IL28B expression depends on a novel TT/-G polymorphism which improves HCV clearance prediction

Stéphanie Bibert,1 Thierry Roger,1 Thierry Calandra,1 Murielle Bochud,3 Andreas Cerny,4 Nasser Semmo,3 François H.T. Duong,6 Tilman Gerlach,7 Raffaele Malinverni,8 Darius Moradpour,2 Francesco Negro,9 Beat Müllhaupt,10 Pierre-Yves Bochud,1 and the Swiss Hepatitis C Cohort Study

1Infectious Diseases Service and 2Division of Gastroenterology, Department of Medicine and Hepatology, University Hospital and University of Lausanne, 1011 Lausanne, Switzerland
2Institute for Social and Preventive Medicine (IUMSP), University Hospital of Lausanne, 1010 Lausanne, Switzerland
3Clinica Moncucco, 6900 Lugano, Switzerland
4Hepatology, Department of Clinical Research, University of Bern, 3010 Bern, Switzerland
5Department of Biomedicine, University of Basel, 4031 Basel, Switzerland
6Division of Gastroenterology, Canton Hospital, 9007 St. Gallen, Switzerland
7Division of Clinical Pathology and Division of Gastroenterology and Hepatology, University Hospitals, 1211 Geneva, Switzerland
8Pourtalès Hospital, 2000 Neuchâtel, Switzerland
9Division of Gastroenterology and Hepatology, University Hospital of Zurich, 8091 Zurich, Switzerland

Approximately 3% of the world population is chronically infected with the hepatitis C virus (HCV), with potential development of cirrhosis and hepatocellular carcinoma. Despite the availability of new antiviral agents, treatment remains suboptimal. Genome-wide association studies (GWAS) identified rs12979860, a polymorphism nearby IL28B, as an important predictor of HCV clearance. We report the identification of a novel TT/-G polymorphism in the CpG region upstream of IL28B, which is a better predictor of HCV clearance than rs12979860. By using peripheral blood mononuclear cells (PBMCs) from individuals carrying different allelic combinations of the TT/-G and rs12979860 polymorphisms, we show that induction of IL28B and IFN-γ-inducible protein 10 (IP-10) mRNA relies on TT/-G, but not rs12979860, making TT/-G the only functional variant identified so far. This novel step in understanding the genetic regulation of IL28B may have important implications for clinical practice, as the use of TT/G genotyping instead of rs12979860 would improve patient management.

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are strongly associated with spontaneous (Rauch et al., 2010) and treatment-induced clearance of HCV infection (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010). IL28B encodes type III IFN-λ3, a cytokine which has antiviral properties both in vitro and in vivo (Kotenko et al., 2003; Sheppard et al., 2003; Meager et al., 2005; Robek et al., 2005; Ank et al., 2006; Hou et al., 2009). Numerous investigators confirmed the importance of IL28B SNPs in the natural course and treatment of HCV infection (Mangia et al., 2010; Rallón et al., 2010; McCarthy et al., 2010; Lange and Zeuzem, 2011).

It remains unknown whether rs12979860 exerts biological effects or whether it is in linkage disequilibrium (LD) with other functional polymorphisms. The expression of IL28B was reported to be lower in whole blood or PBMCs from individuals carrying the mutant alleles than those carrying the WT alleles (Suppiah et al., 2009; Tanaka et al., 2009), but these observations were not unequivocally confirmed in liver biopsies from patients with chronic hepatitis C (Honda et al., 2010; Urban et al., 2010; Dill et al., 2011). The functional effect was proposed to result from a T-to-G substitution (previously described as rs8103142, a nonsynonymous SNP in the IL28B gene (ISG) expression or different inhibition of HCV replication in Huh-7.5 cells (Urban et al., 2010). Other investigators performed gene mapping but failed to detect new SNPs with stronger genetic effect (di Iulio et al., 2011) or with a clear functional mechanism.

