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Basic Study

***PNPLA3* rs139051 is associated with phospholipid metabolite profile and hepatic inflammation in nonalcoholic fatty liver disease**

Ji-Jun Luo, Hai-Xia Cao, Rui-Xu Yang, Rui-Nan Zhang, Qin Pan

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Abstract**AIM**

To investigate the effect of *PNPLA3* polymorphisms on serum lipidomics and pathological characteristics in nonalcoholic fatty liver disease (NAFLD).

METHODS

Thirty-four biopsy-proven NAFLD patients from Northern, Central, and Southern China were subjected to stratification by genotyping their single nucleotide polymorphisms (SNPs) in *PNPLA3*. Ultra performance liquid chromatography-tandem mass spectrometry was then employed to characterize the effects of *PNPLA3* SNPs on serum lipidomics. In succession, correlation analysis revealed the association of *PNPLA3*-related lipid profile and hepatic pathological characteristics on a basis of steatosis, activity, and fibrosis assessment. The variant-based scoring of hepatocyte steatosis, ballooning, lobular inflammation, and liver fibrosis was finally performed so as to uncover the actions of lipidomics-affecting *PNPLA3*

SNPs in NAFLD-specific pathological alterations.

RESULTS

PNPLA3 SNPs (rs139051, rs738408, rs738409, rs2072906, rs2294918, rs2294919, and rs4823173) demonstrated extensive association with the serum lipidomics, especially phospholipid metabolites [lysophosphatidylcholine (LPC), lysophosphatidylcholine plasmalogen (LPCO), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), choline plasmalogen (PCO), phosphatidylethanolamine (PE), ethanolamine plasmalogen (PEO)], of NAFLD patients. *PNPLA3* rs139051 (A/A genotype) and rs2294918 (G/G genotype) dominated the up-regulatory effect on phospholipids of LPCs (LPC 17:0, LPC 18:0, LPC 20:0, LPC 20:1, LPC 20:2) and LPCOs (LPC O-16:1, LPC O-18:1). Moreover, subjects with high-level LPCs/LPCOs were predisposed to low-grade lobular inflammation of NAFLD (ρ : -0.407 to -0.585, $P < 0.05$ -0.001). The significant correlation of *PNPLA3* rs139051 and inflammation grading [A/A vs A/G + G/G: 0.50 (0.00, 1.75) vs 1.50 (1.00, 2.00), $P < 0.05$] further demonstrated its pathological role based on the modulation of phospholipid metabolite profile.

CONCLUSION

The A/A genotype at *PNPLA3* rs139051 exerts an up-regulatory effect on serum phospholipids of LPCs and LPCOs, which are associated with low-grade lobular inflammation of NAFLD.

Key words: Nonalcoholic fatty liver disease; Patatin-like phospholipase domain containing 3; Single nucleotide polymorphism; Phospholipid; Inflammation

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Core tip: *PNPLA3* single nucleotide polymorphisms reflect an important genetic basis of serum lipidomics, especially phospholipid metabolites, in nonalcoholic fatty liver disease (NAFLD) patients. *PNPLA3* rs139051 (A/A genotype) exerts an up-regulatory effect on phospholipids of lysophosphatidylcholines (LPCs) and lysophosphatidylcholine plasmalogens (LPCOs). Moreover, both the A/A genotype at *PNPLA3* rs139051 and high-level LPCs/LPCOs share an association with the low-grade lobular inflammation of NAFLD. Therefore, *PNPLA3* rs139051 may underlie the inflammatory progress of NAFLD with its modulation of phospholipid metabolite profiles.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by excess triglyceride accumulation and lobular inflammatory infiltration and is a leading cause of most chronic liver diseases in European, Asian-Pacific, and American patients^[1]. According to the diagnosis and management guidelines, hyperlipidemia (e.g., hypertriglyceridemia or hypercholesterolemia) has been identified as one of the most important risk factors for NAFLD^[2]. Approximately 50% of patients with hyperlipidemia have ultrasonographic evidence of fatty infiltration in the liver^[3]. Patients with a spectrum of NAFLD, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), show prevalence of dyslipidemia, including increased serum triglycerides and low-density lipoprotein (LDL), and low levels of high-density lipoprotein cholesterol (HDL-C)^[4]. Thus, the lipidemic properties are closely associated with the initiation and progression of NAFLD.

Recently, genome-wide association analysis and clinical investigations have found that single nucleotide polymorphisms (SNPs) of patatin-like phospholipase domain containing 3 (*PNPLA3*) (e.g., rs738409, rs1010023, rs2281135, rs139051 and rs2294918) underlie the genetic susceptibility of NAFLD, independent of gender, age and ethnic background^[5-11]. Functional studies have highlighted that adiponutrin, the encoded product of *PNPLA3*, is a crucial regulator of lipid metabolism in the liver via its activities on triacylglycerol lipase and acyl glycerol O-acyltransferase^[12]. Given the central role of the liver in systemic lipid homeostasis, these loss-of-function SNPs in *PNPLA3* are suggested to predispose individuals to NAFLD, probably by dysregulation of hepatic and serum lipid profiles^[4]. This lipid-metabolism-regulating role of *PNPLA3* SNPs has been confirmed in the liver of NAFLD patients^[13]. However, understanding of the SNP-specific impact on serum lipids and their correlation with pathological characteristics is still limited and controversial^[14-17].

