multivariate logistic regression model adjusted for age, sex and BMI. BMI, body mass index; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; SNPs, single nucleotide polymorphisms.

mon complex traits. The GMDR methodology is widely used in detecting gene-gene and gene-environment interactions in various diseases.[46] Using the GMDR method, our results have identified the *PARVB-rs5764455* as the best single-locus model and the combination of *SAMM50-rs738491* and *PNPLA3-rs738409* as the best double-locus model for predicting the presence of NASH. These data further confirm the correctness of the hypothesis we mentioned above, i.e. the *PNPLA3* and *SAMM50* genes are, respectively, involved in the first and second stages of progression from simple steatosis to NASH, while the *PARVB* gene seems to be implicated in both of these stages. Our study showed that the risk of NASH among patients carrying the haplotype T-A-G was three-times higher than among those carrying the haplotype C-G-C. In addition, the OR of the haplotype T-A-G to C-G-C for predicting the risk of NASH was nearly twice compared to that of haplotype T-G to C-C or A to G, thereby suggesting a synergistic interaction between these three SNPs.

Our study has some important limitations that should be considered. First, we did not include control subjects without NAFLD to examine the allele frequency of *SAMM50, PARVB* and *PNPLA3* polymorphisms in NAFLD vs. non-NAFLD populations. Second, only one SNP in each of the *SAMM50, PARVB* and *PNPLA3* genes was chosen. The limited number of SNPs was therefore not likely to capture most of the genetic information conveyed by these three genes. Third, our results suggesting the existence of a synergistic interaction between the three aforementioned SNPs need further validation in larger cohorts of NAFLD patients of different ethnicity. Fourth, our case-finding strategy could have contributed to the high prevalence of NASH (59.3%) observed in this cohort of subjects with biopsy-proven NAFLD. Therefore, these results need to be further confirmed in populations at lower risk of NASH. Lastly, the underlying mechanisms that may explain the interactions linking these three SNPs to the pathophysiology of NASH need to be better elucidated.

Conclusions

Our results show for the first time that genetic variants of *SAMM50-rs738491*, *PARVB-rs5764455* and *PNPLA3-rs738409* are associated with the presence of NASH in Chinese individuals with biopsy-proven NAFLD, independent of age, sex and BMI. Furthermore, we have also shown that *SAMM50-rs738491*, *PARVB-rs5764455* and *PNPLA3-rs738409* genetic variants may interact synergistically to increase the susceptibility to NASH.

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Table 6. Best models to predict the presence of NASH by GMDR analysis[a]

GMDR model	Training accuracy (%)	Testing accuracy (%)	Sign test (*p*)	CVC
PARVB-rs5764455	57.71	52.05	8 (0.055)	4/10
SAMM50-rs738491, PNPLA3-rs738409	59.90	57.80	8 (0.055)	10/10
SAMM50-rs738491, PARVB-rs5764455, PNPLA3-rs738409	60.59	57.23	9 (0.011)	10/10

[a]Data adjusted for age, sex and BMI. BMI, body mass index; CVC, cross-validation consistency; GMDR, generalized multifactor dimensionality reduction; NASH, nonalcoholic steatohepatitis.

Table 7. Interaction analysis between each best genetic model and risk of having NASH

Genotypes and alleles	*n* (%)		Adjusted OR (95% CI)[a]	*p*
	Non-NASH	NASH		
PARVB-rs5764455				
Genotypes				
GG+GA	147 (86.98)	181 (73.58)	1	
AA	22 (13.02)	65 (26.42)	2.431(1.384, 4.269)	0.002
Alleles				
G	211 (62.43)	238 (48.37)	1	
A	127 (37.57)	254 (51.63)	1.744 (1.293, 2.352)	<0.0001
SAMM50-rs738491, PNPLA3-rs738409				
Genotypes				
CC+CT, CC+CG	129 (84.31)	147 (68.06)	1	
TT, GG	24 (15.69)	69 (31.94)	2.453 (1.413, 4.259)	0.001
Alleles				
C-C	172 (57.33)	185 (42.63)	1	
T-G	128 (42.67)	249 (57.37)	1.733 (1.261, 2.381)	0.001
SAMM50-rs738491, PARVB-rs5764455, PNPLA3-rs738409				
Genotypes				
CC+CT, GG+GA, CC+CG	129 (88.97)	144 (73.85)	1	
TT, AA, GG	16 (11.03)	51 (26.15)	2.751 (1.452, 5.211)	0.002
Alleles				
C-G-C	105 (70.00)	94 (41.23)	1	
T-A-G	45 (30.00)	134 (58.77)	3.157 (1.988, 5.012)	<0.0001

[a]Multivariate logistic regression model adjusted for age, sex and BMI. BMI, body mass index; CI, confidence interval; NASH, nonalcoholic steatohepatitis; OR, odds ratio.

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Conflict of interest

MHZ has been an associate editor of *Journal of Clinical and Translational Hepatology* since 2013. The other authors have no conflicts of interest related to this publication.

