Research Article

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Viral genotype-specific role of *PNPLA3*, *PPARG*, *MTTP*, and *IL28B* in hepatitis C virus-associated steatosis

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Background & Aims: Steatosis is a prominent feature of hepatitis C, especially in patients infected with genotype 3. The analysis of genetic polymorphisms influencing steatosis in chronic hepatitis C has been limited by the studies' small sample size, and important single nucleotide polymorphisms (SNPs), such as those in the patatin-like phospholipase family 3 protein (*PNPLA3*), were never evaluated.

Methods: We analyzed the role of SNPs, from 19 systematically selected candidate genes, on steatosis in 626 Caucasian hepatitis C virus (HCV) infected patients. SNPs were extracted from a genome-wide association-generated dataset. Associations of alleles with the presence and/or different severity of steatosis were evaluated by univariate and multivariate logistic regression, accounting for all relevant covariates.

Results: The risk of steatosis was increased by carriage of I148 M in *PNPLA3*, but only in patients with HCV genotypes non-3 (odds ratio [OR] = 1.9, 95% confidence interval [CI] = 1.6–2.3, p <0.001) and similar, albeit weaker associations were found for SNPs in peroxisome proliferator-activated receptor-γ (*PPARG*) and interleukin-28B (*IL28B*). Carriage of a SNP in the microsomal triglyceride transfer protein (*MTTP*) increased the risk of steatosis, but only in patients with HCV genotype 3 (rs1800803, OR = 3.4, 95% CI = 2.4–4.9, p = 0.001).

Conclusions: The rs738409 SNP in *PNPLA3* is associated with an increased risk of steatosis in patients infected with HCV genotypes non-3. Host genes affect steatosis depending on the infecting HCV genotype, suggesting their interaction with viral factors.

Keywords: Hepatitis C; Steatosis; Genome-wide association study.
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Abbreviations: ADIPOR2, adiponectin receptor-2; BMI, body mass index; CI, confidence interval; HWE, Hardy–Weinberg equilibrium; IL6, interleukin-6; IL10, interleukin-10; IL28B, interleukin-28B; LEPR, leptin receptor; MTTP, microsomal triglyceride transfer protein; MTHFR, methylenetetrahydrofolate reductase; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; PNPLA3, patatin like phospholipase domain-containing protein 3; PPARA, peroxisome proliferator-activated receptor alpha; PPARG, peroxisome proliferator activated receptor-γ; SCCS, Swiss Hepatitis C Cohort Study; SNP, single nucleotide polymorphism; TCF7L2, transcription factor 7-like 2; TGFB1, transforming growth factor beta-1; TNF, tumor necrosis factor; VLDL, very-low density lipoprotein.

Introduction

Liver steatosis is frequent among patients with chronic hepatitis C virus (HCV), with a prevalence ranging from 40% to 86%, i.e. more than twofold the prevalence in the general population and in individuals with other chronic liver infections, such as hepatitis B virus infection [1]. Steatosis occurring in chronic hepatitis C is clinically relevant, since it accelerates liver fibrosis progression and reduces response to antivirals [2–7].

Host, viral, and environmental factors appear to play a role in the pathogenesis of fatty liver. In particular, the direct role of HCV genotype 3 has been repeatedly confirmed [8–10]. Furthermore, some studies have investigated the potential role of host genetic polymorphisms on HCV-associated steatosis. These encompass genes encoding for the microsomal triglyceride transfer protein (*MTTP*) [11–13] and the peroxisome proliferator-activated receptor alpha (*PPARA*) [14] (both involved in lipid metabolism) or for the methylenetetrahydrofolate reductase (*MTHFR*) [15,16], or for cytokines related to the inflammatory response (including their receptors), such as interleukin-10 (*IL10*) [17], interleukin-6 (*IL6*) [17], transforming growth factor beta-1 (*TGFB1*) [17], tumor

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necrosis factor (*TNF*) [18,19], and the leptin receptor (*LEPR*) [17]. Recently, also an interleukin-28B gene (*IL28B*) polymorphism, highly predictive of viral response to therapy, was reported to be independently associated with steatosis in chronic hepatitis C [20], although the mechanism accounting for this association remains at present unclear.

Results are often inconsistent, due to either poor statistical power or lack of adjustment for important cofactors, and warrant validation in larger cohorts. Analyses usually involve a single gene, making it difficult to independently assess the role of several polymorphisms within a multivariate model. Furthermore, the strong association of polymorphisms of the patatin like phospholipase domain-containing protein 3 (*PNPLA3*) with nonalcoholic fatty liver disease (NAFLD) in the general population has never been assessed in HCV-infected patients [21].

Steatosis in HCV infected patients is a complex phenotype. Our previous genome wide association study [22] was unable to detect any marker significantly and independently associated with steatosis. Thus, in order to clarify the role of host genetic polymorphisms in HCV-associated steatosis, we performed a comprehensive analysis on genetic polymorphisms associated with the occurrence and severity of liver steatosis based on a systematic literature review of candidate genes.

Patients and methods

Study definitions

We included all patients in the Swiss Hepatitis C Cohort Study (SCCS) [22] with at least one liver biopsy prior to antiviral treatment and a complete histological and host genetic dataset. The study was approved by local ethical committees and all patients provided written informed consent. Steatosis was expressed as the percentage of affected hepatocytes [23] and graded as absent or, when present, as affecting up to 5% or >5% of hepatocytes.

