PNPLA3 As a Genetic Predictor of Histopathological Severity by Liver Biopsy in Non Alcoholic Fatty Liver Disease

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KEYWORDS

Nonalcoholic Fatty Liver Disease; Patatin-Like Phospholipase Domain-Containing Protein 3; rs738409; Mean Platelet Volume; Neutrophil to Lymphocyte Ratio.

ABSTRACT

Background: Nonalcoholic fatty liver disease (NAFLD) is defined by widespread steatosis within hepatocytes. The pathogenesis of NAFLD is not fully recognised. It is NAFLD is driven by genetic, environmental, and metabolic factors. Moreover, earlier studies have showed that some polymorphisms raise the possibility of NAFLD. The association between the risk of NAFLD and the patatin-like phospholipase domain-containing 3 (PNPLA3) gene was the most consistently observed.

Aim: To evaluate the effect of the rs738409 polymorphism of the PNPLA3 gene (encoding I148m) on NAFLD.

Patients and Methods: This study involved 30 participants, comprising 20 cases with NAFLD and 10 healthy individuals who served as a control group. The diagnosis was based on liver biopsies obtained during surgery from patients undergoing bariatric surgery or as part of the pre-donation evaluation process for liver transplantation candidates. The PNPLA3 gene variant (rs738409 C/G) was genotyped in blood cells using the TaqMan assay and quantitative polymerase chain reaction (qPCR). Results: Analysis of the PNPLA3 gene variant (rs738409 C/G) distribution revealed a greater prevalence of the heterozygous rs738409 CG and homozygous rs738409 GG variants among NAFLD cases compared to the control group ($p = 0.048$). Moreover, NAFLD patients carrying the homozygous GG variant showed a significantly increased incidence of hepatic fibrosis ($p = 0.018$). Additionally, PNPLA3 gene polymorphism emerged as a significant predictor of both liver steatosis and fibrosis in NAFLD cases (p-value equal 0.033 and p-value less than 0.001, correspondingly).

Conclusion: The PNPLA3 rs738409 polymorphism is strongly related to both the risk and severity of non-alcoholic fatty liver disease.

Introduction

Non-alcoholic fatty liver disease is characterised by disseminated hepatocyte steatosis, excluding extreme alcohol consumption and other hepatic diseases [1]. It approximately affects 32.4% of the general population [2,3]. NAFLD has a wide spectrum extending from nonalcoholic fatty liver, which doesn't cause significant health risks, to non-alcoholic steatohepatitis (NASH), hepatic cirrhosis, and finally hepatocellular carcinoma (HCC) [4,5]. Further, Non-alcoholic fatty liver disease is implicated in 36% of liver-related mortality, especially with progressive clinical forms of the disease [6].

The pathogenesis of NAFLD isn't fully recognized [7]. Recently, NAFLD is considered as a multifactorial disease driven by genetic, environmental, and metabolic factors [8]. Substantial inter-individual variations exist in the progression and disease severity in NAFLD cases sharing the same environmental and metabolic risk factors [9]. In addition, inter-ethnic variations and familial clustering point to the substantial impact of genetic factors in the pathophysiology of NAFLD [10]. Moreover, earlier studies have detected that some polymorphisms raise the risk of NAFLD [11-13]. Among these, the relationship between NAFLD risk and the patatin-like phospholipase domain-containing 3 gene is the most robustly observed [11]. The PNPLA3 protein is a transmembrane protein composed of 481 amino a`, predominantly expressed in hepatocytes, with additional expression in adipocytes and skin cells [14,15]. It functions as a lipoatrophic protein, having a key role in hepatic fat metabolism through its triacylglycerol lipase and acylglycerol O-acyltransferase activities. The nonsynonymous gene variant (C > G, rs738409), which lea to the substitution of isoleucine with methionine at position 148 (PNPLA3-I148M), has been recognised as a major genetic risk factor for NAFLD and NASH [16].

