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A Single Nucleotide Polymorphism in IL28B Affects Viral Evolution of Hepatitis C Quasispecies after Pegylated Interferon and Ribavirin Therapy

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Abstract

Interleukin-28B (IL28B) polymorphisms are associated with viral response to peginterferon and ribavirin in chronic hepatitis C (HCV). Their recognition represents a breakthrough in the understanding of the role of the host in viral eradication. How these polymorphisms determine viral eradication is unknown. The IL-28B variants are hypothesized to have a differential impact on HCV quasispecies evolution during treatment with pegylated interferon and ribavirin. In this study, HCV RNA levels were measured at early time points in 33 naïve genotype 1 hepatitis C patients and clonal analysis of the entire NS5A region was performed on sera from baseline and day 7. Site rs12979860 polymorphisms were determined by direct sequencing of PCR products and classified into CC, CT, and TT and were identified in 13, 11 and 9 patients, respectively. The CC polymorphism more commonly was seen in Whites vs. Blacks [12/21 (57%) vs. 1/12 (8%), $P=0.009$] and HIV-infected vs. mono-infected [13/25 (52%) vs. 0/8 (0%), $P=0.009$]. Patients with CC and non-CC had similar baseline viral loads. More patients with the CC polymorphism had amino acid substitutions in NS5A compared to non-CC patients. Despite similar baseline viral diversity, by day 7, significantly more patients with CC had higher non-synonymous substitution values compared to non-CC ($P=0.02$). Chronic hepatitis C patients with the CC IL28B polymorphism have a higher number of amino acid substitutions in the NS5A region and early viral evolution due to greater interferon induced selective pressure during this critical period of treatment.

INTRODUCTION

Treatment of chronic *Flaviviridae Hepacivirus* hepatitis C (HCV) with conventional pegylated interferon (PEG-IFN) combined with ribavirin (RBV) leads to sustained virologic responses in about 50% of patients [Fried et al., 2002; Hadziyannis et al., 2004; Manns et al., 2001] and even lower in those with HIV/HCV co-infection [Torriani et al., 2004] and in

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HJ, MKJ, WML: concept, study design, data interpretation and manuscript preparation

BA: statistical analysis

NL, TP, AW, and NA study implementation and data collection

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Blacks [Muir et al., 2004]. Substantial studies have been performed to identify viral and host factors associated with favorable response to PEG-IFN and RBV therapy [Ghany et al., 2009] in order to minimize undesirable side effects of therapy in those unlikely to respond. Recent genome-wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNP) near the interleukin 28B (IL28B) gene, that encodes for interferon- λ 3, and is associated with an increased likelihood of sustained viral response to PEG-IFN and RBV therapy in patients with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. Patients with favorable IL28B SNP polymorphisms (such as CC at the rs12979860 SNP or TT at the rs8099917 SNP) have a two-fold improvement in sustained viral response rates compared to patients with unfavorable IL28B SNP polymorphisms. Furthermore, studies have shown that IL28B SNP polymorphisms affect viral kinetics after PEG-IFN and RBV therapy [Thompson et al., 2010b] and spontaneous HCV clearance after acute HCV infection [Thomas et al., 2009].

How IL28B SNP polymorphism affects viral response with PEG-IFN and RBV has not been elucidated.. Conflicting data have been reported regarding the IFN-lambda mRNA level in whole blood or peripheral blood mononuclear cells (PBMCs) between patients with the favorable and with the unfavorable SNP [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. Certain amino acid substitutions in HCV core region have been found to be strongly associated with IL28B SNP polymorphisms [Chayama and Hayes, 2011; Kurosaki et al., 2011]. However, no study has examined the difference in viral sequence diversity between patients with different IL28B SNP polymorphism. In a prior study, more patients with lower baseline sequence diversity in HCV NS5A region were able to achieve early viral response [Jain et al., 2009]. In this study, patients with the favorable CC polymorphism at rs12979860 were hypothesized to have lower sequence diversity. In addition, the impact of SNP polymorphism on the early viral evolution was also explored.

METHODS

Patient Population

Patients were recruited prospectively from the HIV and Liver Clinics at Parkland Health and Hospital Systems, a teaching hospital associated with UT Southwestern Medical Center. Patient selection criteria have been described previously [Jain et al., 2009]. All patients signed an informed consent, and were anti-HCV positive, aged 18–65, and had HCV genotype 1 with serum HCV RNA > 1000 IU/mL by either polymerase chain reaction (PCR) or branched DNA (bDNA) assays. Race was self-reported. All patients were HCV treatment naïve. HIV patients had to have a CD4+ T cell counts ≥ 300 cells/mm³ within 12 weeks of study initiation and no evidence of a symptomatic AIDS-defining illness. Subjects could be antiretroviral therapy (ART) naïve; but if on ART, they needed to be on a stable regimen for 12 weeks prior to enrollment. Exclusion criteria included presence of other liver diseases such as chronic hepatitis B, alcoholic liver disease, or evidence of hepatic decompensation. The study protocol and informed consent were approved by the Institutional Review Board at UT Southwestern Medical Center.