We therefore stratified Chinese Han patients with biopsy-proven NAFLD by genotyping their *PNPLA3* SNPs. Ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to characterize the effects of *PNPLA3* SNPs on serum lipidomics of NAFLD patients. Correlation analysis uncovered the association of *PNPLA3*-related lipid profile and hepatic pathological characteristics by assessment of steatosis, activity and fibrosis (SAF). Finally, variant-based scoring of steatosis, ballooning, inflammation, and liver fibrosis was performed to reveal the actions of lipidomics-affecting *PNPLA3* SNPs on NAFLD-specific pathological alterations.

MATERIALS AND METHODS

Study population

Thirty-four Chinese Han patients with biopsy-proven NAFLD were enrolled from Shanghai Xinhua Hospital (n

= 17), Zhengxing Hospital ($n = 8$) and Tianjin Hospital of Infectious Diseases ($n = 9$) between January 2012 and June 2013. The exclusion criteria were as follows: historic or current high alcohol consumption equivalent to > 20 g/d for men and > 10 g/d for women^[18,19], viral hepatitis, drug-induced liver disease, Wilson's disease, autoimmune liver diseases and other diseases that lead to steatosis. The study was approved by the Xinhua Hospital Research Ethics Committee, and informed consent was obtained from all patients.

Anthropometric and biochemical assessments

The baseline data of age (41.03 ± 14.81 years), gender, height (167.44 ± 7.82 cm), weight (75.34 ± 9.49 kg), and body mass index (BMI) (26.90 ± 3.13) were characterized for the study population. Fasting blood samples were collected from the NAFLD patients, and a multichannel automatic analyzer (Advia 1650; Bayer, Moss, Norway) was used to test biochemical indexes of alanine aminotransferase (58.33 ± 33.89 U/L), aspartate aminotransferase (24.05 ± 28.63 U/L), alkaline phosphatase (101.90 ± 110.49 U/L), and γ -glutamyltransferase (115.73 ± 278.59 U/L). Fasting blood glucose (42.70 ± 28.07 μ U/mL), total cholesterol (4.70 ± 0.61 mg/dL), and triglyceride (1.70 ± 0.68 mg/dL) were subjected to assessment using Wako Bioproducts (Wako Pure Chemical Industries, Richmond, VA, United States).

Hepatic histopathological assessment

Liver samples from each NAFLD patient were obtained by needle biopsy after obtaining informed consent. Liver tissues were fixed in formalin, paraffin embedded, and 5- μ m sections were cut. Both hematoxylin and eosin and Masson's trichrome staining were used for pathological characterization, including hepatocyte steatosis, lobular inflammation, ballooning, and liver fibrosis according to the SAF scoring method^[20-22], by three pathologists who were not aware of the study.

Genotyping of *PNPLA3* SNPs

Blood samples from NAFLD patients were treated as follows: (1) centrifugation at 1500 rpm for 10 min; (2) separation of buffy-coat layer; and (3) extraction of genomic DNA from the buffy coat lymphocytes by QiAamp DNA Mini Kit (Qiagen, Venlo, Netherlands). A custom Ion AmpliSeq panel (Life Technologies Thermo Fisher Scientific, Waltham, MA, United States) of human *PNPLA3* was designed for the emulsion polymerase chain reaction of template DNA using a Ion OneTouch 2 System (Life Technologies). *PNPLA3* SNPs were genotyped by the Ion 318 Chip (Life Technologies) according to the Ion PGM 200 Sequencing kit protocol^[23].

UPLC-MS/MS

After 12-h fasting, serum lipidomics of the enrolled NAFLD patients were analyzed with a combination of

UPLC (Waters, Milford, MA, United States) and Triple TOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, United States)^[24]. The column temperature was set to 55°C. The ratios of acetonitrile/water (mobile phase A) and propanol/acetonitrile (mobile phase B) were 3/2 and 9/1, respectively, with 10 mmol/L ammonium acetate added to all mobile phases. The elution gradient program was carried out as follows: 0-1.5 min: 32% B; 1.5-14 min: 85% B; 15.5-15.6 min: 97% B; 15.6-18 min: 97% B; 18-20 min: 32% B (flow rate: 0.26 mL/min). MS was applied by electrospray ionization operating in the positive and negative ion modes. The temperature of the interface heater was 600°C (–) and 500°C (+), with 4500 V (–) and 5500 V (+) ion spray voltage. The declustering potential was 100 V (–) and 100 V (+), and collision energy was 10 V (–) and 10 V (+). Thirteen quality control (QC) samples, being randomly inserted into the sequence, were analyzed for data precision. The raw data obtained from UPLC-MS/MS by Analyst TF 1.6 software (AB SCIEX) were subjected to identification of lipid composition using LipidView/PeakView and quantification of lipid concentrations using MultiQuant 2.0. The relative standard deviation (RSD) of 239 serum lipids in QC samples was evaluated against the internal standards.

