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Liver transcriptional profile of atherosclerosis-related genes in human nonalcoholic fatty liver disease

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ABSTRACT

Objectives and design: Epidemiological studies have suggested a role of nonalcoholic fatty liver disease (NAFLD) in the development of cardiovascular disease. We evaluated liver mRNA expression of 84 genes encoding proteins involved in the atherosclerosis pathway in patients with NAFLD proven through biopsy in a case-control design, and examined the putative role of the histological disease severity in the molecular events associated with the atherogenic profile.
Results: Nonalcoholic steatohepatitis (NASH), when compared with simple steatosis (SS), significantly increases the expression of *TGFB1* (6.8, $p < 0.005$), angiotensin I-converting enzyme (*ACE*) (2.1, $p < 0.007$), *LAMA1* (2.1, $p < 0.007$), *SERPINB2* (2.1, $p < 0.007$), *CSF2* (2.5, $p < 0.002$), *IL1A* (2.5, $p < 0.005$), *IL3* (2.1, $p < 0.007$), *IL4* (2.1, $p < 0.007$), *LIF* (2.1, $p < 0.007$), and *MMP1* (2.1, $p < 0.007$), and decreases the transcript levels of genes involved in the negative regulation of cell-death pathways. A post hoc analysis of liver biopsies of NASH patients who were treated with enalapril monotherapy because of arterial hypertension showed a significant association with lower fibrosis scores in comparison with untreated patients. *BIRC3*, a severe hypoxia-activated gene, was significantly increased in SS (8.2, $p < 0.004$), when compared with the controls. NASH, but not SS, was also associated with a significant increase in platelet abundance of *TGFB1* mRNA. Systems biology analysis revealed highly scored pathways involved in the regulation of programmed cell death, angiogenesis, and immune system, in which *TGFB1* was mostly involved.
Conclusion: NASH, but not SS, may increase atherosclerotic and cardiovascular risk by local overexpression of mediators of atherogenesis, endothelial damage, and regulators of blood pressure; this observation may have therapeutic implications, because ACE inhibitors may improve both cardiovascular outcomes and liver fibrosis. Hepatocyte hypoxia seems to have an important role in the molecular events activated by liver steatosis.

1. Introduction

Epidemiological studies have suggested a possible role of nonalcoholic fatty liver disease (NAFLD) in the development of cardiovascular disease [1]. In fact, in patients with metabolic syndrome (MS), NAFLD is associated with an increased cardiovascular risk and independently predicts the risk of future cardiovascular events [2]. In a recent meta-analysis, we showed that NAFLD patients carry an increase of 13% of carotid intima-media thickness [3]. We also observed that patients with NAFLD not only have increased circulating levels of biomarkers of atherosclerosis, such as soluble intercellular adhesion molecule 1 (sICAM-1), plasminogen activator inhibitor (PAI-1), and soluble CD40 ligand (sCD40L), but the affected liver can even participate in the local expression of these

Abbreviations: ACE, angiotensin I-converting enzyme; *BIRC3*, baculoviral IAP repeat-containing 3; CRP, C-reactive protein; *CSF2*, colony-stimulating factor 2; *IL1A*, interleukin 1 alpha; *IL3*, interleukin 3; *IL4*, interleukin 4; *IL6*, interleukin; sICAM-1, intercellular adhesion molecule 1; *LAMA1*, laminin alpha 1; LIF, leukemia inhibitory factor; *MMP1*, matrix metallopeptidase 1 (interstitial collagenase); MS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PAI-1, plasminogen activator inhibitor; *PPARG*, peroxisome proliferator-activated receptor gamma; *SELPLG*, selectin P ligand; *SERPINB2*, serpin peptidase inhibitor, clade B (ovalbumin), member 2; SS, simple steatosis; sCD40L, soluble CD40 ligand; *TGFB1*, transforming growth factor beta 1.

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proteins [4]. Interestingly, cross-sectional studies showed that cardiovascular complications in patients with NAFLD increase with the histological severity of the disease [2], suggesting a putative role of hepatic necroinflammation in the systemic atherogenic phenotype.

Overall, the current body of evidence raises the possibility that NAFLD is not merely associated with the cardiometabolic risk factors, but is an independent causal factor that promotes by itself a systemic proatherogenic and inflammatory state. In particular, it was suggested that nonalcoholic steatohepatitis (NASH), the clinical form of NAFLD, which is associated with either liver inflammation and/or fibrosis, can contribute to a more atherogenic risk profile over the more benign stage of steatosis alone or simple steatosis (SS). This observation is supported by several independent human case-control studies that have demonstrated that NASH, but not SS, is associated with changes in the hepatic messenger RNA (mRNA) or protein expression levels of molecular mediators of atherosclerosis, such as PAI-1 [5], monocyte chemoattractant protein (MCP-1) [6], interleukin 6 (IL6) [7], and sICAM1 [4]. Nevertheless, the evidence is inconclusive and is still unknown whether SS is just a benign disorder or the trigger event in a cascade of dysregulated molecular pathways.

