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Multiple variants in toll-like receptor 4 gene modulate risk of liver fibrosis in Caucasians with chronic hepatitis C infection☆

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***Background/Aims*: Seven genomic loci, implicated by single nucleotide polymorphisms (SNPs), have recently been associated with progression to advanced fibrosis (fibrosis risk) in patients with chronic hepatitis C virus. Other variants in these loci have not been examined but may be associated with fibrosis risk independently of or due to linkage disequilibrium with the original polymorphisms.**

***Methods*: We carried out dense genotyping and association testing of additional SNPs in each of the 7 regions in Caucasian case control samples.**

***Results*: We identified several SNPs in the toll-like receptor 4 (*TLR4*) and syntaxin binding protein 5-like (*STXBP5L*) loci that were associated with fibrosis risk independently of the original significant SNPs. Haplotypes consisting of these SNPs in *TLR4* and *STXBP5L* were strongly associated with fibrosis risk (global $P = 3.04 \times 10^{-5}$ and 4.49×10^{-6}, respectively).**

***Conclusions*: Multiple variants in *TLR4* and *STXBP5L* genes modulate risk of liver fibrosis. These findings are of relevance for understanding the pathogenesis of HCV-induced liver disease in Caucasians and may be extended to other ethnicities as well.**

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***Keywords*: Liver fibrosis; Risk factor; Single nucleotide polymorphism; Toll-like receptor TLR4; Syntaxin binding protein STXBP5L**

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Abbreviations: SNPs, single nucleotide polymorphisms; HCV, hepatitis C virus; TLR4, toll-like receptor 4; STXBP5L, syntaxin binding protein 5-like; AUC, area under the curve; LD, linkage disequilibrium; CEPH, Centre d'Etude du Polymorphisme Humain; UCSF, University of California at San Francisco; VCU, Virginia Commonwealth University.

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1. Introduction

The development of hepatic fibrosis which leads to cirrhosis in patients with chronic hepatitis C virus (HCV) infection results from inflammatory response. This process is associated with marked inter-patient variation which is difficult to predict [1,2]. The wide spectrum in the rate of fibrosis progression is thought to be modulated by a combination of host genetic factors and other host variables including age, gender, and alcohol intake [3,4]. Recently, a seven gene variant prognostic signature for cirrhosis (CRS7) in patients with chronic hepatitis C has been developed for Caucasian patients and validated in an independent patient cohort with area under the curve (AUC) = 0.73 (95% CI: 0.56–0.89; *P*-value <0.001) [5].

There are strong *a priori* rationale for and functional follow-up study supporting the genes of the prognostic signature, which were initially identified from a large scale case control genetic association study [5]. Among these genes, the toll-like receptor 4 (TLR4), a lipopolysaccharide-receptor, plays a critical role in pathogen recognition and activation of innate and adaptive immunity [6]. Interaction of HCV and TLR4 signaling is robust although complex: HCV infection can directly induce TLR4 expression [7] and interfere with TLR4 signaling in immune cells [8], and TLR4 signaling itself may regulate HCV replication [9]. In hepatic stellate cells, activation of TLR4 results in the down-regulation of the transforming growth factor (TGF)-β pseudoreceptor Bambi, thereby sensitizing cells to TGF-β-induced signaling leading to hepatic inflammation and fibrosis [10]. Furthermore, a mechanistic study initiated because of these results has already demonstrated that the two fibrosis-associated TLR4 missense variants, T339I (rs4986791) and D299G (rs4986790), have a significant impact on the activity of TLR4 in inflammatory and fibrogenic signaling; more specifically disease protective variants lower the apoptotic threshold of hepatic stellate cells [11]. Of the other genes, the antizyme inhibitor AZIN1, considered a tumor suppressor, plays a role in cell proliferation and death [12]. Syntaxin binding protein STXBP5L is likely to be involved in vesicle trafficking and exocytosis [13] and may therefore be involved in the replication of HCV in the liver and indirectly in liver fibrosis via promoting an environment conducive to HCV replication.

In this study, we have carried out additional, dense association testing of the above gene regions, for the following reasons. First, we aim to identify likely causal genes or regulatory elements which may be in linkage disequilibrium (LD: the non-random association of alleles at two or more loci) with the original markers. Second, genetic studies have demonstrated high probability for the existence of other, independent risk variants that impair expression and/or function of genes associated with disease risk (see, for example, [14]). Third, if there are independent risk variants at the same locus [15], then this allelic heterogeneity will likely make an important contribution to the phenotype, i.e. disease risk. In particular, the original *TLR4* and *STXBP5L* variants associated with liver fibrosis are rare or absent in Asians and/or Africans [16,17]; and hence identification of additional risk variants at these loci may explain a portion of the disease risk in those populations. It is conceivable that variants other than those reported may modulate disease risk in these as well as Caucasian populations.

2. Materials and methods

2.1. Study design

The individual SNPs of the CRS7 signature were initially identified from a gene-centric, genome-wide associations study of ~25,000 SNPs [5]. Additional SNPs were tested in this follow-up study to provide better coverage of each region implicated by the signature SNPs so that other potentially causal or independently significant markers could be identified. The extent of fine-mapping regions was determined by examining the LD pattern in the HapMap CEPH (Centre d'Etude du Polymorphisme Humain) dataset (www.hapmap.org); we primarily targeted markers that are present in the same LD region ("main region") as the individual CRS7 markers, although some markers in the adjacent regions were also tested. Markers tested included tagging SNPs (representative SNPs in a region of the genome with high LD), putative functional SNPs, and others such as those in high LD with the individual CRS7 markers. Markers capable of tagging SNP diversity in the main block were selected with the tagger program (http://www.broad.mit.edu/mpg/tagger/server.html) under the following criteria: minor allele frequency $\geqslant 0.05$ and $r^2 > 0.8$; our sample set had 80% power to detect a variant of 0.05 frequency that has an effect size of 2.2 at the allelic level. The putative functional markers, such as non-synonymous SNPs and those in putative transcription factor binding sites, were selected based on both public and Celera annotation. Additional information for the selected SNPs can be found in Table 1.

2.2. Study samples

The 420 Caucasian samples used in this study were collected from the University of California at San Francisco (UCSF) ($N = 187$) and the Virginia Commonwealth University (VCU) ($N = 233$). They consisted of 263 cases and 157 controls where patients with fibrosis stages 3 or 4 were defined as cases and those with fibrosis stage 0 were used as controls; samples with fibrosis stages 1 or 2 were excluded from the study to more effectively delineate genetic factors involved in progression. Fibrosis stages were determined by biopsies read by liver pathologists; the Batts–Ludwig scoring system was utilized in UCSF and the Knodell system in VCU [18]. Cases were sampled at the age of 27–71 years (means ± SD = 49.4 ± 7.4), consisted of 75.3% males, and had daily alcohol intake of 46.2 ± 67.2 g. Controls were sampled at the age of 19–80 years (means ± SD = 47.3 ± 9.1), consisted of 61.1% males, and had daily alcohol intake of 50.5 ± 76.2 g. Sample specific information, including estimated age of infection and duration of infection, is presented in Supplementary Table 1. All patients provided written informed consents, and the study was approved by institutional review boards of UCSF and VCU.

2.3. Genotyping

Cases and controls were individually genotyped by allele-specific, kinetic PCR [19]. For each allele-specific PCR reaction, 0.3 ng of DNA was amplified. Genotypes were automatically called by an in-

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