The role of PNPLA3 (rs738409) c>g variant on histological progression of non-alcoholic fatty liver disease

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Abstract

Background and Aim: The present study aims to investigate the role of genetic variability of the PNPLA3 gene (adiponectin) in predisposition to non-alcoholic fatty liver disease (NAFLD) by comparing NAFLD patients to healthy controls and to investigate any impact of the PNPLA3 genetic variability on the natural course of the disease.

Materials and Methods: This cohort consisted of 174 patients with biopsy-proven NAFLD and 151 healthy controls. DNA was extracted from peripheral blood and the rs738409 C>G single nucleotide polymorphism was assessed using PCR-DNA sequencing.

Results: The frequency distribution of the GG genotype was significantly higher in NAFLD patients than in controls (p=0.01). In patients with NAFLD, the GG genotype was associated with lower platelet counts (p=0.001), the presence of steatohepatitis (p=0.04) and hepatic fibrosis (p=0.016). After adjustment for age, gender, obesity, and diabetes mellitus, the GG genotype was an independent predictor of significant hepatic fibrosis (adjusted odds ratio = 3.031 p=0.012). From the baseline to sequential liver biopsies, the progression of NAS in NAFLD patients was slightly higher in the GG genotype than that of CC and GG genotypes (p=0.18).

Conclusion: The PNPLA3 GG genotype is a predisposing factor for the development of hepatic steatosis in NAFLD patients and related to a more severe liver disease.

Keywords: genome-wide association studies (GWAS); nonalcoholic fatty liver disease; PNPLA3; steatohepatitis.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver disorders worldwide.[1–4] NAFLD is the spectrum of liver disease ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH), hepatic fibrosis, cirrhosis and liver cancer.[1–4] NAFLD affects approximately 25% of the general population.[5–8] Obesity, diabetes, race, ethnic differences and genetic predispositions affect its prevalence and natural course of the disease.[5–8] The prevalence of NAFLD has increased gradually during the past two decades.[9] The increasing prevalence of obesity, DM, and associated metabolic syndromes increases the risk of NAFLD-related cirrhosis.[6] In Turkey, the prevalence of obesity was 36%.[7] Previous studies reported that the NAFLD prevalence in Turkey is around 10.6%–23.2%,[7] however, no reliable data regarding the prevalence of NAFLD are available.

Patinat-like phospholipase domain-containing (PNPLA) proteins, a mammalian family of lipid hydrolase, are members of the patatin family.[10–12] The human genome expresses nine PNPLA proteins (PNPLA1-9), which serve critical roles in lipid metabolism and its signaling, and the maintenance of membrane integrity, and regulation of energy metabolism.[10–14] The nonalcoholic fatty liver disease ranging from hepatic steatosis to non-alcoholic steatohepatitis. The nonsynonymous mutation (1148M) of this gene causes a loss of function that predisposes to steatosis as a result of decreased triglyceride hydrolysis in hepatocytes.[11–13] Several groups reported a mutation in the PNPLA3 gene (adiponutrin, rs738409 C>G) encoding isoleucine to methionine substitution at the 148 position that is associated with the prevalence and severity of NAFLD and alcoholic liver disease, and chronic hepatitis, fibrosis, and cirrhosis and its complications included hepatocellular carcinoma.[15–20] The present study aims to investigate the role of genetic variability of the PNPLA3 gene (adiponutrin, rs738409) in predisposition to NAFLD by comparing NAFLD patients to healthy controls and investigate any impact of the PNPLA3 genetic variability on the natural course of the disease on longitudinal liver biopsies in such patients.
Materials and Methods

Patients

Between May 2010 and February 2014, 174 DNA samples of consecutive patients with NAFLD, who were seen in the Ankara University School of Medicine, Department of Gastroenterology, Liver Diseases Outpatient Clinic, were analyzed. Diagnosis of NAFLD in each case was based on biochemical, radiological and histological criteria. Criteria for inclusion were: 1) age >16 years; 2) convincing evidence of absent or minimal alcohol consumption: <20 g alcohol/day for women and <30 g alcohol/day for men; 3) absence of confounding disease, including acute and chronic viral hepatitis (hepatitis A, B or C); and 4) exclusion of other forms of liver disease, including autoimmune, drug-induced and metabolic liver diseases. Data were obtained from patient visit charts. Population control samples were from 151 healthy individuals who had no evidence of liver disease or any other disease, no alcohol abuse. Controls with high body mass index (BMI, >25 kg/m²) and an abnormal abdominal sonographic pattern were excluded from this study.