Statistical analysis

The clinical data were expressed as mean \pm SD (continuous, normally distributed variables) or medians (interquartile range) (discontinuous, non-normally distributed variables). Differences in serum lipidomics among the groups of *PNPLA3* SNPs were investigated by unpaired Student's independent *t* tests. Correlation analysis of phospholipid metabolite profile and hepatic pathological characteristics was assessed by Spearman's correlation. The Mann-Whitney *U* test was performed to evaluate the differences in pathological grading among groups of *PNPLA3* SNPs. SPSS version 19.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis with a two-side significant criterion at $P < 0.05$.

RESULTS

***PNPLA3* SNPs associated with serum lipidomics in NAFLD patients**

With the stable distribution and limited RSD of QC samples (Figure 1 and Table 1), an obvious association of *PNPLA3* SNPs (rs139051, rs738408, rs738409, rs2072906, rs2294918, rs2294919 and rs4823173) and serum lipidomic characteristics was observed in Chinese Han NAFLD patients by combination of *PNPLA3* genotyping and UPLC-MS/MS (Table 2). In detail, these *PNPLA3* SNPs significantly correlated to the serum level of various members of cholesteryl ester (CE), free fatty acid (FFA), lyso-phosphatidylcholine (LPC), lysophosphatidylcholine plasmalogen (LPCO), lysophosphatidylethanolamine

Table 1 Relative standard deviation distribution in quality control samples

Percentage	Relative SD				
	< 10%	10%-15%	15%-20%	20%-30%	30%-50%
Peak number in total	10	47	32	10	1
Sum of peak number in total	10	57	89	99	100

Table 2 *PNPLA3* single nucleotide polymorphisms associated with serum lipidomics of nonalcoholic fatty liver disease patients

SNPs	Number of SNP-associated serum lipids									
	CE	FFA	LPC	LPCO	LPE	PC	PCO	PE	PEO	TAG
rs139051 (A/A: A/G + G/G)	1	1	5	2	1	0	0	1	1	0
rs738408 (T/T: C/T + C/C)	1	0	0	0	0	0	0	0	0	0
rs738409 (G/G: C/G + C/C)	1	0	0	0	0	0	0	0	0	0
rs2072906 (G/G: A/G + A/A)	1	0	0	0	0	0	1	0	0	0
rs2294918 (G/G: A/A + A/G)	0	0	3	3	1	1	0	9	0	5
rs2294919 (C/C: C/T + T/T)	0	1	0	0	0	0	0	0	0	0
rs4823173 (A/A: A/G + G/G)	1	0	0	0	0	0	0	0	0	0

CE: Cholesteryl ester; FFA: Free fatty acid; LPC: Lysophosphatidylcholine; LPCO: Lysophosphatidylcholine plasmalogen; LPE: Lysophosphatidylethanolamine; NAFLD: Nonalcoholic fatty liver disease; PC: Phosphatidylcholine; PCO: Choline plasmalogen; PE: Phosphatidylethanolamine; PEO: Ethanolamine plasmalogen; *PNPLA3*: Patatin-like phospholipase domain containing 3; SNP: Single nucleotide polymorphisms; TAG: Triacylglycerol.

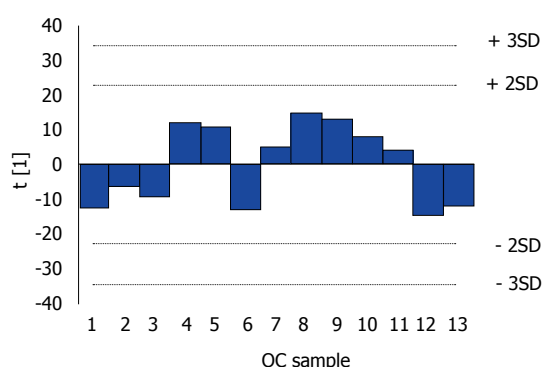


Figure 1 Dynamic distribution of quality control samples throughout the Ultra-high performance liquid chromatography-tandem mass spectrometry analysis. Colored bars indicate the quality control samples that are projected onto the first principal component with a range from -2 SD to +2 SD. QC: Quality control; SD: Standard deviation.

(LPE), phosphatidylcholine (PC), choline plasmalogen (PCO), phosphatidylethanolamine (PE), ethanolamine plasmalogen (PEO), and triacylglycerol (TAG) (Table 2).

