Research Article

Journal of Gastroenterology & Digestive Systems

GG Genotype of Pnpla3 Rs738409 Polymorphism Associated with Nash in Patients of Uzbek Nationality

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Submitted: 03 Nov 2018; **Accepted**: 09 Nov 2018; **Published**: 19 Nov 2018

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. No synonymous coding SNP rs738409 C/G (Ile148Met) in Patatin-like phospholipase domain-containing protein 3 (PNPLA3) genes has been found 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.

***Aim:** The purpose of our research is to investigate the association between polymorphic variant of PNPLA3 gene (rs738409) and susceptibility to non-alcoholic fatty liver disease (NAFLD) in Uzbekistan.*

***Materials and Methods:** In this case-control study, 73 patients a mean age of 55.1 diagnosed with NAFLD (48 patients with simple steatosis and 25 patients with non-alcoholic steatohepatitis (NASH)) and the age, gender and ethnically matched controls (n=37) were recruited. The diagnosis of NAFLD was verified on the basis of anamnesis, clinical and laboratory tests, and liver ultrasound. Genomic DNA was isolated and SNP genotyping was performed by using polymerase chain reaction with specific primers followed by restriction fragment length polymorphism analysis.*

***Result:** Showed significant association between GG genotype of the PNPLA3 rs738409 polymorphisms 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, Pearson's χ2 test). Genotype frequencies of PNPLA3 rs738409 polymorphisms in a subset of patients with simple steatosis and NASH compare to the control group. Comparative analysis of resulting genotypes showed a slight increase of CG and GG genotypes among patient with simple steatosis, then among subjects of the control group, but this did not reach statistical significance. However, statistical analysis of genotype distribution between patients with NASH and controls showed a significant association between GG genotype and NASH assuming an additive model (p<0, 0001, Cochran-Armitage trend test) and recessive model (p<0, 0001, Pearson's χ2 test).*

***Conclusion:** The present study, we confirm the association of PNPLA3 rs738409 GG genotype with susceptibility to NAFLD. After stratification into the two main subtypes of NAFLD, the risk genotype GG was found to be significantly associated with susceptibility to NASH. We also found that the GG genotype is not associated with simple steatosis in Uzbek population.*

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic diseases of the liver and is an escalating medical problem worldwide. The meta-analysis estimated that the overall global prevalence of NAFLD diagnosed by imaging is around 25.24% (95% CI, 22.10-28.65) [1]. The highest prevalence of NAFLD is reported from the Middle East (31.79% [95% CI, 13.48-58.23]) and South America (30.45% [95% CI, 22.74-39.440]) whereas the lowest prevalence rate is reported from Africa (13.48% [5.69-28.69]) [1]. NAFLD has a wide spectrum of clinical manifestations, ranging from simple steatosis, its inflammatory counterpart nonalcoholic steatohepatitis (NASH), fibrosis/cirrhosis, to 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 [2, 3]. NAFLD is considered to be the hepatic component of the metabolic syndrome and is strongly associated with obesity and insulin resistance [47].

Genetic factors play an important role in the development of NAFLD [8-10]. Recently, genome-wide association study showed that a no synonymous sequence variation (rs738409 C>G) that results in an isoleucine to methionine substitution at residue 148 (I148M) in Patatin-like phospholipase domain-containing protein 3(*PNPLA3*) gene, is associated with differences in hepatic lipid content and the susceptibility to NAFLD [11]. *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. Patatin like phospholipase-3 protein, also known as adiponutrin, belongs to the patatin-like phospholipase family of proteins. PNPLA3 is a Tran's membrane protein which, in humans, is highly expressed in hepatocytes and is strongly responsive to changes in energy balance [12].

Wild-type (148I) PNPLA3 possessed lipolytic activity towards triglycerides [13, 14]. The 148M mutation determines a critical amino acid substitution near the catalytic domain, likely reducing the access of substrates and decreasing the PNPLA3 enzymatic activity towards glycolipids, thereby leading to the development of steatosis [13, 14]. 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 [15]. In addition, results deriving from mouse and rat models gave conflicting results [16-19]. The issue regarding functional consequences of the I148M polymorphism is therefore still highly debated, and there is a potential possibility that PNPLA3 could have additional physiological substrates. Human studies have also suggested possible direct or indirect mechanisms by which PNPLA3 genotype exerts its effect on adipose tissue [20-23].

Association of rs738409 I148M polymorphism with NAFLD was confirmed in several ethnic and geographic groups, but to date the assessment of this association has not been performed in the Central Asia populations [24-35]. Uzbeks are the largest, youngest and fastest growing population in Central Asia. Uzbek population is very interesting with regard to 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 offer a unique opportunity for studying the interaction between gene polymorphisms, ethnic-specific genetic backgrounds and environmental contributions to disease occurrence.

