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

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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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Abbreviations used: GWAS, genome-wide association studies; HCV, hepatitis C virus; IP-10, IFN-γ–inducible protein 10; ISG, IFN-stimulated gene; LD, linkage disequilibrium; PBMC, peripheral blood mononuclear cell; SVR, sustained virological response.

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the Flaviviridae family. Among seven distinct genotypes, genotype 1 is the most frequent in the United States and in Western Europe (Lauer and Walker, 2001). 30% of infected individuals spontaneously clear the virus, whereas the others develop chronic infection. Morbidity associated with chronic hepatitis C mainly results from the development of liver fibrosis and cirrhosis, with complications such as hepatocellular carcinoma and liver failure. Treatment of chronic hepatitis C with pegylated IFN-α and ribavirin (PEG-IFN-α/RBV) results in a sustained virological response (SVR) in ∼50% of patients infected with genotype 1/4, and in 70–90% of those infected with genotype 2/3 (Zeuzem et al., 2009). Although new antiviral agents such as telaprevir (Zeuzem et al., 2011) and boceprevir (Bacon et al., 2011; Poordad et al., 2011) improve cure rates, treatment remains long, burdened with adverse effects, costly, with limited availability to many areas, and may lead to the emergence of resistance.

Recent genome-wide association studies (GWAS) showed that single nucleotide polymorphisms (SNPs) nearby *IL28B* (mainly *rs12979860*)

Members of the Swiss Hepatitis C Cohort Study group are listed in the appendix.

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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 *rs8103142*, a nonsynonymous SNP in LD with *rs12979860*, which induces a K-to-R substitution at amino acid position 70 of IL28B (Ge et al., 2009). However, this SNP was not among the most significantly associated with clearance in GWAS. Furthermore, K70 and R70 protein variants did not induce different levels of IFN-stimulated 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 a 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 (R^2 = 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% Cl 0.17–0.45, P = 2.70^{-7}), 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

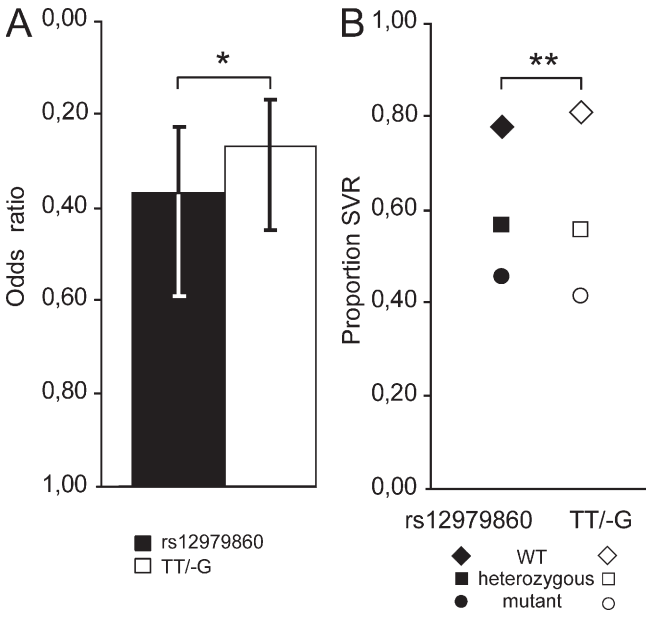

Figure 1. TT/-G is a better predictor of response to treatment than *rs12979860*. (A) Association of the mutant -G allele of TT/-G and the mutant T allele of *rs12979860* with HCV clearance. *, P = 0.04. Error bars represent 95% confidence level. (B) TT/-G versus *rs12979860* in SVR to treatment in chronically HCV-infected patients. **, P = 0.02. Analyses were performed in 540 Caucasian patients from the Swiss Hepatitis C Cohort Study. Numbers of patients in each group are described in Table 1. All genotypes were validated by an independent laboratory as described in the materials and methods. Odds ratios are for an additive model, accounting for the effect of one or two copies of the mutant allele. Multivariate models are adjusted for age and sex, HCV RNA level, fibrosis stage, and viral genotype. P-values were calculated using the integrated discrimination improvement (IDI) test.

