Hepatobiliary & Pancreatic Diseases International 000 (2018) 1–6

Contents lists available at ScienceDirect

Hepatobiliary & Pancreatic Diseases International

journal homepage: www.elsevier.com/locate/hbpd

Association between the CYBA and NOX4 genes of NADPH oxidase and its relationship with metabolic syndrome in non-alcoholic fatty liver disease in Brazilian population

Fabiola Rabelo[a,b], Jose Tadeu Stefano[b], Ana Mercedes Cavaleiro[c], Rodrigo Vieira Costa Lima[a,b], Daniel Ferraz de Campos Mazo[b], Flair Jose Carrilho[a,b], Maria Lúcia Correa-Giannella[c,d], Claudia P. Oliveira[a,b,*]

[a] *Departamento de Gastroenterologia, Faculdade de Medicina FMUSP, Universidade de Sao Paulo, Sao Paulo, SP, BR*
[b] *Hospital das Clinicas HCFMUSP, Departamento de Gastroenterologia, LIM07, Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo, SP, BR*
[c] *Laboratório de Carboidratos e Radioimunensaio, LIM18, Hospital das Clinicas HCFMUSP (LIM07) Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo*
[d] *Programa de Pós-Graduação em Medicina, Universidade Nove de Julho(UNINOVE), São Paulo, Brazil*

ARTICLE INFO

Article history:
Received 15 December 2017
Accepted 13 June 2018
Available online xxx

Keywords:
Non-alcoholic fatty liver disease
Nonalcoholic steatohepatitis
Genetic polymorphism
Metabolic syndrome

ABSTRACT

Background: Oxidative stress has been implicated in the progression of severe forms of non-alcoholic fatty liver disease (NAFLD). NADPH oxidase produces reactive oxygen species. In the present study, we investigated for the first time two single nucleotide polymorphisms (SNPs) in the regulatory region of genes encoding NADPH oxidase 4 (NOX4) and p22phox (CYBA) in NAFLD.
Methods: A total of 207 biopsy-proven NAFLD patients [simple steatosis ($n=27$); nonalcoholic steatohepatitis (NASH) ($n=180$)] were evaluated. Genomic DNA was extracted from peripheral blood cells, and polymorphisms in CYBA (unregistered) and NOX4 (rs3017887) were determined by direct sequencing of PCR.
Results: Associations of CYBA-675 T/A with high-density lipoprotein (HDL) (TT vs TA vs AA; $P<0.01$) and triglycerides (TGL) (TT vs XA; $P<0.01$) were observed only in NASH patients. For polymorphisms in the NOX4 gene, NOX4 (rs3017887) CA + AA genotypes was significant associated with alanine aminotransferase (ALT) (CA + AA vs CC; $P=0.02$). However, there was no association of SNPs in the CYBA and NOX4 genes encoding the NADPH oxidase system proteins and the presence of NASH. Regarding the clinical results, it was observed that the most advanced degrees of fibrosis occurred in patients diagnosed with type 2 diabetes mellitus (66.9% vs 37.5%, $P<0.01$) and those who were more obese (32.2 vs 29.0 kg/m^2, $P<0.01$). In addition, serum glucose and insulin levels increased significantly in the presence of NASH.
Conclusions: There were associations between the presence of the allele A in the NOX4 SNP and a higher concentration of ALT in the NAFLD population; between the presence of the AA genotype in the polymorphism of the CYBA-675 T/A CYBA gene and a higher level of TGL and lower HDL in NASH patients. The presence of metabolic syndrome was associated with advanced degrees of fibrosis in NAFLD patients.

Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a large spectrum of disease, from simple steatosis that demonstrates excessive lipid deposition (> 5%) in hepatocytes of the liver parenchyma, to the potentially progressive form known as nonalcoholic steatohepatitis (NASH), histologically characterized by hepatocyte ballooning, inflammation, and frequently, associated with fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1–3]. NAFLD is a complex entity, with varied development and progression among individuals. The reason for these diversities is not fully known; however, environmental influences, such as eating habits, intestinal microbiota, and multiple genetic factors have been described [4–6]. It is believed that genetic factors contribute to 30–50% of the risk in cases of high prevalence of the disease, such as obesity, cardiovascular disease and cirrhosis [7].

Several enzymatic systems, at different locations, may contribute to the formation of reactive oxygen species (ROS) in

* Corresponding author.
E-mail address: cpm@usp.br (C.P. Oliveira).

https://doi.org/10.1016/j.hbpd.2018.06.005
1499-3872/

the liver, including endothelial cells synthesizing nitric oxide, cytochrome P450, monooxygenases, and the nitrogen oxidative nicotinamide adenine dinucleotide phosphate (NADPH) system. The accumulation of ROS in hepatocytes can cause cell death, which stimulates Kupffer cells to recruit immune cells to produce pro-fibrogenic mediators, including myofibroblasts [8]. NADPH oxidases, also known as NOX, are proteins that transfer electrons through membranes and catalyze the NADPH-dependent reduction of O_2 to O_2^-, generating ROS [9]. Thus, the NADPH system may play a role both in defense against viruses and bacteria, and in acting as a pro-inflammatory agent through the overproduction of ROS.

Among the seven members of the NOX family, the classic phagocytic NOX complex consists of the catalytic subunit gp91phox (the term "phox" derives from "phagocytic oxidase"), renamed NOX2, along with the regulatory subunit p22phox located in the membrane. The other regulatory components are p47phox, p40phox, p67phox and the small Rac GTPase, and they are usually located in the cytoplasm. The p22phox protein is encoded by the CYBA gene (cytochrome b-245α), located on chromosome 16q24, and it contains six exons. P22phox requires the presence of a NOX isoform and NOX p22phox for expression, since they act to mutually stabilize one another on the membrane. Thus, variation in the structure or function of p22phox has the potential to influence the activity of a number of NOX isoforms, and this could influence the generation of ROS in several tissues and under different conditions [10].

