Response to treatment in Brazilian patients with chronic hepatitis C is associated with a single-nucleotide polymorphism near the *interleukin-28B* gene

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A single-nucleotide polymorphism (SNP) upstream of interleukin (IL)28B was recently identified as an important predictor of the outcome of chronic hepatitis C patients treated with pegylated interferon plus ribavirin (PEG-IFN/RBV). The aim of this study was to investigate the association between the IL28B gene polymorphism (rs12979860) and virological response in chronic hepatitis C patients. Brazilian patients (n = 263) who were infected with hepatitis C virus (HCV) genotype 1 and were receiving PEG-IFN/RBV were genotyped. Early virological response (EVR) (12 weeks), end-of-treatment response (EOTR) (48 weeks), sustained virological response (SVR) (72 weeks) and relapse were evaluated using conventional and quantitative polymerase chain reaction (PCR) assays. The frequency of the C allele in the population was 39%. Overall, 43% of patients experienced SVR. The IL28B CC genotype was significantly associated with higher treatment response rates and a lower relapse rate compared to the other genotypes [84% vs. 58% EVR, 92% vs. 63% EOTR, 76% vs. 38% SVR and 17% vs. 40% relapse rate in CC vs. other genotypes (CT and TT), respectively]. Thus, the IL28B genotype appears to be a strong predictor of SVR following PEG-IFN/RBV therapy in treatment-naïve Brazilian patients infected with HCV genotype 1. This study, together with similar research examining other SNPs, should help to define adequate protocols for the treatment of patients infected with HCV genotype 1, especially those with a poor prognosis.

Key words: IL28B - HCV - virological response - treatment - Brazil

Hepatitis C virus (HCV) infection is a global health problem affecting an estimated 170 million people worldwide (WHO 2011). HCV infection is usually persistent and approximately 30% of individuals with persistent infection develop chronic liver diseases, including cirrhosis and hepatocellular carcinoma, after an asymptomatic period of several years (Chevaliez & Pawlotsky 2007, Manzia et al. 2010). Patients with chronic hepatitis C are generally treated for 24-48 weeks with pegylated interferon (PEG-IFN) alpha 2a or 2b in combination with ribavirin (RBV). This treatment is not only expensive, but is also associated with side effects that can result in reduced compliance and even treatment interruption. HCV clearance, or sustained virological response (SVR), is defined as a negative HCV RNA test 24 weeks after the cessation of therapy.

A higher SVR rate has been observed in patients infected with HCV genotypes 2 and 3 compared to those infected with genotype 1, which is the most common genotype found in Europe and the Americas (Manns et al. 2001, Fried et al. 2002, Campiotto et al. 2005, Silva et al. 2007, McHutchison et al. 2009). Furthermore, other host and viral factors, such as advanced liver fibrosis, advanced age and high baseline viral load (VL), have been associated with worse treatment outcomes (McHutchison et al. 2009). For this reason, decisions regarding the inclusion of certain individuals in treatment protocols with the current standard-of-care, i.e., IFN-based therapy, are complex and rely on a balanced assessment of host and viral characteristics (McCarthy et al. 2010, Mangia 2011).

Recently, genome-wide association studies (GWAS) have suggested that several highly correlated, common single-nucleotide polymorphisms (SNPs), which are located on a linkage disequilibrium block in the vicinity of three genes located on chromosome 19, interferon λ1 [interleukin (IL)29], λ2 (IL28A) and λ3 (IL28B), are strongly associated with the response of patients infected with HCV genotype 1 to PEG-IFN/RBV therapy (Ge et al. 2009, Suppiah et al. 2009, Tanaka et al. 2009). Specifi-

cally, SNP rs12979860, which is located three kilobases upstream of the *IL28B* gene, is associated with a greater than two-fold difference in the proportion of patients who achieve SVR (Ge et al. 2009).

The aim of this study was to investigate whether the *IL28B* gene polymorphism (rs12979860) and other variables could predict the outcome of antiviral therapy in a cohort of HCV genotype 1-infected, PEG-INF treatment-naïve Brazilian patients receiving PEG-IFN/RBV therapy.