The exact mechanism and outcomes of PNPLA3-I148M are not yet comprehensively known. Nevertheless, this variant can produce both a loss and gain of function. The loss of function occurs by impairing lipolytic activity which enhance enhancing triglyceride deposition [17]. Additionally, it was suggested that this variant promotes intracellular lipid accumulation through its effects on the excretion of apoB-containing lipoproteins and esterification of very low-density lipoproteins [18]. Moreover, this variant results in impaired triglyceride hydrolysis in hepatocytes [19]. Additionally, PNPLA3 encodes adipo-nutrients located on lipid droplets and the endoplasmic reticulum within hepatocytes, which may represent another mechanism by which the PNPLA3-I148M mutation contributes to increased hepatic triglyceride accumulation [20]. Evidence also indicates increased hepatic triglyceride synthesis by promoting the function of lysophosphatidic acid acyltransferase through the gain of function caused by this variant [21]. Furthermore, this mutation promotes the development of hepatic fibrosis by impairing retinyl-palmitate lipase activity, leading to the upregulation of proinflammatory cytokines, c-Jun N-terminal kinase (JNK), & activator protein-1 (AP-1), as well as increasing matrix metalloproteinase expression [22]. Other studies have confirmed these findings, showing that the PNPLA3-I148M variant contributes to mitochondrial dysfunction by causing cholesterol deposition, which reduces ABCG1 protein expression and inhibits cholesterol efflux, thereby driving the progression of hepatic fibrosis [23]. Hence, we aim to assess the effect of the PNPLA3 (rs738409) gene polymorphism on NAFLD.

Patients and Methods

This study involved 30 participants, 20 NAFLD cases and 10 normal individuals as a control group enrolled from the Internal Medicine and Surgical departments at Ain Shams University Hospitals from December 2021 to February 2023. Participants were diagnosed based on liver biopsies obtained during surgery from bariatric surgery candidates (NAFLD cases) and as part of a pre-donation evaluation for individuals being considered for liver transplantation, serving as the control group.

Patients 18 years and older were involved in the research, excluding those with hepatic diseases of other known aetiologies, such as autoimmune hepatitis, viral hepatitis, and Wilson's disease. In addition, those with alcoholic liver disease, medication use known to induce hepatic steatosis or immunosuppression, and a history of malignancy or haematological diseases or current infection were also excluded. All participants underwent the following:

A complete blood count and calculation of the neutrophil to lymphocyte ratio (NLR) and mean platelet volume (MPV) [24],

Genotyping of the PNPLA3 gene variant (rs738409 C/G) has been carried out utilizing the TaqMan assay qPCR in blood cells. DNA was extracted from all participants with the Genomic Whole Blood Extraction Kit (Puregene, USA) with regard to the manufacturer's instructions. The DNA was then dissolved in TE buffer, and its purity and concentration were assessed using spectrophotometry. Real-time PCR has been utilized to find single nucleotide polymorphisms in the PNPLA3 gene (rs738409), and allelic discrimination of the PNPLA3 polymorphisms was analyzed using the TaqMan assay,

Abdominal ultrasonography,
Liver biopsy specimens analysed using the scoring system in Table 1.
Table 1 The clinical research network system for scoring activity and fibrosis in NAFLD [25]

	Hepatic lobular inflammation	Hepatocellular ballooning
0 = < 5%	0 = None	0 = None
1 = 5-33%	1 = < 2	1 = Few ballooned cells
2 = 34-66%	2 = 2-4	2 = Many ballooned cells
3 = > 66%	3 = > 4	

The protocol of the study complies with the ethics principles of the 1975 Declaration of Helsinki and its appendices. It has been authorized by the Clinical Research Ethics Committee of the Faculty of Medicine, Ain Shams University (FMASU MD 284/2018). Written informed agreement has been acquired from all contributors.

Statistical analysis

Statistical analysis has been conducated, utilizing the Chi-square test, student t- test, Person's correlation coefficient, regression coefficient, and Analysis of variance [ANOVA] test by SPSS V25. Data have been presented as mean ± standard deviation, median and range, or percentage and number. P-value not more than 0.05 has been regarded significant.

