

## Association of rs738409 Polymorphism in the PNPLA3 Gene with Nonalcoholic Fatty Liver Disease

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease and has an estimated incidence of 20%–30% in the general population and 67%–75% in the obese population. Genetic predisposition can play an important role in development of this disease. Nonsynonymous coding SNP rs738409 C/G (I148Met) in the PNPLA3 gene has been found to be associated with the presence of NAFLD in a genome-wide association study. This association has been replicated in several cohorts of different ethnicity, but to date the assessment of this association has not been performed in the Central Asia populations.

**The purpose** of our research was to investigate the association between polymorphic variants of the PNPLA3 (rs738409 C>G) polymorphism and susceptibility to NAFLD in patients of Uzbek nationality.

In this case-control study, we recruited 73 NAFLD patients and 37 controls, matched according to age, gender and ethnicity. Genomic DNA was isolated and SNP genotyping was performed by using a polymerase chain reaction with specific primers followed by restriction fragment length polymorphism analysis. Our result showed significant association between the GG genotype and NAFLD ( $P=0.03$ , OR = 2.99; 95% CI: 1.21–7.42 for the additive model, Cochran-Armitage trend test;  $P=0.02$ , OR = 2.99; 95% CI: 1.21–7.42 for the recessive model).

These findings supported the idea that the PNPLA3 (rs738409 C>G) polymorphism contributes to the susceptibility to NAFLD. Our data suggest the reasonability of including a PNPLA3 rs738409 SNP test to identify high risk groups for NAFLD in Uzbekistan.

**Keywords:** Nonalcoholic fatty liver disease; PNPLA3 (rs738409 C>G) polymorphism.

### Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic disease of the liver and is an escalating medical problem worldwide. The incidence of this disease is estimated to be 20%–30% in the general population and 67%–75% in the obese population. NAFLD has a wide spectrum of clinical manifestations, ranging from simple steatosis and its inflammatory counterpart nonalcoholic steatohepatitis, to fibrosis/ cirrhosis and hepatocellular carcinoma. NAFLD is

a multifactorial disease, the emergence and development of which depends on a number of interrelated factors: genetic polymorphisms, diet, and lifestyle [1,2]. NAFLD is considered to be the hepatic component of the metabolic syndrome and is strongly associated with obesity and insulin resistance [3-6]. Genetic factors play an important role in the development of NAFLD [7-9].

Recently, a genome-wide association study showed that a nonsynonymous sequence variation (rs738409) in PNPLA3 (patatin-like phospholipase domain containing-3) that substitutes methionine for isoleucine at residue 148 (I148M) is associated with differences in hepatic lipid content and the susceptibility to NAFLD [10]. PNPLA3 gene is located in chromosome 22 (22q13.31) and has nine exons; its transcript length is 2805 bp and it is translated to a protein of 481 amino acids. PNPLA3, also known as adiponutrin, belongs to the

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patatin-like phospholipase family of proteins. PNPLA3 is a transmembrane protein which, in humans, is highly expressed in hepatocytes and is strongly responsive to changes in energy balance [11].

Wild-type (148I) PNPLA3 has lipolytic activity towards triglycerides [12,13]. The 148M mutation determines a critical aminoacid substitution near the catalytic domain, likely reducing the access of substrates and decreasing the PNPLA3 enzymatic activity towards glycerolipids, thereby leading to the development of steatosis [12,13]. However, other reported a gain of lipogenic function associated with the 148M variant, which would acquire the ability to synthesize phosphatidic acid from lysophosphatidic acid [14]. In addition, results deriving from mouse and rat models gave conflicting results [15-18]. The issue regarding functional consequences of the 148M polymorphism is therefore still highly debated, and there is a potential possibility that PNPLA3 could have additional physiological substrates. Human studies have also suggested a possible direct or indirect influence of PNPLA3 genotype on adipose tissue biology [19-21].