PATIENTS, MATERIALS AND METHODS

Patient characteristics - A prospective cohort study was carried out between September 2008-August 2009. The patients received free antiviral treatment at the Centre for Injectable Drug Administration and Monitoring (CAMMI) in Porto Alegre, state of Rio Grande do Sul. To receive free treatment, the patients had to meet the following conditions of the Brazilian Ministry of Health (Sander et al. 2002): (i) positive HCV RNA assays, (ii) transaminase [alanine aminotransferase (ALT) and aspartate aminotransferase] levels at least 1.5 times above the upper limit in at least three separate measurements, (iii) recent liver biopsy showing septal fibrosis (Metavir score of F2 or greater), (iv) between 18-70 years of age and (v) a platelet count > 75.000/mm^3 (patients with cirrhosis) or > 90.000/mm^3 (without cirrhosis) and neutrophil/granulocyte counts > 1.500/mm^3. Treatment was initiated only after the determination of each patient's genotype and VL. A few patients failed to fulfil these conditions, but accessed free treatment following judicial decisions. To be enrolled in the study, patients had to be infected with HCV genotype 1 and be naïve to PEG-IFN therapy. Overall, 299 patients received the standard dose of PEG-IFN 2a or 2b (180 µg or 1.5 µg/kg, respectively) plus RBV (1.250 mg/day for body weights > 75 kg or 1.000 mg/day for body weights ≤ 75 kg). The normal duration of antiviral therapy was 48 weeks. However, treatment was interrupted after 12 weeks for non-responders (see below). Furthermore, data analysis was not possible for 36 subjects because of death or the interruption of treatment due to severe side effects or other reasons. Therefore, the results from 263 patients were analysed. Demographic, biochemical and histological data were collected from the patients' clinical charts.

Written informed consent was obtained from each patient. The study protocol was conducted in accordance with the provisions of the ethical guidelines of the Declaration of Helsinki and the study was approved by the Research Ethics Committee of the Public Health School of Rio Grande do Sul, Brazil.

SNP analysis - The three possible IL28B genotypes (SNP rs12979860) were defined as CC, CT and TT. SNP genotyping was performed by direct sequencing after PCR amplification. The following PCR oligonucleotide primer sequences were designed using the National Center for Biotechnology Information DNA database: 5'-CGGAGGATCCCTCCTGGGGC-3' (sense) and 5'-TTCCCACCACGAGACCCCCG-3' (antisense). The polymerase chain reaction (PCR) amplification mixture contained 5 µL of genomic DNA extracted from blood,

20 pmol of each oligonucleotide, 200 mM of deoxyribonucleoside triphosphates, 2 mM MgSO$_4$, 1x high-fidelity PCR buffer (Invitrogen, Carlsbad, CA) and 0.25 U Platinum Taq DNA polymerase (Invitrogen) in a total reaction volume of 50 µL. The following cycling parameters were used for all reactions: one cycle of denaturation at 94ºC for 5 min and 35 cycles of denaturation at 94ºC for 30 s, annealing at 65ºC for 30 s and extension at 72ºC for 45 s. The final extension step was followed by 7-min incubation at 72ºC. The resulting 366-nt PCR product was sequenced in both directions using PCR primers, a Big Dye v1.1 kit (Applied Biosystems, Foster city, CA) and a 3130XL DNA sequencing system (Applied Biosystems). The electropherogram was visualised using Lasergene (DNAstar, Madison, WI) software.

Laboratory and histological tests - Null virological response (NVR) was defined as < 2 log UI/mL reduction in HCV RNA at 12 weeks after the start of treatment (due to treatment interruption) or the presence of detectable HCV RNA by conventional PCR at the end of the course of therapy. Complete early virological response (cEVR) was defined as undetectable levels of HCV RNA at week 12. Partial EVR (pEVR) was defined as an HCV RNA reduction greater than 2 log IU/mL at week *12*. End-of-treatment response (EOTR) was defined as the absence of detectable HCV RNA at the end of the course of treatment and SVR was defined as undetectable levels of HCV RNA 24 weeks after the completion of therapy (72 weeks after the initiation of treatment). Finally, relapse was defined as detectable HCV RNA levels during the follow-up evaluation of patients who had achieved EOTR.