Results

The present research was performed on 30 participants. They were categorized into two groups following histopathology. The NAFLD group comprised of 20 patients, and the control group comprised of 10 healthy participants. Table 2 lists the clinical data of the study participants. NAFLD cases had higher BMI and MPV compared to the control group (p-value less than 0.001 and p = 0.012, Table 2). Additionally, Table 2 highlights the differences in ultrasound and liver biopsy findings between the examined groups (p-value not more than 0.05). A statistically insignificant association has been detected between the NLR and MPV, liver enzymes, lipid panel, body mass index (BMI), the occurrence of fatty liver on ultrasound, or liver steatosis and fibrosis in liver biopsy (p -value not less than 0.05, Supp. Table 1). A significant association existed between the BMI and the existence of fatty liver on ultrasound and steatosis and fibrosis in liver biopsy (p-value equal .04, 0.022, and 0.035, respectively, Supp. table 2).

Table 2 Study participant characteristics

Variable		NAFLD (n=20)	Control (n=10)	P value
Sex	Male	12 (60%)	5 (50%)	0.602
	Female	8 (40%)	5 (50%)	
		Mean ± SD	Mean ± SD	
Age (years)		28.75±5.26	28.50±6.55	0.911
Body mass index		33.50±5.30	22.90±2.18	<0.001
Total leucocytes count (x103)/µL		6.77±2.23	6.51±1.95	0.753
Hemoglobin (g/dL)		13.51±1.33	13.83±1.91	0.596
Platelets count (x103)/µL		269.45±80.81	267±48.78	0.931
Aspartate aminotransferase (U/mL)		21 (10.6 – 63.0)	15 (13.0 – 28.0)	0.42
Alanine aminotransferase (U/mL)		21 (8 – 90)	15 (9.0 – 33.0)	0.28
Serum albumin (mg/dL)		4.49±0.45	4.41±0.42	0.641
Total bilirubin (mg/mL)		0.62 (0.3 – 1.6)	0.5 (0.37 – 1.09)	0.603
Direct bilirubin (mg/mL)		0.2 (0.1 – 0.4)	0.2 (0.1 – 0.4)	0.81
International normalized ratio		1.0 (0.9 – 1.2)	1.0 (0.8 – 1.04)	0.14
Blood urea nitrogen (mg/mL)		14.50±4.75	14.70±4.21	0.911
Creatinine (mg/mL)		0.89±0.14	0.78±0.13	0.050
Total cholesterol (mg/mL)		196.50 ±37.68	174.60±22.32	0.103
High density lipoprotein (mg/mL)		38.90±9.06	45.10±9.04	0.088
Triglycerides (mg/mL)		160.05±66.38	112.60±52.41	0.059
LDL-Cholesterol (mg/mL)		111.35±20.64	106.30±21.56	0.539
Fasting blood glucose (mg/mL)		82.95±10.68	86.20±8.32	0.408
2 hours PP blood glucose (mg/mL)		100.35±15.70	107.60±16.72	0.253
HbA1C		5.39±0.39	5.45±0.37	0.693
Mean platelet volume (fL)		10.39±1.10	9.14±1.39	0.012
Neutrophil to lymphocyte ratio		1.47±0.28	1.49±0.38	0.863
Fatty liver in ultrasound	Grade 0	3 (15%)	10 (100%)	<0.001
	Grade I	12 (60)	0	
	Grade II	4 (20%)	0	
	Grade III	1 (5%)	0	
Hepatic steatosis	Grade I	18 (90%)	0	-
	Grade II	2 (10%)	0	
Lobular inflammation	No	3 (15%)	9 (90%)	0.002
	Mild	12 (60%)	1 (10%)	
	Moderate	1 (5%)	0	
	Severe	4 (20%)	0	
Hepatocyte ballooning	No	3 (15%)	10 (100%)	<0.001
	Mild	7 (35%)	0	
	Severe	10 (50%)	0	
Hepatic fibrosis	No	3 (15%)	10 (100%)	<0.001
	Yes	17 (85%)	0	