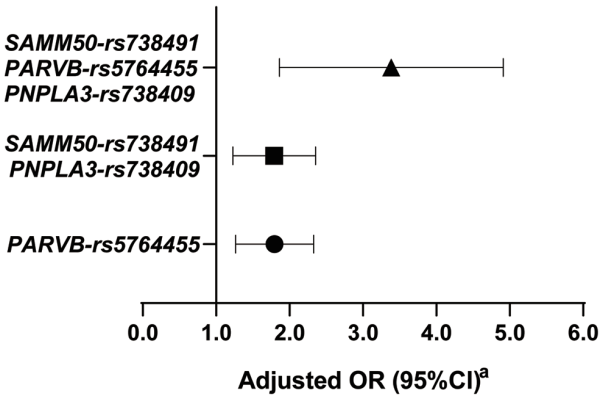

Fig. 1. Interaction analysis between each best genetic model and risk of having NASH. [a]OR (95% CI) adjusted for age, sex and BMI. BMI, body mass index; CI, confidence interval; NASH, nonalcoholic steatohepatitis; OR, odds ratio.

Author contributions

Study concept and design (KX, MHZ), acquisition of data (PWZ, WYL, HLM, GL, LJT, RSR), pathological analyses (XDW), drafting of the manuscript (KX, KIZ), critical revision (GT, CDB), statistical analyses (KX, KIZ), study supervision (YPC, MHZ), and guarantee of the article (MHZ). All authors contributed to the manuscript for important intellectual content and approved the submission.

Ethical statement

Ethical approval was obtained from the First Affiliated Hospital of Wenzhou Medical University Ethics Committee and the study protocol was registered at the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562). Written informed consent was obtained from all participants included in the study.

Data sharing statement

The clinical and histological data used to support the findings of this study are available from the corresponding author upon request.

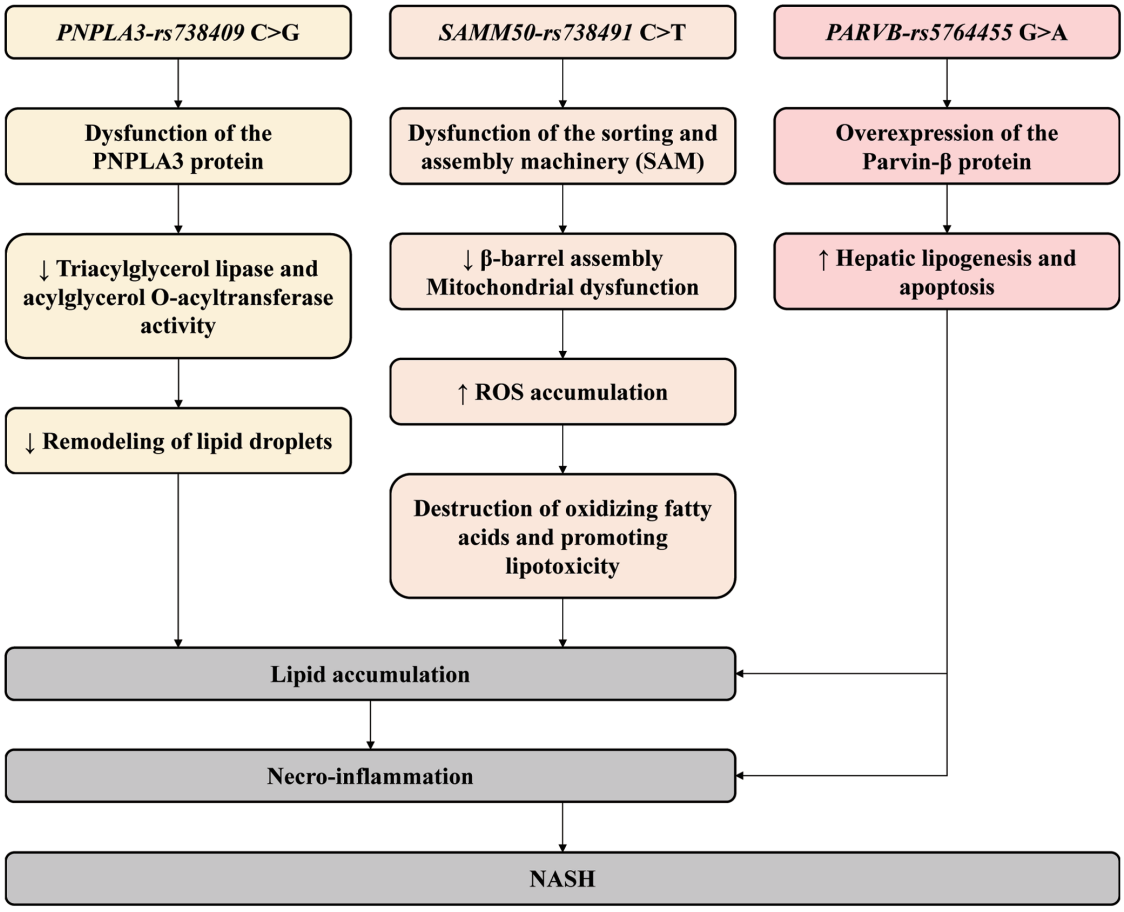

Fig. 2. Putative mechanisms by which these three polymorphisms may affect susceptibility to NASH. NASH, nonalcoholic steatohepatitis.

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