IL-28B genotypes as predictors of long-term outcome in patients with hepatitis C-related severe liver injury

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Abstract

Introduction: Patients with severe fibrosis or cirrhosis are at high risk for liver-related complications, even after successful antiviral treatment and/or regression of fibrosis. These are the first published results concerning the role of IL-28B genotypes as predictors of the durability of sustained virological response (SVR) and long-term outcome, in patients with baseline severe fibrosis and cirrhosis caused by hepatitis C (HCV) infection.

Methodology: Genetic testing for three different single nucleotide polymorphisms (SNP) near the IL28B gene, rs12979860, rs12980275 and rs8099917, was performed in 42 patients with HCV-related advanced fibrosis and cirrhosis, who achieved SVR after successful interferon-based treatment. Baseline clinical and laboratory parameters were analysed, as well as IL28B genotype association with late virological relapse, fibrosis progression and clinical outcomes.

Results: The most prevalent genotypes in all three tested SNP positions were: CCrs12979860 genotype in 69% of patients, GTrs8099917 in 78.6% and GGrs12980275 in 47.6% of patients. The presence of IL28B CCrs12979860 genotype was identified as a negative predictor of late virological relapse. Further analysis did not confirm the association of other IL28B genotypes with the progression of fibrosis and clinical outcomes.

Conclusions: Varying long-term prognosis in patients with HCV-related severe fibrosis and cirrhosis is due to multiple interactions between host genetic factors, virus and environment. These are first published results demonstrating the significance of IL28B CCrs12979860 genotype as a negative predictor of late virological relapse. A further investigation concerning genetic factors is necessary to identify patients under risk for late relapse, complications and unfavorable outcomes, so that they can be reevaluated and offered new treatment options.

Key words: interleukine 28B; late relapse; outcome.

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Introduction

Long-term outcome of chronic hepatitis C (CHC) is variable in both treatment-naive and treatment-experienced patients and influenced by numerous virus-related, environmental and host factors [1]. So far, many genetic markers have been taken in account and examined as possible predictors of outcome, including several single-nucleotide polymorphisms (SNPs) located in various genes, such as interleukine 28B region on chromosome 19, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) [2]. Genome Wide Association Studies (GWAS) have shown that multiple SNPs located near IL28B gene are responsible for the occurrence of different IL28B genotypes and have a significant impact on spontaneous and treatment clearance of hepatitis C virus (HCV) [3,4]. Unfavourable treatment outcomes have been documented in patients with the TT and CT genotypes at the rs12979860 SNP (nonresponder genotypes), including delayed viral decline and lower treatment success rates, after pegylated interferon and ribavirin treatment, contrary to the favourable CC genotype [5]. The impact of IL28B on the natural history of CHC infection is still not well understood [6]. Furthermore, there are conflicting results concerning the predictive role of IL28B genotypes in fibrosis progression, including IL28B CCrs12979860 genotype association with
higher levels of ALT, adverse clinical outcomes and greater hepatic necroinflammation [6].

Estimated seroprevalence of HCV infection in Serbia is higher than in most of the developed European countries, approximately 0.19%, with the predominance of 1b and 3a genotypes [7,8]. Although antiviral treatment (pegylated interferon and ribavirin) has been available since 2003, with high treatment success rates, decades of treatment experience and close follow-up have demonstrated, in our patient population, that liver-related morbidity and mortality is still high in patients with a severe baseline liver damage, in spite of achieving sustained virologic response (SVR). Apart from routine clinical and laboratory outcome predictors, we often suspect that there is a significant impact of host genetic factors on the clinical course and durability of SVR, especially in relation to the presence of IL-28B genotype. In Serbia, there are limited data concerning the role of IL-28B genotypes as treatment response predictors in patients with CHC (only in HCV genotype 1 infection), but with no long-term follow up studies or published data beyond achieving SVR [9].

The aim of this study was to examine if IL-28B genotypes can predict the occurrence of late virological relapse, the progression of fibrosis and to examine its’ association with the long-term outcome in Serbian cohort of patients with CHC-related severe baseline liver damage, who achieved SVR after interferon-based treatment. Host and virus-related factors were also included in the analysis as possible predictors of the durability of SVR and long-term outcome, as well as their association with different IL-28B genotypes.

Methodology

This prospective cohort study was conducted in the referral centre at the Hepatology Department of the Clinic for Infectious and Tropical Diseases, Medical Faculty, University of Belgrade, Clinical Center of Serbia. It included a study group of 42 patients with severe baseline liver damage caused by CHC, in whom IL-28B genotyping was available and who had fulfilled all inclusion criteria. These patients were a part of a larger long-term follow-up study of 325 patients with CHC, treated/retreated with pegylated interferon and ribavirin during a 12 years’ period (from 2003 until November 2015). All patients from this large cohort, who had successfully undergone antiviral treatment and achieved SVR were contacted for a reevaluation during March-July 2018. This included physical exam, liver elastography, HCV RNA loads, liver and genomic biomarkers. Among subjects who responded, 42 patients fulfilled the inclusion criteria and consented to participate in this study. Two patients from this group had been retreated during this period and achieved SVR after retreatment, so they were also recruited. The pre-treatment data were obtained from patient files, including demographic and epidemiological data, baseline clinical findings, laboratory tests and liver histology (before the commencement of antiviral treatment). Regular outpatient monitoring included clinical examinations every 6 months, laboratory tests (haematological, biochemical, HCV RNA loads, HCV genotyping in late relapse), imaging (fibrosan and echosonography yearly) and upper gastrointestinal endoscopy (in patients with cirrhosis). Among these 42 patients, 9 had been tested for HCV RNA prior to our reevaluation during follow-up and already had a confirmed virological relapse, in spite of achieving SVR.