Supplementary table 1 The correlation of Neutrophil to lymphocyte ratio and the laboratory results, presence of fatty liver on the ultrasound, and findings of liver biopsy among NAFLD patients

	Neutrophil to lymphocyte ratio	
	r	P value
Age	0.100	0.674
Aspartate aminotransferase	-0.153	0.519
Alanine aminotransferase	-0.026	0.914
Total leucocytes count	0.038	0.874
Total Cholesterol	0.081	0.735
Low density lipoprotein	0.274	0.243
Triglycerides	0.027	0.911
Body mass index	0.083	0.728
Fatty liver in ultrasound	0.123	0.605
Hepatic steatosis	0.209	0.377
Hepatic fibrosis	0.109	0.648
Mean platelet volume	0.051	0.832

Supplementary table 2 Correlation between Body mass index and existence of fatty liver on the ultrasound and grade of steatosis and fibrosis in liver biopsy

	Body mass index	
	r	P value
Fatty liver in ultrasound	0.619	0.04
Hepatic steatosis	0.508	0.022
Hepatic fibrosis	0.474	0.035

The distribution of PNPLA3 gene variant (rs738409 C/G) revealed a greater prevalence of the heterozygous rs738409 CG & homozygous rs738409 GG variants in NAFLD cases than the control group (p-value equal 0.048, Table 3). Table 4 presents the grades of hepatic steatosis, hepatic fibrosis, and hepatocyte ballooning in NAFLD cases based on PNPLA3 gene variant status. NAFLD patients with the homozygous GG variant exhibited a higher incidence of hepatic fibrosis (p-value equal 0.018). However, no significant variation was found regarding hepatocyte ballooning and hepatic steatosis (p-value not less than 0.05), although those with the homozygous rs738409 GG variant had grade II steatosis with a higher prevalence of hepatocytes ballooning. Further, no significant variations occurred in the NLR, ALT, AST, total cholesterol, LDL cholesterol, and triglyceride values in NAFLD cases according to the PNPLA3 gene rs738409C/G variant status ($p \geq 0.05$, Supp. table 3), while MPV was significantly various between the groups (p-value equal 0.023, Supp. table 3).

Table 3 Distribution of the PNPLA3 polymorphism among NAFLD and control groups

PNPLA3 polymorphism	Total participants (n=30)	NAFLD (n=20)	Control (n=10)	P value
	n (%)	n (%)	n (%)	
Homozygous CC	10 (33.3%)	4 (20%)	6 (60%)	0.048
Heterozygous CG	9 (30%)	7 (35%)	2 (20%)	
Homozygous GG	11 (36.7%)	9 (45%)	2 (20%)	

Table 4 The hepatic steatosis, hepatocellular ballooning, and hepatic fibrosis in NAFLD cases according to the PNPLA3 polymorphism

Variable		Homozygous CC	Heterozygous CG	Homozygous GG	P value
Hepatic steatosis	Grade I	4 (100%)	7 (100%)	7 (77.78%)	0.155
	Grade II	0	0	2 (22.22%)	
Hepatocyte ballooning	No	0	3 (42.86%)	0	0.126
	Mild	1 (25%)	2 (28.57%)	4 (44.44%)	
	Severe	3 (75%)	2 (28.57%)	5 (55.56%)	
Hepatic fibrosis	No	4 (100%)	3 (42.86%)	0	0.018
	Yes	0	4 (57.14%)	9 (100%)	

Supplementary table 3 Laboratory variables in NAFLD patients according to the PNPLA3 polymorphism

Variable	Homozygous CC	Heterozygous CG	Homozygous GG	P value
	Mean ± SD	Mean ± SD	Mean ± SD	
Neutrophil to lymphocyte ratio	1.39±0.10	1.38±0.29	1.56±0.32	0.409
Mean platelet volume	11.51±0.513	9.71±0.93	10.42±1.04	0.023
Aspartate aminotransferase (IU/mL)	17.75±5.25	19.42±3.99	19.44±4.39	0.795
Alanine aminotransferase (IU/mL)	14±3.16	29.14±5.07	27±11.48	0.413
Cholesterol (mg/dL)	193.75±17.01	188.14±27.13	204.22±50.80	0.712
LDL- cholesterol (mg/dL)	119.25±25.60	109±22.88	109.66±18.27	0.715
Triglycerides (mg/dL)	172.50±61.84	130.42±50.08	177.55±77.19	0.358