***PNPLA3* rs139051 and rs2294918 exerted upregulatory effects on LPCs and LPCOs**

PNPLA3 rs139051 and rs2294918, with their effects on 12 and 22 different serum lipids, respectively, dominated the *PNPLA3* SNP-lipidomics association (Table 2). Compared to those with the A/G or G/G genotype, NAFLD patients carrying the A/A genotype at *PNPLA3* rs139051 demonstrated significantly higher serum

levels of LPC 17:0, LPC 18:0, LPC 20:0, LPC 20:1, LPC 20:2, LPC O-16:1, LPC O-18:1, and significantly lower levels of LPE 20:4, PE 34:0 and PE O-36:5 (Table 3). Quantitative analysis showed significantly increasing levels of LPC 17:0, LPC 20:0, LPC 20:1, LPC O-16:0, LPC O-16:1, and LPC O-18:1 in the NAFLD patients with the G/G phenotype compared to the A/A or A/G phenotype at *PNPLA3* rs2294918 (Table 3). Nevertheless, there was lower serum levels of LPE 22:6, PC 32:1, PE 34:0, PE 34:2, PE 36:2, PE 36:4, PE 38:4, PE 38:5, PE 38:6, PE 40:5 and PE 40:6 in these patients (Table 3).

High levels of LPCs and LPCOs demonstrated a correlation with low-grade hepatic inflammation

To shed light on the influence of *PNPLA3* SNP-related lipidomic characteristics, correlations of phospholipid metabolites and NAFLD-specific pathological disorders (hepatocyte steatosis, lobular inflammation, ballooning, and liver fibrosis) were analyzed. Various LPCs (LPC 17:0, LPC 18:0, LPC 20:0, LPC 20:1 and LPC 20:2) and LPCOs (LPC O-16:1 and LPC O-18:1) showed negative correlations with the grade of lobular inflammation. The serum levels of these phospholipid metabolites were high in NAFLD patients with the A/A genotype at *PNPLA3* rs139051 and/or the G/G genotype at rs2294918. Most of them shared similar Spearman's rank correlation coefficients (Table 4). In contrast, pathological indexes other than lobular inflammation correlated to neither high-level phospholipid metabolites

Table 3 Effects of *PNPLA3* rs139051 and rs2294918 on serum profile of phospholipid metabolites in nonalcoholic fatty liver disease patients

Phospholipid metabolites	SNPs					
	rs139051			rs2294918		
	A/A	A/G + G/G	P value	G/G	A/A + A/G	P value
LPC 17:0	0.148 ± 0.119	0.089 ± 0.032	0.045 ^a	0.143 ± 0.113	0.084 ± 0.023	0.023 ^a
LPC 18:0	5.501 ± 4.064	3.482 ± 1.032	0.045 ^a	5.199 ± 3.888	3.564 ± 0.941	0.068
LPC 20:0	0.019 ± 0.012	0.011 ± 0.005	0.013 ^a	0.018 ± 0.011	0.010 ± 0.005	0.008 ^a
LPC 20:1	0.035 ± 0.028	0.020 ± 0.072	0.031 ^a	0.033 ± 0.026	0.018 ± 0.007	0.017 ^a
LPC 20:2	0.046 ± 0.040	0.025 ± 0.090	0.037 ^a	0.042 ± 0.038	0.027 ± 0.010	0.091
LPC O-16:0	0.128 ± 0.109	0.078 ± 0.016	0.058	0.123 ± 0.102	0.075 ± 0.016	0.038 ^a
LPC O-16:1	0.111 ± 0.088	0.068 ± 0.016	0.046 ^a	0.106 ± 0.083	0.067 ± 0.014	0.041 ^a
LPC O-18:1	0.076 ± 0.071	0.041 ± 0.011	0.039 ^a	0.072 ± 0.067	0.040 ± 0.011	0.036 ^a
LPE 20:4	0.087 ± 0.037	0.116 ± 0.035	0.029 ^a	0.092 ± 0.040	0.113 ± 0.034	0.143
LPE 22:6	0.091 ± 0.044	0.113 ± 0.037	0.138	0.090 ± 0.036	0.122 ± 0.046	0.035 ^a
PC 32:1	1.574 ± 0.941	1.912 ± 1.167	0.358	1.450 ± 0.949	2.263 ± 1.036	0.030 ^a
PE 34:0	0.312 ± 0.018	0.325 ± 0.016	0.041 ^a	0.310 ± 0.015	0.332 ± 0.015	0.001 ^a
PE 34:2	0.335 ± 0.187	0.309 ± 0.159	0.673	0.280 ± 0.131	0.418 ± 0.219	0.028 ^a
PE 36:2	0.758 ± 0.349	0.702 ± 0.324	0.634	0.650 ± 0.271	0.913 ± 0.398	0.030 ^a
PE 36:4	0.282 ± 0.142	0.324 ± 0.169	0.435	0.246 ± 0.105	0.410 ± 0.181	0.015 ^a
PE 38:4	0.746 ± 0.349	0.830 ± 0.348	0.493	0.661 ± 0.228	1.031 ± 0.423	0.018 ^a
PE 38:5	0.079 ± 0.036	0.079 ± 0.034	0.960	0.070 ± 0.026	0.098 ± 0.043	0.025 ^a
PE 38:6	0.621 ± 0.431	0.613 ± 0.332	0.953	0.493 ± 0.232	0.878 ± 0.517	0.036 ^a
PE 40:5	0.053 ± 0.026	0.064 ± 0.033	0.305	0.047 ± 0.022	0.080 ± 0.030	0.001 ^a
PE 40:6	0.445 ± 0.316	0.451 ± 0.220	0.952	0.354 ± 0.174	0.644 ± 0.351	0.003 ^a
PE O-36:5	0.529 ± 0.192	0.699 ± 0.276	0.041 ^a	0.547 ± 0.171	0.707 ± 0.331	0.071