Clinical and Laboratory Assessments

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase, bilirubin, fasting glucose, cholesterol, triglycerides levels, and complete blood cell counts were measured by our central laboratory using standard reagents. Insulin was measured by radioimmunoassay.

Histological Assessments

Liver biopsy was performed in patients with normal and abnormal serum aminotransferases levels having an abnormal sonographic pattern as increased liver parenchyma echogenicity. All liver biopsy specimens were retrieved from the archives of the Department of Pathology, Ankara University School of Medicine. Liver biopsy material was mounted on archival slides originally prepared from 10% formalin-fixed paraffin-embedded tissue and stained with hematoxylin-eosin and with Masson’s trichrome stain to evaluate the fibrosis. The liver biopsy specimens were evaluated by one pathologist (BS), who has blinded to the clinical and biochemical data. Histological features of the samples were interpreted based on the criteria of Kleiner et al.[21] Hepatic steatosis was classified from grade 0 to grade 3: Minimal steatosis, grade 0, was defined as <5% steatosis; mild steatosis, grade 1 as 5–33%; moderate steatosis, grade 2, as 33%–66%; and severe steatosis, grade 3, as >66%. Necroinflammation, either lobular or portal, was graded. Fibrosis was scored from 0 to 4 and significant fibrosis was defined as stage 2 to 4 fibrosis. NAFLD Activity Score (NAS) (0–8) was calculated based on the grade of steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2).[21] NASH was defined as patients with NAS ≥5, while patients with NAS <3 were diagnosed as not-NASH.[21]

Genetic Analysis

Genomic DNA samples were extracted from peripheral blood leukocytes using the high salt concentration method.[22] Genomic DNA was amplified by PCR using the primers: 5′-GAGGGTGTTAGTTAGTTCCCGT-3′ and 5′-AGCACACTTCAGAGGCCCTCC-3′ to detect the adiponutrin/GNAT2 and SLC27A5 genetic markers. PCR products were purified by sodium acetate–ethanol precipitation. The purified products were then analyzed using an ABI 3100 genetic analyzer (Perkin Elmer, Foster City, CA, USA).

Definition

BMI was calculated as weight in kilograms divided by height in meters squared. Obesity was defined based on the World Health Organization criteria with a BMI of 25–29.9 kg/m² defining overweight and BMI ≥30 kg/m² defining obesity.[23] 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) x insulin (µu/ml)/405).[24]

After the diagnosis of NAFLD was confirmed, the management was focused in the following areas: the establishment of an appropriate diet, including a conventional diet of 25 kcal/kg x ideal body weight (kg) with three meals per day containing 60% carbohydrate, 25% fat and 15% protein and an exercise program, including walking (initially as 300 steps per day for three days, thereafter adding 500 steps at 3-day intervals until a level of 10000 steps was attained) and/or jogging (20 minutes twice a day).[23,26] improvement in associated conditions, such as diabetes mellitus, IR, and moderate/severe hyperlipidemia; and discontinuation of potentially hepatotoxic drugs, such as herbal medicine. The dietitian interviewed all subjects, and the same diet and exercise program was suggested. The dietitian and one of the authors monitored patient compliance with the diet and exercise program during the follow-up period using verbal communication.

Follow-up

All subjects were seen in the fourth week and in three or six-month intervals thereafter in the Outpatient Clinic. Laboratory tests were performed during the follow-up period. Second liver biopsy was optional and was performed after one year of the follow-up period for the evaluation of the liver histology and treatment effect when the patient was accepted.

