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Variability of the HCV core region and host genetic and epigenetic factors can predict the response to pegylated interferon/ribavirin therapy in genotype 1b hepatitis C patients from Serbia

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Abstract: Variations in the hepatitis C virus (HCV) core sequence have been related to disease progression and response to antiviral therapy. Previously we showed that the methylation status of *RASSF1A* and *p16* genes, and *IL28B* genotypes affects the response to pegylated interferon/ribavirin (PEG-IFN/RBV) therapy. Herein we investigated whether amino acid (aa) substitutions in the HCV core region alone or in combination with *IL28B* genotypes and *RASSF1A/p16* methylation affect the response to PEG-IFN/RBV therapy and liver disease progression. Among 29 examined patients, we found no association between single aa substitutions and response to therapy. However, we observed that patients with the HCV core aa substitution at position 75 and CT/TT *IL28B* genotypes were non-responders (NR), (P=0.023). Moreover, these patients had unmethylated *RASSF1A*. In contrast, most patients (75%) with aa substitutions at position 91 and CC *IL28B* genotype achieved sustained virologic response (SVR), (P=0.030), and 70% of them had methylated *RASSF1A* gene. Our results suggest that combined analysis of aa substitutions in the core protein, the *IL28B* rs12979860 polymorphism, and the methylation status of the *RASSF1A* gene may help in predicting treatment response to PEG-IFN/RBV in genotype 1b chronic hepatitis C patients.

Keywords: hepatitis C virus; variability of HCV core region; *IL28B*; *RASSF1A* and *p16* methylation; therapy response

Abbreviations: chronic hepatitis C (CHC); hepatitis C virus (HCV); pegylated interferon and ribavirin (PEG-IFN/RBV); hepatocellular carcinoma (HCC); sustained virologic response (SVR); non-response (NR)

INTRODUCTION

More than 71 million people worldwide are infected with the hepatitis C virus (HCV), and more than 1.75 million people are newly infected with HCV each year, making it a global health problem [1]. Chronic hepatitis C (CHC) infection can cause liver damage associated with progressive fibrosis and cirrhosis and eventually hepatocellular carcinoma (HCC) [2]. HCV exhibits high genetic variability, leading to the formation of numerous viral quasispecies in one infected person. HCV is classified into eight genotypes, and several sub genotypes [3]. The most prevalent genotype in Serbia, as in Europe, is genotype 1, which is associated with more severe liver disease and poor response to therapy [4,5]. The standard for treating HCV infection in Serbia is combined therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) [4]. The best indicator of effective treatment is a sustained virologic response (SVR), which in the case of this therapy is achieved in only 50% of patients with HCV genotype 1[4]. Therefore, finding reliable markers that will indicate a successful outcome after interferon therapy in genotype 1 patient is of utmost importance.

The molecular mechanisms by which viral and host factors influence disease progression and response to therapy have not yet been fully elucidated [6,7].

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Single-nucleotide polymorphism on chromosome 19, rs12979860, has been shown to strongly affect the response to PEG-IFN/RBV therapy in patients with genotype 1 [8-10]. The polymorphism resides 3 kb upstream of the IL28B gene encoding *IFN-λ 3* [11]. Of the three IL28B genotypes, CC, CT, and TT, genotypes have been associated with SVR in many studies [8-10,12]. In addition, during chronic HCV infection, a combination of direct and indirect factors can lead to epigenetic alterations [13]. For example, the inactivation of different genes in the host genome by methylation of their promoters under the influence of the HCV core protein leads to liver damage and carcinogenesis [6,14-16]. These methylation changes could also affect the response to antiviral therapy [17-19].

The methylation status of the Ras association domain family member 1 (*RASSF1A*) and cyclin-dependent kinase inhibitor 2A (*CDNK2,p16*) genes were shown to be related to fibrosis progression and the response to therapy [17-19]. *RASSF1A* and *p16* are tumor suppressor genes. RASSF1A protein inhibits cell cycle arrest and metastasis, stabilizes microtubules, and induces apoptosis and cell adhesion [20], while the P16 protein prevents activation of the CDK4/cyclin D complex during the G1 phase of the cell cycle [21]. These genes are frequently inactivated by HCV-induced methylation of the promoter region in different states of liver fibrosis, cirrhosis, and HCC [17,22,23]. The core protein may affect the DNA methylation of the RASSF1A gene via histone methylation through an unknown mechanism [24]. At the same time, the core protein inhibits *p16* expression by causing its promoter methylation via upregulation of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3b (DNMT3b) [14,25].

