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Short Communication

Peroxisome proliferator-activated receptors alpha and gamma2 polymorphisms in nonalcoholic fatty liver disease: A study in Brazilian patients

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) refers to the accumulation of hepatic steatosis in the absence of excess alcohol consumption. The pathogenesis of fatty liver disease and steatohepatitis (NASH) is not fully elucidated, but the common association with visceral obesity, hyperlipidemia, hypertension and type 2 diabetes mellitus (T2DM) suggests that it is the hepatic manifestation of metabolic syndrome. Peroxisome proliferator-activated receptor PPARα and PPARγ are members of a family of nuclear receptors involved in the metabolism of lipids and carbohydrates, adipogenesis and sensitivity to insulin. The objective of this study was to analyze the polymorphisms Leu162Val of *PPARα* and Pro12Ala of *PPARγ* as genetic risk factors for the development and progression of NAFLD.
Methods: One hundred and three NAFLD patients (89 NASH, 14 pure steatosis) and 103 healthy volunteers were included. Single nucleotide polymorphisms (SNPs) Leu162Val and Pro12Ala were analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).
Results: NASH patients presented higher BMI, AST and prevalence of T2DM than patients with pure steatosis. A higher prevalence of 12Ala allele was observed in the NASH Subgroup when compared to Control Group. When we grouped NASH and Steatosis Subgroups (NAFLD), we found lower serum glucose and more advanced fibrosis in the Leu162Val SNP. On the other hand, there was no statistical difference in clinical, laboratorial and histological parameters according to the Pro12Ala SNP.
Conclusions: We documented a lower prevalence of 12Ala allele of gene *PPARγ* in the NASH Subgroup when compared to Control Group. In NAFLD patients, there were no associations among the occurrence of Pro12Ala SNP with clinical, laboratorial and histological parameters. We also documented more advanced fibrosis in the Leu162Val SNP. The obtained data suggest that Pro12Ala SNP may result in protection against liver injury and that Leu162Val SNP may be involved in the progression of NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a simple steatosis (fat accumulation in hepatocytes without concomitant inflammation or fibrosis) and non-alcoholic steatohepatitis (NASH), with a necroinflammatory component (Matteoni et al., 1999). The diagnosis of NAFLD requires detection of steatosis by liver histology or imaging modalities, and the exclusion of other liver diseases, such as alcoholic liver disease (AFLD) and viral hepatitis (Bedogni et al., 2005). NAFLD is a condition which cause concern because it may progress to cirrhosis, liver failure and hepatocellular carcinoma (de Alwis and Day, 2008).

NAFLD occurs worldwide and it is present in various ethnic groups, making it the a common liver condition (Das et al., 2010; Fan et al., 2005; Zelber-Sagi et al., 2006). In Brazilian studies, the most common histological finding was NASH with fibrosis in asymptomatic males, and a significant number of cases already presented cirrhosis at the moment of diagnosis (Abdelmalek et al., 2001; Cotrim et al., 2011).

Obesity and insulin resistance (IR) are factors related to NAFLD (Petta et al., 2009; Vuppalanchi and Chalasani, 2009). Others causes include parenteral nutrition, use of hepatotoxic drugs, gastric bypass surgery and disorders associated with fatty acid metabolism (Brunt, 2009; Paschos and Paletas, 2009). Currently, the pathogenesis of NAFLD has been attributed to the "multiparallel hits" hypothesis, which includes

Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PPAR, Peroxisome proliferator-activated receptor; SNPs, single nucleotide polymorphisms.

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the presence of IR, adipocytokines, lipotoxicity, endoplasmic reticulum stress, interaction between adipose tissue with hepatocytes and genetic factors (Smith and Adams, 2011).

Various single nucleotide polymorphisms (SNPs) have been implicated in the predisposition to NAFLD or NASH, including genes encoding adiponutrin/patatin-like phospholipase-3 (PNPLA3), adiponectin, TNFα, microsomal triglyceride transfer protein and peroxisome proliferator-activated receptor (*PPAR*). The *PPARα* gene is located on chromosome 22q13.3. Some SNPs in this gene were associated with dyslipidemia, IR, type 2 diabetes mellitus (T2DM) and cardiovascular disease (Yong et al., 2008).

The Leu162Val *PPARα* SNP (rs1800206) represents a nitrogen base substitution from cytosine (C) to guanine (G), leading to a leucine to valine amino acid exchange in codon 162 (Flavell et al., 2000; Sapone et al., 2000). For this SNP, Leu162Leu characterizes the wild homozygous genotype. The substitution of a single nitrogen base characterizes the heterozygous genotype (Leu162Val), and the substitution of two basis points to the mutated homozygous (Val162Val).

The *PPARγ* gene is located on chromosome 3p25 and encodes a nuclear transcription factor involved in the expression of hundreds of genes. In the Pro12Ala *PPARγ* SNP (rs1801282), the substitution of one nitrogen base alters the coded amino acid (proline to alanine) in codon 12 in exon 2 (Fajas et al., 1997). The association between the 12Ala variant and IR, T2DM, higher BMI, and obesity has been described in several studies (Altshuler et al., 2000; Mori et al., 2001). The role of this SNP in the pathogenesis and progression of fatty liver disease is still debated.