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Table 1. Association of *IL28B* polymorphisms with HCV clearance in chronic hepatitis C

Response to treatment	Genotype	NR	SVR	Prop. SVR[b]	Univariate models		Multivariate models[d]	
					OR(95% CI)	P	OR (95% CI)	P
All viral genotypes[a] (*n* = 540)								
TT/-G	TT/TT	37	152	0.80				
	TT/-G	131	161	0.55				
	-G/-G	35	24	0.41	0.38 (0.28–0.51)	2.51^{-10}	0.27 (0.17–0.45)	2.70^{-7}
rs12979860	CC	43	146	0.77				
	CT	126	163	0.56				
	TT	34	28	0.45	0.46 (0.35–0.62)	1.52^{-7}	0.37 (0.23–0.59)	2.47^{-5}
Viral genotype 1 and 4[a] (*n* = 288)								
TT/-G	TT/TT	22	60	0.73				
	TT/-G	112	62	0.36				
	-G/-G	26	6	0.19	0.25 (0.16–0.40)	4.61^{-9}	0.16 (0.08–0.33)	2.75^{-7}
rs12979860	CC	24	56	0.70				
	CT	109	64	0.37				
	TT	27	8	0.23	0.31 (0.20–0.49)	2.24^{-7}	0.22 (0.11–0.41)	3.46^{-6}
Viral genotype 2 and 3[c] (*n* = 252)								
TT/-G	TT/TT	15	92	0.86				
	TT/-G	19	99	0.84				
	-G/-G	9	18	0.67	0.36 (0.15–0.86)	2.13^{-2}	1.33 (0.22–7.94)	7.57^{-1}
rs12979860	CC	19	90	0.83				
	CT	17	99	0.85				
	TT	7	20	0.74	0.54 (0.21–1.38)	2.01^{-1}	1.48 (0.26–8.53)	6.59^{-1}
Spontaneous clearance (*n* = 633)[a]	**Genotype**	**CRI**	**SC**		**OR(95% CI)**	**P**	**OR (95% CI)**	**P**
TT/-G	TT/TT	189	63					
	TT/-G	292	29					
	-G/-G	59	1		0.28 (0.18–0.43)	8.68^{-9}	0.30 (0.19–0.47)	1.54^{-7}
rs12979860	CC	189	62					
	CT	289	30					
	TT	62	1		0.29 (0.19–0.45)	1.66^{-8}	0.31 (0.20–0.49)	3.64^{-7}

CRI, chronic infection; SC, spontaneous clearance; NR, nonresponse to treatment; OR, odds ratio; CI, confidence interval; Prop, proportion.
[a]Odd ratios and p-values are for an additive model, accounting for increased effect of one or two copies of the mutant allele.
[b]Data indicate the proportion of patients with SVR for indicated host and viral genotypes.
[c]Odd ratios and p-values are for a recessive model, comparing the presence of two copies versus zero or one copies of the mutant allele.
[d]Multivariate models are adjusted for age and sex (spontaneous clearance), as well as HCV RNA level, fibrosis stage, and, whenever appropriate, viral genotype (response to treatment). Multivariate models include a smaller number of patients, due to missing covariates in some patients (*n* = 360 for all genotypes, *n* = 195 for genotypes 1 and 4, and *n* = 165 for genotypes 2 and 3). However, the results of univariate analyses restricted to the subset of individuals with all covariates were concordant with those of the whole group.

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^{-9} vs. OR = 0.31, 95% CI 0.20–0.49, P = 2.24^{-7} for *rs12979860*) or in the multivariate models (OR = 0.16, 95% CI 0.08–0.33, P = 2.75^{-7} vs. OR = 0.22, 95% CI 0.11–0.41, P = 3.46^{-6} 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^{-2}). Finally, TT/-G was also a better marker of spontaneous clearance (Tables 1 and 2, P = 8.68^{-9}), compared with *rs12979860* (P = 1.66^{-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). 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

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Table 2. Independent contribution of *rs12979860* and TT/-G to HCV clearance

Response to treatment	Polymorphisms included in the same model	
	OR (95% CI)	P
All viral genotypes; *n* = 540		
Polymorphisms by pairs		
TT/-G	0.12 (0.03–0.40)	5.94^{-4}
rs12979860	3.41 (1.01–11.4)[a]	4.77^{-2}
Viral genotypes 1 and 4, *n* = 288		
Polymorphisms by pairs		
TT/-G	0.16 (0.05–0.55)	3.60^{-3}
rs12979860	1.61 (0.48–5.40)[a]	4.44^{-1}
Spontaneous clearance[b]		
Polymorphisms by pairs		
TT/-G	0.42 (0.14–1.30)	1.31^{-1}
rs12979860	0.66 (0.22–1.99)	4.59^{-1}

