Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Common polymorphism in the *PNPLA3*/adiponutrin gene confers higher risk of cirrhosis and liver damage in alcoholic liver disease

Eric Trépo[1,2], Thierry Gustot[1,2], Delphine Degré[1,2], Arnaud Lemmers[1,2], Laurine Verset[3], Pieter Demetter[3], Romy Ouziel[1,2], Eric Quertinmont[2], Vincent Vercruysse[2], Leila Amininejad[1], Pierre Deltenre[4], Olivier Le Moine[1,2], Jacques Devière[1,2], Denis Franchimont[1,2,†], Christophe Moreno[1,2,†,*]

[1]*Department of Gastroenterology, Hepatopancreatology and Digestive Oncology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium;* [2]*Laboratory of Experimental Gastroenterology, Université Libre de Bruxelles, Brussels, Belgium;* [3]*Department of Pathology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium;* [4]*Service d'Hépato-Gastroentérologie, Hôpital de Jolimont, Haine-Saint-Paul, Belgium*

Background & Aims: A recent genome-wide association study identified genetic polymorphism (rs738409 C>G) in the *PNPLA3*/adiponutrin gene associated with liver steatosis. This variant has also been linked to increased risk of alcoholic liver disease (ALD) and cirrhosis in Mestizo Mexicans with excessive alcohol intake. Our aim was to study the influence of this polymorphism on European Caucasian patients with histologically suggestive ALD.

Methods: Three-hundred-and-twenty-eight healthy controls and 330 ALD patients, among whom 265 had cirrhosis, were genotyped for the rs738409 polymorphism. We studied the impact of rs738409 on clinical and biological parameters, together with histological staging of steatosis and fibrosis. *PNPLA3* messenger RNA (mRNA) levels were measured by quantitative real-time PCR according to the patient's phenotype.

Results: The G-allele was significantly more frequent in ALD patients than in controls (odds ratio [OR] = 1.54, 95% confidence interval [CI] = 1.12–2.11 $p = 0.008$) and was, among ALD patients, significantly associated with steatosis ($p = 0.048$), fibrosis ($p = 0.001$), and greater risk of cirrhosis ($p = 0.001$). In multivariate analysis, rs738409 remained the strongest independent factor associated with risk of cirrhosis (OR = 2.08; 95% CI = 1.15–3.77; $p = 0.02$). Furthermore, the *PNPLA3* mRNA liver expression level was significantly lower in patients with more advanced fibrosis ($p = 0.03$) and negatively correlated with the hepatic venous pressure gradient ($r = -0.41$, $p = 0.006$).

Conclusions: In European Caucasians, the rs738409 variant is associated with increased risk of ALD, liver damage, and cirrhosis. Further prospective studies are required to confirm these results and to evaluate the potential of *PNPLA3* as both a predictor and a therapeutic target in ALD.

Keywords: Polymorphism; *PNPLA3*; Steatosis; Fibrosis; Caucasian; Alcoholic liver disease.

Received 1 June 2010; received in revised form 20 December 2010; accepted 6 January 2011; available online 18 February 2011

* Corresponding author. Address: Department of Gastroenterology, Hepatopancreatology and Digestive Oncology, Erasme Hospital, Université Libre de Bruxelles, 808, route de Lennik, 1070 Brussels, Belgium. Tel.: +32 2 555 37 12; fax: +32 2 555 46 97.

E-mail address: christophe.moreno@erasme.ulb.ac.be (C. Moreno).

† These authors contributed equally to this work.

Abbreviations: ALD, alcoholic liver disease; BMI, body mass index; SNP, single nucleotide polymorphism; *PNPLA3*, Patatin-like phospholipase-3; NAFLD, non-alcoholic fatty liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; *HVPG*, hepatic venous pressure gradient; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; *COL1A1*, collagen, type 1, α 1; *α-SMA*, α-smooth muscle actin; *TGFβ1*, transforming growth factor β 1; *PPAR-α*, peroxisome proliferator-activated receptor alpha; *SREBP-1c*, sterol regulatory element binding transcription factor 1; *MTP*, microsomal triglyceride transfer protein; *HPRT*, hypoxanthine–guanine phosphoribosyltransferase; OR, odds ratio; CI, confidence interval.