The association of rs738409 148M polymorphism with NAFLD was confirmed in several ethnic and geographic groups [22,33], but to date the assessment of this association has not been performed in the Central Asia populations. Uzbeks are the largest, youngest, and fastest growing population in Central Asia. The Uzbek population is very interesting from cultural, socioeconomic, and genetic perspectives. It is remarkable to note that this population has been formed by admixture of two or more ancestral populations, thus it offers a unique opportunity for studying the interaction between gene polymorphisms, ethnic-specific genetic backgrounds, and environmental contributions to the occurrence of disease.

**The purpose** of our research was to investigate the association between polymorphic variants of the PNPLA3 (rs738409 C>G) polymorphism and susceptibility to NAFLD in patients of Uzbek nationality.

## Materials and Methods

The study included 73 patients diagnosed with non-alcoholic fatty liver disease, who underwent the treatment at the Republican Specialized Scientific-Practical Medical Center of Therapy and Rehabilitation of the Ministry of Health of the Republic of Uzbekistan. The control group constituted 37 healthy, age-matched, randomly selected persons.

The diagnosis of NAFLD was established on the basis of clinical history, clinical examination, laboratory tests, and liver ultrasound. All patients consumed less than 20 g/day of alcohol and showed no signs of chronic viral hepatitis (B, C, D); autoimmune hepatitis and drug-induced hepatitis, Wilson's disease, idiopathic hemochromatosis,  $\alpha$ 1-antitrypsin deficiency.

Study was conducted in accordance with the guidelines of the Helsinki Declaration of the World Medical Association's "Ethical Principles for Medical Research Involving Human Subjects" with amendments (2013). All patients who participated in this study gave written informed consent and the protocol was approved by the National Ethics Committee

of Uzbekistan. Molecular genetic studies were performed in Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan.

DNA samples were isolated from peripheral blood leucocytes by using DNA extraction kit Diatom™ DNA Prep 200 ("IsoGen Laboratory", Moscow, Russia). PNPLA3 rs738409 148M variant was genotyped by means of previously described PCR-RFLP method [34].

A 333-bp region of the PNPLA3 gene was amplified by PCR using specific primers (forward primer: 5'-TGGGCCTGAAGTCCGAGGGT-3' and reverse primer: 5'-CCGACACCAGTGCCTGCAG-3'). PCR mixture (25  $\mu$ l) consisted of 13  $\mu$ l of ddH<sub>2</sub>O, 2.5  $\mu$ l 10xPCR buffer, 2.5  $\mu$ l 25 mM MgCl<sub>2</sub>, 2.5  $\mu$ l 2.5 mM dNTP Mix, 1.5  $\mu$ l (10pkmol/ $\mu$ l) of each oligonucleotide primer, 0.3  $\mu$ l (1.5 units.) "hot-start" Taq-polymerase and 3  $\mu$ l of DNA. PCR amplification was carried out in GeneAmp 9700 (Applied Biosystems).

The PCR conditions were as follows: 95 °C for 5 min, and then 37 cycles of 94°C for 30 sec, 66°C for 30 sec, and 72 °C for 40 sec and a final extension step of 72°C for 5 minutes.

Then PCR products were digested overnight at 65°C with BstF5 I. Digested PCR products were subjected to horizontal electrophoresis in 1.5 % ethidium bromide-stained agarose gels in 1X TBE buffer at 120 V for 1 hr and were visualized using WiseDoc WGD-30 (DAIHAN, Korea).

Interpretation of genotyping results was performed on the basis of different patterns of bands: CC genotype 200 and 133 bp, CG genotype - 333, 200 and 133 bp, GG genotype - 333 bp.

**Statistical analysis:** The Hardy-Weinberg equilibrium was tested by a goodness-of-fit  $\chi^2$  test to compare the observed genotype frequencies with the expected ones among the control subjects. Genotypic associations of SNPs were evaluated by Pearson's  $\chi^2$  test and logistic regression analysis under additive, dominant and recessive models of inheritance, followed by risk assessment using odds ratio and 95% confidence of interval (CI) computation. All statistical analyses were performed by using STATA software version 12.0 for Windows (Stata Corporation, USA).

