The influence of RS738409 I148M polymorphism of patatin-like phospholipase domain containing 3 gene on the susceptibility of non-alcoholic fatty liver disease

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Abstract

We aimed to elucidate the frequency of polymorphic genotypes and alleles of patatin-like phospholipase domain containing 3 rs738409 polymorphism and its possible associations with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis in a cohort from Turkey.

We enrolled 200 patients diagnosed with NAFLD and genotyped for rs738409 I148M polymorphism by real-time polymerase chain reaction, particularly by melting curve analysis. SPSS analysis software was used for statistical significance. Continuous variable values were expressed as mean ± standard deviation. Significant statistical level was chosen as \( p=0.05 \).

Our results demonstrate in a cohort from Turkey that rs738409 C > G polymorphism (I148M) of patatin-like phospholipase domain containing 3 gene is significantly able to affect individuals to have NAFLD in unadjusted regression model.

Consistent with the previous studies in other populations, our study group showed a significantly higher risk of having NAFLD in unadjusted regression model but not in the adjusted model indicating that non-genetic factors such as age and sex may be responsible for the association. However, independent studies need to validate our findings with a larger group of NAFLD patients, as well as in different ethnic cohorts.

Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase, BMI = body mass index, GGT = gamma-glutamyl transferase, IR = insulin resistance, NAFLD = non-alcoholic fatty liver disease, NASH = non-alcoholic steatohepatitis, PNPLA3 = patatin-like phospholipase domain containing 3, SNPs = single nucleotide polymorphisms.

Keywords: NAFLD, NASH, PNPLA3, rs738409, Turkish patients

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a group of liver diseases including the conditions from simple steatosis to steatohepatitis. This disease could progress into the advanced fibrosis/cirrhosis along with its subsequent conversion to hepatocellular carcinoma with significant mortality rates. It is characterized by accumulated fat content in the liver, which is unrelated to alcohol consumption. NAFLD affects ∼25% to 30% of the adult population in the world, particularly with highest prevalence in the Middle East and South America.\textsuperscript{1–3} However, progression and severity of the disease varies in different geographic regions and ancestry groups, indicating the influence of environmental and genetic factors in NAFLD pathogenesis. Clinical studies provide strong evidences about the association of type 2 diabetes mellitus and obesity with NAFLD.\textsuperscript{4,5} Thus, accelerating proportion of diabetic or obese population in the world enhances the prevalence of NAFLD and its induction to inflammatory phase, namely non-alcoholic steatohepatitis (NASH). The emergence of NASH in NAFLD patients is characterized by inflammation, hepatocellular damage, and/or fibrosis. Recently, NASH has gained much attention due to the findings, indicating that it is an important risk factor for the development of hepatocellular carcinoma.\textsuperscript{6} In fact, the induction of NAFLD into NASH is a multifactorial process, including not only obesity or diabetes but also some genomic instability.\textsuperscript{7,8}

Clinical and phenotypic variations among NAFLD patients could arise from heritable genetic factors, which were shown by many epidemiological, familial, twin, and clinical based studies.\textsuperscript{9–13} Similarly, inconstancy of clinical data such as alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), and insulin resistance (IR) levels in different studies also...
showed heritable properties.\textsuperscript{14–17} Therefore, although liver biopsy is the gold-standard diagnostic method for NAFLD, recently genetic methods, specifically single nucleotide polymorphisms (SNPs), are gaining interest due to their non-invasive applications.

Genome-wide association studies have provided insights into the genetic background of NAFLD, indicating significant associations between patatin-like phospholipase domain containing \textit{3} (\textit{PNPLA3}) variants and increased risk of NAFLD and NASH, as well as hepatocellular carcinoma. Currently, extensive research has been focused on the role of rs738409 polymorphism of \textit{PNPLA3} gene.

\textit{PNPLA3} is a 481-amino-acid protein containing a conserved patatin-like domain at the N terminus. This protein is a multifunctional enzyme, highly expressed in liver and adipose tissues. It has an important role in hepatocellular remodeling of lipid droplets and very low-density lipoprotein (VLDL) secretion, which is a major determinant of differential hepatic fat content between individuals. Rs738409 polymorphism disrupts phospholipase activity of \textit{PNPLA3}, leading to the impairment of lipid catabolism, lipid droplets remodeling, and VLDL secretion, result in accumulation of triacylglycerol in hepatocytes.\textsuperscript{13–19} Non-invasive risk prediction scoring system uses SNPs as a risk component and to date, convincing data have demonstrated the link between \textit{PNPLA3} SNPs with the risk for NAFLD, NASH, and their severity. Nevertheless, the differences in genotyping methods or diagnostic standards and the limitations of sample sizes arise heterogeneous data, presenting significant difference between allele frequencies of \textit{PNPLA3} variants in populations.\textsuperscript{20–24}