The purpose of our research is to inves¬tigate the association between polymorphic variant of PNPLA3 gene (rs738409) and susceptibility to NAFLD in Uzbek population.

Materials and Methods

The study included 73 patients with mean age of 55.1 diagnosed with NAFLD, 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. Group of patients with NAFLD included 48 patients with simple steatosis and 25 patients with non-alcoholic steatohepatitis (NASH). The control group constituted 37 healthy, age-matched, randomly selected persons.

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.

The diagnosis of NAFLD was established on the basis of clinical history, clinical examination, laboratory tests and liver ultrasound.

Inclusion criteria

Inclusion criteria were persistently elevated serum alanine aminotransferase (ALT > 1. 2 times the upper limit of normal for more than 6 months), diffusely hyper echogenic liver on abdominal ultrasonography (USG).

Exclusion criteria

The patients who were positive for hepatitis B surface antigen, hepatitis C or hepatitis D antibody tests were excluded from the study. Alcohol intake of more than 20g/wk, liver cirrhosis, primary biliary cirrhosis, biliary obstruction, autoimmune hepatitis, Wilson disease, idiopathic hemochromatosis, celiac disease and α-1-antitrypsin deficiency were also criteria for patient exclusion due to the possible elevation of their liver enzymes. Additionally, patients were excluded from the study if they ingested following drugs for a period of more than 4 weeks during past 6 weeks: amiodarone, мethotrexate, perhexiline, glucocorticoids, estrogens, tamoxifen, nifedipine, diltiazem.

Anthropometric Assessment

All participants underwent anthropometric measurements: weight, stature and waist circumference (WC) and hip circumference (HC). Body mass index (BMI) was calculated using standard formula: BMI= weight in kilograms/ (height in meters) [2].

Abdominal ultrasonography (USG)

NAFLD was diagnosed using abdominal USG, according to standardized criteria. Diagnosis of NAFLD was based on the presence of "bright liver" echo pattern and hepatorenal echo contrast. Abdominal USG was performed by the same operator using Accuvix V20 apparatus (Samsung Medison Co., Ltd., Seoul, South Korea).

Laboratory data

All blood samples were collected from the median cubital vein vein in the morning, after an overnight fast. Alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ glutamyl Trans peptidase (γ GTP), Serum triglycerides (TGs), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL) were measured.

Genotyping

DNA samples were isolated from peripheral blood leucocytes by using DNA extraction kit Diatom™ DNA Prep 200 ("IsoGen Laboratory", Moscow, Russia). PNPLA3 rs738409 I148M variant was genotyped by means of previously described PCR-RFLP method [34]. A 333-bp region if the PNPLA3 gene was by PCR using specific primers (forward primer: 5'-TGGGCCTGAAGTCCGAGGGT-3' and reverse primer: 5'- CCGACACCAGTGCCCTGCAG-3'). PCR mixture(25 µl) consisted of 13 µl of ddH2O, 2.5 µl 10xPCR buffer, 2.5 µl 25 mM $MgCl_2$, 2.5 µl 2.5 mM dNTP Mix, 1,5 µl (10pkmol/µl) of each oligonucleotide primer, 0.3 ul (1.5 units.) "hot-start" Taq-polymerase and 3 µl of DNA. PCR amplification was carried out in Gene Amp 9700(Applied Bio systems). The PCR conditions were as follows: 95 °C for 5 min, and then 37 cycles of 94°C for 30 s, 66°C for 30 s, and 72 °C for 40 s 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). A P value <0.05 (two-sided) was considered statistically significant.

Results

Genotype frequencies of PNPLA3 rs738409 polymorphisms in patients with NAFLD and controls are shown in (Figure 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 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 recessive model.

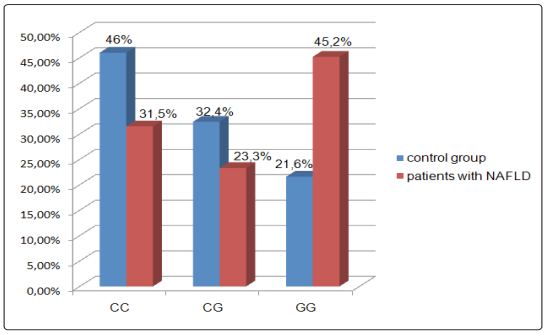

Figure 1: Genotype distribution of PNPLA3 rs738409 polymorphisms in patients with NAFLD and control group.