The stage of liver fibrosis was scored according to the Metavir scoring system (F0-F4) and coded as a three-level variable (F0-F2, mild/moderate; F3-F4, severe; cirrhosis) for analysis. Serum HCV RNA quantitation results were classified as low (< 600.000 IU/mL) or high (≥ 600.000 IU/mL) VLs for analysis.

Statistical analysis - Allele frequencies were estimated by gene counting. Both deviation from Hardy-Weinberg equilibrium and comparisons between groups were assessed using the chi-squared test or Fisher's exact test with GraphPad InStat software version 2.04a (GraphPad Software, San Diego, CA). Quantitative variables between non-SVRs and SVRs were compared using the Student´s *t* test. Univariate logistic regression analyses were used to determine the predictors of treatment success. Age, gender, stage of liver fibrosis and VL were included into a multivariate logistic regression model to estimate adjusted odds ratios (ORs) and 95% confidence intervals (CIs). p values less than 0.05 were considered to be statistically significant. SPSS software v.16.0 (SPSS Inc, Chicago, IL) was used for these analyses.

RESULTS

Demographic, biochemical, virological and histological data and response to treatment - First, the patients were asked about possible modes of HCV infection. Blood transfusion (27.5%), the use of illicit drugs (13.1%), surgery (13.1%) or a combination of these factors (10.3%) were the most frequently cited routes. Tattoos, sexual transmis-

sion and other modes of transmission were also reported. HCV-human immunodeficiency virus co-infection was mentioned in the clinical records of 57 patients. An analysis of the patient response to PEG-IFN/RBV treatment, according to their baseline characteristics, is shown in Table I. Overall, 114/263 (43.3%) patients experienced SVR, whereas 149 (56.7%) did not. The mean age of the patients was 50.4 years and 152/263 (58.6%) individuals were male. No significant differences were observed with respect to gender, age or ALT levels between the SVR and non-SVR groups (p = 0.257, 0.435 and 0.827, respectively), although the mean age of the patients who achieved SVR was slightly lower. As expected, responders to PEG-IFN/RBV treatment had a significantly lower VL (p = 0.001). Regarding the stage of liver fibrosis, no significant differences were found between the two groups coded as F0-F2 (mild/moderate) and F3-F4 (severe) (p = 0.509). However, when comparing patients with cirrhosis to other patients, cirrhotic patients were less likely to respond (p = 0.121).

IL28B genotype prevalence and baseline VLs - The frequencies of the various IL28B genotypes were in Hardy-Weinberg equilibrium and the frequency of the C allele was 39%. The IL28B genotypes were found to be CC, CT and TT in 38 (14.4%), 128 (48.7%) and 97 (36.9%) patients, respectively. Patients with the IL28B CC genotype had a slightly lower mean VL at baseline in comparison to patients with a T allele (6.20 ± 0.99, 6.39 ± 0.74 and 6.27 ± 0.74 log IU/mL for patients with CC, CT and TT genotypes, respectively). However, when VL was categorised according to the threshold of 600.000 IU/mL, the proportion of patients with a low baseline VL did not differ in relation to their IL28B genotype (data not shown).

Association between IL28B genotype and PEG-IFN/RBV response - Fig. 1 illustrates the association between the IL28B genotype and the percent of SVRs among HCV genotype 1 patients receiving PEG-IFN/RBV treatment. The C allele was significantly associated with SVR status, particularly when two copies were present, with an OR of 5.31 as compared to non-CC (CT and TT) genotypes (95% CI, 2.29-13.30; p < 0.001).

