Impact of *IL28B*-Related Single Nucleotide Polymorphisms on Liver Histopathology in Chronic Hepatitis C Genotype 2 and 3

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Abstract

*Background and Aims:* Recently, several genome-wide association studies have revealed that single nucleotide polymorphisms (SNPs) in proximity to *IL28B* predict spontaneous clearance of HCV infection as well as outcome following peginterferon and ribavirin therapy among HCV genotype 1 infected patients. The present study aimed to evaluate the impact of *IL28B* SNP variability on liver histology in the context of a phase III treatment trial (NORDynamIC) for treatment-naïve patients with chronic HCV genotype 2 or 3 infection, where pretreatment liver biopsies were mandatory.

*Methods:* Three hundred and thirty-nine Caucasian patients had samples available for *IL28B* genotyping (rs12979860) of whom 314 had pretreatment liver biopsies that were evaluated using the Ishak protocol, allowing for detailed grading and staging of liver histopathology.

*Results:* *IL28B* CC<sub>rs12979860</sub> genotype in HCV genotype 3 infected patients was associated with higher ALT levels (p<0.0001), higher AST to platelet ratio index (APRI; p=0.001), and higher baseline viral load (p<0.0001) as compared to patients with the CT or TT genotypes. Additionally the CC<sub>rs12979860</sub> genotype entailed more pronounced portal inflammation (p=0.02) and steatosis (p=0.03). None of these associations were noted among HCV genotype 2 infected patients.

*Conclusion:* This study shows that the CC<sub>rs12979860</sub> SNP is associated with more pronounced liver histopathology in patients chronically infected with HCV genotype 3, which may be secondary to higher viral load. The finding that *IL28B* variability did not impact on liver pathology or viral load among genotype 2 infected patients implies that *IL28B* may differentially regulate the course of genotype 2 and 3 infection.

Introduction

Hepatitis C virus (HCV) infects 170 million people worldwide [1] and is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma [2]. Treatment with pegylated interferon-α (peg-IFN) and ribavirin results in sustained viral response (SVR) in approximately 50% of patients infected with HCV of genotype 1 and in 80% of those with HCV genotypes 2 or 3 [3,4,5]. Recently, several genome-wide association studies have revealed that single nucleotide polymorphisms (SNPs) in the 19q13 region, in close proximity to three genes (*IL28A*, *IL28B*, and *IL29*) encoding cytokines of the IFN-α (i.e., type III IFN) family, predict spontaneous clearance of HCV infection [6,7] as well as SVR following peg-IFN/ribavirin therapy among patients infected with HCV genotype 1 [6,8,9,10]. Additionally, these polymorphisms are strongly associated with the first phase viral decline (i.e., reduction of HCV RNA during the first days of treatment) irrespective of HCV genotype [11], and concomitant assessment of pretreatment levels of systemic IP-10 and *IL28B*-related SNPs augments the prediction of the first phase decline in HCV RNA and the final therapeutic outcome [12,13].

Carriage of the favorable C allele at rs12979860 also is associated with higher baseline viral load (8, 11, 14), which otherwise is an established negative predictor of response to peg-IFN/ribavirin therapy [3,4,5], possibly secondary to major allele carriage being associated with lower intrahepatic expression of interferon-stimulated genes (ISGs) [14,15]. A similarly unexpected observation is that a C allele at rs12979860 is more common in Caucasians
injected with HCV genotype 2 and 3 than with genotype 1 [13,16,17].

In a study of Japanese patients infected with HCV genotype 1 or 2, those homozygous for the IL28B major allele had significantly higher inflammatory activity; also, a higher proportion of these patients had fibrosis stage F2-4 as compared with F0-1, but interestingly these patients also had significantly lower gamma-GTP levels [18]. Similarly, among HCV genotype 3 infected patients of Scandinavian descent, CC carriers at rs12979860 had significantly higher normalized alanine aminotransferase (ALT) levels as well as aspartate aminotransferase platelet ratio index (APRI) than T allele carriers indicating a higher degree of inflammation and fibrosis [19]. The aim of the present study was to evaluate the impact of IL28B SNP variability on liver histology in the context of a phase III treatment trial for treatment naïve patients with chronic HCV genotype 2 or 3 infection. In this trial, pretreatment liver biopsies were mandatory and were centrally evaluated using the Ishak protocol, allowing for detailed grading and staging of liver histopathology.