Genotype frequencies of PNPLA3 rs738409 polymorphisms in a subset of patients with simple steatosis and NASH compare to the control group are shown in (Figure 2 & 3). Comparative analysis of resulting genotypes showed slight increase of CG and GG genotypes among patient with simple steatosis, then among subjects of control group, but this did not reach statistical significance (Table 1 & 2). However, statistical analysis of genotype distribution between patients with NASH and controls showed significant association between GG genotype and NASH assuming an additive model (p<0, 0001, Cochran-Armitage trend test) and recessive model (p<0, 0001, Pearson's $\chi 2$ test).

To investigate whether the genotypes of rs738409 were associated with biochemical characteristics, we compared ALP, AST, ALT, γ GTP, TGs, TC, HDL, and LDL between different genotypes in the group of patients with simple steatosis, NASH and control group. Statistical analysis showed that G-allele of rs738409 was significantly associated with increases in AST and ALT (P < 0, 05) (Table 3 & 4).

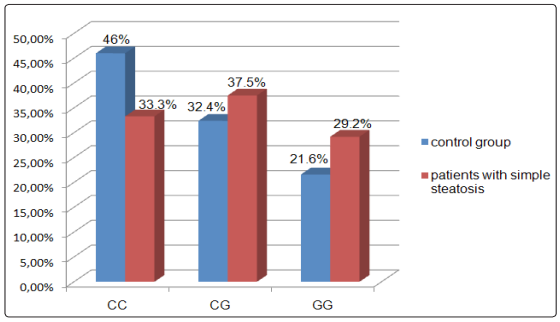

Figure 2: Genotype distribution of PNPLA3 rs738409 polymorphisms in patients with simple steatosis and control group

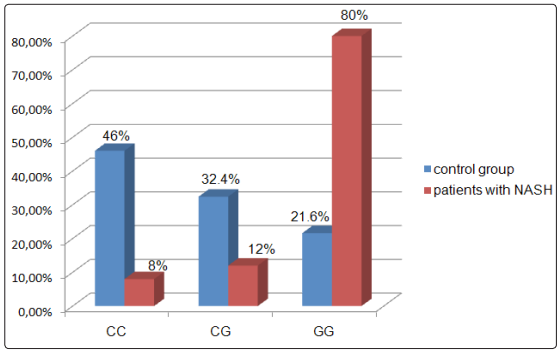

Figure 3: Genotype distribution of PNPLA3 rs738409 polymorphisms in patients with NASH and control group

Table 1: Results of statistical analysis of genotype distribution between patients with NAFLD and controls

Model of inheritance	Genotypes	Cases	Controls	χ2	p	OR	
		n = 73	n = 37			value	95% CI
Additive model (Cochran-Armitage trend test xi = [0,12], df = 1)	C/C	31.5%	46%	4.82	0.03	0.54	0.24-1.22
	C/G	23.3%	32.4%			0.63	0.26-1.52
	G/G	45.2%	21.6%			2.99	1.21 -7.42
Recessive model (χ2 test, df = 1)	C/C+C/G	54.8	78.4	5.84	0.02	0.33	0.13-0.83
	G/G	45.2	21.6			2.99	1.21 -7.42

Table 2: Results of statistical analysis of genotype distribution between patients with simple steatosis and controls

Model of inheritance	Genotypes	Cases	Controls	χ2	p	OR	
		n = 48	n = 37			value	95% CI
Additive model (Cochran-Armitage trend test xi = [0,12], df = 1)	C/C	33.3%	46%	1.35	0.25	0.59	0.24-1.42
	C/G	37.5%	32.4%			1.25	0.51 -3.08
	G/G	29.2%	21.6%			1.49	0.55-4.06
Recessive model (χ2 test, df = 1)	C/C+C/G	70.8%	78.4	0.62	0.43	0.67	0 25-1.82
	G/G	29.2%	21.6			1 49	0.55-4.06

Table 3: Results of statistical analysis of genotype distribution between patients with NASH and controls

Model of inheritance	Genotypes	Cases	Controls	χ2	p	OR	
		n = 25	n = 37			value	95% CI
Additive model (Cochran-Armitage trend test xi = [0,12], df = 1)	C/C	8%	46%	18.78	>0,0001	0.10	0.02-0.50
	C/G	12%	32.4%			0.28	0.07-1.14
	G/G	80%	21.6%			14.50	4.14-50.82
Recessive model (χ2 test, df = 1)	C/C+C/G	20%	78.4	20.53	>0,0001	0.07	0.02-0.24
	G/G	80%	21.6			14.50	4.14-50.82