As shown in Table II, the overall rates of cEVR and pEVR (measured at week 12) were 59% and 21%, respectively (total 80%). One hundred seventy-five out of 270 (65%) patients reached EOTR after 48 weeks of treatment. However, among the subjects who initially achieved SVR with PEG-IFN/RBV therapy, 64 (35%) relapsed.

Of the patients with the CC genotype, 84% achieved cEVR and 13% achieved pEVR. Thus, only one (3%) patient did not achieve a two-log reduction in VL after 12 weeks of treatment. Greater than 90% of the patients showed a virological response at the end of the treatment period and 76% of the patients achieved SVR.
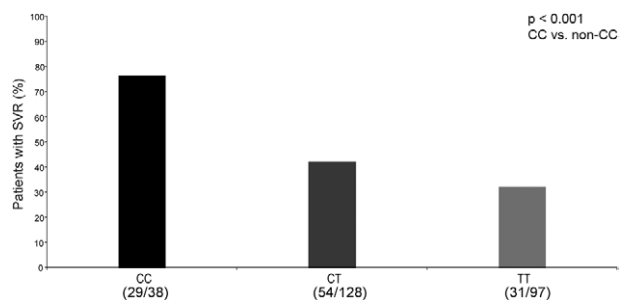

Fig. 1: association of interleukin-28B genotype with sustained virological response (SVR) in hepatitis C virus genotype 1 patients receiving pegylated interferon plus ribavirin treatment. For each genotype, the proportion of patients with SVR out of the total number of patients is shown.

TABLE I

Baseline characteristics of genotype 1 chronic hepatitis C patients treated with PEG-IFN/RBV and rate of sustained virological response

Variable	All (n = 263)	Non-SVR (n = 149)	SVR (n = 114)	p
Age (years) (mean ± SD)	50.4 ± 10.7	50.8 ± 10.1	48.9 ± 11.5	0.435[a]
Sex (male) [n (%)]	152 (58.6)	92 (61.7)	62 (54.4)	0.257[b]
ALT level (IU/L) (mean ± SD)	96.3 ± 71.9	97.2 ± 70.8	95.2 ± 73.5	0.827[a]
Viral load (IU/mL) [n (%)]				0.001[b]
< 600.000	56 (21.8)	20 (13.8)	36 (32.1)	-
≥ 600.000	201 (78.2)	125 (86.2)	76 (67.9)	-
Metavir fibrosis stage [n (%)]				
F0-F2	146 (60.1)	80 (58)	66 (62.9)	0.509 (F0-F2 vs. F3-F4)[b]
F3-F4	97 (39.9)	58 (42)	39 (37.1)	-
Cirrhosis	41 (16.9)	28 (20.3)	13 (12.4)	0.121 (cirrhosis vs. others)[b]

a: ANOVA; *b*: Student's *t* test; ALT: alanine aminotransferase; F0-F2 and F3-F4: Metavir score (mild/moderate and severe and cirrhosis, respectively); PEG-INF/RBV: pegylated interferon plus ribavirin (PEG-IFN/RBV); SD: standard deviation; SVR: sustained virological response.

The IL28B CC genotype was associated with higher response rates to PEG-IFN/RBV treatment at all time points (12, 48 and 72 weeks). Moreover, in comparison to all other patients, the cEVR, EOTR and SVR rates were significantly higher for carriers of the CC genotype (all p-values ≤ 0.005). Furthermore, relapse occurred at a lower frequency among CC patients. Statistically significant differences were not observed between T heterozygotes and homozygotes (all p-values ≥ 0.05); however, the rate of SVR was higher among CT patients (42%) compared to TT subjects (32%) and the relapse rate was also lower in the former group (34% vs. 49%).