The purpose of this study was to analyze the SNPs Leu162Val and Pro12Ala of *PPARα* and *PPARγ* as genetic risk factors for the development and progression of the NAFLD.

2. Materials and methods

2.1. Subjects

This study included 103 outpatients with NAFLD, diagnosed according to the histopathological findings from the liver biopsy. The Control Group consisted of 103 healthy subjects without laboratorial signs of hepatitis or other liver dysfunctions, and presenting normal liver ultrasound. All volunteers were registered in a Brazilian university hospital and provided informed consent according to the institutional research ethics committee (Proc#7697/2007).

2.2. Liver histology

The grading and staging of all liver biopsy specimens were conducted by one pathologist specialized in hepatology, and followed Brunt's criteria. Liver samples were prepared with hematoxylin-eosin, Masson's trichrome and silver reticulin stains. The presence of more than 5% steatotic hepatocytes in a liver tissue section was accepted as the minimum criterion for the histological diagnosis of NAFLD (Brunt, 2010; Brunt et al., 2011). Briefly, histological activity was graded based on steatosis [grade 0 (no steatosis), grade 1 (5-33%), grade 2 (33-60%) and grade 3 (>60%)]; lobular inflammation (0-3); portal chronic inflammation (0-2); ballooning (0-2) and fibrosis scores (0-3). The NAFLD activity score (NAS) was used to classify NAFLD into Steato7sis (NAS $\leq$ 4) and NASH (NAS $\geq$ 5) Subgroups. For the present study, the grading of steatosis, lobular inflammation, ballooning and fibrosis was used to rank the histological findings into moderate (0 $\leq$ score $\leq$ 1) or severe (score $\geq$ 2).

3. Methods

3.1. Biochemical analyses

Venous blood samples were obtained after a 12-hour overnight fast. Plasma glucose, serum aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase, iron, ferritin, tryglicerides, total and HDL-cholesterol were measured in a conventional automated analyzer. Low-density lipoprotein cholesterol (LDL-c) was calculated according to the equation of Friedewald et al. (1972). Serum adiponectin, leptin and insulin levels were measured using enzyme linked immunosorbent assays (Millipore, Missouri, USA).

3.2. Clinical and nutritional assessment

The subjects' weight and height were measured with standard techniques. The body mass index (BMI) was calculated by the formula: body weight (kg) / [height (m)]^2. A BMI over 27 kg/m^2 defined obesity (Bray, 1992). We selected 30 individuals of each group randomly and they answered a semi-quantitative food consumption frequency questionnaire, which described food consumption within 6 months prior to the study. Diabetes mellitus was established in patients with a prior diagnosis of T2DM and those with glucose concentrations above 100 mg/dL (Deurenberg et al., 1998; Genuth et al., 2003). Dyslipidemia was defined when total cholesterol was above 240 mg/dL or triglycerides above or equal to 150 mg/dL or LDL-c above or equal to 160 mg/dL or HDL-c below 40 mg/dL for men and below 50 mg/dL for women (JAMA, 2001). IR was calculated according to the homeostasis model assessment of the insulin resistance index (HOMA-IR), computed through the formula: [fasting serum insulin (μU/mL) × fasting serum glucose (mg/dL)] / 405 (Matthews et al., 1985). HOMA above 2.5 defined IR (Guidorizzi de Siqueira et al., 2005).

3.3. Genotyping of Leu162Val of PPAR-α and Pro12Ala of PPARγ

DNA was isolated from peripheral blood leukocytes by the salting-out method (Miller et al., 1988). The Leu162Val and Pro12ALA SNPs were detected through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) following previously described protocols (Al-Shali et al., 2004; Vohl et al., 2000). PCR products were digested overnight at 37 °C with 2 U of *Hinf I* and 1 U of *Hae III* restriction enzymes to analyze Leu162Val and Pro12ALA SNPs, respectively. In both cases, the DNA fragments were visualized on 10% polyacrylamide gels followed by silver staining (Sanguinetti et al., 1994).

3.4. Statistical analysis

Statistical analysis was performed using the software SAS 9.2 (SAS Institute Inc, Cary, USA). Continuous variables were presented as means ± standard deviation. Simple comparison of the clinical data between NAFLD and Control Groups was performed using the Mann-Whitney U test. The comparison of the clinical data among the different genotypes was carried out using the analysis of variance test (ANOVA) after logarithmic transformation. Genotype and allele distributions in NAFLD and Control Groups were analyzed by Fisher's exact test. Concordance to the frequency predicted by the Hardy-Weinberg equilibrium was assessed by the chi-square test. Logistic regression analyses were used to assess the association between these SNPs with disease using sex, age and BMI as covariates at the model. Odds ratio (OR) and the 95% confidence interval (CI) were estimated. Moreover, a logistic regression analysis was included for the evaluation of the association between genotypes and histological disease severity. To verify the association between SNPs and comorbidities, Fisher's exact test was used and the variables were expressed as percentages. The level of significance was set at $p \leq 0.05$ in all analyses.

4. Results

The energy (1838.8 ± 281.1 vs 2013.3 ± 539.3 kcal/day, $p = 0.14$) and carbohydrate (261.5 ± 54.8 vs 230.7 ± 1;66.3 g/day, $p = 0.06$) consumption was similar between the volunteers of NAFLD and Control Group,