Statistical Analysis

Categorical variables were evaluated using Chi-square test or Fisher’s exact test, where applicable. Differences among the groups for baseline clinical and laboratory characteristics were evaluated using Kruskal-Wallis variance analysis. When there were two groups, the Mann-Whitney U test was used. To define risk factors of the outcome variable (steatosis, NAS), multiple logistic regression analysis was used. SPSS for Windows 11.5 was used for statistical analysis. P-value less than 0.05 was considered significant.

Results

The present cohort consisted of 325 subjects from 174 individuals with biopsy-proven NAFLD (M/F: 89/85, mean age: 47.9±10.5 years) and 151 healthy controls (M/F: 45/106, mean age: 31.1±8.8 years). NAFLD patients were older with male predominance (p<0.001 and p<0.001, respectively). At the time of the diagnosis of NAFLD, the median BMI was 29.2 kg/m² (36% were obese), and 23% of the patients had diabetes mellitus, 58% IR (HOMA score >2.7), 49% hyperlipidemia, and 24% hypertension. Median serum AST, ALT and GGT levels were 32...
U/L (range: 10–133 U/L), 50 U/L (range: 16–235 U/L) and 38 (range: 10–442 U/L), respectively. The characteristics of all patients with NAFLD are shown in Table 1.

The median NAS of the initial 174 liver biopsies was 5.0 (range: 1 to 8), median hepatic steatosis 2 (0–3), ballooning 2 (0–3), lobular inflammation 1 (0–3), portal inflammation 0 (0–2), and fibrosis 0.5 (0–4). Of those, 22.4% of the biopsy samples revealed grade 1, 25.3% grade 2 and 41.4% grade 3 hepatic steatosis. Ballooning was observed 59.2% of the samples: few balloon cells (mild-moderate) in 57.5% and many balloon cells (severe) in 1.7%, whereas 40.8% of the samples showed no ballooning cell. Lobular inflammation was graded as follows: 8.6% of the patients (n=15) had no necroinflammation, 77.6% had grade 1, 12.1% had grade 2, and the remaining 1.7% had grade 3 necroinflammation. Of note, 50.0% of the samples (n=87) revealed the presence of fibrosis: 57.5% of those (50/87) had F1 and 42.5% (37/87) showed significant fibrosis (≥F2). A histological diagnosis of NASH (NAS ≥5) was obtained in 60% of the patients (104/174). The histological features of the patients are shown in Table 2.

### Association with Clinical and Biochemical Parameters

Among NAFLD patients, 47.7% (n=83) had GG, 27.6% (n=48) had CC and 24.7% (n=43) had CG genotype, compared with 31% (n=47) GG, 36% (n=54) CC and 33% (n=50) CG genotype frequencies among healthy controls, respectively (Fig. 1). The frequency distribution of the GG genotype was significantly higher in NAFLD patients than in healthy controls (48.0% vs. 31.0%, p=0.01) (Fig. 1). Among patients’ baseline characteristics, the GG genotype was only associated with low platelet counts (226.8±57.1 vs. 263.5±81.2, p=0.001).

### Association with Liver Histology

The GG genotype was significantly associated with the presence of steatohepatitis (56/83, 67.5% vs. 48/91, 52.7%; p=0.048) and hepatic fibrosis (1.01±1.10 vs. 0.06±0.77, p=0.016). No association was found with the severity of steatosis (grades 2 and 3), hepatocellular ballooning, or lobular or portal inflammation. In addition, significant fibrosis (≥F2) was significantly associated with the GG genotype (25/83, 30.1% vs. 12/91, 13.2%; p=0.006). After adjustment for age, gender, obesity and diabetes mellitus, the GG genotype was significantly associated with significant fibrosis [adjusted odds ratio [OR]= 3.0, 95% confidence interval (CI)= 1.279 – 7.183; p=0.012] (Table 3).