LPC: Lysophosphatidylcholine; LPCO: Lysophosphatidylcholine plasmalogen; LPE: Lysophosphatidylethanolamine; NAFLD: Nonalcoholic fatty liver disease; PC: Phosphatidylcholine; PCO: Choline plasmalogen; PE: Phosphatidylethanolamine; PEO: Ethanolamine plasmalogen; *PNPLA3*: Patatin-like phospholipase domain containing 3. Values are expressed as mean ± SD. ^a*P* < 0.05.

Table 4 Phospholipid metabolites correlated with pathological characteristics of nonalcoholic fatty liver disease

Phospholipid metabolites	Steatosis		Lobular inflammation		Ballooning		Fibrosis	
	rho	P value	rho	P value	rho	P value	rho	P value
LPC 17:0	0.149	0.399	-0.525	0.001 ^a	0.093	0.599	0.095	0.592
LPC 18:0	0.024	0.892	-0.478	0.004 ^a	0.136	0.442	0.089	0.618
LPC 20:0	-0.071	0.692	-0.585	0.000 ^a	0.010	0.956	0.082	0.645
LPC 20:1	-0.061	0.734	-0.489	0.003 ^a	-0.165	0.352	-0.072	0.686
LPC 20:2	0.092	0.604	-0.453	0.007 ^a	0.145	0.415	0.042	0.812
LPC O-16:0	0.297	0.088	-0.296	0.089	0.025	0.886	0.146	0.410
LPC O-16:1	0.070	0.695	-0.425	0.012 ^a	0.028	0.874	0.027	0.881
LPC O-18:1	-0.114	0.521	-0.407	0.017 ^a	-0.018	0.921	0.107	0.546
LPE 20:4	-0.079	0.658	0.107	0.547	0.071	0.690	-0.071	0.692
LPE 22:6	-0.274	0.117	0.054	0.764	0.125	0.481	0.173	0.327
PC 32:1	0.020	0.913	0.086	0.630	0.107	0.547	-0.069	0.699
PE 34:0	-0.012	0.946	0.193	0.275	-0.173	0.327	-0.146	0.410
PE 34:2	-0.054	0.762	-0.025	0.888	-0.043	0.809	-0.153	0.388
PE 36:2	-0.050	0.777	-0.079	0.659	-0.118	0.506	-0.257	0.143
PE 36:4	-0.091	0.608	0.150	0.398	0.121	0.495	-0.052	0.770
PE 38:4	-0.112	0.529	0.214	0.224	0.110	0.537	-0.065	0.716
PE 38:5	-0.234	0.182	0.178	0.313	-0.026	0.885	-0.096	0.587
PE 38:6	-0.151	0.393	0.096	0.588	0.176	0.320	0.105	0.554
PE 40:5	-0.013	0.944	0.132	0.457	0.049	0.783	-0.194	0.273
PE 40:6	-0.192	0.277	0.132	0.457	0.150	0.397	0.067	0.705
PE O-36:5	0.021	0.905	0.178	0.313	-0.205	0.245	-0.131	0.461

LPC: Lysophosphatidylcholine; LPCO: Lysophosphatidylcholine plasmalogen; LPE: Lysophosphatidylethanolamine; NAFLD: Nonalcoholic fatty liver disease; PC: Phosphatidylcholine; PCO: Choline plasmalogen; PE: Phosphatidylethanolamine; PEO: Ethanolamine plasmalogen; rho: Spearman's rank correlation coefficient. ^a*P* < 0.05.

(LPCs and LPCOs) nor low-level ones (LPEs, PC 32:1, PEs and PEO 36:5) in these NAFLD patients (Table 4). Given their impact on the serum profile of phospholipid metabolites, both *PNPLA3* rs139051 and rs2294918 are proposed to act in hepatic inflammation of NAFLD by

targeting, at least to a large extent, LPCs and LPCOs.