Despite the large amount of data from various populations in different parts of the world, the frequency and significance of \textit{PNPLA3} rs738409 polymorphism in Turkish subjects is still debated. In this study, we aimed to determine the frequency of polymorphic genotypes and alleles of \textit{PNPLA3} rs738409 polymorphism and its possible associations with NAFLD and NASH as well as severity of liver disease in a cohort from Turkey. Since the majority of NAFLD patients have simple or advanced steatohepatitis, a subgroup of NASH patients is also included as a case, due to their high risk of having fibrosis/cirrhosis and hepatocellular carcinoma.

2. Methods

2.1. Study subjects

Two hundred NAFLD patients and 62 control subjects were recruited from Balcali Hospital of Cukurova University, Adana, Turkey. Eighty of 200 patients had biopsy-proven NASH. Being diagnosed with NAFLD was taken as inclusion criteria for case group while having no disease such as NAFLD or similar metabolic disease was taken as inclusion criteria for control group. High alcohol consumption, having hepatitis B virus and hepatitis C virus infection, autoimmune hepatitis, Wilson disease, hereditary hemochromatosis and previously subjected to steatosis treatment, as well as being in the same family were exclusion criteria. Before obtaining specimens for DNA analysis, informed consent was taken from all subjects. Medical ethics committee of Cukurova University was approved this study.

2.2. Histological data

Patients with NAFLD were classified according to their liver histology data determined by the NASH Clinical Research Network classification.\textsuperscript{25} All liver biopsy specimens, obtained from enrolled patients were placed in formalin solution for fixation; embedded in paraffin blocks and stained with hematoxylin–eosin and Masson’s trichrome. An experienced liver pathologist who was blinded to clinical and laboratory data of participants analyzed all the biopsies. The histologic features of NAFLD were scored according to the NASH Clinical Research Network classification.

2.3. Biochemical analysis

Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. IR was calculated on the basis of fasting plasma glucose and insulin values using the homeostasis model assessment-insulin resistance method (HOMA-IR: plasma glucose [mg/dL] × insulin [\textmu g/mL]/405).\textsuperscript{26} Serum ALT, aspartate aminotransferase (AST), GGT, alkaline phosphatase (ALP), total bilirubin, total cholesterol (TC), triglycerides levels (TG), high-density and low-density lipoprotein (HDL, LDL) were measured by central laboratory using standard reagents. The interpretation of test results was based on the reference range recommended by the manufacturers’ instructions (Siemens ADVIA Centaur XP and ADVIA 2400 systems).

2.4. Genotyping

For \textit{PNPLA3} C/G polymorphism, 2.5 mL of blood samples was taken into separate tubes and stored at -4°C until DNA isolation. Genomic DNA extraction from blood samples was performed with high pure PCR template preparation kit (Roche). Case and control group subjects were genotyped by real-time PCR method (Roche LightCycler 480 Instrument). Genomic DNA was amplified by using the primers: 5’-GGAGGTGATGTTAGTTCCCCGT-3’ and 5’-AGCA-CACCTCCAGGGCCC-3’ to detect the \textit{PNPLA3} rs738409 C→G SNP, encoding I148M. The PCR conditions were as follows: initial denaturation step at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute, followed by a final step at 72°C for 7 minutes. Subsequent analyses were performed using the BigDye Terminator v3.1 cycle sequencing system (PE Applied Biosystems, Foster City, CA). The amplified products were then analyzed using an ABI 3100 genetic analyzer (Perkin Elmer, Foster City, CA).

2.5. Statistical analysis

SPSS (SPSS Inc., IL, Chicago) software was used for statistical analyses. Continuous variable values were expressed as mean± standard deviation. Significant statistical level was chosen as \( p < 0.05 \). Gene counting was performed for genotype and allele frequencies. Normal distribution was checked by Kolmogorov–Smirnov test. Chi-square analysis was performed for genotype and allele frequency differences between NAFLD and control groups. Differences of clinical characteristics between NAFLD and control groups were evaluated with Kruskal–Wallis and Mann–Whitney U tests. Effect of polymorphic genotypes on individuals to have NAFLD was evaluated by logistic regression analysis. Chi-square analysis was used for testing the Hardy–Weinberg equilibrium.
3. Results

Baseline clinical characteristics of case and control group subjects were shown in Table 1. Recruiting age matched control group could not be achieved due to patient population applied to gastroenterology clinic as subjects. BMI of NAFLD patients was significantly higher than controls ($p<0.001$). AST and ALT levels were also significantly higher in NAFLD patients ($p<0.001$). IR value of CC+GG control subjects was significantly higher than IR value of CC subjects ($p<0.001$).

