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A *Toll-like receptor 7* single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection☆

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***Background/Aims*: HCV-infection leads to development of liver fibrosis, causing morbidity and mortality. Multiple factors influence the progression of fibrosis, including genetic factors. Since HCV is an RNA virus, a role for TLR7 in the immune response against HCV is likely. No systematic analysis of *TLR7* single nucleotide polymorphisms (SNPs) has been published.**

***Methods*: We sequenced *TLR7* in 52 women and investigated SNPs with an allele frequency >5% in 807 patients with chronic HCV-infection by melting curve analysis. We analyzed the effect of *TLR7* SNPs on grade of inflammation and stage of fibrosis as determined by liver biopsy.**

***Results*: We detected five *TLR7* SNPs, three of which showed a frequency >5%. One variant, c.1-120T > G, was more common in patients with no or little inflammation than in patients with grades 2–4 (10.7% vs. 6.1%; $P = 0.034$). The variant was also enriched in patients with no or little fibrosis compared to those with higher stages (12.6% vs. 6.6%; $P = 0.005$). The difference was fully attributable to male patients.**

***Conclusions*: This is the first analysis of *TLR7* SNPs in patients with chronic HCV-infection. Our data suggest that the *c.1-120G TLR7* allele offers protection from the development of inflammation and fibrosis in male patients with chronic HCV-infection.**

***Keywords*: HCV; Fibrosis; Toll-like receptor; Genetic polymorphism; Innate immunity**

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☆ The authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Abbreviations: HCV, hepatitis C virus; TLR, Toll-like receptor; SNP, single nucleotide polymorphism; HBV, hepatitis B virus; UTR, untranslated region; pDC, plasmacytoid dendritic cell.

1. Introduction

HCV-infection leads to chronic liver inflammation in the majority of patients. A substantial proportion of patients develop fibrosis or cirrhosis, causing HCV-related morbidity and mortality. Multiple factors influence the progression of fibrosis, including gender, age at infection, and alcohol consumption [1,2]. In addition,

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genetic factors influence progression of fibrosis. Association of genetic polymorphisms with progression of fibrosis was demonstrated among others for the hemochromatosis [3], transforming growth factor-β1 [4], complement factor-5 [5], angiotensinogen [6], monocyte chemotactic protein-1 [7], and microsomal epoxide hydrolase genes [8]. Fibrosis is the result of defective repair of liver damage resulting from inflammation caused by effector cells of the immune system. The cytokine interferon-α is a key mediator at the interface between innate and adaptive immunity. It is mainly produced by plasmacytoid dendritic cells (pDCs) after the engagement of Toll-like receptors (TLRs) [9]. TLR7 is a promising candidate for an immune mediator in HCV-infection since it is expressed on pDCs, binds single stranded RNA, and its activation stimulates secretion of interferon-α [10–12]. Two lines of evidence support a role for TLR7 in HCV-infection: first, a clinical study demonstrated an antiviral effect of the TLR7 agonist, isatoribine [13], which is mediated by immune cells [14]. Second, activation of TLR7 on hepatocytes exerts an antiviral effect independent of interferon [15]. Binding of the TLR7 ligand SM360320 to hepatocytes leads to expression of antiviral genes and suppression of HCV-replication. Thus, two compartments are involved in the TLR7-dependent antiviral response in HCV-infection: immune cells such as pDCs [16,17], which produce interferon-α and hepatocytes, which up-regulate antiviral proteins. The *TLR7* gene is located on the X-chromosome [18], spanning three exons. Single nucleotide polymorphisms (SNPs) have been described for most TLRs, but no analysis of *TLR7* SNPs has been published. We determined the prevalence of *TLR7* SNPs in a cohort of patients with chronic HCV-infection and analyzed the effect of these SNPs on histology as determined by liver biopsy.

2. Methods

2.1. Study subjects

The study was approved by the Ethics Committee and all patients gave informed consent. Chronic HCV-infection was confirmed by persistence of anti-HCV antibodies and HCV-RNA over 6 months. All patients were negative for HBs-antigen and HIV-antibodies. We performed HCV-genotyping by reverse hybridization assay (Inno Lipa HCV II, Innogenetics).

Patients from the sequencing cohort were female with a median age of 54 years (range 32–75). Forty-five patients were German, three were Turkish, and four were of other Caucasian ethnicity. Patients suffered from chronic HCV-infection ($n = 35$), primary biliary cirrhosis ($n = 6$), and alcoholic or non-alcoholic fatty liver disease ($n = 3$ each). One patient each suffered from autoimmune hepatitis, chronic HBV-infection, drug-induced liver disease, arteriovenous malformation, and cryptogenic liver disease, respectively.

The test cohort consisted of 807 patients with chronic HCV-infection, data regarding histological staging and grading were available in 801 and 651 patients, respectively. Hepatic inflammation and fibrosis were classified according to the semiquantitative scoring system described by Scheuer [19]. The degree of inflammation was graded on a scale of 0–4 (0: absent; 1: minimal; 2: mild; 3: moderate; 4: severe). The degree of fibrosis was staged on a scale of 0–4 (0: absent; 1: mild without septa; 2: moderate with few septa; 3: numerous septa without cirrhosis; 4: cirrhosis). Liver biopsies were performed before treatment for HCV-infection. Patient characteristics are listed in Table 1.

2.2. Detection of TLR7 SNPs

We isolated genomic DNA from EDTA–blood using spin columns (Qiagen) and analyzed *TLR7* exons by bi-directional DNA-sequencing. DNA was selected from 52 consecutive females from our DNA-bank regardless of diagnosis and ethnicity. DNA-fragments were amplified by PCR, generating a fragment (primers 1F/2R) covering the 5′-UTR, exons 1 and 2, and complete intron 1. Two fragments were generated covering exon 3 (primers 31F/34R, 35F/38R). Primers were designed based on published sequence (AC005859) as depicted in Table 2 and were synthesized by TIB MOLBIOL.

We performed PCR (0.75 U AmpliTaq Gold (Applied Biosystems), 400 μM dNTPs, 1.5 $MgCl_2$, 0.1 μM of each primer) in a volume of 25 μl. The reaction mix was denatured (95 °C for 12 min) followed by 40 cycles of 95 °C for 20 s, 58 °C for 40 s, 72 °C for 90 s, and a final extension step (72 °C for 2 min) in a Biometra thermocycler. We digested PCR products (shrimp alkaline phosphatase (USB), exonuclease I (USB)) and performed cycle sequencing using BigDye terminator mix (Applied Biosystems). Sequencing was performed using 10 primer pairs (Table 2) that covered the whole sequence and generated fragments of at least 400 bases. Reaction products were purified with Sephadex G-50 (Amersham) and loaded onto an ABI 3100 fluorescence sequencer (Applied Biosystems).

Table 1
Patient characteristics

Sex	
Male	427 (52.9%)
Female	380 (47.1%)
Age	
Mean (range)	47 (18–78)
Ethnicity	
German	487 (60.3%)
Turkish	42 (5.2%)
Other Caucasian	49 (6.1)
Unknown	229 (28.4%)
HCV genotype	
1	455 (56.4%)
2	25 (3.1%)
3	74 (9.2%)
4	8 (1.0)
Unknown	245 (30.4%)
Grade of inflammation	
0	25 (3.1%)
1	282 (43.3%)
2	289 (44.4%)
3	52 (8.0%)
4	3 (0.5%)
Stage of fibrosis	
0	85 (10.5%)
1	233 (28.9%)
2	183 (22.7%)
3	82 (10.2%)
4	218 (27.0%)