The inclusion criteria for the study group were: patients with a detectable HCV RNA by polymerase chain reaction (PCR) and advanced fibrosis or cirrhosis (METAVIR score F3 and F4), who underwent a complete treatment with pegylated interferon and ribavirin, achieved SVR (e.g. undetectable HCV RNA 6 months after the completion of antiviral treatment) and gave informed consent for participation. The exclusion criteria for study group were: age (subjects younger than 16 or older than 65 years), mild liver damage with METAVIR < 2 confirmed by histopathology, coinfections (hepatitis B, HIV), other liver diseases (Wilson’s disease, haemochromatosis, autoimmune hepatitis, primary biliary cirrhosis, baseline decompensated cirrhosis and hepatocellular carcinoma-HCC), severe comorbidities (decompensated cardiomyopathies, thyroid dysfunctions, autoimmune diseases, epilepsy, depression, severe neutropenia and thrombocytopenia), active abuse of narcotics and alcohol, pregnancy. The study protocol was designed according to the ethical guidelines of the Helsinki declaration and was approved by the Ethical Committee of Medical Faculty, University of Belgrade.

Baseline liver damage assessment included non-invasive (liver elastography) and invasive (aspiration liver biopsy, Institute for Pathology, Medical Faculty Belgrade University) diagnostic methods, and METAVIR score was used to quantify the degree of inflammation and fibrosis of the liver. Viral loads were measured with quantitative PCR HCV RNA (Cobas Amplicor HCV Test version 2.0, Roche Diagnostics, Menheim, detection: 50 IU/mL) and hepatitis C virus genotyping (Linear Array HCV genotyping test, Roche Diagnostics, Mannheim, Germany) in Virology
laboratory, Microbiology Department, Clinical Center of Serbia. Biochemical testing was performed using Siemens Dimension Xpand biochemistry analyzer in the Centre for Medical Biochemistry, Department of the Clinic for Infectious and Tropical Diseases, Clinical Centre of Serbia.

Study endpoints during follow-up period included late virological relapse, increase of alanine aminotransferase (ALT) and unfavourable clinical outcomes. Late relapse was considered in patients who had detectable HCV RNA during follow up, in spite of achieving SVR, and in whom repeated virus genotyping matched initial pretreatment HCV genotype. The progress of fibrosis was assessed using repeated non-invasive liver elastography. Clinical outcomes, which were taken into consideration, included cirrhosis decompensation, ascites, variceal bleeding, spontaneous bacterial peritonitis, hepatic encephalopathy and hepatocellular carcinoma (HCC).

All analyzed subjects were Caucasians of Serbian origin. Molecular genetic analysis was performed at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade.

Genomic DNA was extracted from peripheral blood samples of the patients using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The detection of three SNPs near the IL28B gene, rs12979860, rs12980275 and rs8099917, was performed using sequence specific primer – polymerase chain reaction (SSP-PCR), as previously described [10].

Statistical analysis was performed using SPSS® v. 17 and included both descriptive and analytical methods. Data were presented as a percent or mean value with SD. Pretreatment data were analyzed using parametric (Student T-test, ANOVA) and nonparametric tests (Mann-Whitney, χ², Kruskal Wallis), depending on the normality of variables. Further genotype associations with different endpoints were described using relative risks, and tested with logistic regression and beta coefficient, including Cox regression for a time-dependent association. Values at the p ≤ 0.05 level were considered statistically significant, the confidence interval (CI) was 95% and all tests were 2-tailed.

**Results**

The study group included a total of 42 patients who had achieved SVR, 46.2 ± 9.4 years old (ranging from 27 up to 63), including 24 (57.1%) males, with a variable pretreatment duration of infection ranging from 6 months to 13 years. The predominant route of infection in 40.5% of patients was blood transfusion (P = 0.005).

### Table 1. Comparison of baseline (pretreatment) clinical, laboratory and histology findings in patients with three rs12979860 polymorphism genotypes (CC, CT and TT), including a comparison of patients with CC vs. non-CC genotype.

| Variable                        | CC (%) | CT (%) | TT (%) | P      | CC vs. non CC |
|---------------------------------|--------|--------|--------|--------|---------------|
| patients, n (%)                 | 29 (69%) | 7 (16.7%) | 6 (14.3%) | < 0.0001 | 0.014         |
| male sex, n (%)                 | 17 (58.6%) | 4 (57.1%) | 3 (50%)   | 0.077  | 0.733         |
| age, years*                     | 47±9.5 | 39.4 ± 7.69 | 50.3 ± 7  | 0.07   | 0.418         |
| duration of infection, years*   | 3.4 ± 4.1 | 2 ± 1.6  | 3 ± 4.4  | 0.704  | 0.469         |
| BMI*                            | 21.45 ± 1.9 | 21.5 ± 1.6 | 21.2 ± 1.7 | 0.969  | 0.926         |
| cirrhosis (METAVIR F4), n (%)   | 7 (24.1%) | 5 (71.4%) | 4 (66.7%) | 0.445  | 0.05          |
| high activity, n (%)            | 7 (24.1%) | 5 (71.4%) | 4 (66.7%) | 0.02   | 0.029         |
| HCV genotype 1, n (%)           | 21 (72.4%) | 3 (42.9%) | 4 (66.7%) | 0.581  | 0.501         |
| logHCV RNA IU/mL *              | 5.95 ± 0.65 | 6.1 ± 0.7 | 6.56 ± 0.5 | 0.12   | 0.124         |
| HCV RNA > 800.000 IU/mL, n (%)  | 18/29 (62.1%) | 5/7 (71.4%) | 5/6 (83.3%) | 0.578  | 0.014         |
| Hgb (g/L)*                      | 138 ± 18   | 153 ± 6.9 | 151.2 ± 9.5 | 0.249  | 0.135         |
| RBC (×10^12) cells/µL*          | 4.5 ± 0.63  | 4.5 ± 0.59 | 4.8 ± 0.5  | 0.286  | 0.16          |
| WBC (×10^3) cells/µL*           | 7 ± 1.8    | 6.9 ± 0.56 | 8.95 ± 2.96 | 0.039  | 0.36          |
| Platelets (×10^12) cells/µL*    | 192.5 ± 70  | 185 ± 33.9 | 222 ± 86.6 | 0.515  | 0.633         |
| AST (U/L)*                      | 65.7 ± 47.7 | 64.2 ± 19.5 | 80.83 ± 52.6 | 0.702  | 0.639         |
| ALT (U/L)*                      | 132.46 ± 128.7 | 110.4 ± 47.9 | 157 ± 151 | 0.783  | 0.882         |
| GGT (U/L)*                      | 61.5 ± 64.5 | 81.6 ± 39.1 | 78.2 ± 72  | 0.55   | 0.28          |
| total bilirubin (µmol/L)*       | 12.7 ± 6.8  | 12.5 ± 6.5 | 14.45 ± 6.11 | 0.374  | 0.869         |
| ferritin (µg/mL)*               | 155.9 ± 144.3 | 209.6 ± 136 | 68.5 ± 31.65 | 0.227  | 0.748         |
| total proteins (g/L)*           | 72.7 ± 7    | 75 ± 7    | 66.8 ± 14  | 0.199  | 0.658         |
| albumin (g/L)*                  | 39.2 ± 8.6  | 44.7 ± 5.8 | 39.5 ± 6.8 | 0.272  | 0.257         |