As shown in Table 2, genotype and allele frequencies were significantly different between groups ($p<0.01$). Homozygous polymorphic genotype (GG) frequency was importantly higher in NAFLD patients than control group in both additive and recessive models ($p<0.001$). Frequency of polymorphic allele carriers (CG+GG) was also significantly higher in NAFLD group ($p<0.001$). In addition, polymorphic (G) allele frequency was higher in NAFLD group than control group ($p<0.0001$).

In Table 3, comparison of frequency between NASH patients and controls was shown. No significant difference detected between groups.

Table 4 demonstrated intragroup genotype frequencies of NAFLD and NAFLD–NASH patients; however, any significant difference between genotype frequencies in both groups could not be obtained.

Tables 5 and 6 show intragroup clinical characteristic comparison between NAFLD genotypes as well as NASH genotypes. AST, ALT, and IR values of polymorphic homozygous control subjects (GG) were higher in recessive model than homozygous normal plus heterozygous subjects (CC+CG) ($p<0.05$, $p<0.05$, and $p<0.001$, respectively). Similarly, IR value of CC+GG control subjects was significantly higher than IR value of CC subjects ($p<0.001$).

In Table 7, regression results were shown. Our cohort showed a significantly higher risk of having NAFLD, when the individual had a homozygous polymorphic genotype with an odds ratio of 3.563 ($p<0.01$, 95% CI = 1.724–7.365) compared to homozygous normal individuals. Likewise, if the individual had a heterozygous genotype, they placed in the case group with odds ratio of 2.432 ($p<0.05$, 95% CI = 1.198–4.935) compared to homozygous normal individuals in the unadjusted model. Both recessive and dominant models gave close odds ratios as 2.462 (95% CI = 1.253–4.834) and 2.957 (95% CI = 1.639–5.333) ($p<0.01$ and $p<0.001$, respectively) for having NAFLD. However when adjusted for sex and age, the association disappears.

Unfortunately, we could not confirm the control group is in concordance with Hardy–Weinberg equilibrium ($p<0.05$), most probably due to a small population structure that causes the low chance of recruiting a balanced and homogeneous cohort.

4. Discussion

PNPLA3 gene consists of 23,777 nucleotides and locates on the long arm of the 22th chromosome (22q13.31). Although, it is
thought that the PNPLA3 enzyme (triacylglycerol lipase) regulates the development of adipocytes and hepatocytes by catalyzing the hydrolysis of triacylglycerol, its exact function is not clearly understood. PNPLA3 is 1 of the 5 genes that has been extracted from genome-wide association studies related to NAFLD.\textsuperscript{24} Rs738409 (I148M) polymorphism is a nucleotide C to G substitution at position 444 of PNPLA3 gene that leads to change of isoleucine to methionine in PNPLA3 protein (synonymous adpinonitrin) at position 148, which is close to catalytic domain of the enzyme.\textsuperscript{24} This substitution restricts the binding of substrates to the catalytic domain of the protein, thus reducing the enzyme activity on glycerolipids to a degree.\textsuperscript{27} On the other hand, clues indicate that this enzyme leads to acquired feature of synthesizing phosphatidic acid from lysophosphatidic acid.\textsuperscript{28}

In a population of 9229 individuals, including African Americans, European Americans, and Hispanics, rs738409 was found to be strongly associated with increment of hepatic fat content and hepatic inflammation. Besides, in this study, the highest frequency of the G allele was detected in Hispanic populations, we showed that a genotype consists of at least 1 polymorphic allele significantly increased the susceptibility of NAFLD in unadjusted regression model ($P < 0.05$). However, significant results could not be obtained in the adjusted model for sex and age ($P > 0.05$); may be due to undetermined or