### Association with Histological Progression

Fifty-one NAFLD patients had sequential liver biopsies; 34 patients had NASH on their initial biopsies. The median interval between the two biopsies was 44 months (range, 12–120 months). The median NAS of the initial 51 liver biopsies was 4.96 (range: 2–8), hepatic steatosis 2 (0–3), ballooning 2 (0–3), lobular inflammation 1 (0–3), portal inflammation 0 (0–2), and fibrosis 0 (0–4). On their second liver biopsies, 29 patients had NASH; the median NAS was 5.0 (range: 2 to 7), hepatic fibrosis 1.01±1.10 vs. 0.06±0.77, p=0.016). No association was found with the severity of steatosis (grades 2 and 3), hepatocellular ballooning, or lobular or portal inflammation.

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**Table 1. Characteristics of all patients with NAFLD**

| Characteristic | Mean±SD (median) | Range |
|---------------|-----------------|-------|
| Age (years)   | 47.9±10.5 (49)  |       |
| Gender (Male/Female) | 89 (51.1%)/85 (48.9%) |       |
| BMI (kg/m²)   | 30.0±5.5 (29.2) |       |
| Diabetes mellitus | 23.3% |       |
| Hypertension  | 23.6% |       |
| Hyperlipidemia | 49% |       |
| Insulin resistance (HOMA score>2.7) | 58% |       |
| Serum AST levels (normal range: 10–37 U/L) | 38.8±21.5 (32) |       |
| Serum ALT levels (normal range: 10–37 U/L) | 61.6±40.1 (50) |       |
| Serum GGT levels (normal range: 0–55 U/L) | 55.7±54.5 (38) |       |
| Serum total bilirubin levels (normal range: 0.2–1.1mg/dL) | 0.84±0.46 (0.7) |       |
| Serum platelet counts (normal range: X10⁹/L) | 245.8±72.8 (234) |       |

Mean±SD (median); SD: Standard deviation; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Serum alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase.

**Table 2. The baseline histopathological characteristics of 174 patients with NAFLD**

| Characteristic | Mean±SD | Median |
|---------------|---------|--------|
| NAS           | 4.7±1.5 | 5.0    |
| Steatosis     | 1.97±1.04 | 2.0    |
| Ballooning    | 1.61±0.52 | 2.0    |
| Lobular inflammation | 1.07±0.52 | 1.0    |
| Fibrosis      | 0.79±0.96 | 0.5    |
| NASH diagnosis (NAS ≥5) | 104 | 59.8 |

NAS: NAFLD Activity Score; NAFLD: Non-alcoholic fatty liver disease.

**Table 3. With logistic regression analysis, the association of the GG genotype with advanced hepatic fibrosis**

| Variables | Adjusted OR | 95.0% CI | p     |
|-----------|-------------|----------|-------|
| Lower     | Upper       |          |       |
| Age       | 1.023       | 0.978    | 1.070 | >0.05 |
| Gender    | 1.686       | 0.669    | 4.253 | >0.05 |
| Diabetes  | 2.377       | 0.968    | 5.841 | >0.05 |
| BMI       | 1.435       | 0.607    | 3.390 | >0.05 |
| GG genotype | 3.0       | 1.279    | 7.183 | 0.012 |

OR: Odd ratios; CI: Confidence interval; BMI: Body mass index.

**Figure 1. The frequency distributions of the PNPLA3 gene, CC, CG and GG genotypes in NAFLD patients compared to healthy controls.**

GG, 36% (n=54) CC and 33% (n=50) CG genotype frequencies among healthy controls, respectively (Fig. 1). The frequency distribution of the GG genotype was significantly higher in NAFLD patients than in healthy controls (48.0% vs. 31.0%, p=0.01) (Fig. 1). Among patients’ baseline characteristics, the GG genotype was only associated with low platelet counts (226.8±57.1 vs. 263.5±81.2, p=0.001).