Materials and Methods

The NORDynamIC trial

Three hundred and eighty-two treatment naïve patients with HCV genotype 2/3 infection were randomized at baseline to either 12 or 24 weeks of combination treatment with peg-IFN α-2a 180 μg once weekly and ribavirin 800 mg daily. Baseline characteristics of the 339 Caucasian patients that could be analyzed for IL28B polymorphisms are detailed in Table 1. The first dose of study medication was administered under the supervision of a study nurse, and a patient diary monitored subsequent dosing. Further details regarding this trial are provided elsewhere [20].

Classification of response

Patients were classified as having a rapid virological response (RVR) if HCV RNA was undetectable (i.e., <15 IU/mL) day 29, and as achieving SVR if plasma HCV RNA was undetectable 24 weeks after completion of therapy.

IL28B genotyping

SNP rs12979860 was determined in plasma by allelic discrimination using Taqman MGB (minor groove binding) probes. The following primers and probes were used: rs12979860; Forward, GTGCTGCTGTGACTCAACCA, Reverse, AGCGCGGAGTGCAATTCA, Probe_C, FAM-CCTGGTTCGCGCCTT-MGB, Probe_T, VICCCTGGTTCACGCCT-MGB. All SNPs were at Hardy-Weinberg equilibrium. SNP rs12979860 has previously been reported to have a stronger association with both first phase decline and SVR than rs8099917 and rs12980275 among Caucasian HCV infected patients, and was thus analyzed in the present study [11].

HCV RNA quantification

Plasma was obtained using PPT-tubes and HCV RNA was determined by RT-PCR of plasma using Cobas AmpliPrep/COBAS TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ), which quantifies HCV RNA with a limit of detection of ≤15 IU/mL. HCV RNA quantification was performed on days 0, 3, 7, 8, 9, week 8, week 12, week 24 (for those receiving 24 weeks of therapy), and 24 weeks after completion of therapy. All samples were frozen (−70°C) and subsequently analyzed at the central laboratory.

Liver biopsies

Liver biopsies were obtained from all patients within 24 months prior to study entry. Only biopsies with a length exceeding 1.5 cm and containing more than 6 portal tracts were evaluated. The evaluation was performed in a blinded fashion by two independent observers according to the Ishak protocol [21]. Equivocal issues were debated after the independent scores were noted, and a consensus score was obtained. In addition, steatosis was graded as follows: absent = 0, less than 30% of hepatocytes involved = 1, 30–70% of hepatocytes involved = 2, and more than 70% of hepatocytes involved = 3 [22]. In total 314 patients could be evaluated for both liver histology and IL28B genotype.

Fibrosis index

APRI was calculated as the ratio of normalized aspartate aminotransferase, i.e., value divided by the upper limit of normal, to the platelet count as previously detailed [23].

Statistical methods

Wilcoxon-Mann-Whitney U-test, Kruskal-Wallis test, and Chi squared (χ²) tests were utilized to evaluate relationships between groups. All statistical analyses were performed using IBM SPSS statistics version 19.0 (IBM Corporation, Somers, NY) and StatView (version 3.0, SAS Institute Inc., Cary, NC, USA) software packages. All reported p-values are two-sided, and p-values<0.05 were considered significant.

Ethical considerations

Written informed consent was obtained from each participating patient. Ethics committees in each participating country approved

### Table 1. Baseline Characteristics with Patients Grouped According to HCV genotype and IL28B Genetic Variations (rs12979860).

|                    | HCV genotype 2 |                  | HCV genotype 3 |                  |
|--------------------|----------------|-----------------|----------------|-----------------|
|                    | CC  | CT  | TT  | n = 48 | n = 40 | n = 10 | p  | CC  | CT  | TT  | n = 101 | n = 113 | n = 27 | p  |
| **Age (years)**    |     |     |     |        |        |        |     |     |     |     |        |        |        |     |
| Male/Female        |     |     |     | 28/20 | 24/17 | 6/4    | NS  | 69/37 | 68/49 | 14/13 | NS  |
| BMI (kg/m²)        |     |     |     | 25 (22–27) | 25 (24–28) | 25 (23–26) | NS  | 25 (23–28) | 26 (23–29) | 27 (23–28) | NS  |
| Fibrosis Stage (Ishak 0/1/2/3/4/5/6) |     |     |     | 2.7 (1.4–4.4) | 2.4 (1.7–7.0) | 2.5 (1.3–9.8) | NS  | 2.2 (1.4–5.0) | 2.9 (1.9–6.6) | 3.8 (2.0–7.7) | NS  |
| Alcohol consumption (drinks/day) |     |     |     | 0.0 (0.0–0.5) | 0.1 (0.0–0.4) | 0.1 (0.0–0.4) | NS  | 0.1 (0.0–0.4) | 0.0 (0.0–0.4) | 0.0 (0.0–0.3) | NS  |

Data presented as median (25th–75th percentile) or n. 