Introduction

Alcoholic liver disease (ALD) is one of the most common causes of cirrhosis, and is the main indication for liver transplantation in Europe and North America [1,2]. It is estimated that 3.8% of overall deaths are related to alcohol consumption. More specifically, alcoholic cirrhosis accounts for about 15% of all alcohol-attributable net deaths [3]. Despite a mortality decline in many Western countries, ALD remains significantly lethal [4]. While the threat of cirrhosis increases in proportion to daily alcohol consumption, with the highest risk found above 120 g/day [5], the causal association has been only partially unraveled, since 10–20% of heavy drinkers ultimately develop liver cirrhosis [6]. It is recognized that other factors, including gender [7], insulin resistance, body mass index (BMI), steatosis [8–10], and environmental factors, such as chronic viral infection [11], play a role in the genesis of alcohol-induced liver fibrosis. Nevertheless, these factors fail to explain the extreme variability in ALD progression.

Genetic factors have long been suspected to play a role in ALD liver damage [12]. Studies on ethnic influence [13], familial history, and in twins suggest that ALD might be genetically determined [14]. In earlier candidate gene studies, very few single nucleotide polymorphisms (SNPs) within genes and/or gene

regions of cytokines, alcohol-metabolizing, and antioxidant enzymes were shown to be associated with progression of alcohol-induced liver fibrosis [15].

Recently, a genome-wide association study identified a non-synonymous sequence variation (rs738409 C>G) encoding for an isoleucine to methionine substitution at position 148 in the adiponutrin/patatin-like phospholipase-3 (*PNPLA3*) gene which appeared to be the strongest determinant of human steatosis [16]. This SNP was also found to be associated with elevated levels of liver enzymes in healthy subjects [17], and with disease severity, especially steatosis and fibrosis, in non-alcoholic fatty liver disease (NAFLD) [18]. *PNPLA3* encodes for a transmembrane protein which, in humans, is most prominently expressed in hepatocytes [19]. It exhibits hydrolase activity against triglycerides *in vitro* [20]. *In vivo*, the rs738409 variant alters catalytic activity, leading to triglyceride accumulation in hepatocytes [21]. A study by Tian *et al.* suggested that the rs738409 G-allele was associated with clinically evident liver disease in a subethnic group of Hispanics already prone to ALD following heavy alcoholism [22].

The aim of the present study was to evaluate the impact of rs738409 polymorphism on ALD risk in the general population and upon disease severity, by studying the SNP influence on clinical, biochemical, and histological parameters in a European Caucasian cohort with histologically documented ALD.

Patients and methods

Patients

Three-hundred-and-thirty DNA samples were collected prospectively among patients who had ALD-suggestive histology and underwent liver biopsy between January 2002 and January 2010 at Erasme Hospital, Brussels, Belgium. These patients were unrelated European Caucasians with a mean age of 53 (27–78) years at biopsy, a history of excessive alcohol ingestion of >30 g/d for males and >20 g/d for females, and abnormal alanine aminotransferase (ALT) and aspartate aminotransferase (AST) or suspected cirrhosis related to ALD. Two-hundred-and-sixty-five (80%) of our ALD patients had cirrhosis. Patients with coexisting liver disease, including viral or autoimmune hepatitis, hemochromatosis or co-infection with human immunodeficiency virus by blood screening or suggestive imaging were excluded from the study.

In addition, we included 328 European Caucasian healthy controls who were social workers 21- to 65-years-old (birth date hidden for confidentiality reasons) recruited from the Occupational Medicine Department during a routine physical examination. Written informed consent was obtained from all patients and this study was approved by the local ethics committee.

Histological assessment

Ninety-seven percent of histological specimens were obtained by transjugular biopsy and, when feasible, associated with measurement of the hepatic venous pressure gradient (HVPG). Only patients with adequate biopsy suggestive of histological diagnosis of ALD were selected [23]; pathologists were unaware of the purpose of the study. Depending on the disease spectrum, analysis included the percentage of steatosis among the total number of hepatocytes, ballooning degeneration with assessment of Mallory bodies, neutrophilic inflammation, degree of lobular inflammation, vascular changes, and iron overload. Degree of fibrosis was assessed according to the Brunt score [24], using picrosirius staining. Since NAFLD is histologically similar to ALD, the distinction is based on a history of chronic alcohol consumption, i.e. according to the NAFLD definition, less than 20/30 g/d for women/men, respectively [25]. The diagnosis of cirrhosis was based on liver biopsy or both unequivocal clinico-biochemical data and compatible imaging findings.

