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Molecular profiling of early stage liver fibrosis in patients with chronic hepatitis C virus infection

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Abstract

The molecular mechanisms of acute hepatitis C virus (HCV) infection, end-stage hepatitis (cirrhosis), and hepatocellular carcinoma have been extensively studied, but little is known of the changes in liver gene expression during the early stages of liver fibrosis associated with chronic HCV infection, that is, the transition from normal liver (NL) of uninfected patients to the first stage of liver fibrosis (F1-CH-C).

To obtain insight into the molecular pathogenesis of F1-CH-C, we used real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to study the mRNA expression of 240 selected genes in liver tissue with F1-CH-C, in comparison with NL.

The expression of 54 (22.5%) of the 240 genes was significantly different between F1-CH-C and NL; 46 genes were upregulated and 8 were downregulated in F1-CH-C.

The most noteworthy changes in gene expression mainly affected the transcriptional network regulated by interferons (IFNs), including both IFN-α/β-inducible genes (*STAT1*, *STAT2*, *ISGF3G/IRF9*, *IFI27*, *G1P3*, *G1P2*, *OAS2*, *MX1*) and IFN-γ-inducible genes (*CXCL9*, *CXCL10*, *CXCL11*). Interesting, upregulation of IFN-α/β-inducible genes (but not IFN-γ-inducible genes) was independent of histological scores (grade and stage of fibrosis) and HCV characteristics (hepatic HCV mRNA levels and the HCV genotype), and was specific to HCV (as compared to hepatitis B virus (HBV)).

Other genes dysregulated in F1-CH-C, albeit less markedly than IFN-α/β- and IFN-γ-inducible genes, were mainly involved in the activation of lymphocytes infiltrating the liver (*IFNG*, *TNF*, *CXCL6*, *IL6*, *CCL8*, *CXCR3*, *CXCR4*, *CCR2)*, cell proliferation (*p16/CDKN2A*, *MKI67*, *p14/ARF*), extracellular matrix remodeling (*MMP9*, *ITGA2*), lymphangiogenesis (*XLKD1/LYVE*), oxidative stress (*CYP2E1*), and cytoskeleton microtubule organization (*STMN2/SCG10*).

Thus, a limited number of signaling pathways, and particularly the transcriptional network regulated by interferons, are dysregulated in the first stage of HCV-induced liver fibrosis. Some of the genes identified here could form the basis for new approaches aimed at refining IFN-based therapies for chronic HCV infection.
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Keywords: Chronic hepatitis C; Liver fibrosis; Real-time RT-PCR quantification; Signaling pathways

Abbreviations: Ct, cycle threshold; HCV, hepatitis C virus; HBV, hepatitis B virus; IFN, interferon; NL, normal liver; RT-PCR, reverse transcriptase-polymerase chain reaction.

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Introduction

Hepatitis C virus (HCV) infects approximately 170 million people worldwide (Poynard et al., 2003). Disease severity ranges from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma. Chronic HCV infection is typically characterized by slowly progressive liver fibrosis: progression from stage 0 (no fibrosis) to stage 4 (cirrhosis) takes place at approximately 0.10–0.15 fibrosis METAVIR units per decade (Marcellin et al., 2002). However, some patients progress rapidly to severe fibrosis, while others do not develop fibrosis.

The molecular mechanisms of acute HCV infection, end-stage HCV disease (cirrhosis), and hepatocellular carcinoma have been extensively studied, but little is known of the changes in liver gene expression during the early stage of liver fibrosis associated with chronic hepatitis C virus infection, that is, the transition from normal liver (NL; no fibrosis, no HCV infection) to the first stage of liver fibrosis (F1-CH-C; portal fibrosis without septa, chronic HCV infection). Such study could give clues to both early mechanisms of liver fibrogenesis and liver cell response to chronic HCV infection.

The recent advent of efficient tools for large-scale gene expression studies has already provided new insights into the involvement of gene networks and regulatory pathways in various pathological processes (DeRisi et al., 1996). CDNA microarrays can be used to test the expression of thousands of genes at a time, while real-time reverse transcriptase-polymerase chain reaction (RT-PCR) is a more accurate and quantitative assay method applicable to smaller numbers of selected genes (Latil et al., 2003; Paradis et al., 2003).