Single nucleotide polymorphism measurement and imputation

Candidate single nucleotide polymorphisms (SNPs), potentially relevant for the pathogenesis of steatosis in HCV infection, were identified through a systematic literature search (Supplementary Table 1) via the PubMed database, using keywords as fatty liver/hepatitis C, chronic/genetics. We included also SNPs of candidate genes previously shown to be associated with NAFLD and potentially influencing the steatosis occurring in HCV-infected patients with elevated body mass index (BMI) [1,9]. Selected SNPs were extracted from previously established dataset using Illumina human 1 M-Duo chip [22]. SNPs that were not directly measured were imputed using the MACH software [24]. The reference phased datasets for the imputation were downloaded from HapMap (http://hapmap.org, release 22) or from the 1000 Genome Project (ftp://ftp.sanger.ac.uk/pub/1000genomes/REL-0908/LowCov/, August 2009).

Statistical analysis

The effect of SNPs was estimated by logistic regression using an additive model (the host genotype was coded as 0, 1, or 2 based on the number of minor alleles). For imputed SNPs, the uncertainty of imputation was considered using the fractional allele count (imputed dosage of minor allele) [24]. We analyzed the interaction between HCV genotype (3 *vs.* non-3) and each SNP for their effect on steatosis using an appropriate multiplicative term. The haplotype inference was conducted using the *haplo.stats* package (in R version 2.8.1) with the default parameters. Although most investigators agree that multiple testing correction is not a major issue in validation studies, we used Pollard and van der Laan (2004) algorithm [25,26] to correct for multiple testing, which is suitable to handle the underlying unknown correlation structure across multiple hypotheses (Supplementary Fig. 1).

Results

Patient characteristics

Of the 1068 patients included in the SCCS genetic project [22], 626 Caucasian patients fulfilled our inclusion criteria (Table 1). Most patients were males (61.8%), the median age was 44.7 years (IQR = 13.3 years), the median BMI was 23.7 kg/m^2 (IQR = 5.3 kg/m^2). One third of patients (33.0%) reported significant alcohol consumption (>20 g/day) and 6.5% had diabetes. The majority were infected with HCV genotype 1 (n = 324, 51.8%), followed by genotype 3 (n = 183, 29.2%), genotype 4 (n = 55, 8.8%), and genotype 2 (n = 52, 8.3%). Severe activity (Metavir score A3) was found in 18.9% of patients, and 30.3% had advanced fibrosis (Metavir score F3 or F4). Steatosis was present in 395 patients (63.1%), and, when present, affected <5% of hepatocytes in 98 (15.7%) and >5% of hepatocytes in 297 (47.4%) cases. The factors significantly associated with presence and different degrees of steatosis were male sex (p <0.001), age (p <0.001), HCV genotype (p <0.001), BMI (p <0.001), diabetes (p = 0.002), significant alcohol intake (p = 0.002), severe activity (p = 0.03), and advanced fibrosis (p <0.001) (Table 1).

Univariate analysis

Nineteen genes comprising 31 SNPs were retrieved from the literature search (Supplementary Table 1). Twenty-one SNPs were directly measured in the GWA dataset and 10 were imputed. Genotype frequencies were consistent with those observed among other Caucasian populations and did not deviate from Hardy–Weinberg equilibrium (HWE), except for rs2278422 in *TGFB1* (HWE test p <0.001), a SNP for which the imputation quality was relatively low (Rsq = 0.39). The SNP minor allele frequencies reported in the present study were similar to those in the general population from the same geographical area and those described in the literature (Supplementary Table 1).

The significance of the association of each SNP with steatosis, the direction of the association, and the potential interactions between each SNP and the viral genotype (3 versus non-3) are shown in Table 2. For five out of the 19 genes, there was evidence of an interaction between SNP and HCV genotypes (Supplementary Table 2). Thus, further analyses were stratified according to HCV genotypes.

Only two SNPs were significantly associated with the presence of steatosis (any degree) in the whole study population, i.e. rs738409 in *PNPLA3* (p = 0.001) and rs2069837 in *IL6* (p = 0.03). After stratification by HCV genotypes, the *PNPLA3* association disappeared in genotype 3 patients (p = 0.3), but remained significant in genotypes non-3 patients (p = 0.002), while the *IL6* association was lost in both viral genotype groups (p = 0.2 in genotype 3 and p = 0.2 in genotypes non-3). SNP rs12980275 in *IL28B* was only associated with steatosis in genotypes non-3 (p = 0.003).

When using a 5% cutoff (i.e. lack of steatosis or steatosis affecting <5% of hepatocytes *vs.* all other cases), only rs738409 in *PNPLA3* (p = 0.03) was associated with steatosis. After stratification by HCV genotype, the *PNPLA3* association was lost among genotype 3 patients (p = 0.5), but remained significant in genotype non-3 patients (p = 0.008). Other SNPs were associated with steatosis, i.e. rs1029629 in *ADIPOR2*, but solely in genotype 3 (p = 0.03) and rs12980275 in *IL28B*, but solely in genotypes non 3 (p = 0.009).