**Association with Liver Histology**

The GG genotype was significantly associated with the presence of steatohepatitis (56/83, 67.5% vs. 48/91, 52.7%; p=0.048) and hepatic fibrosis (1.01±1.10 vs. 0.06±0.77, p=0.016). No association was found with the severity of steatosis (grades 2 and 3), hepatocellular ballooning, or lobular or portal inflammation. In addition, significant fibrosis (≥F2) was significantly associated with the GG genotype (25/83, 30.1% vs. 12/91, 13.2%; p=0.006). After adjustment for age, gender, obesity and diabetes mellitus, the GG genotype was significantly associated with significant fibrosis [adjusted odds ratio [OR]= 3.0, 95% confidence interval (CI)= 1.279 – 7.183; p=0.012] (Table 3).
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Table 4. The GG genotype did not influence disease progression in patients with NAFLD between the two sequential liver biopsies

| NAFLD Patients | 51 NAFLD patients | 34 NASH patients | p |
|----------------|-------------------|------------------|---|
|                | GG (%)            | CC+CG (%)        | GG (%) | CC+CG (%) | p |
| NAS            |                   |                  |       |
| Stabilized and regressed | 64.0 | 80.8 | 72.2 | 75.0 | p=0.180 |
| Progression    | 36.0 | 19.2 | 27.8 | 25.0 | p=1.0 |
| Steatosis       |                   |                  |       |
| Stabilized and regressed | 88.0 | 80.8 | 88.9 | 81.3 | p=0.703 |
| Progression    | 12.0 | 19.2 | 11.1 | 18.8 | p=0.648 |
| Ballooning      |                   |                  |       |
| Stabilized and regressed | 72.0 | 84.6 | 77.8 | 87.5 | p=0.274 |
| Progression    | 28.0 | 15.4 | 22.2 | 12.5 | p=0.660 |
| Lobular Inflammation |      |      |       |
| Stabilized and regressed | 88.0 | 80.8 | 88.9 | 81.3 | p=0.703 |
| Progression    | 12.0 | 19.2 | 11.1 | 18.8 | p=0.648 |
| Fibrosis        |                   |                  |       |
| Stabilized and regressed | 60.0 | 61.5 | 61.1 | 56.3 | p=0.910 |
| Progression    | 40.0 | 38.5 | 38.9 | 43.8 | p=0.774 |

NAFLD: Non-alcoholic fatty liver disease; NAS: NAFLD Activity Score; NASH: Non-alcoholic steatohepatitis.

steatosis 2 (0–3), ballooning 2 (1–3), lobular inflammation 1 (0–2), portal inflammation 0 (0–2), and fibrosis 1 (0–4).

Between the two biopsies, hepatic steatosis regressed in 15.7% of the samples, showed no change in 52.9% and progressed in 31.4%; hepatic ballooning regressed in 21.6%, stabilized in 49.0% and progressed in 29.4%; lobular inflammation regressed in 15.7%, stabilized in 58.8% and progressed in 25.5%; NAS regressed in 27.5%, stabilized in 31.4% and progressed in 41.1%, and fibrosis regressed in 39.2%, stabilized in 45.1% and progressed in 15.7%.

In NAFLD patients, from the baseline to sequential liver biopsies, the progression of NAS slightly higher in the GG genotype than that of CC and GG genotypes (36% vs. 19.2%, p=0.180). However, progression of fibrosis did not differ (40.0% vs. 38.5%, p=0.910). No significant differences concerning NAS and fibrosis progression among genotypes of NASH patients were observed (p=0.05). The progression of NAS and fibrosis in NASH patients with GG and other genotypes were 27.8% and 38.9%, and 25% and 43.8% (p=1.0 and p=0.774), respectively (Table 4).

Discussion
This study aimed to investigate the genetic variability of the PNPLA3 gene in a Turkish NAFLD patient cohort with variations in demographic, anthropometric and environmental factors. The frequency distributions of the PNPLA3 gene, CC, CG and GG genotypes in NAFLD patients and controls were 28% and 25%, 48% and 36%, and 33% and 31%, respectively. The GG genotype was more prevalent in NAFLD patients compared to a healthy population (48.0% vs. 31.0%, p=0.01). This result confirms the previous studies that showed a significant association between the GG genotype of the PNPLA3 gene and fatty liver disease.\[15–18,20,27–29\] This result suggests that the GG genotype exerts a strong influence on liver fat accumulation.