Patients received peg-IFN alfa 2a weekly and ribavirin 1000 mg (<75kg) or 1200 mg (>75kg) in two divided doses daily for a total of 48 weeks. Patients were allowed to use growth factors for anemia and neutropenia. Some patients were co-enrolled in the Roche Paradigm study which was a randomized multicenter double blind trial comparing high dose ribavirin 1000/1200 mg per day to the approved dose of 800 mg per day with Peg-IFN alfa 2a 180 μ g per week in HIV/HCV co-infected genotype 1 patients. Patients who co-enrolled in both studies agreed to have additional samples taken for the viral kinetics studies. Only one patient who participated in both studies was randomized to 800 mg of ribavirin.

Sample Collection and Laboratory Evaluation

Blood was collected on day(s) 0, 1, 2, 3, 4, 7, 9, 11, 14, 21, 28, 42, and 56 days after the first dose. HCV RNA level quantification on stored sera was performed using the VERSANT® 3.0 branched DNA technology, (Siemens Medical Solutions Diagnostics, Tarrytown, N.Y., USA).

Quasispecies Analysis

Sequence data of 12 White patients had been reported in a previous paper [Jain et al., 2009]. HCV RNA was extracted from 140 µl serum with QIAamp viral RNA Mini kit (QIAGEN Inc. Valencia, CA, USA). The entire NS5A gene was amplified as described [Jain et al., 2009]. The amplification products were purified with QIAquick Gel extraction kit (QIAGEN Inc. CA) and ligated into the TA-cloning plasmid pGEM-T Easy (Invitrogen, Carlsbad, CA, USA) and cloned independently. Ten to twelve clones were selected from each sample and sequenced in the sequence core facility at UT Southwestern Medical Center. The clones were submitted to GenBank and the accession numbers are JX112366-JX112605.

Within each sample, the mean within-sample sequence genetic distance (SGD) (measuring diversity within each sample), synonymous substitution rate per synonymous substitution site (dS) (measuring substitutions which do not lead to amino acid change), and non-synonymous substitution rate per non-synonymous substitution site (dN) (measuring substitutions which lead to a amino acid change) at baseline and at 7 days were calculated with MEGA software (version 4 <http://www.megasoftware.net>) [Tamura et al., 2007]. Each clone from baseline samples was compared with sub-genotype reference sequence (H77 as HCV 1a reference sequence, HCV-J as HCV 1b reference sequence), the number of amino acid substitutions in NS5A region was counted and the average number of amino acid substitutions for each patient was calculated.

The rs12979860 polymorphism was determined by sequence analysis. Human genomic DNA was extracted from serum samples or stored PBMCs. The 592bp fragment overlapped the SNP rs12979860 was amplified by polymerase chain reaction with the sense primer (5'-CAGGACTGCGGGGACGAG-3') and anti-sense primer (5'-GGCGCTGAGGGACCGCTA-3'). The PCR products were visualized on agar gel. The amplification products were purified with QIAquick Gel extraction kit (QIAGEN Inc. Valencia, CA, USA). The polymorphism at rs12979860 was determined based on direct sequencing the PCR products and classified into CC, CT and TT.

Statistical Analysis

For continuous baseline variables, two group comparisons were made with the Mann-Whitney U test. Categorical variables were compared using the Fisher's Exact test. For parametric analyses, data were log transformed as needed to meet analysis assumptions. Results are presented as median (range). Statistical analysis was performed with SAS version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Demographic data

Thirty-three patients were recruited in this study, including 12 Black and 21 White patients. The median age was 41.0 (22–59) years. The male to female ratio was 2:1. Twenty-seven patients were infected with HCV genotype 1a and 6 patients were infected with HCV genotype 1b. Clonal analysis was performed in 11 Black and 12 White patients. No

significant differences were found between patients with and without HCV quasispecies analysis with regard to age, gender, and race.