Several polymorphisms have already been described in the promoter region and in the coding region of the CYBA gene, and some of them are able to influence gene expression, which results in significant functional variation in the level of oxidative stress among individuals. Some polymorphisms are associated with systemic arterial hypertension, coronary artery disease, myocardial infarction, and diabetic and non-diabetic nephropathy [11, 12].

On the other hand, the NOX4 gene is located on chromosome 11 and contains 19 exons. NOX4 is expressed in hepatocytes, Kupffer cells and culture-activated (but not quiescent) hepatic stellate cells (HSC) from mice [13]. The physiological functions of NOXs in the liver are still only partially understood.

Considering the association of hepatic fibrosis in NAFLD patients and the role of oxidative stress in its evolution, it is important to search for genetic markers capable of identifying individuals with a greater or lesser risk of disease progression. We designed a cross-sectional study to evaluate for the first time the influences of these polymorphisms in Brazilian patients with NAFLD. This study aimed to evaluate the possible association of SNPs of genes encoding proteins of the NADPH oxidase system in the CYBA (unregistered) gene and the NOX4 gene (rs3017887), and the presence of NASH in NAFLD, and also to estimate the possible association between these polymorphisms and hepatic fibrosis, insulin resistance, metabolic syndrome and NASH.

Methods

Subjects and study design

This was a cross-sectional study with biopsy-proven NAFLD patients who were followed at the Hepatology Outpatient Unit in the Hospital das Clínicas of School of Medicine of University of São Paulo (HCFMUSP), São Paulo, Brazil, between January 2009 and August 2012. A total of 207 patients were selected. They all agreed to participate in the study and signed the informed consent form. The study was approved by the Ethics Committee of the Hospital das Clínicas (435.621) and was conducted following the ethical guidelines of *the Declaration of Helsinki*.

The diagnosis of NAFLD was made by a liver biopsy. The inclusion criteria were as follows: 1) NAFLD patients with age of 18–75 years; 2) hepatic biopsy compatible with NAFLD. The exclusion criteria were as follows: 1) presence of other causes of chronic liver disease, such as schistosomiasis, viral hepatitis, autoimmune hepatitis, Wilson's disease, alpha-1-antitrypsin deficiency, hemochromatosis; 2) alcohol intake (>100 g ethanol/week); 3) refusal to consent to the research.

Clinical variables

On the day of liver biopsy or near the date of the biopsy (within 3 months), demographic data such as gender, age and anthropometric data [weight, height, body mass index (BMI) and waist circumference] were collected. Metabolic syndrome was defined using the National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATPIII) criteria: fasting glucose ≥ 100 mg/dL; systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; triglycerides ≥ 150 mg/dL; low high-density lipoprotein cholesterol (HDL-cholesterol: men <40 mg/dL, women <50 mg/dL); abdominal obesity, given as waist circumference (men >102 cm/40 in., women >88 cm/35 in.). The definition used for type 2 diabetes was fasting glucose ≥ 126 mg/dL according to American Diabetes Association 2017 [14]. For the evaluation of insulin resistance, Homeostasis Model Assessment (HOMA) was used [fasting glucose (mg/dL)/$18 \times$ fasting insulin (μU/mL)/22.5]. The HOMA, when greater than or equal to 2.513, was used as the marker of insulin resistance [15].

Biochemical analysis

Serum tests are routinely performed in patients who undergo liver biopsy. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), total bilirubin and fractions, glucose, insulin, markers for hepatitis B and C, hepatic autoantibodies, copper and serum ceruloplasmin, and an iron profile were all measured. In addition, a parasitological examination of feces and a search for eggs of *Schistosoma mansoni* (Kato-Katz) were requested if this had not yet been performed.

Samples of blood collected after a 12-hour fast were centrifuged within one hour after collection for separation of plasma, serum and leukocyte cells and stored at -80 °C. Biochemical analyses were performed at the HCFMUSP central laboratory. Glucose concentrations were determined by the enzymatic method of hexokinase (Cobas, Roche, Switzerland); insulin by the chemiluminescence method (Cobas, Roche); total cholesterol, HDL cholesterol and triglycerides by the enzymatic colorimetric method (Cobas, Roche); and low-density lipoprotein (LDL) cholesterol by using the Friedwald equation.

NADPH oxidase genotype analysis

Genomic DNA was extracted from peripheral blood leukocytes by the salting-out method. Two SNPs in the putative regulatory region of the genes encoding NADPH oxidase 4 catalytic subunit (NOX4) (rs3017887) and its regulatory subunit p22phox (CYBA) were selected based on previous studies [16, 17]. The samples were genotyped by real-time PCR using fluorescent-labeled probes according to the procedures recommended by the manufacturer. NOX4 rs3017887 was genotyped by assay type validation (C_15762095; Applied Biosystems, Foster City, USA), and CYBA-675 T→A (unregistered) was genotyped by PCR using specific primers (sense: 5′-GCGCTGGCTCACCAC-3′ and antisense: 5′-ACTGGGAAAGCACAGAATGCA-3′) and fluorescent-labeled probes (VIC: 5′-CCTCCCGAACCCAGG-3′ and FAM: 5′-CCTCCCGTACCCAGG-3′) (TaqMan, Applied Biosystems). Amplifications were performed

Please cite this article as: F. Rabelo et al., Association between the CYBA and NOX4 genes of NADPH oxidase and its relationship with metabolic syndrome in non-alcoholic fatty liver disease in Brazilian population, Hepatobiliary & Pancreatic Diseases International (2018), https://doi.org/10.1016/j.hbpd.2018.06.005