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A Duffy antigen receptor for chemokines (*DARC*) polymorphism that determines pro-fibrotic chemokine serum concentrations is not directly associated with severity of hepatitis C infection

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ABSTRACT

Genetic host factors influence the progression of hepatitis C infection (HCV). Chemokines play important roles in HCV-induced liver fibrosis. Recently, a single nucleotide polymorphism in the Duffy antigen receptor for chemokines (*DARC*) was identified which strongly determines the serum concentrations of pivotal pro-fibrotic chemokines, including CCL2. We here tested the hypothesis that this genetic variant (rs12075 A/G) is a risk factor for liver fibrosis in HCV infection. Overall, 880 patients with HCV from three cohorts and 108 controls were genotyped for rs12075. Although serum CCL2 levels were associated with early liver fibrosis, rs12075 itself was not associated with HCV infection or the severity of liver disease in any of the cohorts. The lack of association was evident in qualitative and quantitative analysis despite sufficient statistical power. We conclude that gene variations that strongly determine serum concentrations of chemokines are not necessarily risk markers of the disease traits in which these molecules play pathophysiological roles.

1. Introduction

Liver cirrhosis resulting from chronic hepatitis C (HCV) infection is a leading cause of mortality worldwide and the main indication for liver transplantation in Europe and the United States [1]. Notably, only about half of all infected patients progress from chronic hepatitis to liver cirrhosis and are at risk to develop cirrhosis-related complications [2]. This interindividual variation has been attributed to exogenous (*e.g.*, alcohol consumption, co-infection with other viruses) and endogenous, *i.e.*, genetic factors [3]. The systematic identification of gene variants contributing to HCV disease progression has been hampered by lack of reproducibility and missing functional data of the identified polymorphisms. Recently, large-scale association studies have been performed which could identify single nucleotide polymorphism (SNP) with a high diagnostic power to detect individuals with a high risk of advanced liver cirrhosis [4,5]. Nevertheless, these studies also lack functional data.

During the progression of HCV infection, various leukocyte subsets are recruited into the liver. Their recruitment is governed by chemokines, a class of small molecular chemoattractants which are known to orchestrate the immune response during HCV infection [6,7]. We and others have recently provided evidence that genetic variation in chemokines and their specific receptors contribute to the genetic basis of progressive inflammation and fibrosis in HCV infection [8–12].

Among all chemokines, CCL2 (monocyte chemoattractant protein-1 [MCP-1]) is considered as a prototypic pro-fibrotic chemokine as its expression is strongly correlated to liver fibrosis [13] and the chemotaxis of hepatic stellate cells [14]. Functionally, genetic deletion of the CCL2 receptor CCR2 leads to reduced fibrosis in animal models of chronic liver damage [15,16]. Furthermore, a functional promoter SNP in *CCL2*, which increases its transcription, has already been associated with increased HCV-induced fibrosis [17]. Thus, other genetic factors contributing to CCL2 concentrations might be good candidates for genetic risk assessment of HCV disease progression.

Chemokine serum concentrations are determined not only by their transcriptional activity, but also by posttranslational regulation. Chemokine scavenger receptors have recently been identified to contribute to such posttranslational regulation. These scavenger or decoy receptors include D6, CCX-CKR, and the Duffy antigen receptor for chemokines (DARC) [18].

DARC has initially been described as the antigen of the Duffy blood group system, which consists of the two different variants, Fy^a and Fy^b, which differ by a single point mutation encoded by two

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co-dominant alleles [19]. In Duffy-positive individuals, DARC is expressed on venular endothelium and on the erythrocyte surface and mediates the neutralization of chemokines from the circulation [20]. The Duffy negative phenotype is very rare among Caucasian individuals but is more prevalent in individuals of African American black ethnicity [21,22]. This phenotype is mainly due to a single T to C substitution at nucleotide -46 (rs2814778) leading to an impaired promoter activity in erythroid cells [23]. Individuals that lack *DARC* expression on erythroid cells are known to be resistant to *Plasmodium vivax* erythrocyte infection [24] and display a decreased risk of human immunodeficiency virus–1 (HIV-1) infection and a slower disease progression [25]. Moreover, this SNP has been associated with Asthma bronchiale and serum IgE levels [22] and with a reduced white blood cells and neutrophil count in African Americans [24]. In a recent report, a nonsynonymous SNP in *DARC* (rs12075, Asp42Gly) has been shown to be a major determinant of CCL2 serum concentration in a genome-wide association study and family-based linkage analysis [26]. Overall, this SNP in *DARC* accounted for 20% of the variability in CCL2 serum concentrations and showed a high minor allele frequency (45.6%) among Caucasian populations [26].

Based on this strong association of this *DARC* SNP and the well known pro-fibrotic role of CCL2, we investigated here the association of rs12075 (Asp42Gly) with liver fibrosis in HCV infection in control subjects and three independent cohorts of HCV-infected Caucasian patients.