BMI- the body mass index; Hgb-hemoglobin; RBC-red blood cell count; WBC-white blood cell count; AST-aspartate aminotransferase; ALT-alanine aminotransferase; GGT- gamma-glutamyl transferase; *mean ± SD.
Table 2. Comparison of baseline (pretreatment) clinical, laboratory and histology findings in patients with three rs8099917 polymorphism genotypes (GT, GG and TT), including a comparison of patients with TT rs8099917 genotype versus non-TT genotype.

| Variable                        | G/T       | G/G       | T/T       | P         | TT vs. non TT |
|---------------------------------|-----------|-----------|-----------|-----------|---------------|
| patients, n (%)                 | 33 (78.6%)| 5 (11.9%) | 4 (9.5%)  | 0.000     | 0.000         |
| male sex, n (%)                 | 17 (51.5%)| 3 (60%)   | 4 (100%)  | 0.179     | 0.070         |
| age, years*                     | 46.1 ± 9.3| 47.6 ± 13 | 45.5 ± 6.1| 0.938     | 0.871         |
| duration of infection, years*   | 3.4 ± 4   | 2.5 ± 3.1 | 1.1 ± 0.7 | 0.584     | 0.359         |
| BMI*                            | 20.9 ± 1.7| 23.3 ± 1.1| 23 ± 0.8  | 0.003     | 0.069         |
| cirrhosis (METAVIR F4), n (%)   | 12 (36.4%)| 2 (40%)   | 0 (0%)    | 0.327     | 0.137         |
| high activity, n (%)            | 13 (39.4%)| 1 (20%)   | 2 (50%)   | 0.544     | 0.617         |
| HCV genotype 1, n (%)           | 21 (63.6%)| 3 (60%)   | 4 (100%)  | 0.466     | 0.331         |
| logHCV RNA IU/mL*               | 6.03 ± 0.64| 5.98 ± 0.78| 6.33 ± 0.8| 0.376     | 0.394         |
| HCV RNA > 800,000 IU/mL, n (%)  | 20 (60.6%)| 4 (80%)   | 4 (100%)  | 0.229     | 0.283         |
| Hgb (g/L)*                      | 141.8 ± 16.4| 143.6 ± 21.3| 143 ± 17.4| 0.931     | 0.847         |
| RBC (×10⁶ cells/µL)*            | 4.5 ± 0.6 | 4.5 ± 0.7 | 4.5 ± 0.5 | 0.972     | 0.820         |
| WBC (×10⁹ cells/µL)*            | 6.9 ± 1.9 | 7.6 ± 1.3 | 7.47 ± 3.6| 0.741     | 0.688         |
| Platelets (×10⁹ cells/µL)*      | 192.5 ± 69| 196.6 ± 35| 208 ± 96  | 0.886     | 0.645         |
| AST (UL)/L*                     | 62.6 ± 32.8| 76 ± 79.4| 88.5 ± 69 | 0.479     | 0.297         |
| ALT (UL)/L*                     | 116 ± 77 | 164 ± 248| 170 ± 193 | 0.547     | 0.455         |
| GGTT (UL)/L*                    | 60.8 ± 61| 101 ± 64 | 53.5 ± 15.2| 0.390     | 0.643         |
| total bilirubin (µmol/L)*       | 12.2 ± 6.2| 15.8 ± 9.35| 11.7 ± 2.8| 0.482     | 0.772         |
| ferritin (µg/mL)*               | 162.5 ± 138.5| 114 ± 125.6| 91.25 ± 92| 0.551     | 0.390         |
| total proteins (g/L)*           | 72.68 ± 9.3| 71.8 ± 9.2| 70.5 ± 2.4| 0.900     | 0.671         |
| albumin (g/L)*                  | 41.5 ± 7.9| 35.6 ± 8 | 35.3 ± 9.2| 0.143     | 0.206         |

BMI- the body mass index; Hgb- hemoglobin; RBC- red blood cell count; WBC- white blood cell count; AST- aspartate aminotransferase; ALT- alanine aminotransferase; GGTT- gamma-glutamyl transferase; *mean ± SD.