CC, CT and TT polymorphism at rs12979860 was identified in 13, 11 and 9 patients respectively. Patients with CC and non-CC polymorphisms had comparable median age [median (range): 41(29–49) vs. 43(22–59), $P=0.51$]. Fifty-five percent (12/22) of those with CC polymorphism were male and 9% (1/11) were female ($p=0.02$). As expected, Black patients had lower frequency of CC polymorphism compared to White patients (1/12 vs. 12/21, $p=0.009$). Fifty-two percent (13/25) of patients with HCV-HIV co-infection had the CC polymorphism whereas none of the 8 HCV mono-infected patients were found to possess CC ($p=0.012$). More patients with CC polymorphism had higher baseline serum ALT levels compared to those with non-CC [median (range): 86(34–310) vs. 59(25–118), $p=0.049$]

Virological response to pegylated interferon and ribavirin therapy

Patients with CC and non-CC polymorphism had comparable baseline serum HCV RNA levels (Table 1). Compared to patients with non-CC polymorphism, patients with the CC polymorphism tended to have a faster decline of serum HCV RNA levels within the first two days after administration of PEG-IFN and RBV. Greater viral decline was seen in the patients with CC polymorphism by the end of the first week, which continued through week 4, these differences were not statistically significant. Similar level of viral decline was seen in patients with CC and non-CC polymorphisms between weeks 4 and 8. No differences in the rate of RVR, EVR, and sustained viral response were found between CC and non-CC polymorphisms as shown in Table 1.

Phylogenetic analysis of baseline sequences

Phylogenetic analysis was performed using baseline consensus sequences from 23 patients. Compared to patients with non-CC, sequences from patients with CC are distant from the HCV reference sequence (H77 and HCV-J). Even though HCV sequences from patients with CC and non-CC polymorphisms were intermingled, there was a relative cluster of sequences from patients with CC in one branch. Four of 5 sequences in this branch were from patients with CC polymorphism. No specific cluster was found according to race and viral response (Figure 1).

HCV quasispecies

Of the 23 patients in whom clonal analysis was performed, patients with CC or non-CC polymorphisms had similar baseline sequence genetic diversity (SGD) (Figure 2). When synonymous substitution rate per synonymous substitution site (dS) and non-synonymous substitution rate per non-synonymous substitution site (dN) were analyzed, patients with CC and non-CC had similar dS and dN value at baseline. On day 7, patients with CC and non-CC still had similar SGD and dS value, but significantly more patients with CC had higher dN value compared to patients with non-CC ($p=0.02$, Figure 2). If limited to those with HIV/HCV co-infected, SGD, dS, and dN were not different between CC patients ($n=7$) and non-CC patients ($n=8$) at baseline; and by day 7, CC patients showed a trend toward higher dN values compared to non-CC patients ($p=0.07$).

When compared to the genotype-specific reference sequence (H77 for HCV genotype 1a and HCV-J for genotype 1b), significantly more patients with CC had higher baseline amino acid substitutions in the whole NS5A compared to patients with non-CC [median (range): 26.6 (21.9–43.2) vs. 22.3(10.9–39.8), $P=0.045$]. When individual regions were compared, patients with CC polymorphism had a higher number of amino acid substitutions in V3 region and its carboxyl terminal region compared to patients with non-CC [median (range):

5.5(2.0–8.2) vs 3.3(1.0–6.0), $P=0.071$ for V3 region, 3.0(2.0–4.0) vs. 2.0(0–4), $P=0.055$ for carboxyl terminal of V3 region]. No significant difference was found in the number of amino acid substitutions in the ISDR-PKR region between the different polymorphism groups ($p=0.29$). No specific amino acid substitution in the entire NS5A region was found to be associated with IL28B SNP polymorphism.

Impact of race

Black and White patients had comparable baseline serum HCV RNA levels. Compared to Blacks, HCV RNA levels declined faster during the first week of PEG-IFN therapy in White patients, and this group also had faster second phase viral decline (Table 1). Blacks had a slightly lower SGD, dS and dN value at baseline (Figure 3 $p=0.06$, 0.09 and 0.08, respectively) compared to Whites. By day 7, Black patients had significantly lower SGD, dS and dN value (Figure 3, $P=0.004$, 0.009 and 0.024 respectively) compared to Whites.

Of the 23 patients in whom HCV clonal analysis was performed, 6 of 12 White patients had the CC polymorphism, and only one of 11 Black patients were found to have the CC polymorphism ($p=0.07$). In Whites, patients with CC and non-CC had similar baseline SGD, dS and dN value. By the end of first week, White patients with CC polymorphism had significantly higher dN values compared to White patients with non-CC polymorphism, as seen in Figure 4. Among Blacks, a comparison between CC and non-CC could not be done. Blacks with non-CC polymorphism did have similar dN value on day 7 [median (range): 0.0040(0.0020–0.0181)] when compared to White patients with a non-CC polymorphism.