Low-grade hepatic inflammation occurred in NAFLD patients with the A/A genotype at *PNPLA3* rs139051

After the stratification of NAFLD patients by *PNPLA3*

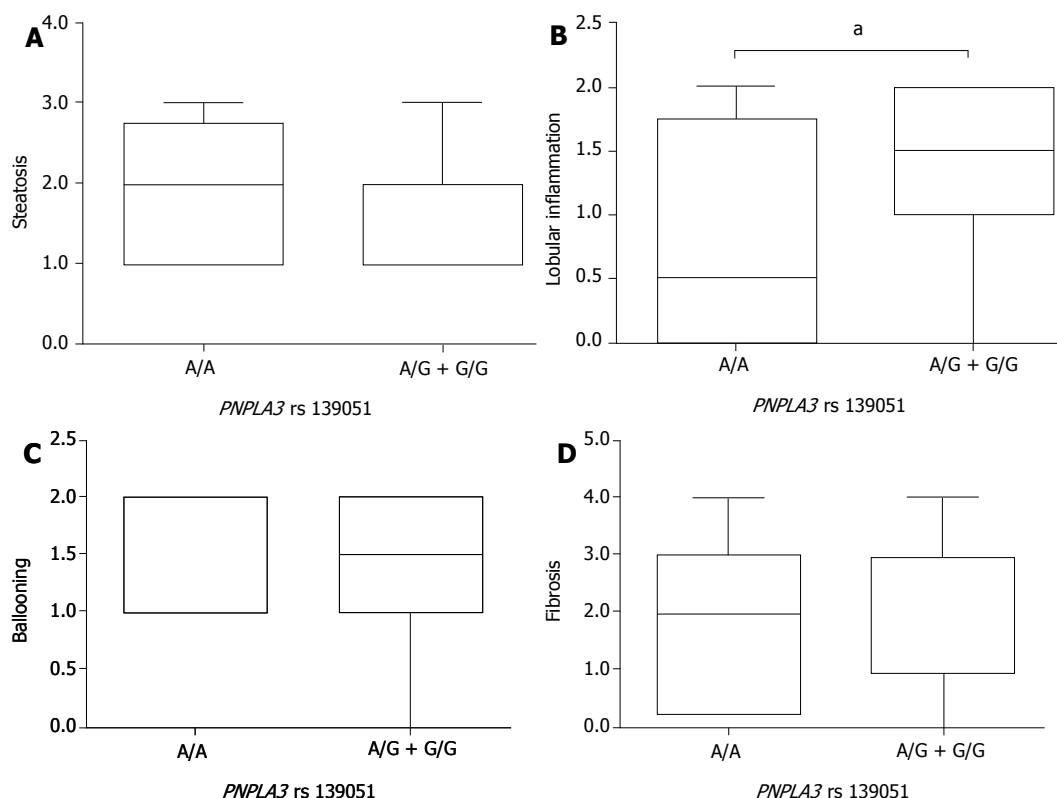


Figure 2 Nonalcoholic fatty liver disease patients carrying the A/A genotype at *PNPLA3* rs139051 demonstrated low-grade lobular inflammation. Box plots indicate the difference in pathologic characteristics of steatosis (A); lobular inflammation (B); ballooning (C); and fibrosis (D) between nonalcoholic fatty liver disease patients with the A/A or A/G + G/G genotype at *PNPLA3* rs139051. Results are presented as medians and Interquartile Range. ^a $P < 0.05$.

genotypes, comparison of steatosis, lobular inflammation, ballooning, and liver fibrosis was carried out to assess the pathological role of *PNPLA3* rs139051 and rs2294918. By the SAF-based scoring, the A/A genotype at *PNPLA3* rs139051 conferred significantly lower lobular inflammation than the A/G + G/G genotypes (Figure 2). However, *PNPLA3* rs139051 did not seem to exert any significant impact on NAFLD-specific steatosis, ballooning, and fibrosis (Figure 2). NAFLD patients with *PNPLA3* rs2294918, another SNP related to the inflammation-associated phospholipid metabolites, showed a comparable grade in each of these pathological indexes regardless of genotype (Figure 3).

DISCUSSION

PNPLA3 is the liver-enriched member of the PNPLA family, which is located on the membrane of lipid droplets and endoplasmic reticulum in hepatocytes^[25,26]. Its conserved patatin-like domain demonstrates hydrolase activity against TAG, diacylglycerol and monoacylglycerol^[15]. Activities of thioesterase and acylglycerol transacylase reflect the other aspects of the lipometabolic action of *PNPLA3*^[15]. *PNPLA3* was recently identified to promote the transfer of very-long-chain polyunsaturated fatty acids from TAG to phospholipids^[27]. These effects shed light on an important regulatory role of *PNPLA3* in lipid homeostasis^[13], which is supposed to be affected by

PNPLA3-related genetic variants.

In the present study, genotype-based, widespread effects of *PNPLA3* SNPs in lipid profiles were found in the sera of NAFLD patients from Northern (Tianjin), Central (Shanghai) and Southern China (Zhangzhou, Fujian). Being similar to previous reports^[13,28,29], members of TAG, CE and FFA were identified in the differential serum lipids associated with *PNPLA3* SNPs (e.g., rs738408, rs738409, rs2072906, rs2294919 and rs4823173). In contrast, *PNPLA3* rs139051 and rs2294918 primarily exerted their lipidomic impact on phospholipid metabolites. LPCs, LPCOs and PEs were confirmed to dominate the differential serum lipids because of their high abundance. Therefore, dysregulation of phospholipid metabolite profile reflects the major role of *PNPLA3* SNPs in serum lipidomics.