RESULTS AND DISCUSSION

By sequencing the putative regulatory region upstream of IL28B, we identified a T deletion (previously described as rs67272382 NT_011109.16:g.12007372delT) adjacent to a T-to-G substitution (previously described as rs74597329 NT_011109.16:g.12007373T>G), located at position 39739154 and 39739155 in chromosome 19 (Genome Build 37.1). The presence of the TT/-G substitution, as well as the previously known rs12979860, was explored in a cohort of 540 Caucasian patients with chronic HCV infection and in 93 patients with spontaneous HCV clearance (Table S1). The rs12979860 and TT/-G polymorphisms were in strong LD (R2 = 0.91) and both had a minor allele frequency of 0.38. Despite such strong correlation, individuals who were discordant at both SNPs allowed us to explore which of the two SNPs is most strongly associated with the outcome and therefore more likely to be the functional variant.

We assessed whether IL28B polymorphisms were associated with response to PEG–IFN-α/RBV therapy among chronically infected patients. When considering all viral genotypes together, both polymorphisms were associated with response to treatment (Fig. 1, Table 1, and Table S2). The strongest and most significant association was found for TT/-G (OR = 0.38, 95% CI 0.28–0.51, P = 2.51−10), compared with rs12979860 (0.46, 95% CI 0.35–0.62, P = 1.52−7). TT/-G still provided the strongest and most significant association in a multivariate model, after adjustment for age, sex, HCV RNA level, fibrosis stage, and viral genotype (OR = 0.27, 95% CI 0.17–0.45, P = 2.70−5), compared with rs12979860 (OR = 0.37, 95% CI 0.23–0.59, P = 2.47−5). When both polymorphisms were included in the same model, the mutant -G allele of TT/-G was still associated with reduced clearance, whereas the mutant T allele of rs12979860 was associated with increased clearance (OR > 1, Table 2). TT/-G had a better ability than rs12979860 to discriminate SVR rates (P = 0.04, Fig. 1 A). The SVR proportion was 0.80, 0.55, and 0.41 for patients carrying the TT/TT, TT/-G, and -G/-G genotypes, compared with 0.77, 0.56, and 0.45 for those carrying rs12979860 CC, CT, and TT genotypes (P = 0.02, Fig. 1 B).

Altogether, this confirms that TT/-G is a better marker for HCV clearance than rs12979860 and that the mutant -G allele, but not the rs12979860 mutant T allele, is associated with reduced clearance. The rs12979860 mutant T allele likely represents a proxy for the effect of the mutant -G allele.

Similar observations were found after stratification by viral genotypes (Tables 1 and 2). In patients infected with HCV
expression in PBMCs from individuals carrying different allelic combinations of rs12979860 and TT/-G that were stimulated with the dsRNA analogue poly(I:C) (Fig. 2, A and C). Among individuals WT for rs12979860, PBMCs from those carrying one or two copies of the mutant allele -G of TT/-G had lower expression of IL28B mRNA compared with those from TT/-G WT individuals (P < 0.001). In contrast, among individuals WT for TT/-G, those carrying one or two copies of the mutant allele T of rs12979860 had similar expression of IL28B mRNA than those WT for rs12979860. Finally, among individuals carrying one or two copies of the mutant allele T of rs12979860, those carrying one or two copies of the mutant allele -G of TT/-G had lower expression of IL28B mRNA compared with those from TT/-G WT individuals (P = 0.007). The pattern of IL28B expression after stimulation genotype 1/4, the strongest and most significant association was also found for TT/-G either in the univariate (OR = 0.25, 95% CI 0.16–0.40, P = 4.61 × 10^{-9} vs. OR = 0.31, 95% CI 0.20–0.49, P = 2.24 × 10^{-7} for rs12979860) or in the multivariate models (OR = 0.16, 95% CI 0.08–0.33, P = 7.57 × 10^{-1} for rs12979860). In patients infected with HCV genotype 2/3, TT/-G was the only SNP associated with response to treatment, but only in a recessive model (OR = 0.36, 95% CI 0.15–0.86, P = 2.13 × 10^{-3}). Finally, TT/-G was also a better marker of spontaneous clearance (Tables 1 and 2, P = 8.68 × 10^{-9}), compared with rs12979860 (P = 1.66 × 10^{-8}).