Table 3. Comparison of baseline (pretreatment) clinical, laboratory and histology findings in patients with three rs12980275 polymorphism genotypes (GG, AG and AA), including a comparison of patients with AA rs12980275 genotype versus non-AA genotype.

| Variable                        | G/G       | A/G       | AA        | P         | AA vs. non AA |
|---------------------------------|-----------|-----------|-----------|-----------|---------------|
| patients, n (%)                 | 20 (47.6%)| 16 (38.1%)| 6 (14.3%) | 0.024     | 0.000         |
| male sex, n (%)                 | 12 (60%)  | 8 (50%)   | 4 (66.7%) | 0.733     | 0.685         |
| age, years*                     | 47 ± 9.4  | 46.7 ± 10 | 43.6 ± 9  | 0.777     | 0.475         |
| duration of infection, years*   | 2.4 ± 2.9 | 4.2 ± 4.6 | 2.8 ± 4   | 0.400     | 0.832         |
| BMI*                            | 21.7 ± 1.8| 20.8 ± 1.8| 21.8 ± 1.9| 0.280     | 0.616         |
| cirrhosis (METAVIR F4), n (%)   | 7 (35%)   | 6 (37.5%) | 1 (16.7%) | 0.638     | 0.096         |
| high activity, n (%)            | 9 (45%)   | 4 (25%)   | 3 (50%)   | 0.381     | 0.021         |
| HCV genotype 1, n (%)           | 14 (70%)  | 12 (75%)  | 2 (33.3%) | 0.440     | 0.168         |
| logHCV RNA IU/mL*               | 6.1 ± 0.4 | 6.07 ± 0.7| 5.8 ± 1   | 0.748     | 0.450         |
| HCV RNA > 800,000 IU/mL, n (%)  | 15 (75%)  | 11 (68.8%)| 2 (33.3%) | 0.161     | 0.000         |
| Hgb (g/L)*                      | 138.5 ± 19.7| 142.7 ± 13.8| 147.33 ± 15.6| 0.512     | 0.364         |
| RBC (×10⁶ cells/µL)*            | 4.48 ± 0.7| 4.5 ± 0.5 | 4.86 ± 0.5| 0.419     | 0.188         |
| WBC (×10⁹ cells/µL)*            | 7 ± 2.2   | 6.96 ± 1.3| 7.4 ± 3.2 | 0.897     | 0.654         |
| Platelets (×10⁹ cells/µL)*      | 207.9 ± 70| 162.75 ± 54.2| 207.95 ± 70| 0.060     | 0.227         |
| AST (UL)/L*                     | 50 ± 20  | 78 ± 48   | 89.6 ± 68.35| 0.060     | 0.164         |
| ALT (UL)/L*                     | 81.4 ± 42.9| 143.8 ± 147.5| 228.5 ± 139.6| 0.022     | 0.021         |
| GGTT (UL)/L*                    | 81.42 ± 75.3| 51.75 ± 42.8| 48.5 ± 17.3 | 0.191     | 0.422         |
| total bilirubin (µmol/L)*       | 12.7 ± 6.6| 12.6 ± 7.2| 12.1 ± 3.3| 0.978     | 0.845         |
| ferritin (µg/mL)*               | 176.8 ± 146| 140 ± 99  | 87.8 ± 164| 0.427     | 0.249         |
| total proteins (g/L)*           | 71.1 ± 11.5| 73.25 ± 5.5| 74 ± 6   | 0.659     | 0.608         |
| albumin (g/L)*                  | 40.8 ± 7.6| 39.37 ± 9.6| 40.2 ± 6.6| 0.877     | 1.000         |

BMI- the body mass index; Hgb- hemoglobin; RBC- red blood cell count; WBC- white blood cell count; AST- aspartate aminotransferase; ALT- alanine aminotransferase; GGTT- gamma-glutamyl transferase; *mean ± SD.
Baseline demographic, clinical and laboratory findings

Baseline analysis of rs12979860 polymorphism genotypes frequencies revealed the predominance of CC rs12979860 genotype in 29/42 (69%) patients, compared to other less favourable genotypes CT (16.7%) and TT (14.3%) (P < 0.0001). There were no significant host-related differences (such as sex distribution, age, duration of infection, BMI) between patients with different genotypes (Table 1). However, in patients with non-CC genotypes compared to patients with CC rs12979860 genotype, higher histological activity of hepatitis (69.2% vs. 24.1%) and cirrhosis (53.8% vs. 24.1%) were more prevalent (P < 0.05). High baseline viraemia was noted in patients with non-CC genotype, with more patients having HCV RNA loads above 800.000 IU/mL compared to those with CC genotype (P = 0.014). Patients with TT genotype had higher baseline white blood cell (WBC) counts compared to CT and CC genotypes (8.95 ± 2.9 vs. 6.9 ± 0.56 vs. 7 ± 1.8, P = 0.039), but there was no statistically significant difference between patients with CC and non-CC genotypes (P = 0.360). There were no significant differences in average values of other haematological and biochemical findings between patients with CC and non-CC genotypes (Table 1).

Further baseline analysis of rs8099917 polymorphism genotype frequency (Table 2) revealed a predominance of GT rs8099917 genotype in 33/42 patients (78.6%) (P < 0.0001). The only significant differences among three groups of patients, according to their genotype, was in average BMI, but there were no significant differences between TT and non-TT genotypes.

Study group baseline analysis of rs12980275 polymorphism (Table 3) showed a predominance of GG rs12980275 genotype in 20/42 patients (47.6%, P = 0.02). A more favourable AA rs12980275 genotype found in 6/42 of patients (14.3%) was the most infrequent, compared to non-AA genotypes (P < 0.000). Furthermore, patients with AA genotype had a higher baseline histological activity of hepatitis compared to non-AA genotypes (50% of patients had high activity vs. 36.1%. P = 0.021), and higher pretreatment activity of ALT (P = 0.022).

Association of IL28B genotypes with virological relapse, increase in ALT levels, progression of fibrosis and unfavourable clinical outcomes

The follow-up period of patients, after achieving SVR, ranged from 20 months to 11.3 years (66.9 ± 37.5 months), but during the last follow-up visit, HCV RNA testing confirmed detectable viremia in 14 patients (33.3%), who were considered late relapers. Non-CC genotype was more common in patients with late relapse, in 8/14 (57.1%) patients compared to 6/14 (42.9%) of patients with CC rs12979860 genotype (P = 0.015), with 6 times greater relative risks for virological failure (RR = 3.2; 95%CI 1.278-7.982 vs. RR = 0.522; 95%CI 0.278-0.979) respectively. Further analysis (Table 4) supported by logistic regression revealed a significant negative association of CC rs12979860 genotype with late virological relapse (β = -1.814, P = 0.013). Genotype TT rs12979860 presence had the highest relative risk for late relapse (RR = 2.000, 95% CI 0.462-8.664), but no statistically significant association was established including other three examined genotypes (Table 4).