To gain further insight into the *PNPLA3*-related phospholipid characteristics, differences in serum levels of LPC, LPCO and PE were analyzed between various genotypes of *PNPLA3* rs139051 and rs2294918. NAFLD patients with the A/A instead of A/G + G/G genotype at *PNPLA3* rs139051 exhibited significantly higher levels of LPCs and LPCOs. The G/G, but not the A/A + A/G, genotype of *PNPLA3* rs2294918 also predisposed NAFLD patients to statistical elevation of serum LPCs and LPCOs. Various types of LPCs (e.g., LPC 17:0 and LPC 18:0) have been shown to play a critical role in the spectrum of NAFLD^[30,31]. Their reduction in blood samples was detected in patients with ¹H-MRS- or

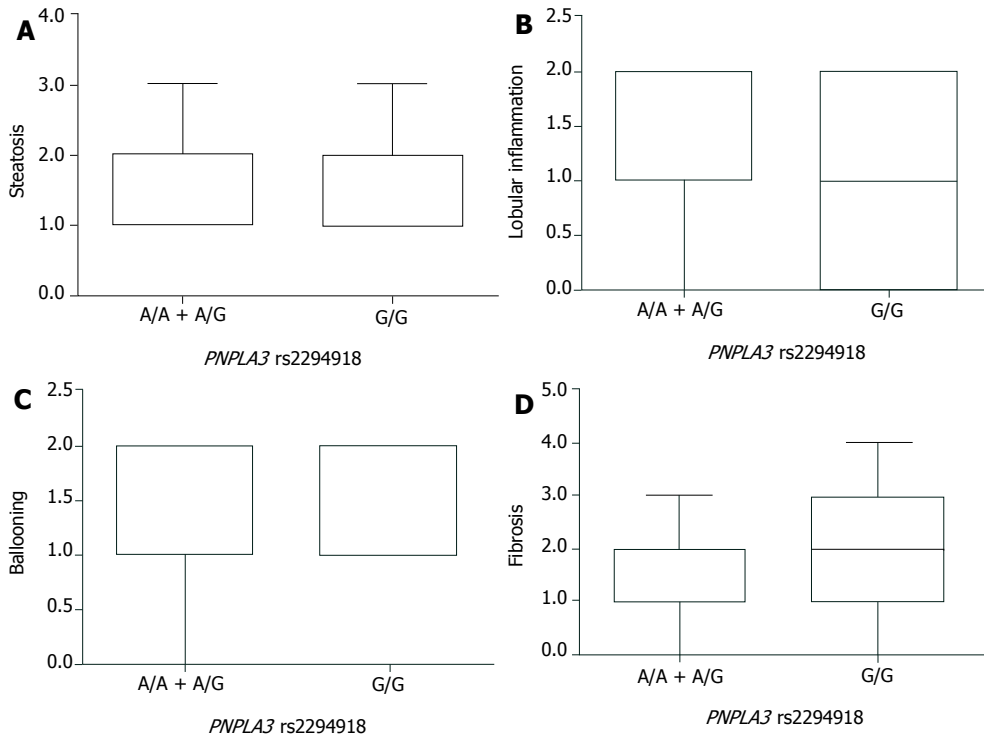


Figure 3 *PNPLA3* rs2294918 showed no association with pathologic characteristics of nonalcoholic fatty liver disease patients. Box plots indicate the grade of steatosis (A); lobular inflammation (B); ballooning (C); and fibrosis (D) in nonalcoholic fatty liver disease patients with the G/G or A/A + A/G genotype at *PNPLA3* rs2294918. Results are presented as medians and Interquartile Range.

biopsy-proven hepatic steatosis^[30]. Similarly, significant decreases in serum palmitoyl-, stearoyl- and oleoyl-LPC were obtained in an experimental rodent model of NASH^[32]. In contrast, uptake of LPC metabolite (PC) ameliorates the hepatic injury of NAFLD with a reduction of serum aspartate aminotransferase and alanine aminotransferase^[33]. Given the similarity in LPC- and LPCO-based lipid metabolism^[32,34-37], *PNPLA3* rs139051 and rs2294918 are suggested to protect the liver from NAFLD-related impairment by their upregulatory effect on both LPCs and LPCOs.

In contrast to its correlation with LPCs and LPCOs upregulation, the G/G genotype at *PNPLA3* rs2294918 conferred significantly lower levels of PEs in the NAFLD patients compared to those with the A/A + A/G genotype. A similar decrease in serum PE 34:0 was found in these NAFLD patients in association with the A/A genotype at *PNPLA3* rs139051. Recent studies have highlighted a growing increase in PE concentration during progression of NAFLD (healthy control < simple steatosis < NASH)^[38]. Nevertheless, individuals carrying the Val175Met variant allele of PE N-methyltransferase (PEMT), which inhibits PE to PC conversion, show high susceptibility to NASH^[39]. PEMT^{-/-} mice also suffer from NASH after being fed a choline-deficient or high-fat diet^[40,41]. Thus, *PNPLA3* rs139051 and rs2294918 represent a novel layer of genetic regulation that down-regulates the harmful components of lipidomics.