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the study (i.e. Regional Ethical Review Board, Gothenburg, Sweden (Regionala etikprövningsnämnden i Göteborg), Regional Committee for Ethics in Medical Research, Bergen, Norway (Regionala etikprövningsnämnden i Göteborg), The Scientific Ethical Committee for the Region of Middle Jylland, Viborg, Denmark (Regional etikprövningsnämnden i Region Midtjylland), The Scientific Ethical Committee for the Region of South Denmark, Vejle, Denmark (Regional etikprövningsnämnden i Region Syddanmark), and the Ethics Committee, Department of Medicine for the Hospital District of Helsinki and Uusimaa, Finland (Etiska kommitéen för invärtsemedicin)). The study has been registered at the NIH trial registry (ClinicalTrials.gov Identifier: NCT00143000).

Results

A strong association was noted between IL28B genotype and pretreatment normalized ALT (median 2.4, 2.3, and 1.6 for CC, CT, and TT respectively) and as well as APRI, a biomarker of liver fibrosis [23], among HCV genotype 3 infected patients, with homozygous TT carriers having the lowest levels (p = 0.0001 and p = 0.001 respectively, Kruskal-Wallis test; Figure 1). With regards to ALT, CT carriers were more similar to CC than TT (CC vs. CT p = 0.6, CT vs. TT p = 0.0001, and CC vs. TT p < 0.0001, Mann-Whitney U-test), but when evaluating APRI, CT patients were intermediary (CC vs. CT p = 0.03, CT vs. TT p = 0.07, and CC vs. TT p = 0.0015, Mann-Whitney U-test). This was mirrored by a significantly higher proportion of these patients having lower portal inflammation grades in their pretreatment liver biopsies, in contrast to homozygous CC carriers who had the highest grades (p = 0.02, Chi-squared test; Figure 2C). A similar non-significant trend was noted for interface hepatitis grade and fibrosis stage, with CC carriers having the highest grades and stages. No such trend was noted for the grade of lobular inflammation or confluent necrosis. In accordance with the observation for portal inflammation, homozygous CC carriers had the most pronounced steatosis and homozygous TT carriers had the least (p = 0.03, Chi squared test; Figure 2D). Because APRI is a continuous variable in contrast to fibrosis stage, which is ordered categorical data derived from liver biopsies prone to sampling error, different statistical methods are applicable, which may explain the significant association between IL28B genotype and APRI for genotype 3 in contrast to a non-significant trend for fibrosis stage.

The associations between IL28B genotype and ALT, APRI, portal inflammation grade, and steatosis observed among HCV genotype 3 infected patients, were not significant among HCV genotype 2 infected patients nor were any such trends noted among these latter patients. It should, however, be noted that the

Figure 1. Impact of IL28B (rs12979860) on normalized ALT and AST to platelet ratio index (APRI) among HCV genotype 2 and 3 infected patients. Box plots displaying the 10th, 25th, 50th, 75th, and 90th percentiles and p-values obtained using Kruskal-Wallis test. doi:10.1371/journal.pone.0029370.g001
HCV genotype 2 infected patients enrolled in the present study were significantly older than those infected with HCV genotype 3 (mean age 47.2 vs. 39.8 years for HCV genotype 2 and 3 respectively, \( P < 0.0001 \), Mann-Whitney U-test), and the number of patients enrolled with HCV genotype 2 was smaller than genotype 3 (98 vs. 241).

The HCV genotype 3 infected homozygous CC carriers had significantly higher baseline viral load (mean 6.3, 5.9, and 5.9 log10 IU/mL for CC, CT, and TT respectively, \( P < 0.0001 \), Kruskal-Wallis test; Figure 3C) as well as first phase decline as measured by the decline in HCV RNA during the first three days of interferon/ribavirin therapy (mean 2.1, 1.7, and 1.9 log10 IU/mL for CC, CT, and TT respectively, \( P = 0.0005 \), Kruskal-Wallis test; Figure 3D). In contrast, no such associations were noted among HCV genotype 2 infected patients. Additionally no significant associations were noted among IL28B genotype and the likelihood of achieving SVR irrespective of HCV genotype. Further details regarding the impact of IL28B genotype on the viral kinetic during therapy in this trial are provided elsewhere [24].