The elevation of ALT was observed in 10.3% of patients with CC rs12979860 genotype, and in 15.4% with non-CC genotype. Although the difference in frequencies between these two groups did not reach significance level (P = 0.637), relative risks for the ALT elevation (independent of viraemia), were 1.5 times greater in non-CC genotype vs. CC genotype patients (RR = 1.345, CI 95% 0.412-4.389 vs. RR = 0.854 CI 95% 0.405-1.800). Cox regression did not reveal a time-dependent association between CC rs12979860 genotype and elevation of ALT (β = -0.353 P = 0.723).

Among patients with CC rs12979860 genotype estimated progression of fibrosis was observed in 6/29 (20.7%) patients, similar to patients with non-CC genotype 5/13 (38.5%), but with 2.4 times lower

Table 4. Association between IL28B genotypes and the occurrence of late virological relapse evaluated by logistic regression.

|                | beta  | P    | RR*  | 95% CI     |
|----------------|-------|------|------|------------|
| CC rs12979860  | -1.814| 0.013| 0.522| 0.278-0.979|
| TT rs12979860  | 0.821 | 0.358| 2.000| 0.462-8.664|
| GG rs8099917   | -0.329| 0.737| 1.333| 0.251-7.084|
| TT rs809917    | 20.64 | 0.999| 1.167| 1.003-1.357|
| AA rs12980275  | 0.423 | 0.968| 1.000| 0.208-4.814|

*RR-relative risk.
relative risks (RR = 0.538 CI95% 0.2-1.448 vs. RR = 1.289 CI95% 0.807-2.058). However, a time-dependent association between CC<rs12979860> genotype and the progression of fibrosis was not established (P = 0.519).

Unfavourable clinical outcomes were noted in a total of three cases, during follow up period, including an occurrence of ascites in two and HCC in one patient. The incidence of unfavourable clinical outcomes among patients with CC<rs12979860> genotype was 2/29 (6.9%) and in non-CC genotype 1/13 (7.7%) (P = 0.926). Relative risks were slightly greater for patients with non-CC genotypes (RR = 1.009 CI 95% 0.089-9.029 vs. RR = 0.897 CI 95% 0.089-9.029). There was no significant time-dependent association between CC<rs12979860> genotype and unfavourable outcome during follow-up (P = 0.764).

**Discussion**

There is a significant geographical difference in allelic frequencies of the rs12979860 C allele (although it is established as the most common allele worldwide), ranging from the lowest of 38% in African, intermediary (50-85%) in European/Caucasian populations and up to 100% in East Asian population [11]. These host genetic variants offer a plausible explanation for otherwise clinically inexplicable differences in treatment response rates among different ethnicities. Large-scale studies have shown that each population has a relatively unique pattern of gene polymorphism, which may have a significant effect on disease susceptibility, immunogenetics and pharmacogenetics [12]. Reports of the protective CC<rs12979860> genotype frequencies are also diverse, ranging from 44-49% in a European population, up to 78.5% in East Asian subjects who also have higher reported SVR rates than patients of European ancestry [13,14].

In our study group, the most prevalent genotypes in all three SNP positions were: CC<rs12979860> genotype in 69% of patients, GT<rs8099917> in 78.6% of patients and GG<rs12990275> in 47.6% of patients. These frequencies differ from the only available published data concerning Serbian population by Lazarevic et al., who had shown lower frequencies of CC<rs12979860> genotype-24.5% in the whole study group of 106 patients with genotype 1 HCV infection and in 33.9% of patients who achieved SVR [9]. Among authors from our surroundings, a group of Croatian authors Grbic et al. analysed SNP of rs12979860 in 595 patients with CHC genotype 1 and showed predominance of CT<rs12979860> (56.3 %) genotype, as well as lower frequencies of CC<rs12979860> (29.1%) and TT<rs12979860> (14.6%) genotypes, in treatment naive patients with genotype 1 HCV infection [15]. Our results differ, due to the size and specificity of our patient group and partially selection/inclusion criteria, as we included only patients who had achieved SVR, with HCV genotypes other than genotype 1 (genotypes 2 and 3 were also present), as well as differences in genotyping methods. These advantageous frequencies of favourable genotypes in our study may also explain very high treatment success rates in Serbia approximately 79.7% of end of treatment response (EOT) and 70.5% SVR (60.7% in genotype 1 HCV infection) [16,17].

The association between IL28B genotypes and liver injury has been substantially studied but remains inconclusive. In our study group, patients with a favourable CC<rs12979860> genotype had also a lower degree of necroinflammation and frequency of cirrhosis, in agreement with the data from Falleti et al. [18]. Di Marco et al. in addition demonstrated that apart from non-CC<rs12979860> genotypes, patients’ age and non-TT<rs8099917> genotypes were also significantly associated with F3-F4 META VIR scores [19]. Fabricio-Silva et al. observed, in a larger multiethnic study, that CT<rs12979860> genotype and younger age presented protective factors against inflammatory activity [20]. However, results from other authors have shown contradictory results on associations of CC<rs12979860> genotype with fibrosis and cirrhosis, as well as the absence of any kind of association [6,18,21]. This is probably due to differences in studies’ designs and the fact that liver fibrosis in chronic hepatitis C is multicausal, including host related factors-genetics, age, insulin resistance, body mass index, but also the duration of HCV infection, oxidative stress and environmental factors [22].