Metabolically, LPCs are catabolized into PCs by the lysophosphatidylcholine acyltransferase 1/2/4^[32].

Glycerophosphocholine, the lysoplasmalogenase-dependent catalysate of LPCOs, serves as another precursor of PCs^[36,37]. As a result, the upregulation of LPCs and LPCOs, together with the downregulation of PEs, may lead to an increased ratio of PC to PE in NAFLD patients with the A/A genotype at *PNPLA3* rs139051 and/or G/G at *PNPLA3* rs2294918. Acting as the major source of serum phospholipids, hepatic PCs and PEs play an essential part in the integrity of cellular and organelle membranes^[41,42]. An abnormally low molar ratio of PC/PE has been linked to steatohepatitis due to its induction of membrane leakage^[43]. On the contrary, administration of polyene PC prevents patients from hepatic injury, with an improvement in PC/PE ratio^[44]. Because of the antioxidant propensity of plasmalogen, LPCO deficiency is responsible for additional effects of NASH that sensitize patients to reactive-oxygen-species-dependent peroxidation, and successive lobular inflammation^[45,46]. An inflammation-attenuating effect of *PNPLA3* rs139051 and rs2294918 is then proposed on the basis of LPCs and LPCOs augmentation.

Indeed, our experimental observations relating to NAFLD patients revealed that an increase in LPCs and LPCOs significantly correlated with an attenuation of hepatic inflammation. Both high-level LPCs/LPCOs and low-grade lobular inflammation characterized patients with the A/A genotype at *PNPLA3* rs139051 and/or the G/G genotype at rs2294918. However, pathological characteristics other than hepatic inflammation, including hepatocyte steatosis, ballooning, and liver

fibrosis, were not associated with either of these phospholipid metabolites. *PNPLA3* genotyping and SAF-based pathological grading confirmed the phospholipid-mediated effect of *PNPLA3* SNPs on NAFLD. There was a lower grade of lobular inflammation in patients with the A/A vs A/G + G/G genotype at *PNPLA3* rs139051. Similar, yet mild, amelioration of lobular inflammation occurred in NAFLD patients carrying the G/G rather than other genotypes at *PNPLA3* rs2294918.

In conclusion, *PNPLA3* SNPs reflect an important genetic basis of lipidomic characteristics in NAFLD patients, with the focus on phospholipid metabolite profile. The A/A genotype at *PNPLA3* rs139051 exerts an upregulatory effect on serum LPCs and LPCOs. Both the genotype of *PNPLA3* rs139051 and the increased levels of LPCs/LPCOs share an association with the low-grade lobular inflammation of NAFLD. *PNPLA3* rs139051, therefore, may underlie the inflammatory progress of NAFLD by its modulation of the phospholipid metabolite profile.

ARTICLE HIGHLIGHTS

Research background

Genome-wide association analysis and clinical investigations have found that single nucleotide polymorphisms (SNPs) of patatin-like phospholipase domain containing 3 (*PNPLA3*) underlie the genetic susceptibility of nonalcoholic fatty liver disease (NAFLD), independent of gender, age and ethnic background.

Research motivation

The understanding of the SNP-specific impact on serum lipids and their correlation with pathological characteristics is still limited and controversial. In this study, the authors stratified Chinese Han patients with biopsy-proven NAFLD by genotyping their *PNPLA3* SNPs.

Research objectives

In this study, the authors investigated the effect of *PNPLA3* polymorphisms on serum lipidomics and pathological characteristics of NAFLD.

Research methods

Thirty-four biopsy-proven NAFLD patients from China were subjected to stratification by genotyping their SNPs in *PNPLA3*. Ultra-performance liquid chromatography-tandem mass spectrometry was employed to characterize the effects of *PNPLA3* SNPs on serum lipidomics. The variant-based scoring of hepatocyte steatosis, ballooning, lobular inflammation, and liver fibrosis was performed to uncover the actions of lipidomics-affecting *PNPLA3* SNPs in NAFLD-specific pathological alternations.

Research results

PNPLA3 SNPs demonstrated extensive association with the serum lipidomics, especially phospholipid metabolites of NAFLD patients. The significant correlation of *PNPLA3* rs139051 and inflammation grading further convinced its pathological role that was based on the modulation of phospholipid metabolite profile.

Research conclusions

The authors found that the A/A genotype at *PNPLA3* rs139051 exerts an up-regulatory effect on serum phospholipids of lysophosphatidylcholine (LPC) and lysophosphatidylcholine plasmalogen (LPCO), which are associated with low-grade lobular inflammation of NAFLD.

Research perspectives

These experimental observations relating to NAFLD patients revealed that

an increase in LPCs and LPCOs significantly correlated with an attenuation of hepatic inflammation. However, the pathological characteristics other than hepatic inflammation displayed no association with either of these phospholipid metabolites.

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