Both TT/-G and *rs12978960* were introduced in the same logistic regression model. OR, odds ratio; CI, confidence interval. OR represent the additive effect of the mutant allele (-G for TT/-G and T for *rs12979860*).
[a]When TT/-G was entered into the model, the direction of the association of the T allele of *rs12979860* was reversed (OR > 1).
[b]93 individuals with spontaneous HCV clearance were compared to 540 patients with chronic infection

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 ($R^2 = 0.71$) compared with Caucasian ($R^2 = 0.92$). 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

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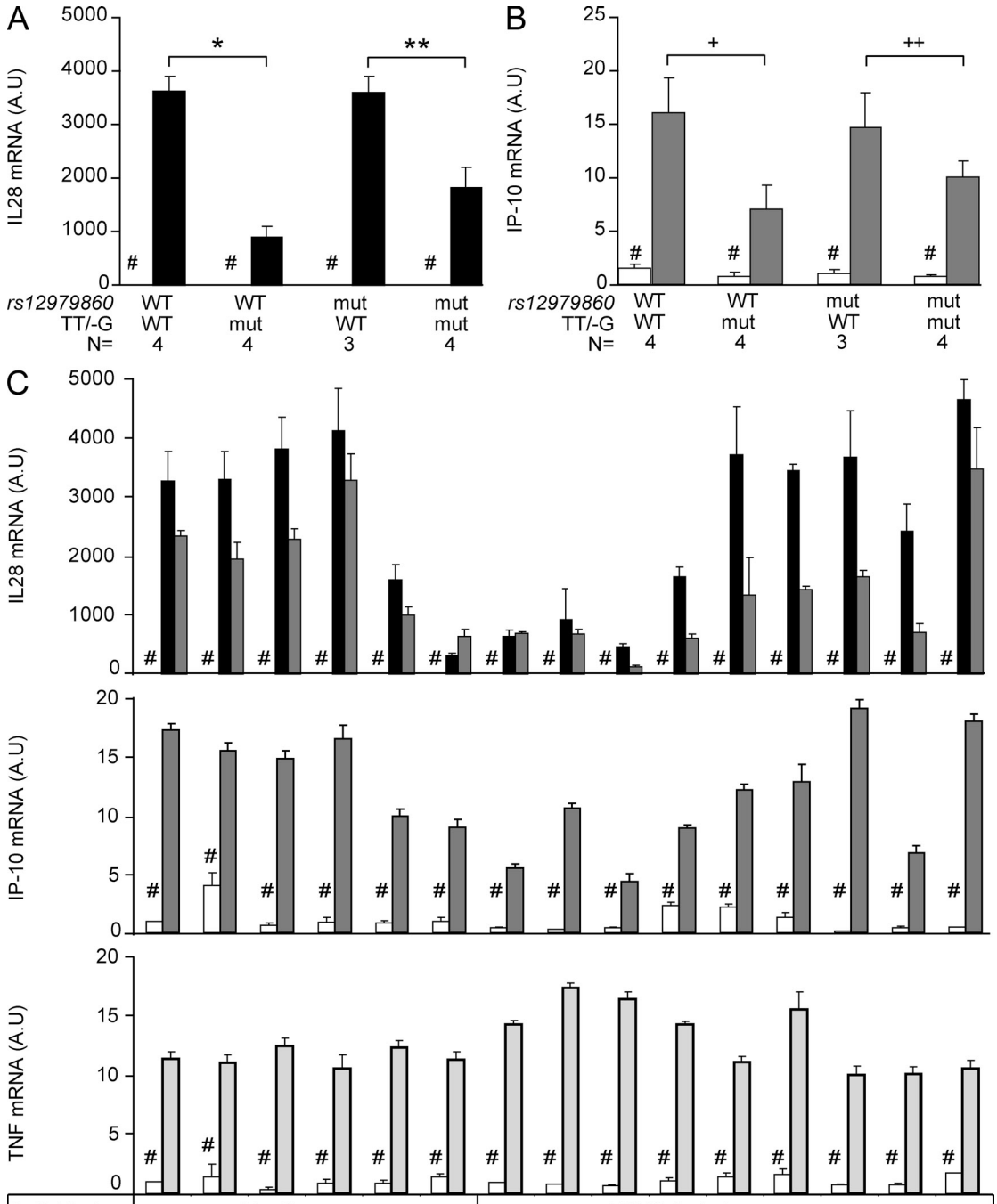