To obtain further insight into the molecular pathogenesis of early stage liver fibrosis in patients with chronic HCV infection, we used real-time quantitative RT-PCR to quantify the mRNA expression of a large number of selected genes in pooled F1-CH-C specimens, in comparison with pooled NL specimens of uninfected patients. We examined the expression level of 240 genes known to be involved in various cellular and molecular mechanisms associated with fibrogenesis and inflammation. We particularly focused on the expression of genes related to hepatic stellate-cell activation (Friedman, 2000). Genes of interest were further investigated in 21 individual F1-CH-C specimens, in comparison with 16 NL specimens. We also sought a possible link between the mRNA levels of these genes of interest and viral characteristics related to HCV (hepatic HCV mRNA level and HCV genotype), *IFNA1*, *IFNA2*, *IFNB1*, and *IFNG* mRNA levels, and histological features (grade of necroinflammation and stage of fibrosis).

Results

We first determined the mRNA expression level of 240 genes (Fig. 1) in a pool of liver specimens from patients with chronic HCV infection and minimal fibrosis (F1-CH-C) and in a pool of normal liver specimens (NL) of uninfected patients. Genes whose expression in the F1-CH-C pool differed by at least 2-fold from their expression in the NL pool were then examined for their mRNA expression levels in 21 individual F1-CH-C samples relative to 16 NL samples.

MRNA expression of the 240 genes in the F1-CH-C pool relative to the NL pool

The F1-CH-C and NL pools were each prepared by mixing equal amounts of liver tissue RNA from eight patients. The mean *TBP* gene threshold cycle (Ct) values for the F1-CH-C pool and the NL pool were 26.44 ± 0.36 and 26.52 ± 0.31, respectively.

Seven (2.9%) of the 240 genes were detectable but not reliably quantifiable in both the F1-CH-C and NL pools, that is, Ct > 32 by RT-PCR with TaqMan (or SYBR Green) fluorescence methodology.

The mRNA expression of 68 (29.2%) of the remaining 233 genes showed at least a 2-fold difference between the F1-CH-C pool and the NL pool; 54 (79.4%) genes were upregulated and 14 (20.6%) were downregulated.

MRNA expression of the 54 upregulated genes in 21 F1-CH-C samples and 16 NL samples

The expression level of the 54 upregulated genes, identified by pooled sample analysis, was then determined individually in 21 F1-CH-C and 16 NL samples. Forty-six (85.2%) of these 54 genes were significantly upregulated in the 21 F1-CH-C samples ($P < 0.05$; Table 1). The eight genes, identified in the pool analysis but not significantly upregulated in the individual sample analysis, mainly corresponded to genes that showed high upregulation in only one sample within the F1-CH-C pool (prepared by mixing eight samples).

The 46 upregulated genes were mainly interferon (IFN)-inducible genes encoding proteins with known functions (*CXCL10*, *CXCL11*, *ISGF3G*, *CXCL9*, *HLAB*, *OAS2*, *MX1*, *STAT1*, *PLSCR1*, *PSMB9*, *LGALS3B*, *OAS1*, *STAT2*, *MDK*, and *IRF7*) or unknown functions (*IFI27*, *G1P3*, *G1P2*, *IFITM1*, *IFIT4*, *IFI35*, *IFIT1*, and *PRKR*). The other upregulated genes mainly encoded growth factors (*CXCL6*, *IL6*, *FGF7*, *IFNG*, *TNF*, *CCL8*, *TNFSF6/FASL*, and *HGF*) or growth factor receptors (*CXCR3*, *CXCR4*, and *CCR2*), or were involved in extracellular matrix remodeling (*MMP9*, *MMP7*, *ITGA2*, and *CHI3L1*) or cell cycle regulation (*p16/CDKN2A*, *MKI67*, and *p14/ARF*).

The capacity of each of these 46 genes to discriminate between F1-CH-C and NL was then tested by using receiver operating characteristic (ROC) curve analysis. The overall diagnostic value of the 46 molecular markers was assessed in terms of their area under curve (AUC) values (Table 1). Fig. 2 shows the mRNA levels of the three most discriminatory genes, namely *CXCL10* (AUC-ROC, 1.000), *IFI27* (AUC-

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