### Table 5
Comparison of intragroup clinical characteristics, recessive model.

| Genotype | OR (95% CI) | $P$ |
|----------|-------------|-----|
| Controls (mean rank) BMI (kg/m$^2$) | 30.7 (1.938–4.935) | $< 0.05$ |
| AST (U/L) | 28.5 (1.938–4.935) | $< 0.05$ |
| ALT (U/L) | 28.3 (1.938–4.935) | $< 0.05$ |
| IR (HOMA) | 25.5 (1.938–4.935) | $< 0.001$ |
| NASH (mean rank) BMI (kg/m$^2$) | 102.1 (1.938–4.935) | $< 0.05$ |
| AST (U/L) | 98.4 (1.938–4.935) | $< 0.05$ |
| ALT (U/L) | 95.8 (1.938–4.935) | $< 0.05$ |
| IR (HOMA) | 104.1 (1.938–4.935) | $< 0.05$ |
| TG (mg/dL) | 94.6 (1.938–4.935) | $< 0.05$ |
| GGT (U/L) | 80.8 (1.938–4.935) | $< 0.05$ |
| TC (mg/dL) | 94.4 (1.938–4.935) | $< 0.05$ |
| LDL (mg/dL) | 93.3 (1.938–4.935) | $< 0.05$ |
| ALP (U/L) | 83.0 (1.938–4.935) | $< 0.05$ |
| Total bilirubin (mg/dL) | 78.5 (1.938–4.935) | $< 0.05$ |
| HDL (mg/dL) | 100 (1.938–4.935) | $< 0.05$ |

### Table 6
Comparison of intragroup clinical characteristics, dominant model.

| Genotype | OR (95% CI) | $P$ |
|----------|-------------|-----|
| Controls (mean rank) BMI (kg/m$^2$) | 30.7 (1.938–4.935) | $< 0.05$ |
| AST (U/L) | 28.5 (1.938–4.935) | $< 0.05$ |
| ALT (U/L) | 28.3 (1.938–4.935) | $< 0.05$ |
| IR (HOMA) | 25.5 (1.938–4.935) | $< 0.001$ |
| NASH (mean rank) BMI (kg/m$^2$) | 105.2 (1.938–4.935) | $< 0.05$ |
| AST (U/L) | 93.1 (1.938–4.935) | $< 0.05$ |
| ALT (U/L) | 96.1 (1.938–4.935) | $< 0.05$ |
| IR (HOMA) | 106.1 (1.938–4.935) | $< 0.05$ |
| TG (mg/dL) | 93.1 (1.938–4.935) | $< 0.05$ |
| GGT (U/L) | 77.1 (1.938–4.935) | $< 0.05$ |
| TC (mg/dL) | 85.8 (1.938–4.935) | $< 0.05$ |
| LDL (mg/dL) | 84.4 (1.938–4.935) | $< 0.05$ |
| ALP (U/L) | 80.7 (1.938–4.935) | $< 0.05$ |
| Total bilirubin (mg/dL) | 90.1 (1.938–4.935) | $< 0.05$ |
| HDL (mg/dL) | 94.3 (1.938–4.935) | $< 0.05$ |

### Table 7
Logistic regression analysis.

| SNP number | Genotype | OR (95% CI) | $P$ |
|------------|----------|-------------|-----|
| Additive model |  |  |  |
| rs738409 | CC | 1 | 1 |
| | CG | 2.432 (1.198–4.935) | $< 0.05$ |
| | GG | 3.563 (1.724–7.365) | $< 0.01$ |
| Recessive model |  |  |  |
| rs738409 | CC + CG | 1 | 1 |
| | CG | 2.462 (1.253–4.834) | $< 0.01$ |
| | GG | 2.957 (1.639–5.333) | $< 0.001$ |
| Dominant model |  |  |  |
| rs738409 | CC | 1 | 1 |
| | CG + GG | 1.449 (0.388–6.411) | $< 0.05$ |

SNPs = single nucleotide polymorphisms.

* Adjusted for sex and age.
non-genetic factors that we did not investigate in this study. Considering the effect of I148M on NAFLD and liver degeneration in different populations all over the world, it was not a surprise to see the same effect in a population from Turkey.

Our logistic regression analysis with both recessive and dominant models demonstrated that heterozygosity did not prevent NAFLD pathogenesis ($P < 0.01$, $P < 0.001$, respectively). Particularly, inconsistent results were obtained from different ethnic populations of different countries, similar to many other case-control disease-polymorphism association studies. In our analysis, for unadjusted model the odds ratio of having NAFLD was 3.563 (95% CI = 1.724−7.365), resembling to the odds ratio 3.3 from Italy and UK according to their additive models.\(^{24}\) GG genotype frequencies of patients were higher than controls in our cohort compared to same study (40% vs 14% in cases and 13% vs 3% in controls), suggested that impact of the I148M polymorphism on having NAFLD was independent of genotype frequencies in investigated populations. Additionally, in a recent meta-analysis, GG genotype was associated with NAFLD with an odds ratio of 4.01 (95% CI = 2.93−5.49) ($P < 0.05$). Consistent with our study ($P < 0.0001$), the frequency of the polymorphic G allele was higher in the NAFLD group than controls in the same meta-analysis.\(^{29-33}\) However, often, sample sizes of case-control studies that evaluates associations between diseases and polymorphisms are not large enough to represent precisely the population from which they were recruited.