Individual	Healthy						HCV-infected								
Sex	M	M	M	F	M	F	F	F	M	M	F	F	F	M	F
Age	48	56	43	49	50	37	63	58	44	54	41	44	43	44	64
Response to PIFN/RIB	NA	NA	NA	NA	NA	NA	SVR	NR	NR	NR	SVR	SVR	SVR	SVR	SVR
rs12979860	CC	CC	CC	CC	CT	CT	CC	CC	CC	CC	CT	CT	TT	TT	TT
TT/-G	TT/TT	TT/TT	TT/TT	TT/TT	TT/-G	TT/-G	TT/-G	TT/-G	TT/-G	-G/-G	TT/TT	TT/TT	TT/TT	TT/-G	TT/-G

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;

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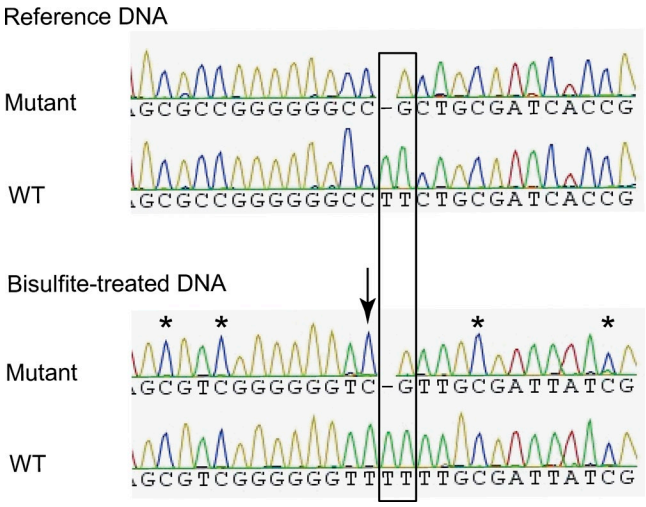

Figure 3. The TT/-G creates a methylation site in the CpG region. The TT/-G substitution is associated with the methylation of the adjacent cytosine residue (indicated by arrow). As opposed to methylated cytosine residues (indicated by asterisks), unmethylated cytosine residues, C, are replaced by thymine, T, after bisulfite treatment and subsequent PCR amplification.

cells, unless by transfection of an IFNL4 construct. Furthermore, the low secretion level of IFNL4 together with the lack of demonstration of its effective binding to the IFNL receptor and/or another specific receptor raises questions about its physiological function.

Using a large European cohort, we identified a new TT/-G polymorphism nearby *IL28B* that influences both IL28B and IP-10 mRNA expression and improves HCV clearance prediction in patients infected with HCV viral genotype 1/4 or 2/3. Because IL28B has antiviral properties, reduced IL28B expression may impair individual ability to clear HCV by itself. Whether this phenomenon is further influenced by another protein remains to be demonstrated. TT/-G genotyping may have an important impact on the management of both African American and Caucasian patients with 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.

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 infection with HCV genotypes 1, 2, 3, or 4 and were assessable for response to therapy with pegylated-IFN-α and ribavirin, i.e., who received >80% of the recommended dose of each drug. Demographic characteristics including 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 databases. 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 METAVIR score F3 or higher.

SNP genotyping. TT/-G *and rs12979860* 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 *rs12979860*, 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 missing 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 pwld and genhw, respectively, both implemented in Stata. Strong LD was defined as an $R^2 > 0.7$. 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 $2^{(-\Delta\Delta Ct)}$ 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).

NA, not assessed; NR, nonresponse to treatment. For each individual, stimulation was performed in triplicate. P-values are calculated by linear regression. *, P < 0.001; **, P = 0.007; +, P < 0.001; ++, P = 0.006. N stands for the number of individuals. Error bars represent standard error. #, unstimulated cells.

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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 *rs12979860* 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 *rs12979860* allele). Specifically, 250 ng of genomic DNA were treated with sodium bisulfite using the EpiTect 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 BigDye 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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