Because TT/-G is a better marker of HCV clearance, we speculated that it might correlate with IL28B mRNA expression. To address this issue, we measured IL28B mRNA expression in PBMCs from individuals carrying different allelic combinations of rs12979860 and TT/-G that were stimulated with the dsRNA analogue poly(I:C) (Fig. 2, A and C).
was similar among patients with cured hepatitis C (SVR) and those with chronic infection (nonresponse) according to TT/-G, suggesting that the viremic state does not significantly influence acute response to stimulation in vitro (Fig. 2 C). Altogether, these data demonstrate that IL28B mRNA expression is driven by the presence of one or two mutant alleles of TT/-G but not by rs12979860. Because IL28B has a strong homology with IL28A due to ancestral gene duplication events, we sequenced previously cloned amplicons to unambiguously confirm that they were part of IL28B but not IL28A (unpublished data).

Although the number of individuals carrying discrepant genotypes was low, the reduction in IL28B mRNA expression among samples from individuals carrying the mutant TT/-G allele was strongly significant. Recently, Prokunina-Olsson et al. (2013) also identified TT/-G as a better predictor of response to treatment than rs12979860 among African Americans, whereas it did not improve this prediction among Caucasians. This can be explained by the lower level of LD between the two polymorphisms among African Americans (R² = 0.71) compared with Caucasian (R² = 0.92). Thus, the high proportion of rs12979860 and TT/-G discrepant individuals in the African American population would allow further validation of the functional role of TT/-G on IL28B mRNA expression.

Infection with HCV activates the endogenous IFN system, which leads to the induction of ISGs and contributes to viral clearance. Pretreatment plasmatic levels of ISGs, such as IFN-γ-inducible protein 10 (IP-10), are predictors of HCV clearance. Because the induction of IP-10 is thought to rely on both type I and type III IFNs, we analyzed IP-10 mRNA expression in PBMCs stimulated with poly(I:C) for 8 h (Fig. 2, B and C). As for IL28B, IP-10 expression was lower among individuals carrying the mutant allele of TT/-G but not in those carrying the mutant allele of rs12979860 (Fig. 2 B), indicating that IL28B expression could be a determinant for the induction of some ISGs. These observations suggest a strong link between the mutant -G allele of TT/-G, reduced expression of IL28B, lower induction of ISGs, and HCV treatment failure. However, the mechanisms by which IL28B TT/-G genotypes are related to ISG induction and clinical phenotypes remain to be elucidated. As a control, TNF mRNA expression after LPS stimulation was not influenced by both polymorphisms (Fig. 2 C).

Genetic polymorphisms can influence gene function through different mechanisms. For instance, they can disrupt or create DNA regulatory elements affecting gene expression. In addition, SNPs can influence the ability of DNA sequences to undergo methylation, thereby influencing gene expression. Both rs12979860 and TT/-G are located within a CpG island which has been identified on the UCSC human genome draft by the Miklem and Hillier method. As assessed by bisulfite sequencing, TT/-G polymorphism was found to promote the methylation of a cytosine residue which is unmethylated in WT DNA sequences (Fig. 3). Further studies are needed to determine the relationship between TT/-G and surrounding CpG methylation and IL28B and/or ISG expression.