Table 4: Biochemical data of the subjects

Genotype	Simple steatosis			NASH			Controls		
	CC	CG	GG	CC	CG	GG	CC	CG	GG
ALT	**32, 6+3,0*	**35, 3+2,8*	**48,7+ 3,3*	48, 8+2,6*	69, 6+2,8*	114, 2+4,8*	16, 6+2,5	20, 4+3,0	28, 5+ 1,9
AST	14, 5+ 1,7*	**16, 7+2,0	**22,5+2,8*	20,1+2,3*	32,2+3,7*	48,7+4,8*	8,7+1,6	11,4+1,4	14,8+2,1
ALP	188,0+12,5*	**265,4+21,5*	**322,2+20,5*	255,5+18,4*	333,4+19,0*	438,0+20,7*	135,4+12,7	145,8+15,8	140,4+20,0
γ –GTP	**35,6+2,1*	**36,6+2,5*	**67,4+3,3*	66,5+3,6*	78,6+3,8*	135,4+8,9*	22,0+2,7	24,8+3,6	27,3+2,7
TC	5,9+0,6	6,6+0,7	7,0+0,5	6,0+0,9	6.9+ 1,1	7,5+1,3	4,0+0,9	5,5+0,7	5,8+0,8
HDL	1,5+0,1	1,3+0,1	1,2+0,007	1,2+0,07	1,1+0,1	0,9+0,1	1,71 ± 0.3	1.68. ± 0,4	1,32 ± 0,2
LDL	2,9+0.4	3,0+0,5	3,5+0,8	3,4+0,9	4,0+0,6	4,6+0,5	2,19 ± 0,6	3,3 ± 0,7	3,22 ± 0,8
TGs	1,7+0,2	1,9+0,5	2,2+0,8	2,1+0,3	2,2+0,5	2,4+0,2	1,44 ± 0,3	1,82 ± 0,6	1,73 ± 0,5

* - (p<0, 05) statistical significant differences compare to the control group
** - (p<0, 05) statistical significant differences between NASH and simple steatosis

Discussion

In genome-wide association study, rs738409 polymorphism of PNPLA3 was found to be associated with hepatic fat content and NAFLD [11]. 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 [11, 24]. Since then, several studies and a recent meta-analysis have replicated the association between the rs738409 polymorphism and NAFLD in several ethnic groups [24-34]. There is no report on the association between rs738409 and NAFLD in the Uzbek population.

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 origin of Uzbek population, which postulates early occupation by western Caucasian peoples followed by East Asian admixture [36-38]. This genetic admixture suggested that Uzbeks have the genetic affinity towards both Asians and Europeans.

In the present study, we confirm the association of *PNPLA3* rs738409 GG genotype with susceptibility to NAFLD. After stratification into the two main subtypes of NAFLD the risk genotype GG was found to be significantly associated with susceptibility to NASH. Thus, we also replicated the genetic association of the *PNPLA3* variant with NASH in our population as was observed in several studies [31, 28, 5].

We also found that the GG genotype is not associated with simple steatosis in Uzbek population. Two resent Asian studies revealed that G allele is not associated with the simple steatosis [33, 39]. Taking into consideration the presence of an Asian component in genome of Uzbek people our results are consistent with these findings. From the other side slight increase of CG and GG genotypes in patients with simple steatosis, compare to the control group (but not having reached statistical significance) may be a reflection of presence of hidden Caucasian component in the genome of Uzbek people since many European studies did show association with simple steatosis.

There are some conflicting reports regarding association of rs738409 with ALT and AST levels. The rs738409 G allele was shown to be associated with increased levels of both serum AST and ALT in the Argentinian, Italian, Japanese and the Taiwanese populations [26, 27, 33, 34]. In the Hispanic and Italian population, the G allele was found to be associated only with increased serum ALT [11, 40]. In the Finnish population the G allele was revealed to be associated only with increased serum AST level [24]. However, the levels of AST and ALT appeared not to be significantly associated with the G allele in the African-Americans, European Americans and Germans [11, 41, 42].

Results of our study showed that G-allele of rs738409 was significantly associated with increases in both AST and ALT, which is consistent with Argentinian, Italian, Japanese and the Taiwanese studies.

Complex genetic diseases, such NAFLD are likely to be due to multiple, potentially interacting, genes 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 indicated that genetic effect of PNPLA3 rs738409 polymorphisms is so powerful that despite of potential existence of ethnic-specific genetic and environmental modifiers it still exerts significant impact on the development of NASH in a population with such historically mixed genetic background as Uzbeks.

Since predicting steatohepatitis is very important as NASH can potentially progress into life-threatening conditions and treatment should be advised at an early stage of NASH, our data suggest the reasonability of inclusion PNPLA3 rs738409 SNP test for the identification of high risk groups for NASH in Uzbekistan for the targeted organization of effective preventive lifestyle and medical interventions on the level of the national healthcare system.

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