Discussion

In spite of the beneficial treatment effects among patients carrying the C allele carriage at rs12979860, little is known regarding its impact on the natural history of chronic HCV. Abe et al. reported that among Japanese patients infected with HCV genotype 1 or 2, patients with homozygous carriage of the IL28B major allele had significantly higher inflammatory activity and higher fibrosis stage, indicating that this SNP genotype may not be beneficial outside the context of therapeutic intervention [18]. Similarly in a treatment trial by Moghaddam et al. involving HCV genotype 3 infected Caucasian patients, CC carriers at rs12979860 had significantly higher normalized ALT levels and APRI scores than CT or TT carriers, however, liver biopsy evaluations were not reported [19]. In this context the main finding of the present study was a significant association between CC carriage at
rs12979860 and ALT, APRI score, portal inflammation grade, and steatosis among HCV genotype 3 infected patients, and that these associations appeared to be secondary to a significantly higher baseline viral load. Indeed it has previously been reported that among HCV genotype 3 infected patients, steatosis is more prevalent and pronounced than in other HCV genotypes [22,25] due to impaired lipid export from infected hepatocytes [26,27] possibly mediated by inhibition of microsomal triglyceride transfer protein (MTP) [28,29] or due to increased availability of free fatty acids by reduced oxidation or by increased de novo synthesis [30,31,32,33] of the genotype 3 core protein. Additionally the presence of steatosis in HCV genotype 3 infection is associated with higher baseline HCV RNA levels [25,34], negatively impacts the clearance of HCV RNA during the initial 12 weeks of therapy [34], and entails accelerated fibrosis progression [22] indicative of a likely cytopathic effect of HCV genotype 3 virus. Moreover, it was recently reported that the cumulative mortality of HCV genotype 3 infected US Department of Veterans Affairs (VA) patients failing to achieve SVR after therapy was higher than among non-SVR patients infected with genotypes 1 or 2 [35] possibly secondary to HCV genotype 3 associated steatosis and ensuing liver damage.

In the setting of therapeutic intervention for HCV genotype 2 or 3, uncertainty prevails regarding the benefit of favorable IL28B allele carriage. Sarrazin et al. reported increased SVR rates following therapy among HCV genotype 2 or 3 infected Caucasian CC rs12979860 carriers as compared to carriers of the T allele [36]. In contrast Mangia et al. noted an association between IL28B genotype and SVR only among HCV genotype 2 or 3 infected patients failing to achieve RVR [37]. Yu et al. reported a significantly higher rate of achieving RVR but not SVR among Asian homozygous TT rs8099917 carriers infected with HCV genotype 2 [38] and Moghaddam et al. noted similar results among HCV genotype 3 infected Caucasian CC rs12979860 carriers [19]. In the present study a significantly improved first phase decline in HCV RNA was noted among homozygous CC carriers infected with HCV genotype 3 but not among genotype 2. However, the higher baseline viral load among these genotype 3 infected patients essentially negated most of the favorable decline in HCV RNA observed during the initial three days of therapy. The reason for failing to observing an association between CC rs12979860 and the first phase decline in HCV RNA among the HCV genotype 2 infected patients enrolled in the present study is not clear, but may indicate that IL28B SNP polymorphisms have a more pronounced therapeutic impact among HCV genotype 3 than genotype 2 infected Caucasian patients of Scandinavian descent.

In conclusion, the present study demonstrated an association between CC carriage at rs12979860 and ALT, APRI, portal inflammation grade, and steatosis among HCV genotype 3 infected patients, and that this association may be secondary to the significantly higher baseline viral load. In this light, analysis of IL28B genotype may be beneficial among HCV genotype 3 infected patients so as to encourage homozygous CC rs12979860 carriers to initiate therapy. Additionally the finding that IL28B variability did not impact on liver histopathology or viral load...
among genotype 2 infected patients implies that \textit{IL28B} may differentially regulate the course of genotype 2 and 3 infection.

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Author Contributions
Conceived and designed the experiments: KR M. Lindh KH JW M. Lagging. Performed the experiments: KR AA M. Lindh JW M. Lagging. Analyzed the data: KR M. Lindh M. Lagging. Contributed reagents/materials/analysis tools: GN M. Lindh M. Lagging AA PBC MF NL MRB CP KM. Wrote the paper: KR M. Lagging.