Prokunina-Olsson et al. (2013) showed that TT/-G introduces a frame shift in the DNA sequence, which transiently induces mRNA expression of an IFN analogue (IFNL4) in human hepatocytes stimulated with poly(I:C). Only patients carrying the mutant allele -G express IFNL4. The authors suggest that this protein could be a direct marker of HCV treatment failure and a new target for therapeutic intervention, raising major interest in the medical community. Nevertheless, issues regarding the molecular functions of IFNL4 remain to be clarified, such as the inability of recombinant IFNL4 to directly induce the Jak–STAT pathway in HepG2.
Figure 2. Expression of IL28B and IP-10 mRNA relies on TT/-G but not rs12979860 polymorphism. PBMCs from healthy and HCV-infected individuals were stimulated with poly(I:C) for 4 h (black) and 8 h (dark gray). The mRNA expression of IL28B and IP-10 was measured by RT-PCR. Results grouped according to different allelic combinations of the mutant T allele of rs12979860 and the mutant -G allele of TT/-G are shown in A (IL28B) and B (IP-10). Individual results are shown in C. As a control, TNF expression was measured after stimulation with LPS (C, bottom). AU, arbitrary units; mut, mutant allele;
age, sex, HCV risk factors, HCV genotypes, alcohol consumption, markers of chronic infection with the hepatitis B virus and HIV, HCV viral load, liver biopsy data, and HCV treatment were extracted from clinical database. SVR was defined as an undetectable HCV RNA in serum more than 24 wk after treatment termination. Severe fibrosis was considered in patients with a METAIR score F3 or higher.

**SNP genotyping.** TT/-G and n12979860 were extracted from a GWAS-generated dataset (Rauch et al., 2010) or genotyped by TaqMan (Applied Biosystems), using the ABI 7500 Fast real time thermocycler, according to manufacturer’s protocols. For TT/-G and n12979860, TT and C were defined as WT and -G and T as mutant alleles, respectively. TaqMan probes and primers were designed and synthesized using Applied Biosystems software (Table S3). Automated allele calling was performed using SDS software (Applied Biosystems). For quality control, all samples were also genotyped in an independent laboratory (KBioscience, Unit 7, Maple Park, Hoddesdon, Herts, UK) using KASP SNP genotyping system, a competitive allele-specific PCR incorporating a FRET quencher cassette. Patients with at least one matching genotype and/or discordant results regarding one polymorphism were excluded from the analyses.

**Statistical analysis.** Statistical analyses were performed using Stata (version 11.1, StataCorp LP). LD and Hardy-Weinberg equilibrium test were assessed using the programs gwm and genew, respectively, both implemented in Stata. Strong LD was defined as an R² > 0.7. The association of IL28B polymorphisms with response to treatment and spontaneous clearance was performed by univariate and multivariate logistic regression. Age, duration of infection, and body mass index (BMI) were treated as continuous variables. Multiple logistic regression models were adjusted for age, sex, HCV RNA level, fibrosis stage, and, whenever appropriate, viral genotype. For IL28B SNPs, comparisons were made using an additive model (considering a similar effect for each additional copy of the minor allele), a neutral model (comparing separately patients with heterozygous and homozygous mutant genotypes to patients with WT genotypes), and, when appropriate, a recessive model (comparing patients with homozygous mutant genotypes to the others). Discrimination ability between two different logistic regression models was compared using the integrated discrimination improvement test (IDI) implemented in Stata (Pencina et al., 2008).

**PBMCs isolation and RT-PCR analysis.** PBMCs were prepared from 1.6 mg/ml of fresh EDTA blood from nine patients and six healthy donors with written consent and approval of Ethics committee. In brief, whole blood diluted in PBS was overlaid above Ficoll-Paque Plus (GE Healthcare) and mononuclear cells extracted by gradient density centrifugation. Viability, determined by trypan blue exclusion, was >90%. PBMCs were stimulated with 50 µg/ml poly(I:C) and 0.1 µg/ml LPS for 4 or 8 h. Total RNA was isolated from PBMCs using an RNeasy Mini kit and the automated QIAcube (QIAGEN). 250 ng of total RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (QIAGEN). The relative levels of IL28B, IP-10, and TNF transcripts were determined by RT-PCR, with a 7500 Fast real-time PCR system (Applied Biosystems), using the Power SYBR green PCR master mix (Applied Biosystems) with primers described in Table S3. Primers were designed using the Primer 3 software and validated by BLAST analysis. The relative levels of mRNA expression to HPRT were determined by the 2⁻ΔΔCt method and expressed in arbitrary units. HPRT expression was not influenced by cell stimulation. The PCR products were run on a 2% agarose gel, purified (QIAquick Gel extraction kit; QIAGEN), cloned into a pGem-T Easy vector (Promega), and sequenced on an ABI3130XL Sequencer (Applied Biosystems).