DISCUSSION

In patients with HCV, the exciting finding of a single nucleotide polymorphism around IL28B has opened up a new avenue of research [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. In the present study, the rs12979860 polymorphism appears to affect the number of amino acid substitutions in NS5A region at baseline and viral evolution in the first week of PEG-IFN and RBV therapy.

Patients with the favorable CC polymorphism had a higher number of amino acid substitutions in the entire NS5A region and its carboxyl terminal region including V3 region. This is in line with the phylogenetic observations that sequences from patients with CC were clustered and far away from the reference genotype. Patients with higher numbers of amino acid substitutions in the NS5A region have been reported to have better responses to treatment [Enomoto et al., 1996; Chayama and Hayes, 2011]. Patients with the CC polymorphism also had significantly higher serum ALT levels, suggesting that these patients may experience stronger immune pressure, a phenomenon which may lead to selection of HCV sequences with more amino acid substitutions in the NS5A region.

In patients with HCV, recent studies of interferon stimulating gene (ISG) expression activity in liver tissue have found that patients with favorable IL28B polymorphism had lower ISG activity [Honda et al., 2010]. Similarly, prior studies have shown lower baseline intrahepatic ISG activity was associated with response to PEG-IFN and RBV therapy [Chen et al., 2005; Sarasin-Filipowicz et al., 2008]. The ISGs in patients with higher expression may be maximally activated, and thus are less likely to be further stimulated with interferon exposure. So these patients will have a poorer response to PEG-IFN treatment [Sarasin-Filipowicz et al., 2008].

When HCV quasiespecies were analyzed in individual patients, those with CC and non-CC polymorphisms had similar baseline SGD, dS and dN values. However after initiation of PEG-IFN therapy, patients with the favorable CC polymorphism, thought to have lower

baseline ISG activity [Honda et al., 2010], appear to be capable of mounting higher expression of associated ISGs which would lead to increased interferon pressure and changes in the quasispecies environment. In line with this assumption, significantly more patients with CC polymorphism had a higher dN value by day 7 compared to those lacking CC, even though both groups had comparable dN value at baseline. In non-CC patients, who are expected to have higher ISG expression and are not further induced by interferon, no significant change in dN values occurred in this group by day 7. This result further supports a previous study that showed that patients with early viral evolution are more likely to achieve viral response [Jain et al., 2009]. Early HCV viral evolution may occur even before there is a significant viral decline in patients who are receiving PEG-IFN and RBV therapy.

It has been well established that lower baseline viral load correlates with better viral response to interferon-based antiviral therapy. Recent clinical studies of a large number of patients have found that patients with the favorable IL28B polymorphism had higher baseline serum HCV RNA level compared to patients with a less favorable SNP polymorphism [Ge et al., 2009]. After treatment with PEG-IFN and RBV, patients with the favorable CC polymorphism will have a faster viral decline during the first two weeks of treatment, as well as higher rapid viral response rate at week 4, early viral response rate at week 12 and eventual sustained viral response [Thompson et al., 2010a; Thompson et al., 2010b]. Studies have shown that, for patients who achieved an RVR, the IL28B polymorphism did not affect the eventual sustained viral response rate. Of interest, among patients who did not achieve RVR, those with the CC polymorphism demonstrate higher sustained viral response rates compared to patients with non-CC polymorphism [Thompson et al., 2010b].

In this cohort of 33 patients, viral decline within the first week of treatment has been further studied. Patients with CC polymorphisms had a trend toward a rapid first phase and second phase of viral decline compared to those with non-CC polymorphism. But patients with CC and non-CC polymorphism had similar viral decline after week 4, suggesting the IL28B SNP polymorphism mainly affects the early viral response, during the first 4 weeks [Thompson et al., 2010b].

Clinical studies have demonstrated that Blacks have significantly lower prevalence of CC compared to Whites. In this small study, White patients had a faster second phase viral decline compared to Black patients. However, the rate of decline between CC and non-CC was not significantly different. Compared to Blacks, Whites have significantly higher median SGD, dS and dN value on day 7. Among Whites, those with CC had significantly higher median dN value on day 7 compared to Whites with non-CC. Interestingly, Black patients with non-CC had a similar median dN value as White patients with non-CC. These observations supports the suggestion that the racial difference in the prevalence of IL28B SNP polymorphism only explains partially the differences in response to PEG-IFN and RBV therapy that has been observed between Blacks and Whites.