P for One Way ANOVA test

Table 5 shows that hepatic steatosis correlated positively with PNPLA3 gene polymorphism and BMI ($p = 0.033$ and $p < 0.001$, correspondingly). Additionally, they were predictors for hepatic steatosis ($p = 0.033$ and $p < 0.001$, correspondingly, Supp. table 4). Table 6 shows that hepatic fibrosis correlated positively with PNPLA3 gene polymorphism, BMI, and total cholesterol ($p < 0.001$, $p < 0.001$, and $p = 0.049$, correspondingly). However, PNPLA3 gene polymorphism was the only predictor for hepatic fibrosis ($p < 0.001$, Supp. table 5).

Table 5 Correlations between hepatic steatosis and other studied parameters

	Hepatic steatosis	
	r	P value
Neutrophil to lymphocyte ratio	0.209	0.377
Mean platelet volume	0.168	0.479
Total cholesterol	0.436	0.055
LDL-Cholesterol	0.246	0.295
Body mass index	0.863	<0.001
PNPLA3 gene polymorphism	0.391	0.033

Supplementary table 4 Predictors of hepatic steatosis

	Unstandardized Coefficients		Standardized Coefficients	t	P value
	B	Std. Error	Beta		
Neutrophil to lymphocyte ratio	2.438	5.176	0.064	0.471	0.646
Mean platelet volume	-0.324	1.338	-0.033	-0.242	0.813
Total cholesterol	-0.095	0.053	-0.331	-1.779	0.101
LDL-Cholesterol	0.113	0.080	0.216	1.422	0.180
Body mass index	2.076	0.377	1.019	5.511	<0.001
Fatty liver in ultrasound	-0.097	3.688	-0.005	-0.026	0.980
PNPLA3 gene polymorphism	5.318	2.365	0.391	2.249	0.033

Table 6 Correlations between hepatic fibrosis and other studied parameters

	Hepatic fibrosis	
	r	P value
Neutrophil to lymphocyte ratio	0.094	0.623
Mean platelet volume	0.127	0.503
Total cholesterol	0.363	0.049
LDL-Cholesterol	0.067	0.724
Body mass index	0.642	<0.001
PNPLA3 gene polymorphism	0.689	<0.001

Supplementary table 5 Predictors of hepatic fibrosis

	Unstandardized Coefficients		Standardized Coefficients	t	P value
	B	Std. Error	Beta		
Neutrophil to lymphocyte ratio	0.239	0.194	0.149	1.231	0.971
Mean platelet volume	-0.046	0.051	-0.121	-0.899	0.566
Total cholesterol	-0.002	0.003	-0.086	-0.483	0.635
LDL-Cholesterol	0.000	0.004	-0.015	-0.098	0.586
Body mass index	0.040	0.013	0.543	3.205	0.367
PNPLA3 gene polymorphism	0.310	0.078	0.522	3.983	<0.001

Discussion

The 1st genome-wide association study (GWAS) regarding NAFLD found that the allele of PNPLA3 rs738409 is significantly correlated with hepatic steatosis and inflammation [26]. This finding gave new insight into the pathogenesis of NAFLD. Since then, knowledge regarding the genetic constituents of NAFLD has substantially increased. Several studies have reported that this variant leads to the progress of NAFLD by increasing triglyceride production and deposition in hepatocytes, increasing the risk of hepatic hepatocyte ballooning, steatosis, and lobular inflammation [27,28]. Furthermore, PNPLA3 rs738409 polymorphism is strongly linked to NASH, cirrhosis, HCC, and death [29-31]. The impact of this polymorphism is observed even in individuals with lean NAFLD [32].

