Variants in the *LGALS9* Gene Are Associated With Development of Liver Disease in Heavy Consumers of Alcohol

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BACKGROUND & AIMS: **Alcohol consumption is a major cause of chronic liver disease and contributes to a large proportion of cirrhosis-related deaths worldwide. However, only a fraction of heavy consumers of alcohol develop advanced alcoholic liver disease (ALD), so there are likely to be other risk factors. We investigated whether polymorphisms in the gene encoding galectin-9 (LGALS9), previously shown to mediate liver injury, were associated with the development of ALD.**

METHODS: **We isolated DNA from peripheral blood mononuclear cells (PBMCs) of 554 individuals with at-risk alcohol consumption but no other risk factors for chronic liver disease; all subjects were white Europeans who had consumed more than 80 grams ethanol per day. Of the subjects, 375 had ALD (268 with cirrhosis and 74 with alcoholic hepatitis; mean age, 49 y; 71% male) and 179 had normal liver function with no biochemical or clinical evidence of liver disease (controls; mean age, 42 y; 73% male). Select *LGALS9* polymorphisms were genotyped using allelic discrimination. We also genotyped and measured expression of *LGALS9* messenger RNA in PBMCs from individuals who were not heavy consumers of alcohol.**

RESULTS: **We used data from the HapMap project to identify 5 single-nucleotide polymorphisms (SNPs) that tag all the common haplotypes. When we looked for these SNPs in individuals with vs without liver disease, 4 (rs3751093, rs4239242, rs732222, and rs4794976) were associated with an increased risk of developing ALD. We found that levels of *LGALS9* messenger RNA and protein expressed were associated with an allele carried by PBMCs. Multivariate analysis confirmed that rs4239242 and rs4794976 were associated with an increased risk of ALD.**

CONCLUSIONS: **In a genetic analysis of heavy consumers of alcohol, we associated 2 SNPS in *LGALS9* with the development of ALD. Although larger studies are required, this information could be used to determine the risk of individuals developing ALD or to develop therapeutic agents.**

Keywords: Genetic; Fibrosis; Human; Susceptibility.

Alcohol consumption is a major cause of chronic liver disease, accounting for a large proportion of cirrhosis-related deaths worldwide.[1] Alcoholic liver disease (ALD) comprises a broad spectrum of liver injury, ranging from simple steatosis to more severe forms, including alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma. Less than a third of heavy alcohol drinkers develop advanced ALD, indicating that other factors likely contribute to the phenotypic heterogeneity; these factors include sex, obesity, drinking patterns and duration, and non–sex-linked genetic factors.[1,2] In this regard, genetic variation in the *PNPLA3* gene has been associated with an increased risk of both alcohol-related cirrhosis and cancer.[3–6] Candidate gene case-control studies also have found associations between ALD and single-nucleotide polymorphisms (SNPs) within genes coding for alcohol-metabolizing enzymes, antioxidant enzymes, and cytokines.[7] Excessive tumor necrosis factor α (TNF-α) production is characteristic of ALD. Two TNF-α SNPs have been associated with the progression of ALD, although results in subsequent studies have been discrepant.[7]

Galectins are evolutionarily conserved glycan-binding proteins with wide tissue distribution and critical functions in immune tolerance and inflammation.[8] Emerging data have implicated galectin-9 (Gal-9), a tandem repeat-type galectin with 2 carbohydrate recognition domains, in hepatic immune homeostasis.[9] We had first reported

Abbreviations used in this paper: Acon, alcoholic controls; ALD, alcoholic liver disease; EtOH, ethanol; Gal-9, galectin-9; IFN, interferon; MAF, minor allelic frequency; PBMC, peripheral blood mononuclear cell; SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor.

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that Gal-9 was up-regulated by Kupffer cells in patients with chronic hepatitis C virus infection, induced apoptosis of viral-specific $CD8^+$ T cells, and that it increased the production of proinflammatory cytokines (TNF-α, interleukin 1β) from liver-derived and peripheral mononuclear cells,[10] implicating Gal-9 as a key mediator in pathways that lead to liver injury. Subsequent reports have ascribed central roles for Gal-9 in hepatitis B virus–related hepatocellular carcinoma,[11] autoimmune hepatitis,[12] and ischemic-reperfusion injury in the liver.[13] Based on the broad functions of Gal-9 in a wide range of hepatic disorders, we hypothesized that functional genetic polymorphisms in the gene coding for galectin-9 (*LGALS9*) would be associated with the development of ALD.