Fig. 2 shows the proportion of CC and non-CC genotypes among patients who reached SVR, relapsed or were classified as non-responders. The frequency of the IL28B CC genotype in the relapse group was intermediate between the frequencies observed for the SVR and NVR groups. By univariate analysis, statistically significant differences were found between patients who reached SVR and who relapsed (p = 0.01; OR = 3.3, CI = 1.23-10.28), but not between those who relapsed or were classified as NVRs, which suggested that the IL28B CC genotype influenced SVR, but could not be used to accurately distinguish patients who relapse from those with NVR (p = 0.173; OR = 2.83, CI = 0.57-18.50). As shown in Table III, after making adjustments for age, gender, stage of liver fibrosis and VL in the multivariate analysis, the difference between patients who reached SVR and who relapsed remained statistically significant (p = 0.011; OR = 3.98, CI = 1.38-11.48).

DISCUSSION

Chronic hepatitis C has been recognised as a progressive fibrotic liver disease. However, little is known about the molecular and cellular mechanisms responsible for inter-individual heterogeneity in the virological response to PEG-IFN/RBV treatment among patients infected with HCV genotype 1. A better understanding of these mechanisms may facilitate the development of treatment strategies and could delay disease progression, especially for patients who fail to achieve SVR after undergoing current standard therapy.

The *IL28B* gene encodes a protein also known as IFN lambda 3, which is thought to suppress the replication of various viruses, including HCV (Robek et al.
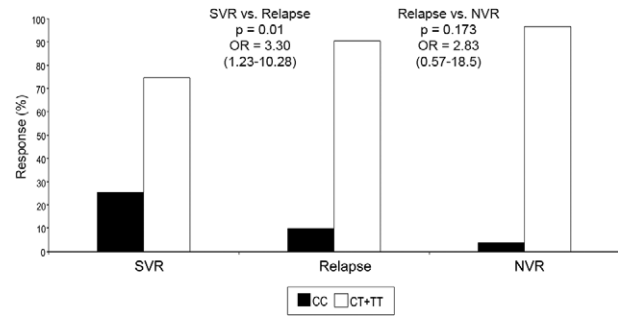

Fig. 2: association between interleukin (IL)28B genotype and three-level treatment outcome in chronic hepatitis C virus patients. The rates of null virological response (NVR), relapse and sustained virological response (SVR) are shown. Odds ratios (OR) and 95% confidence intervals are for IL28B CC vs. CT/TT genotypes, comparing SVR patients to relapsers and relapsers to NVR patients. The p value shown is for the effect of the IL28B CC genotype in a univariate logistic regression analysis.

TABLE III

Association between interleukin-28B genotype and three-level treatment outcome with multivariate logistic regression adjusted for age, gender, viral load and stage of liver fibrosis

Variable	OR	95% CI	p
SVR x relapse			
Age (years)	0.974	0.938-1.010	0.155
Sex (male)	1.976	0.928-4.210	0.077
Viral load < 600.000 IU/mL	0.291	0.115-0.735	0.009
Fibrosis	1.343	0.620-2.905	0.454
IL28B CC genotype	3.981	1.381-11.480	0.011
Relapse x NVR			
Age (years)	1.031	0.99-1.073	0.137
Sex (male)	0.842	0.385-1.840	0.666
Viral load < 600.000 IU/mL	1.243	0.431-3.586	0.687
Fibrosis	0.354	0.168-0.748	0.007
IL28B CC genotype	2.531	0.531-12.062	0.244

CI: confidence interval; NVR: null virological response; OR: odds ratio; SVR: sustained virological response.

TABLE II

Rates of virological response and interleukin-28B genotypes

Treatment response	Overall n (%)	CC n (%)	CT n (%)	TT n (%)	CC vs. CT p	CC vs. TT p	CT vs. TT p
cEVR - week 12	158/269 (59)	32/38 (84)	76/132 (58)	58/99 (59)	0.002	0.005	0.894
pEVR - week 12	58/269 (21)	5/38 (13)	30/132 (23)	25/99 (25)	0.257	0.167	0.755
EOTR - week 48	175/270 (65)	36/39 (92)	83/130 (64)	64/101 (63)	0.001	0.001	1.000
Relapse	64/178 (35)	6/35 (17)	28/82 (34)	30/61 (49)	0.077	0.002	0.086
SVR - week 72	114/263 (43)	29/38 (76)	54/128 (42)	31/97 (32)	< 0.001	< 0.001	0.128

cEVR: complete early virological response; EOTR: end-of-treatment response; pEVR: partial EVR; SVR: sustained virological response. Comparisons between groups were assessed by Fisher's exact test.