We agree with the current hypothesis that an abnormal intrahepatic accumulation of triglycerides along with a global disruption of the metabolic homeostasis may play a causative role in the development of the proatherogenic state associated with the MS phenotype [1]. Hence, to gain insights into the molecular events occurring in the liver that may explain how NAFLD participates in the systemic phenotype associated with atherogenesis and cardiovascular disease, two sequential approaches are proposed. First, the liver mRNA gene expression signature of 84 genes encoding proteins involved in the atherosclerosis pathway in patients with NAFLD proven through biopsy in a case-control design was evaluated, and further the putative role of the histological severity of NAFLD in the molecular events associated with the atherogenic profile was examined. Second, a systems biology approach was used to integrate a large set of functional association data and identity common pathways in the liver-related proatherogenic phenotype. Finally, as platelets are involved in the systemic atherosclerotic process, we examined NAFLD patients and control subjects for differences in the abundance of platelet mRNA of the most significantly dysregulated liver transcript.

2. Methods and study design

2.1. Selection of patients and controls

The mRNA expression of the 84 genes in 12 liver samples of NAFLD patients (6 SS and 6 NASH) and 6 control subjects was analyzed in a cross-sectional case-control study involving untreated patients with NAFLD proven through biopsy. We studied platelet-circulating mRNA expression of a target gene in additional 84 individuals; see the following description.

In addition, 66 patients, who were diagnosed for NASH and prescribed either enalapril (an ACE inhibitor) monotherapy ($n = 36$) as an antihypertensive medication or metformin monotherapy ($n = 30$) for type 2 diabetes mellitus, were included to compare fibrosis and grading scores with a subset of 56 NASH patients who did not receive any concomitant medication by the time of liver biopsy (details given later). All the patients mentioned in the enalapril, metformin, and untreated group were participating in an unsponsored observational study about the natural history of NAFLD and genetic factors associated with the disease, and were included in a post hoc analysis. This analysis was performed because when compared with SS patients, the abundance of the liver transcript of angiotensin-converting enzyme (ACE) was found to be increased in NASH patients.

Control subjects were selected from patients attended by the Liver Unit, whose age and sex matched the NAFLD patients. In addition to the standard health assessment, a careful ultrasonographic (US) examination of the liver was performed in all the control individuals. For examining the gene expression, control liver specimens were obtained by percutaneous liver biopsy. Details about patients and controls selection can be seen in Supplementary material.

2.2. Physical, anthropometric, biochemical, and cardiovascular evaluation

Health examinations included anthropometric measurements, a questionnaire on health-related behaviors, and biochemical determinations, including C-reactive protein (CRP). Determination of a 10-year risk of developing coronary heart disease outcomes (myocardial infarction and coronary death) was carried out using Framingham risk scoring.

Complete details about the inclusion and exclusion criteria, physical, anthropometric, and biochemical evaluation in all the studied groups are shown in Table 1 and Supplementary material; patients with alcohol consumption above 20 or 30 g of intake daily for women and men, respectively, were not included in the study.

All the investigations performed in this study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from individuals was obtained in accordance with the procedures approved by the Ethical Committe of the authors' institution.

2.3. Liver biopsy and histopathological evaluation

The degree of steatosis was assessed according to the system developed by Kleiner et al., based on the percentage of hepatocytes containing macrovesicular fat droplets [8]. NASH was defined as steatosis plus mixed inflammatory-cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline, and any stage of fibrosis, including absence of fibrosis [8]; details can be seen in Supplementary material.

2.4. Evaluation of liver gene expression by quantitative PCR (qPCR)

We evaluated the hepatic expression profile of 84 genes encoding proteins involved in the atherosclerosis pathway using the pre-designed Human Atherosclerosis RT^2 Profiler^{TM} PCR Array (SABiosciences, Frederick MD, USA), according to the manufacturer's instructions; a complete list of the gene expression assay can be found in Appendix Table 2. Genes on PCR array were selected by the manufacturer based on previous knowledge about published association with the atherosclerosis pathway and specific information gathered from multiple accessible databases and text mining relevant literature. The array included genes involved in the processes of blood coagulation and circulation as well as genes involved in cell-adhesion and lipid transport and metabolism, stress response, cell growth and proliferation, and apoptosis. Details about calculation of the threshold cycle (Ct) values for all the genes on the PCR array can be seen in Supplementary material.

2.5. Platelet isolation and evaluation of mRNA expression

Evaluation of mRNA expression was performed on platelets of 44 NAFLD patients and 40 control subjects whose clinical and biochemical characteristics are shown in Table 1; neither patients nor the control individuals were prescribed any medication. Briefly, blood (8 ml) was drawn from the cubical vein in a plastic syringe