2. Subjects and methods

2.1. Study cohorts

Overall, 880 patients with chronic hepatitis C infection and 108 control subjects were genotyped in this study. All included subjects were Caucasian. Patients were recruited at the University Hospital in Aachen (168 patients), the Department of Gastroenterology and Hepatology (523 patients) and the Department of Surgery (189 patients) of the Charité University Hospital, Berlin, Germany. The diagnosis was based on a positive anti-HCV test (Abbott) and a positive HCV-RNA (Cobas Taqman). None of the study subjects had detectable HBsAg (Abbott) or was anti-HIV positive. Other chronic liver diseases were excluded by appropriate serologic tests, *i.e.*, measurement of serum ferritin, alpha 1-antitrypsin, ceruloplasmin and autoantibody titers. None of the patients admitted an alcohol intake of more than 40 g per day.

The first two cohorts were recruited before antiviral therapy and all underwent percutaneous liver biopsy with a Menghini needle for assessment of liver inflammation and fibrosis. The histologic samples were evaluated by experienced pathologists according to the scoring system proposed by Desmet and Scheuer [27]. All patients from the third cohort from the Department of Surgery in Berlin underwent liver transplantation because of HCV-related cirrhosis. This cohort was included in the study to minimize false-negative results resulting from the known interobserver variation in the histologic scoring of liver biopsies [28].

We also included 108 control subjects with negative anti-HCV test results, selected according to criteria proposed by Schulz and Grimes to minimize false-positive results in case-control studies [29]. Informed consent was obtained from patients and the protocol was approved by the local ethics committees.

2.2. Genotyping of rs12075 in study cohorts

Genotyping of study subjects for the SNPs rs12075 (Asp42Gly) was performed with 5′-endonulease (Taqman) assays with an ABI PRISM 7000 sequence detection system (Applera). Primers and probes were obtained from http://www.appliedbiosystems.com. Each 25 μl of polymerase chain reaction (PCR) medium contained 20 ng of genomic DNA, 900 nmol/l primers, 250 nmol/l probes, and 12.5 μl of TaqMan Universal PCR master mix (Applied Biosystems).

2.3. CCL2 serum concentrations

Serum concentrations of CCL2 (MCP-1) were assessed in 76 HCV-infected Caucasian patients with different histologic stages of liver fibrosis recruited from the Department of Gastroenterology and Hepatology of the Charité University Hospital, Berlin, Germany, by Cytometric Bead Assay (Becton Dickinson, Heidelberg, Germany) in duplicate according to the manufacturer's instructions.

2.4. Statistical analysis

Genetic data were analyzed on the allelic and genotype level. For qualitative analysis, patients were separated into 2 groups according to the severity of histologic inflammation and fibrosis (group 1 with histologic grades 0–2 representing mild to moderate inflammation and fibrosis, and group 2 with grades 3 and 4 representing severe inflammation and fibrosis). Accordance of genotype distribution with Hardy–Weinberg equilibrium was assessed by an exact test. The groups with mild and severe fibrosis and the transplanted cohort were analyzed by software provided by http://ihg.gsf.de for difference in allele frequency, the effect of the presence of 1 risk allele versus no presence of the risk allele mimicking a dominant model (AG/GG vs AA) and the effect of homozygous presence of the risk allele against all other genotypes in a recessive model (GG vs AG/AA) with G as a minor allele. Quantitative analysis of fibrosis scores in relation to rs12075 genotype in the two cohorts with liver biopsies was performed with Kruskal–Wallis Test. In this analysis mean (±SEM) fibrosis scores were analyzed in relation to rs12075 genotype. In all analyses p values <0.05 were considered as significant.

3. Results

The demographic data and the distribution of inflammation and fibrosis scores in the study cohorts are depicted in Table 1. We first assessed validity of the genotyping assay by analyzing 108 control subjects for rs12075. Overall, genotype distribution was in accordance with Hardy–Weinberg equilibrium in this cohort and the minor allele frequency (49.1%) was not different from the published minor allele frequency (45.6% [26]). We next assessed

Table 1
Demographic data of study cohorts

Parameter	Controls (n = 108)	HCV-infected patients Aachen (n = 168)	HCV-infected patients Berlin (n = 523)	Transplanted HCV cohort Berlin (n = 189)
Age (years ± SEM)	39.8 ± 0.1	42.3 ± 0.14	41.6 ± 0.21	53.9 ± 0.6
Gender (% male)	57.4	58.3	56.9	61.9
Fibrosis score (mean ± SEM)	NA	1.96 ± 0.08	1.64 ± 0.2	NA
Inflammatory score (mean ± SEM)	NA	1.97 ± 0.08	1.82 ± 0.04	NA
Fibrosis score 0/1 (n/%)	NA	67/39.8	245/46.8	NA
Fibrosis score 2 (n/%)	NA	51/30.4	171/32.7	NA
Fibrosis score 3 (n/%)	NA	27/16.1	66/12.6	NA
Fibrosis score 4 (n/%)	NA	23/13.7	40/7.6	189/100

NA, not applicable.