In addition, the HCV core protein has different biological functions, such as controlling cell growth, apoptosis, oxidative stress, and immunomodulation during hepatocyte infection [6,26]. Therefore, variations in the HCV core sequence are associated with liver disease progression and response to PEG-IFN/RBV therapy [6,27-31]. Previous studies have shown that aa core region substitutions at positions 70 and 91 may be associated with the outcome of interferon-based therapy [32-34], and with disease progression and development of HCC [30,31,35-37]. Although there are novel and more effective approaches to the treatment of HCV, the risk of complications still exists [38]. Therefore, variability of the HCV genome in relation to *IL28B* gene polymorphism and epigenetic alterations in chronic HCV infection could have clinical implications for the detection and prevention of liver fibrosis, cirrhosis, and the development of HCC.

This study was a continuation of our previous work in which we demonstrated that the methylation status of *RASSF1A* and *p16* genes, and *IL28B* genotypes affect the response to therapy with PEG-IFN/RBV [19]. However, as the variability in the HCV core region has not been examined in the Serbian population thus far, the main objective of this study was to investigate whether there is a relationship between aa substitutions in the HCV core region, *IL28B* genotypes and promoter methylation of *RASSF1A* and *p16* genes in patients with genotype 1b HCV infection. We also aimed to determine whether HCV core variability alone or in combination with *IL28B* gene polymorphism and *RASSF1A*/*p16* methylation status affects the response to therapy and disease progression.

MATERIALS AND METHODS

Ethics statement

All procedures were carried out with the prior informed consent of the patients. The study complied with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Vinča Institute of Nuclear Sciences – National Institute of the Republic of Serbia, University of Belgrade (Approval No. 116-8-2/2021-000). Our study included 29 patients (13 females, 16 males; median age 42.9, range 22-67 years) with chronic hepatitis C genotype 1b.

Plasma samples were collected before the start of therapy. A sustained virologic response (SVR) was defined as a therapeutic response in which there was no HCV RNA detectable in plasma 6 months after the end of treatment, whereas non-response (NR) was defined as a therapy response characterized by the presence of HCV RNA in plasma 6 months after treatment. The METAVIR scoring system was used to evaluate histological activity grade and fibrosis [39]. The liver histological staging was based on five degrees of fibrosis: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis with few septa), F3 (severe fibrosis with numerous septa without cirrhosis), and

F4 (cirrhosis). Methylation analyses of *RASSF1A* and *p16* genes and *IL28B* genotypes were performed as in our previous study [19].

HCV RNA extraction

The Ribo-Sorb-100 (HCV Quant) RNA/DNA Extraction Kit (Sacace Biotechnologies, Como, Italy) was used for the extraction of total ribonucleic acid (RNA) from 100 μL of plasma according to the manufacturer's protocol. The isolated RNA was stored at -80° until required.

Baseline viral parameters and HCV genotyping

The copy number of HCV RNA was determined by real-time polymerase chain reaction (PCR) (Applied Biosystems 7500, Foster City, USA) using the commercially available R-TMQ HCV Kit (Sacace Biotechnologies, Como, Italy) according to the manufacturer's instructions (quantitation limit, 250 IU/mL).

Reverse-transcription (RT) nested PCR and amplification of the core fragment

The C region was amplified by one-step RT-PCR which was followed by a 2nd round of nested PCR. The One-Step RT-PCR Kit (QIAGEN GmbH, Hilden, Germany) was used for reverse transcription and first PCR with 5 μL of isolated total HCV RNA. RT-PCR was carried out with the following thermal profile: reverse transcription at 50°C for 30 min and 95°C for 15 min, followed by 35 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 45 s and final extension at 72°C for 7 min. For the RT-PCR and the 2nd PCR reaction, primers for the C region were designed using Primer-BLAST (Supplementary Table S1). One μL of the PCR product was subjected to a 2nd round of PCR. Twenty-five μL of the amplification mix for nested PCR reactions contained 1.75 units of DreamTaq Polymerase (Thermo Fisher Scientific, Lithuania), 2.5 μL of 10×PCR buffer with $MgCl_2$, 2 μL of dNTP (10 mM each), 0.25 μL of each internal primer (40 pmol, final concentration 0.6 μM). The thermal PCR protocol included initial denaturation for 3 min at 95°C, followed by 30 cycles of amplification at 94°C for 45 s, 60°C for 45 s and 72°C for 60 s, followed by a final extension at 72°C for 7 min. RT-PCR and nested PCR were performed on a thermal cycler (Applied Biosystems Gene Amp® PCR System 9700). The final PCR products (length 433 base pairs) were separated by electrophoresis on 6% acrylamide gels and stained with silver nitrate and sodium carbonate.