Patients and Methods

Study Subjects

To be included in the study patients had to have been consuming more than 80 grams ethanol per day for at least 10 years at the time of presentation, as detailed previously.[14–16] After obtaining informed consent, blood (10 mL) was collected. All patients were Caucasian and originated in the northeast of England, as did their parents and grandparents. Heavy drinkers were recruited from 2 sources: referrals to the hospital liver unit with suspected ALD, and referrals to the Regional Alcohol Addiction Unit with alcohol dependency. As described previously, patients who presented to the liver unit had their detailed lifetime alcohol history taken by a specific alcohol research nurse, and those who presented to the Alcohol Addiction Unit by a community psychiatric nurse trained in drug and alcohol addiction.[14] The presence and severity of ALD initially was determined by standard liver blood tests. Patients with either alanine aminotransferase, alkaline phosphatase, or bilirubin that was more than twice the upper limit of normal on 2 occasions within a 6-month period were investigated further with ultrasonography. If there was no evidence of biliary obstruction, a liver biopsy was performed unless contraindicated by coagulopathy (prothrombin time $>$ 3 seconds prolonged). The criteria for inclusion into the ALD group were either liver histology compatible with ALD or clinical evidence of hepatic decompensation. For inclusion into the alcoholic control (Acon) group, patients had to be actively drinking, have no clinical evidence of liver disease, and have either a normal liver blood test result on 2 occasions (not including an isolated increase in γ-glutamyl transferase) or, for patients with abnormal liver blood test results, liver histology showing either normal liver or steatosis with no evidence of steatohepatitis or fibrosis. Exclusion criteria for this study were as follows: positivity to hepatitis B virus surface antigen or antibody to hepatitis C virus, and other type of liver disease, including primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis, hemochromatosis (and other metabolic diseases), or α1-antitrypsin deficiency.

In addition, 34 healthy subjects with no significant alcohol history were included to study the effect of peripheral blood mononuclear cell (PBMC) stimulation on Gal-9 transcription to determine if SNPs were associated with differences in transcript and protein expression.

Single-Nucleotide Polymorphism Analysis

The selection of a candidate gene for an association study usually is based on the biological plausibility in chosen gene(s) that play a putative role in the pathogenesis of the disease of interest. The *LGALS9* gene is located on human chromosome 17 (Chr17:25,958,174-25,976,586). By using data from the HapMap project, 5 SNPs that tag all the common haplotypes (frequency $>$ 0.01) were identified using Haploview. Genomic DNA was isolated from PBMCs from each of the study subjects. Genotyping for the selected *LGALS9* polymorphisms was performed using allelic discrimination by LGC Genomics (Herts, UK). To determine whether the allelic frequencies varied according to ethnicity, we used data from the 1000 Genomes Project (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes). The average frequencies were determined from multiple groups: for whites (GBR, British in England and Scotland; FIN, Finnish in Finland; IBS, Iberian population in Spain; TSI, Toscani in Italy; CEU, Utah residents with Northern and Western European ancestry); Hispanics (CLM, Columbians from Medellin, Columbia; MXL, Mexican Ancestry from Los Angeles; PUR, Puerto Ricans from Puerto Rico); for African Americans (ASW, Americans of African ancestry in the southwest United States).

In Vitro Assays

PBMCs from control individuals who did not consume significant amounts of alcohol (n = 34), at a concentration of 1 million/mL in 24-well plates, were cultured with interferon (IFN)-γ (25 ng/mL; R&D Systems), ethanol (EtOH, 25 nmol/L; Sigma), or a combination of both (IFN/EtOH) for 24 hours. Cells were harvested and RNA was isolated using the RNeasy Mini kit (Qiagen), quantified using a Nanodrop microspectrometer, and 1 μg of RNA was transcribed to complementary DNA using the Quantitect RT kit (Qiagen). The expression of *LGALS9* was assessed by the Step One Plus real-time polymerase chain reaction system using the Fast SYBR Green Master Mix Protocol (Applied Biosystems). The QuantiTect primer assays for use with SYBR Green were purchased from Qiagen/Superarray. For 8 selected individuals, cells were cultured as described earlier and harvested for analysis of cell surface Gal-9 expression by monocyte subsets (% positive compared with isotype control) using flow cytometry. Acquisition was performed using a