In another meta-analysis, rs738409 was shown to be strongly associated with NAFLD and NASH. This study further identified that GG genotype was more frequent than CC in NASH patients. Moreover, 2 G alleles had no effect on developing more severe histopathology.\(^{25}\) Likewise, we did not detect any effect of polymorphic genotypes on histopathological grades ($P > 0.05$).

Our analysis revealed that BMI, AST, ALT, and IR values of the NAFLD patients were significantly higher than controls ($P < 0.001$). In a review study, higher ALT and AST levels were found to be associated with G allele.\(^{18}\) Similar to our cohort, NAFLD was associated with BMI in different cohorts.\(^{18,22,23,34}\) To the contrary, in Dallas Heart Study, BMI and G allele was not found to be associated.\(^{19}\) BMI level is an informative and clinically valuable parameter for diagnosis and follow up for NAFLD as well as obesity regarding PNPLA3, since this gene is expressed mainly in abdominal and subcutaneous tissues.\(^{26}\) Considering increased liver fat and liver degeneration in NAFLD patients, these significant differences are expected. When intragroup clinical characteristics of control patients were compared between genotypes, AST, ALT, and IR levels GG genotype were significantly higher than CC+CG genotypes according to recessive model ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively), indicating that GG genotype was associated with increased AST, ALT, and IR independent of NAFLD and NASH. Similarly, compared to control with dominant model intragroup, only IR of GG+GG genotypes was significantly higher than CC genotype ($P < 0.001$). Recessive and dominant model comparison of clinical characteristics showed that homozygosity of the polymorphism had a higher impact than heterozygosity on AST and ALT levels. Clinical characteristics in different populations often give contradictive results due to high variability among individuals and ethnicity. For instance, HDL cholesterol levels were inconsistently associated with I148M polymorphism in various study populations.\(^{18,24,35}\) Unlike our study, NAFLD patients with rs738409 polymorphism were previously associated with increased AST, ALT, and GGT levels.\(^{36}\) With respect to statistical testing for associations between clinical characteristics and polymorphisms, it should be noted that levels of biochemical parameters may vary largely among individuals and cohort mean values depending on age, lifestyle, other genetic factors, and diseases.\(^{37}\) Thus, special attention must be taken when analyzing the biochemical characteristics.

An informative risk assessment score using multivariate logistic regression was carried and showed that besides biochemical parameters, addition of PNPLA3 rs738409 into the model improved accurate prediction only by 1%.\(^{38}\)

Throughout the last 15 years, genome-wide association studies and case-control association studies accumulate a great deal of knowledge about NAFLD. Rs738409 of PNPLA3 was first shown to be associated with NAFLD in 2008.\(^{18}\) Additionally, recent studies revealed that this polymorphism was also associated with liver damage diseases such as NASH, fibrosis, cirrhosis, and hepatocellular carcinoma.\(^{8,12,21,22,24,39}\) In order to show the involvement of rs738409 in other liver damages in our cohort, further studies are required with a larger group of patients and controls.

We classified all patients according to their liver degeneration (grades 1, 2, 3, 4, and 5) and when we compared genotype frequencies between grade groups, we did not see any significant difference ($P > 0.05$). Conversely, in Japanese population PNPLA3 gene was found to be strongly associated with NASH progression.\(^{29,40}\)

It should be mentioned that this study has 2 limitations: first, we did not add correction for multiple testing since the statistical methods used in this study were eligible for case-control polymorphism studies. Second, sample size is not as large as similar studies. Consequently, our results show a significant association between I148M polymorphism and NAFLD in a cohort from Turkey in only unadjusted regression model, which is consistent with other similar studies carried out in various ethnic populations. Here, we also demonstrate that in only unadjusted regression model rs738409 C>G polymorphism (I148M) of PNPLA3 gene is significantly able to affect individuals to have NAFLD. Nevertheless, for our study cohort, final consideration of association between rs738409 and NAFLD may be negative due to disappearing significance $P$ level in adjusted regression model. However, to obtain precise genotype and allele frequencies of NAFLD patients in Turkey, larger and more accurate cohorts require as a complementation of this study.

**Author contributions**

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