References
1. WHO (1999) Hepatitis C- global prevalence (update). Weekly Epidemiological Report 74: 423–427.
2. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, et al. (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci U S A 87: 6547–6549.
3. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.
4. Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, et al. (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 140: 346–355.
5. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, et al. (2001) Peginterferon alpha-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358: 938–943.
6. Rauh A, Katulik Z, Descombes P, Cai T, Di Iulio J, et al. (2010) Genetic variation in \textit{IL28B} is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138: 1338–1345, 1345 e1331–1337.
7. Thomas DL, Tiao CL, Martin MP, Qi Y, Ge D, et al. (2009) Genetic variation in \textit{IL28B} and spontaneous clearance of hepatitis C virus. Nature 461: 786–801.
8. Ge D, Fellay J, Thompson AJ, Simons JS, Shianna KV, et al. (2009) Genetic variation in \textit{IL28B} predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
9. Suppiah V, Moldovan M, Ahlendsted G, Berg T, Weltman M, et al. (2009) \textit{IL28B} is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
10. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of \textit{IL28B} with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
11. Bochud PY, Bibert S, Negro F, Haagmans B, Soulier A, et al. (2011) \textit{IL28B} polymorphisms predict reduction of HCV RNA from the first day of therapy in chronic hepatitis C. J Hepatol 55: 980–8.
12. Darling JM, Aersens J, Fanning G, McHutchison JG, Goldstein DB, et al. (2011) Quantitation of pretreatment serum interferon-gamma-inducible protein-10 improves the predictive value of an \textit{IL28B} gene polymorphism for hepatitis C treatment response. Hepatology 53: 14–22.
13. Lagging M, Askarich G, Negro F, Bibert S, Soderholm J, et al. (2011) Response Prediction in Chronic Hepatitis C by Assessment of IP-10 and \textit{IL28B}-Related Single Nucleotide Polymorphisms. PLoS One 6: e17292.
14. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, et al. (2010) Hepatic IFN-g expression is associated with genetic variation in interferon 28B...
and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 139: 499–509.

15. Dill MT, Duong FH, Vogt JE, Bibert S, Bochud PY, et al. (2011) Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. Gastroenterology 140: 1021–1031.

16. McCarthy JJ, Li JH, Thompson A, Suchindran S, Lao QX, et al. (2010) Replicated association between an IL28B gene variant and a sustained response to pegylated interferon and ribavirin. Gastroenterology 138: 2307–2314.

17. Montes-Cano MA, Garcia-Louzano JR, Ahad-Molina C, Romero-Gomez M, Barroso N, et al. (2010) Interleukin-28B genetic variants and hepatitis virus infection by different viral genotypes. Hepatology 52: 35–43.

18. Abe H, Ochi H, Mekawa T, Hays CN, Tsuchi M, et al. (2010) Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol 53: 439–455.

19. Moghaddam A, Melum E, Reinton N, Ring-Larsen H, Verbaan H, et al. (2011) IL28B genetic variation and treatment response in patients with hepatitis C virus genotype 3 infection. Hepatology 53: 746–754.

20. Lagging M, Langeland N, Pedersen C, Farkkila M, Buhl MR, et al. (2008) Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 patients. J Hepatol 47: 1837–1845.

21. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, et al. (1995) Histological grading and staging of chronic hepatitis. J Hepatol 22: 606–609.

22. Westin J, Nordlander H, Langeland N, Farkkila M, Buhl MR, et al. (2002) Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 infected patients. J Hepatol 37: 837–842.

23. Wai CT, Greenson JK, Fontana RJ, Kalbleisch JD, Marrero JA, et al. (2003) A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology 38: 518–526.

24. Lind M, Langeland N, Pedersen C, Farkkila M, Buhl MR, et al. (2008) Randomized comparison of 12 or 24 weeks of peginterferon alpha-2a and ribavirin in chronic hepatitis C virus genotype 2/3 infection. Hepatology 47: 1837–1845.

25. Backus LI, Boothroyd DB, Phillips BR, Belperio P, Halloran J, et al. (2011) A novel interferon-α receptor substrate 1 through genotype-specific mechanisms. Hepatology 45: 812–821.

26. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Uliello, et al. (2001) Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. Hepatology 33: 1086–1095.