The GG genotype represents a loss of function of adiponutrin, which causes failure in the breakdown of triglycerides during hyperinsulinemic states.\[16–14\] The reported data regarding the association between the GG genotype and abnormalities in biochemical parameters, including BMI, hypertriglyceridemia and elevated serum aminotransferases levels in NAFLD patients are conflicting. Some data were able to confirm these findings,\[13,15,27–31\] but other data were unable.\[17,29,30,32,33\] Romeo et al.\[13\] found a significantly increased hepatic triglyceride content in individuals with GG genotype. Kollerits et al.\[13\] reported a significant association of the GG genotype with serum total cholesterol, non-high-density lipoprotein and low-density lipoprotein levels and suggested that adiponutrin might disturb lipid metabolism. Interestingly, in contrast to these findings, other studies\[17,29,30,32,33\] demonstrated the dissociation among the GG genotype, gender and metabolic abnormalities, including obesity, fasting glucose, insulin levels, IR and hypertriglyceridemia in NAFLD patients. In the present study, among the patients’ characteristics, the GG genotype was only significantly associated with low platelet counts in patients with NAFLD (p=0.001). This discrepancy may explain the differences between the recruited patients in various studies.

An association between the GG genotype and the severity of disease in the adult and pediatric populations was reported previously.\[20,29,34–17\] Valenti et al.\[34\] showed that the GG genotype was strongly associated with the presence of severe steatosis (>1), NASH and fibrosis stage (>1). A meta-analysis study reported that NAFLD patients with GG genotype had a 3.24-fold greater risk of higher inflammation scores and a 3.20-fold greater risk of developing liver fibrosis when compared with CC genotype patients.\[29\] In the present study, the GG genotype was significantly associated with the presence of steatohepatitis and hepatic fibrosis (p=0.048, p=0.016, respectively) in NAFLD patients. However, no association was found with the severity of hepatic steatosis, hepatocellular ballooning or lobular inflammation. More importantly, significant fibrosis was more common in the GG genotype than in the other genotypes (p=0.006). After adjustment for age, gender, obe-
sity, and diabetes mellitus, the risk of significant fibrosis was 3.0-fold higher in NAFLD patients with the GG genotype. These results suggest that the GG genotype of the PNPLA3 gene is associated with both liver fat accumulation and also a higher disease severity.

Advanced age (>50 years), male gender, obesity, diabetes mellitus and hepatic necroinflammation have been identified previously as predictive factors associated with the severity of fibrosis and progression of disease in patients with NAFLD.28,29 Grimaudo et al.20 reported that NAFLD patients carrying PNPLA3 rs738409 G>C variant are at higher risk of liver-related events and death. In the present study, from baseline to second liver biopsies, the progression of NAS slightly higher in NAFLD patients with GG genotype than those of CC and GG genotypes (p=0.18). However, no significant differences were observed in NASL patients (p>0.05). This result indicates that a lack of association between the GG genotype of the PNPLA3 gene and disease progression in NAFLD patients. Several other factors, such as advanced age, obesity, alcohol intake, insulin resistance and diabetes, are also exit in the disease progression.

Conclusion

In conclusion, the PNPLA3 GG genotype is a predisposing factor for the development of hepatic steatosis in NAFLD patients and related to a more severe liver disease. GG genotype is one of the predictive factors for disease progression.

Ethics Committee Approval: The Ankara University School of Medicine Clinical Research Ethics Committee granted approval for this study.

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Author Contributions: Concept – RI, MB; Design – RI, SCK, AMB; Supervision – RI; Resource – RI, SCK, AMB; Materials – RI, SCK, GK, BS; Data Collection and/or Processing – RI, SCK, GK, AMB; Analysis and/or Interpretation – RI, SCK, GK, BS, AHE, AMB; Literature Search – RI, AMB; Writing – RI, AHE; Critical Reviews – RI, BS, AHE, MB.

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