*P* value <0.05 (two-sided) was considered statistically significant.

## Results

Genotype frequencies of PNPLA3 rs738409 polymorphism in patients with NAFLD and controls are shown in Table 1. The genotype distributions of the PNPLA3 rs738409 polymorphisms were in Hardy-Weinberg equilibrium in control groups ( $P > 0.05$ ).

Comparative analysis of resulting genotypes between patients and controls showed significant association between the GG genotype and nonalcoholic fatty liver disease, assuming an additive model ( $P = 0.03$ , Cochran-Armitage trend test) and recessive model ( $P = 0.02$ , Pearson's  $\chi^2$  test). The odds ratio (OR) of increased relative risk of developing NAFLD for GG genotype carriers was OR=2.99 (95% confidence interval (CI): 1.21–7.42) under the additive as well as under the recessive model.

## Discussion

In a genome-wide association study, rs738409 polymorphism of PNPLA3 was found to be associated with hepatic fat content and NAFLD [10]. It is remarkable to note that association between rs738409 and liver fat was independent of major differences in body composition, diabetes and serum lipoprotein levels. Furthermore, the prevalence of rs738409 risk allele was higher in Hispanics (MAF:0.49) than in Europeans (MAF:0.23), and less common in Afro-Americans (MAF:0.17) that could explain a significant fraction of the inter-ethnic variability concerning susceptibility NAFLD [10,22]. Since then, several studies and a recent meta-analysis have replicated the association between the rs738409 polymorphism and NAFLD in several ethnic groups [21-32].

**Table 1.**

*Association between the genotypes of PNPLA3 rs738409 polymorphism and risk of NAFLD*

Genetic model of inheritance	Genotypes	NAFLD	Control	$\chi^2$	P	OR	
		n = 73	n = 37			Value	95% CI
Additive model (Cochran-Armitage trend test $\chi^2 = [0,1,2]$ , df = 1)	C/C	0.315	0.459	4.82	0.03	0.54	0.24-1.22
	C/G	0.233	0.324			0.63	0.26-1.52
	G/G	0.452	0.216			2.99	1.21 -7.42
Recessive model ( $\chi^2$ test, df = 1)	C/C+C/G	0.548	0.784	5.84	0.02	0.33	0.13-0.83
	G/G	0.452	0.216			2.99	1.21 -7.42

Though this SNP predisposes susceptibility to NAFLD, there are some conflicting results regarding its association with steatosis grade [26-28].

Whereas some studies showed that SNP is associated with steatosis grade, there was no significant association found between rs738409 and steatosis grade in two East Asian-based studies [31,33].

There is no report on the association between rs738409 and NAFLD in the Uzbek population. The Uzbek population is very interesting with regard to dietary habits, lifestyle and genetic structure. Historical, archaeological and genetic evidence indicated the “hybrid zone” scenario of the origin of the Uzbek population, which postulates early occupation by western Caucasian peoples followed by an East Asian admixture [35,36].

Complex genetic diseases, such as NAFLD, are likely to be due to multiple, potentially interacting, genetic and environmental factors and therefore are more challenging to study than the simple monogenic diseases. Presumably, many of these environmental and genetic risk factors are contextual, meaning that other factors, such as ethnic-specific genetic background, are likely to be key modifiers of these risk factors. This general phenomenon is known as effect modification and represents an interaction between two or more variables.

The results of the present study indicate that the genetic

effect of PNPLA3 rs738409 polymorphisms is so powerful that despite the potential existence of ethnic-specific genetic and environmental modifiers, it still exerts a significant impact on the development of nonalcoholic fatty liver disease in a population with such a historically mixed genetic background as the Uzbeks.

Our data suggest the reasonability of including the PNPLA3 rs738409 SNP test to identify high risk groups for NAFLD in Uzbekistan in order to organize an effective preventive lifestyle and medical interventions on the level of the national healthcare system.

## Competing interests

The authors declare that they have no competing interests.

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