The current investigation observed a greater occurrence of the heterozygous rs738409 CG and homozygous rs738409 GG PNPLA3 gene variants in the NAFLD group compared to the control group. Moreover, NAFLD patients with the heterozygous rs738409 CG & homozygous rs738409 GG variants had a greater incidence of hepatic fibrosis than those with the homozygous rs738409 CC variant. Additionally, the PNPLA3 rs738409 polymorphism was identified as a significant predictor of both hepatic steatosis and fibrosis. The results align with a meta-analysis consisted of 16

investigations that examined 2,124 patients and detected that the homozygous PNPLA3 rs738409 GG had a 73% higher hepatic fat content in comparison to the homozygous rs738409 CC polymorphism. In addition, rs738409 GG had a greater risk of developing fibrosis in contrast to homozygous CC [16].

In agreement with the current study, a case–control research including the genomic data of 2,950 NAFLD patients and 12,907 healthy controls detected that the incidence of the phospholipase domain-containing 3 rs738409 GG allele was higher in NAFLD cases in comparison with the control group. Furthermore, the PNPLA3 rs738409 GG and GC variants had a greater percentage of NAFLD patients compared to the PNPLA3 rs738409 CC variant (19.9% vs 16%). In addition, the PNPLA3 rs738409 GG allele was an independent risk factor for NAFLD [33].

In agreement with the current results, a GWAS by Anstee et al. stated that the PNPLA3 rs738409 variant is a risk factor for full pathological spectrum of NAFLD [28]. Another study found that the BMI, serum triglyceride, and AST concentration were risk factors for NAFLD [34]. In addition, a meta-analysis consisting of 20 investigations recorded a significant correlation between NAFLD susceptibility and PNPLA3 rs738409 polymorphism [20]. Nevertheless, in contrast to our outcomes, Li et al. [35] detected no correlation between hepatic steatosis and PNPLA3 rs738409 gene polymorphism.

Many studies have confirmed a strong relationship between metabolic syndrome and the grade of fibrosis and steatosis in NAFLD [36,37]. In agreement with earlier studies [33,34,38], NAFLD patients had higher BMI values than those of the control group. Moreover, a strong association has been observed between the BMI and hepatic steatosis and fibrosis in liver biopsy.

Substantial evidence suggests that the stimulation of the immune system with proinflammatory mediators secreted by visceral adipose tissue is a crucial element of disease severity and progression. In addition, intrahepatic lipids are implicated to induce oxidative stress, triggering portal and lobular inflammation, characterising the severe histological forms of the disease [39]. Furthermore, growing evidence suggests that the NLR is a useful marker for assessing the severity of NAFLD [40]. In contrast to our findings, Lesmana et al. [38] stated that cases with moderate to severe steatosis exhibited a higher NLR compared to those with mild steatosis. Additionally, Yilmaz et al. [41] noted that cases with NASH had a greater NLR compared to controls. However, in agreement with the present results, a large cohort study found that the NLR is not related to fibrosis and inflammation in NAFLD [42].

In agreement with an earlier study [43], NAFLD patients had a greater MPV than the control group. However, MPV wasn’t statistically different between the NAFLD group and controls, nor was a predictive factor for NAFLD in another study [34].

A major strength of the present research is the evaluation of hepatic disease with biopsy-proven NAFLD, which is the golden standard for evaluating the liver disease severity. However, the present research is restricted by the small sample size. From the current results, PNPLA3 genotyping may improve risk stratification and prognostication and allow prioritisation of intensive interventions in NAFLD patients [44]. Additionally, with this knowledge, it has become possible to apply its effects on NAFLD into clinical applications, such as innovative drugs, and discover potential targets to treat the disease [45,46]. In this context, antisense oligonucleotides-mediated silencing of PNPLA3 gene was investigated in a mice model. Notably, there was a decline in the NAFLD activity score, hepatic inflammation, steatosis, and fibrosis grade [47].

Conclusions

The PNPLA3 rs738409 polymorphism is strongly related to to both the susceptibility & severity of non-alcoholic fatty liver disease.

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