2005, Marcello et al. 2006). A polymorphism near the *IL28B* gene on chromosome 19, rs12979860, has recently been described as a determining factor in the response to treatment, as individuals with the CC genotype carry a more favourable prognosis than those with the CT or TT genotypes. At this genomic position, the frequency of the C allele may vary depending on geographical region and ethnicity (Thomas et al. 2009). The Brazilian genetic structure is considered to be quite complex and is one of the most heterogeneous in the world due to the ethnic admixture of people classified as European-derived, African-derived, Brazilian mulattos and Asian-derived, according to their reported ethnicity (IBGE 2000, 2008). The current study was the first to investigate a large number of Brazilian patients and the frequency of the C allele was assessed in a cohort of 263 patients with liver fibrosis due to infection with HCV genotype 1. The C allele was present in 39% of patients, which is lower than the frequencies reported from Europe, Asia and Oceania, but similar to that observed in Africa (IBGE 2000, Thomas et al. 2009, Lin et al. 2011), and this finding may be due to the large proportion of African descendants in the Brazilian population.

The present study confirmed that the IL28B polymorphism is a good predictor of SVR, independent of other factors, such as age, gender, stage of liver fibrosis and VL. Due to the admixture of the Brazilian population over time, it is not possible to classify Brazilian individuals into ethnic groups, at least those based on skin colour (Pena et al. 2009). For this reason, it was not possible to exclude ethnicity as a confounding factor. Indeed, SNP rs12979860 was strongly associated with the response to standard-of-care PEG-IFN/RBV therapy, as demonstrated by higher virological response rates at weeks 12, 48 and 72 and lower rates of post-treatment relapse compared to other genotypes. The findings demonstrated in the current study are consistent with previous reports showing that PEG-IFN/RBV therapy is more effective at blocking the production of HCV in patients with the IL28B CC genotype and that the rate of viral decline is increased in patients with the CC compared to the CT or TT genotypes, which correlates with higher rates of SVR (Thompson et al. 2010, Lindh et al. 2011, Stättermayer et al. 2011). Here, the SVR rate was 76% for patients with the CC genotype and 38% for those with the CT or TT genotypes. Regardless, the SVR rate for non-CC patients was higher than the rates previously reported for Caucasian (23%), African-American (9%) and European (28%) patients (Rauch et al. 2010).

Because we focused on a unique SNP, the impact of the present study may be limited. However, these findings, together with the results of GWAS (Ge et al. 2009, Suppiah et al. 2009, Tanaka et al. 2009), provide a rationale for the development of prognostic tests and customised IFN-based therapies for patients with chronic hepatitis C. Well-adapted therapies should be based on the baseline characteristics of the patient and should follow a response-guided approach. According to our data, the presence of the IL28B CC genotype may indicate a high probability of achieving SVR. In contrast, patients with non-CC geno-

types and those with other predictors of poor response, such as advanced fibrosis or high VL, would be unlikely to achieve SVR. Along with previous reports, our results may be used to decide whether to implement a PEG-IFN/RBV treatment regimen or to wait for more effective therapeutic protocols, such as those currently in development that are based on HCV protease and polymerase inhibitors (Jacobson et al. 2011, Poordad et al. 2011).

In conclusion, the IL28B genotype appears to be the strongest predictor of SVR following PEG-IFN plus RBV therapy in treatment-naïve patients infected with HCV genotype 1. Recently, important genetic findings related to the antiviral response in patients with chronic hepatitis C suggest that customised therapy is likely to be implemented in the near future. Further clinical trials are needed to determine whether HCV genotype 1-infected patients with the favourable IL28B CC genotype could be treated for a shorter duration with equal clinical benefit, which would reduce the cost of long-term treatment and the burden of adverse side effects.

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