We also observed that significantly more patients with a favourable CC<rs12979860> genotype had lower baseline viral loads (HCV RNA < 800.000 IU/mL), but without a significant genotype-associated difference in average baseline viraemia. A possible effect of IL28B genotypes on the baseline viral load is still poorly understood, and only a few authors reported significant associations. Boglione et al. reported TT/CC<rs12979860> genotypes association with a higher baseline viral load (HCV RNA >800.000 IU/mL), and the presence of the G allele at rs8099917 with lower viral loads [23]. In a prospective international cohort study of injectable drug users with acute HCV infection, HCV RNA levels 12 months following infection were independently associated with male gender, IL28B CC<rs12979860> genotype and HCV genotype 1 [24]. The importance of these results remains to be elucidated, as some studies
have refuted the effect of HCV RNA loads on disease progression, but it is probable that high levels of HCV RNA may have an impact on immunological response and hepatic inflammation especially in advanced liver injury [25,26].

The relevance of rs12980275 polymorphism remains to be elucidated, but our results confirmed that patients with AA genotype had more unfavourable baseline characteristics- higher pretreatment levels of ALT and level of necroinflammation. Lazarevic et al. had shown that AA genotype in combination with other favourable genotypes has a significant impact on achieving SVR [9]. In patients with this genotype, this may be due to a more vigorous immune response and greater liver injury marked by increased hepatic inflammation and higher serum ALT, but a larger scale study is needed in order to evaluate this hypothesis.

In the DAA era the role of genotyping IL28B may be diminished, but in resource-limited countries, particularly in Serbia, where interferon-based treatment is still the only available treatment for CHC, it is useful as a predictor of the natural course of HCV infection and treatment success [26]. In this study, we evaluated its role in patients who had baseline liver damage and had undergone successful treatment, but in whom, in our clinical practice, in spite of achieving SVR we anticipate and observe virological relapse and/or progression of liver damage. To our knowledge, these are the first published results concerning this particular group of patients in Serbia and the association of IL28B genotypes with any clinical events beyond SVR- long term outcomes and late virological relapse.

SVR is considered a reliable endpoint of CHC treatment, although there are numerous reports of patients with detectable levels of HCV RNA even after achieving SVR [27-29]. However, a minority of authors differentiated a relapse after SVR from reinfection and/or a possible occult HCV infection. A comprehensive systematic review showed that SVR appears durable in the majority of patients at 5 years post-treatment, driven mostly by an increased reinfection risk, with 5-year recurrence rates of 0.95%, 10.67%, and 15.02% in the low-risk, high-risk, and coinfection groups, respectively [30]. Furthermore, published reports of late virological relapses described mostly asymptomatic patients with self-limited viraemias, detected after years of careful monitoring and investigation of de novo elevated liver enzyme levels. Lu et al. speculated that this may be due to the fact that in these cases SVR represented HCV suppression, rather than HCV eradication [27]. Unfortunately, in Serbia, there is no involvement in any European monitoring systems of HCV infection and no long-term follow-up studies, except for our own clinical observations and patients’ feedback during the past decade. We maintained long-term outpatient monitoring of all patients with severe liver injury, including those who achieved SVR, in spite of guidelines that have changed over the years and mostly advocated cost-effective time-limited follow up and selective screening for HCC. Due to intermittent stock outs, the availability of HCV RNA testing was also often limited.

There is overwhelming evidence that patients with severe fibrosis or cirrhosis are still at high risk of liver-related complications, even beyond SVR and/or regression of fibrosis (or reversal of cirrhosis) [31-33]. Several large scale follow-up studies of treatment-experienced patients, with baseline severe liver injury caused by CHC, have shown that comorbidities, such as diabetes mellitus type 2, high levels GGT, high BMI and non-alcoholic steatohepatitis (NASH), are associated with liver fibrosis progression and development of HCC in cirrhotic patients in spite of antiviral treatment outcome [34,35]. Our clinical policy of long-term follow up of patients with baseline severe liver injury is also supported by results from a Scottish cohort showing that liver-related morbidity in patients who achieved SVR may be caused by recurrent alcoholism and other comorbidities, and suggesting that patients ought to be managed even after completion of successful antiviral treatment [32].

In our study, after a significant follow-up period, reevaluation showed that patients with non-CC genotype had a 6 times greater risk for detectable viraemia and were predominant among late relapers. We also showed that CC>rs12979860 genotype was a negative predictor of late relapse. So far there have been no published data concerning this subject, as rs12979860 polymorphism was evaluated only as a predictor of SVR and spontaneous HCV clearance. Reported risk factors for late relapse are mostly due to immunosuppression (which was not the case in our patients), but there are no other conclusive predictors. This may be due to the durability of SVR, restrictive guidelines concerning monitoring patients with SVR, loss to follow-up, all resulting in a very limited number of patients for such studies. One of the weaknesses of our study was that we were not able to exclude reinfection by RNA sequencing, as only retesting for virus genotypes was available. Nevertheless, none of the patients had risks for reinfection and none of them were previous or active injectable drug users. The main route of HCV transmission was through a blood
transfusion, a trend in the 1990s, before the routine screening of blood derivatives was introduced.

We did not find a time-dependent association between CC<rs12979860> genotype and fibrosis progression, in accordance to results by Noureddin et al., who had additionally observed an association with hepatic inflammation and clinical outcomes, but not with fibrosis, suggesting that the mechanisms for fibrogenesis are inflammation-independent and multicausal, as previously mentioned [6].

Unfavourable clinical outcomes in our study were very infrequent, occurring in three patients and with no evidence of rs12979860 genotype association, probably due to a small number of patients. Similar to our results, Savino et al. in a larger 15-year cohort study of patients with HCV-related cirrhosis consisting of treatment naïve and experienced patients with low SVR, did not confirm any influence of IL28B genotypes on the clinical outcome, such as decompensation, hepatocellular carcinoma and death, but without considering virological relapse [36].