Viral evolution occurred in those with CC polymorphism. Some differences between Whites and Blacks in terms of viral diversity did exist. However, after pegylated interferon and ribavirin, the differences became more significant. Likely these differences are due to viral evolution occurring in White patients but not Blacks. Again, a better understanding of why Blacks do not undergo selective decline in quasispecies is needed to help understand the mechanism of viral response in Blacks.

IL28B SNP polymorphism has also been found to be associated with spontaneous clearance of HCV in patients with acute HCV infection [Thomas et al., 2009]. HIV co-infection impairs the adaptive immune response, making HCV clearance rare among HIV patients

with acute HCV infection [Soriano et al., 2007]. In this study, HIV co-infected patients had a higher prevalence of CC polymorphism compared with those with HCV mono-infection. This higher prevalence may result from HIV hindering the natural (enhanced) natural clearance of HCV infection in patients with CC polymorphism.

Since the patient numbers are small, these observations need to be further validated in larger studies. Differences in viral decline between CC and non-CC are likely to be seen in larger studies, and this difference in viral decline between Whites and Blacks will be more significant. This study does not have the power to determine differences in decline of serum HCV RNA levels in the first week. Confounding factors such as race and HIV co-infection might also affect the role of rs12979860 SNP in the viral response to antiviral therapy. Another limitation of the study was that we were unable to determine if the amino acid variations were influenced by HLA restricted epitopes. The small number of clones and the short period between sequencing time points did not allow for a conclusive analysis.

In summary, this study demonstrates that patients with the favorable IL28B CC polymorphism have a higher number of amino acid substitutions in NS5A region compared to patients with less favorable IL28B polymorphisms. These patients more often have early viral evolution after pegylated interferon and ribavirin treatment, well before the appearance of viral decline. Viral decline was faster in Whites but this was not due to CC polymorphism alone. Further studies are needed to understand the relationship between host polymorphism, race and viral sequence changes.

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Figure 1.

Figure. Evolutionary relationships of HCV NS5A consensus sequences from patients with CC, CT and TT polymorphism.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.03173567 is shown. The percentage of replicate trees in which the associated sequence clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4. The red circle represent the HCV genotype 1a reference sequence (H77); the red square represent HCV 1b reference sequence (HCV-J); the black circle represent patients with CC polymorphism, grey circle represent patients with CT polymorphism and white circle represent patients with TT polymorphism.

Figure 2. Sequence diversity at baseline and day 7 in patients with CC and non-CC polymorphisms. Black box represents patients with CC and white box represent patients with non-CC. SGD, genetic distance within quasispecies; dS, synonymous substitutions, dN, non-synonymous substitutions.

Figure 3. Sequence diversity at baseline and day 7 in Black and White patients: Black box represents Black patients and white box represents White patients. SGD, genetic distance within quasispecies; dS, synonymous substitutions, dN, non-synonymous substitutions.

Figure 4. dN value in Whites with CC, Whites with non-CC and Blacks with CC polymorphisms, dN, non-synonymous substitutions.

Table 1

Viral response to peginterferon and ribavirin therapy by CC polymorphism and race.

	CC	CT/TT	P value	Black	White	P value
No of patients	13	20		12	21	
Baseline viral load	5.94(4.50~6.89)	6.11(4.32~6.73)	0.94	6.13(4.32~6.89)	5.94(4.50~6.89)	0.50
Viral decline						
within 2 days	-1.08(-2.16~-0.01)	-0.71(-1.75~-0.06)	0.29	-0.66(-1.75~-0.06)	-0.90(-2.36~0.01)	0.23
day 7	-1.13(-3.11~-0.05)	-0.45(-1.82~-0.15)	0.11	-0.37(-1.54~-0.14)	-0.62(-3.11~-0.05)	0.11
Week 1 to 4	-0.95(-1.99~0)	-0.55(-1.34~-0.06)	0.11	-0.29(-1.20~-0.06)	-0.67(-1.99~0)	0.082
Week 4-8	-0.73(-1.73~-0.34)	-0.497(-2.20~-0.01)	0.78	-0.43(-2.2~-0.01)	-0.68(-1.74~-0.34)	0.93
RVR, n (%)	5/13(38.5%)	3/20(15%)	0.21	1/12(8.3%)	7/21(33.3%)	0.21
EVR, n (%)	12/13(92.3%)	12/19(63.2%)	0.10	7/12(58.3%)	17/20(85.0%)	0.12
SVR, n (%)	5/13(38.5%)	6/20(30.0%)	0.71	2/12(16.7%)	9/21(42.9%)	0.25

RVR, rapid viral response- undetectable at 4 weeks; EVR, early viral response, undetectable or ≥ 2 log decline from baseline at 12 weeks; SVR, sustained viral response, undetectable 24 weeks after completing therapy.