**MATERIALS AND METHODS**

**Study patients.** Patients were included from the Swiss Hepatitis C Cohort Study (SCCS), a multicenter study of >3,700 HCV-infected patients enrolled at eight major Swiss hospitals and their local affiliated centers since 2001 (Prasad et al., 2007; Bochud et al., 2009). Caucasian patients enrolled in the SCCS before August 1, 2010, with available DNA and written consent for genetic studies were selected. The study included patients with spontaneous HCV clearance (defined as presence of anti-HCV but undetectable HCV RNA without previous antiviral treatment) and those who had chronic hepatitis C. Altogether, the identification of this TT/-G functional variant provides a new step in understanding the role of IL28B polymorphisms in the prediction of the response to chronic hepatitis C treatment.

**Figure 3.** The TT/-G creates a methylation site in the CpG region. The TT/G substitution is associated with the methylation of the adjacent cytosine residue (indicated by arrow). As opposed to methylated cytosine residues (indicated by asterisks), unmethylated cytosine residues, C, are replaced by thymine, T, after bisulfite treatment and subsequent PCR amplification.
Methylation analysis by bisulfite sequencing. The TT/G-containing region was amplified by PCR using primers detailed in Table S3. Regular sequencing was performed in eight patients (four homozygous for the WT C allele and four homozygous for the mutant T = 12979860 allele). Polymorphic region was also sequenced after bisulfite treatment of DNA, with a subsequent cloning step, in 26 additional patients (13 homozygous for the WT C allele and 13 homozygous for the mutant T = 12979860 allele). Specifically, 250 ng of genomic DNA were treated with sodium bisulfite using the Epit T-Bisulfite kit (QIAGEN) per manufacturer’s recommendations and the polymorphic TT/G region was amplified using bisulfite sequencing primers designed using MethPrimer (http://www.urogene.org/methprimer/index1.html). PCR amplifications from 10 ng of DNA consisted of an initial activation of HotStartTaq DNA polymerase (QIAGEN) at 95°C for 15 min, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 1 cycle at 72°C for 10 min. Amplicons were gel purified with the QIAquick Gel Extraction kit (QIAGEN) and cloned into a pGEM-T Easy vector (Promega). Five clones from each sample were sequenced using an ABI Big-Dye Terminator v.3.0 Cycle sequencing kit (Applied Biosystems) and an ABI3130XL Sequencer (Applied Biosystems).

Online supplemental material. Table S1 shows patient characteristics. Table S2 shows genotypic association of IL28B polymorphisms with response to pegylated IFN-α and ribavirin in chronically infected HCV patients. Table S3 shows primers. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130012/DC1.

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S. Bibert and P.-Y. Bochud are inventors on a patent application filed by the Centre Hospitalier Universitaire Vaudois on the basis of these genetic findings but they have no additional financial interests. The other authors have no conflicting financial interests.

Members of the Swiss Hepatitis C Cohort Study Group: Francesco Negro (Geneva, Chairmain), Antoine Hadengue (Geneva, Chairman of Scientific Committee), Laurent Kaiser, and Laura Rubbia-Brandt (Geneva); Darius Moradpour, Giuseppe Fantale, and Patrick Francioli (Lausanne); Martin Rickenbach (Lausanne Data Center); Gladys Martinetti and Andreas Cerny (Lugano); Virginie Masserey Spicher, Meri Gorgievska, and Jean-François Dufour (Berne); Hans Hirsch and Markus Heim (Basel); Beat Hembing, Beat Mühlbacht, and Stephan Regenss (Zurich); Raffaele Malinverni (Neuchatel); Christa Meyerberg, Tilman Gerlach, and Guenter Dollenmaier (St. Gallen); and Gieri Cathomas (Liecal).

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