As this was a pilot study, major limitations are the sample size and the lack of a healthy control group, for a more reliable population study of IL28B genotype frequencies. In the group of patients with late relapse, there was a response-bias, as some of the patients from this group had previously been evaluated and aware of detectable HCV RNA, so they were motivated to respond to our invitation for reevaluation, and may have caused an overestimation of late relapse rates. We used non-invasive methods such as fibroscan and ultrasonography to monitor patients for the progress of fibrosis and liver-related complications, as repeated liver biopsies are not ethically justified.

Conclusion

Careful long-term monitoring of patients with severe baseline liver damage is very important and needs to include both screenings for HCC as well as HCV RNA detection. We demonstrated that, in this group of patients who additionally achieved SVR after interferon-based treatment, IL28B CC<rs12979860> genotype can be useful as a negative predictor of late virological relapse, but not for prediction of fibrosis progression and clinical outcomes. Varying long-term prognosis in patients with HCV-related severe fibrosis and cirrhosis is due to complex multicausal mechanisms and interactions between host genetic factors, virus and environment. A further investigation concerning genetic factors is necessary, so that patients who are under risk for late relapse, complications and unfavourable outcomes, can be identified and reevaluated, especially as they are candidates for new treatment options in the DAA era.

Authors’ contributions

Jordovic Jelena, Bojovic Ksenija-study design, writing manuscript, collecting data and patients’ clinical follow-up. Simonovic-Babic Jasmina, Delic Dragan -manuscript revision, clinical data collection, patients’ clinical follow up. Katanic Natasa, Nikolic Natasa, Urosevic Aleksandar, Nestorov Jelena –manuscript drafting. Gasic Vladimir, Kotur Nikola, Zukić Branka, Pavlovic Sonja-revision and manuscript drafting, laboratory analysis. Lazarevic Ivana, Karalic Danijela-manuscript revision and laboratory analysis.

References

1. Pawlotsky JM (2011) Has genetics eradicated the good old predictors of hepatitis C treatment response? Clin Res Hepatol Gastroenterol 35: 157-158.
2. Gatselis N, Zachou K, Saitis A, Samara M, Dalekos GN (2014) Individualization of chronic hepatitis C treatment according to the host characteristics. World J Gastroenterol 20: 2839-2853.
3. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J (2009) IL28B is associated with response to chronic hepatitis C interferon and ribavirin therapy. Nat Genet 41: 1100–1104.
4. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuurra K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M (2009) Genome-wide association of IL28B with response to pegylated interferon-a and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
5. Lagging M, Askarieh G, Negro F, Biber S, Söderholm J, Westin J, Lindh M, Romero A, Missale G, Ferrari C, Neumann AU, Pawlotsky JM, Haagmans BL, Zeuzem S, Bochud PY, Hellstrand K (2011) Response prediction in chronic hepatitis C by assessment of IP-10 and IL28B-related single nucleotide polymorphisms. PLoS ONE 6: e17232.
6. Noureddin M, Wright EC, Alter HJ, Clark S, Thomas E, Chen R, Zhao X, Conry-Cantilena C, Kleiner DE, Liang TJ, Ghany MG (2013) Association of IL28B genotype with fibrosis progression and clinical outcomes in patients with chronic hepatitis C: a longitudinal analysis. Hepatology 58: 1548-1557.7. Mitrović N, Delić D, Marković-Denić L, Jovičić M, Popović N, Bojović K, Simonović Babić J, Švirtlih N (2015) Seroprevalence and risk factors for hepatitis C virus infection among blood donors in Serbia: A multicentre study. Dig Liver Dis 47: 572-576.
7. Švirtlih N, Delić D, Simonović J, Jevtović D, Doklić L, Gvozdenović E, Boričić I, Terzić D, Pavić S, Nešković G, Žerjav S, Urban V (2007) Hepatitis C virus genotypes in Serbia and Montenegro: the prevalence and clinical significance. World J Gastroenterol 13: 355-360.
8. Lazarević I, Đordjević J, Ćupić M, Karalić D, Delić D, Švirtlih, Simonović J, Svorcan P, Mišić N, Jovanović T (2013)
The influence of single and combined IL28B polymorphisms on response to treatment of chronic hepatitis C. J Clin Virol 58: 254-257.

9. Hori K, Shin WS, Hemmi C, Toyoo-oka T, Makino T (2003) High fidelity SNP genotyping using sequence-specific primer elongation and fluorescence correlation spectroscopy. Curr Pharm Biotechnol 4: 477-484.

10. Moini M, Azarpira N, Darai M, Sabet S, Geramizadeh B (2015) Allele and genotype frequency of IL28B (rs12979860) in South Iranian population. Middle East J Dig Dis 7: 261-262.

11. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O’Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461: 798–801.

12. Sarrazin C, Sussur S, Doehring A, Lange CM, Müller T, Schlecker C, Herrmann E, Lötsch J, Berg T (2011) Importance of IL28B gene polymorphisms in hepatitis C virus genotype 2 and 3 infected patients. J Hepatol 54: 415–421.

13. Tomokazu K, Nelson HC, Waka O, Hidenori O, Maekawa T, Hiromi A, Masatake T (2011) Predictive value of the IL28B polymorphism on the effect of interferon therapy in chronic hepatitis C patients with genotypes 2a and 2b. J Hepatol 54: 408–414.

14. Grigic I, Gorenc L, Gašpar M, Ćerina M, Paliminić A, Trupković M, Vince A, Židovec S (2016) Interleukin-28B polymorphism in persons with chronic hepatitis C in Croatia. Croat Med J 57(2):69–73.

15. Delić D (2012) Epidemiology of chronic hepatitis C. In Jovanović T, Stojković Švirtlih N, editors. Chronic hepatitis C infection. Belgrade: Institute for textbook publishing and teaching aids. 324-325. [Book in Serbian]

16. Jordović J, Bojović K, Simonović-Babić J, Gasić V, Kotur N, Zukić B, Vuković M, Pavlović S, Lazarević I, Bekić I, Nikolić N, Urošević A, Mitrović N, Delić D (2019) Significance of UGT1A1*28 genotype in patients with advanced liver injury caused by chronic hepatitis C. J Med Biochem 38: 45-52.