Genotyping

For each patient, DNA was isolated by the phenol–chloroform method, from a whole blood sample collected concomitantly with the liver biopsy. Twenty nanograms of DNA was used to assay the *PNPLA3* rs738409 C>G variant with the TaqMan assay ID C7241_10 (Applied Biosystems, Foster City, CA) on a LightCycler® 480-Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). We included DNA samples of known genotype as internal positive and negative controls (water) to secure the genotyping procedure. Additional procedure's details are available in Supplementary data material. The success rate for genotyping was nearly 99%. Allele frequencies were in Hardy–Weinberg equilibrium.

Hepatic mRNA expression

From 47 patients among those selected for genotyping analysis during the biopsy process, an adequate-sized piece of tissue was kept for gene expression analysis and the remaining tissue sample was sent for histological assessment. We evaluated the expression of *PNPLA3* and genes involved in fibrogenesis (collagen, type 1, α 1 [*COL1A1*]; α-smooth muscle actin [*α-SMA*]; transforming growth factor β 1 [*TGFβ1*]) [26], and adipogenesis/lipogenesis (peroxisome proliferator-activated receptor alpha [*PPAR-α*]; sterol regulatory element binding transcription factor 1 [*SREBP-1c*]; microsomal triglyceride transfer protein [*MTP*] [27]. Hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) was used as a housekeeping gene. The procedure concerning the gene expression analysis is further explained in the Supplementary data material.

Endpoints and statistical analysis

Influence of rs738409 polymorphism on the risk of ALD

To assess the risk of ALD in the general population, we compared the genotype for rs738409 of ALD patients with that of healthy controls using the 2-sided Chi-square test.

Association between rs738409 polymorphism and clinical/biochemical and histological variables

Analyses were performed in ALD patients. Categorical variables were studied using the 2-sided Chi-square or Fisher exact test when necessary, whereas quantitative variables were analyzed using the Student *t* test or non-parametric Mann–Whitney or Kruskall–Wallis tests when appropriate, and post hoc adjustment for multiple testing was performed when necessary for all analyses.

Association between rs738409 polymorphism and cirrhosis risk/severity

Univariate analysis was performed to study factors potentially associated with the presence of cirrhosis among ALD patients. Then, a multivariate logistic regression model was conducted to support the hypothesis of rs738409 G-allele as a cause of cirrhosis. Inclusion of variables in the model was based on subject matter knowledge. Thus, variables known to be potential risk factors for ALD fibrosis/cirrhosis were included regardless of statistical significance in univariate analysis. Steatosis was categorized using the median value of 15% as a cut-off, a value close to that previously reported (10%) as a risk factor for fibrosis progression in ALD [10]. The Hosmer–Lemeshow test was used to verify goodness of fit of the model to the data (a non-significant result was indicative of satisfactory calibration). Odds ratios (OR) were calculated together with their 95% confidence interval (CI).

Association between rs738409 polymorphism and PNPLA3 mRNA expression

Levels of *PNPLA3* expression and both rs738409 genotype and phenotype for several quantitative factors were studied in ALD patients such as mRNA levels of other genes involved in fibrogenesis (*COL1A1*, *α-SMA*, and *TGFβ1*) and adipogenesis/lipogenesis (*PPAR-α*, *SREBP-1c*, and *MTP*) using correlation analysis. Spearman or Pearson correlation coefficients were calculated and tested using a two-tailed significance test.

All statistical analyses were performed using SPSS v.17.0 for Windows. A p value below 0.05 was considered statistically significant.

Results

Influence of rs738409 polymorphism on the risk of ALD

Three-hundred-and-thirty Caucasian ALD patients were studied; 71% were males and 80% had cirrhosis. Healthy controls consisted of 328 Caucasians, among whom 52% were males, the proportion of G-allele carriers (homozygotes (GG) and heterozygotes (CG)),