17. Falleti E, Bitetto D, Fabris C, Cussigh A, Fornasiere E, Cmet CA, Babić J, Gasic V, Kotur N, Zukić B, Vuković M, Pavlović S, Lazarević I, Běčk ̌i I, Nikolić N, Urošević A, Mitrović N, Delić D (2019) Significance of UGT1A1*28 genotype in patients with advanced liver injury caused by chronic hepatitis C. J Med Biochem 38: 45-52.

18. Di Marco V, Bronte F, Calvaruso V, Capra M, Borsellino Z, Maggio A, Renda MC, Pitrolo L, Lo Pinto MC, Rizzo M, Fiorenza F, Gerardi C, Grimaudo S, Di Cristina A, Levrero M, Craxi A (2012) IL28B polymorphisms influence stage of fibrosis and spontaneous or interferon-induced viral clearance in thalassemia patients with hepatitis C virus infection. Haematologica 97: 679-686.20.

19. Fabricio-Silva GM, Poschetzky BS, de Mello Perez R, Dos Santos RC, Cavalini LT, Porto LC (2015) Association of cytokine gene polymorphisms with hepatitis C virus infection in a population from Rio de Janeiro. Brazil Hepat Med 7: 71-79.

20. Maraština F, Aghemo A, De Nicola S, Rumi MG, Cheroni C, Scavelli R, Crimi M, Soffredini R, Abrignani S, De Francesco R, Colombo M (2011) Genetic variation in the interleukin-28B gene is not associated with fibrosis progression in patients with chronic hepatitis C and known date of infection. Hepatology 52: 1127-1134.

21. Mallat A, Hezode C, Lotersztajn S (2008) Environmental factors as disease accelerators during chronic hepatitis C. J Hepatol 48: 657-665.

22. Boglione L, Cusato J, Di Perri G, D’Avolio A (2016) The Role of IL28B Genotype in HCV-RNA Baseline Levels. Intervirology 59: 67-68.

23. Grebely J, Morris MD, Rice TM, Bruneau J, Cox AL, Kim AY, McGovern BH, Shoukry NH, Lauer G, Maher L, Lloyd AR, Hellard M, Prins M, Dore GJ, Page K (2013) Cohort profile: the international collaboration of incident HIV and hepatitis C in injecting cohorts (InCS) study. Int J Epidemiol 42: 1649-1659.

24. Poynard T, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J (2001) Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis C. J Hepatol 34: 730-739.

25. Berger K, Kim A (2012) IL28B polymorphisms as a pre-treatment predictor of response to HCV treatment. Infect Dis Clin North Am 26: 863–877.

26. Lu Y, Andonov A, Wong D (2014) Hepatitis C virus late relapse after sustained virologic response from interferon and ribavirin treatment as confirmed by RNA sequencing. J Clin Microbiol 52: 367-369.

27. Radkowski M, Gallegos-Orozco JF, Jablonska J, Colby TV, Walewska-Zielecka B, Kubicka J, Wilkinson J, Adair D, Rakela J, Laskus T (2005) Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology 41: 106–114.

28. Giannini EG, Basso M, Savarino V, Picciotto A (2010) Sustained virological response to pegylated interferon and ribavirin is maintained during long-term follow-up of chronic hepatitis C patients. Aliment. Pharmacol. Ther 31: 502–508.

29. Simmons B, Saleem J, Hill A, Riley RD, Cooke GS (2016) Risk of late relapse or reinfection with hepatitis C virus after achieving a sustained virological response: A systematic review and meta-analysis. Clin Infec Dis 3: 683–694.

30. Serfaty L (2016) Follow-up of patients with chronic hepatitis C and a sustained viral response. Liver Int 36: 67-71.

31. Innes HA, Hutchinson SJ, Allen S, Bhattacharyya D, Bramley P, Delahooke TE, Dillon JF, Forrest E, Fraser A, Gillespie R, Goldberg DJ, Kennedy N, McDonald S, McLeod A, Mills PR, Morris J, Hayes P (2011) Excess liver-related morbidity of chronic hepatitis C patients, who achieve a sustained virological response, and are discharged from care. Hepatology 54: 1547–1558.

32. Tachi Y, Hirai T, Miyata A, Ohara K, Iida T, Ishizu Y, Honda T, Kuzuya T, Hayashi K, Ishigami M, Goto H. (2015) Progressive fibrosis significantly correlates with hepatocellular carcinoma in patients with a sustained virological response. Hepatol Res 45: 238–246.

33. Bedossa P, Mouchari R, Chelbi E, Asselah T, Paridis V, Vidaud M, Cazals-Hatem D, Boyer D, Valla D (2007) Evidence for a role of nonalcoholic steatohepatitis in hepatocellular carcinoma in patients with a sustained virological response. J Hepatol 47: 74–87.

34. Huang CF, Yue ML, Tsai PC, Hsieh MH, Yang HL, Hsieh MY, Yang JF, Lin ZY, Chen SC, Wang LY, Dai CY, Huang JF, Chiang WL, Yu ML (2014) Baseline gamma-glutamyl transferase levels strongly correlate with hepatocellular carcinoma development in non-cirrhotic patients with successful hepatitis C virus eradication. J Hepatol 61: 67–74.

35. Savino B, Facchiotto S, Cossignani A, Savojardo D, Cariani E, Critelli R, Maisonneuve P, Villa E, Rossi-Savino B, Facciotto S, Crosignani A, Savojardo D, Cariani E, Critelli R, Maisonneuve P, Villa E, Rossi-Savino (2016) Interleukin-28B (IL-28B) polymorphism has no impact on the progression of chronic hepatitis C patients. J Infect Dev Ctries 13(6):526-535.
long-term outcome of patients with HCV-related cirrhosis: A 15-years retrospective-prospective cohort study.
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