Sequencing and analysis of the variability of the core region

After amplification, the PCR products were purified using a MinElute PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. All sequences were determined by the Institute of Microbiology and Immunology, University of Belgrade, Faculty of Medicine, Serbia. Amplicons were sequenced in both directions by dye-terminator sequencing on an ABI 310 automated DNA sequencer (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's protocol. For analysis of aa substitutions and similarities between the sequences, we performed programs by National Center for Biotechnology Information (NCBI) Blastp (protein-protein BLAST) and Constraint-based Multiple Alignment Tool (COBALT).

Statistical analysis

Differences in frequency distribution between two categorical variables were evaluated by Pearson's $\chi 2$ test and Fisher's exact two-tailed test when the expected frequencies were lower than five. The means of normally distributed continuous variables were compared using Student's t-test, while the Mann-Whitney U Test was used for means of skewed continuous variables. The results are presented as the mean±standard deviation (SD) or numbers (percentages). P values less than 0.05 were considered statistically significant. All statistical analyses were performed using the Sigma Plot 14.0 licensed statistical analysis software package.

RESULTS

The mean age of all examined patients was 42.9±12.4 years. Sixteen out of 29 (55.2%) patients were male and 44.8% (13/29) were female. Early-stage fibrosis was present in 37.9% (11/29), whereas late-stage fibrosis was found in 62.1% (18/29) of the patients. SVR was achieved in 37.9% (11/29) of patients, while 62.1%

(18/29) were NR. Considering the status of the *IL28B* rs12979860 polymorphism, the CC genotype occurred in 53.6% (15/28) of patients, while 46.4% (13/28) of patients had the CT/TT genotypes. Aberrant methylation of the *RASSF1A* gene was present in 37% (10/27) of patients, while aberrant methylation of the *p16* gene was present in 25.9% (7/27) of cases. The promoter methylation status was missing in two patients due to insufficient material for analysis. Data on the *IL28B* polymorphism rs12979860 was not available for one patient.

Amino acid substitutions in the HCV core protein

Analysis of aa substitutions in the viral core protein was successfully performed in all 29 cases. All sequences from the HCV core region were aligned to a reference sequence (HCV-J, GenBank ID: D90208) for genotype 1b, deposited in the GenBank database following accession numbers (OQ607636-OQ607663).

Overall, aa substitutions at position 70 occurred in 41.4% (12/29) of cases, substitutions at position 75 occurred in 44.8% (13/29), whereas substitutions at position 91 occurred in 34.5% (10/29), and substitutions at position 110 were observed in 13.8% (4/29). We observed multiple aa substitutions in some cases (Table 1). The most frequently observed change at position 70 was R70Q, which was noted in 75% (9/12) of cases, whereas the most frequent substitution at position 75 was T75A, which was observed in 53.8% (7/13). The most common aa substitution at position 91 was M91C which was observed in 70% (7/10) of cases, while the most common aa substitution at position 110 was T110S which was detected in 50% (2/4) of cases. The sequences of all isolates with aa substitutions at specific positions are shown in Fig. 1.

Table 1. Amino acid substitutions in HCV core protein in patients with genotype 1b chronic hepatitis C infection.

amino acid substitution position	amino acid substitution	number of patients (frequency in %)
Position 70	R70Q	9/12 (75)
	R70H	1/12 (8.3)
	R70P	1/12 (8.3)
	R70K	1/12 (8.3)
Position 75	T75A	7/13 (53.8)
	T75S	4/13 (30.8)
	T75V	1/13 (7.7)
	T75P	1/13 (7.7)
Position 91	M91C	7/10 (70)
	M91L	3/10 (30)
Position 110	T110S	2/4 (50)
	T110A	1/4 (25)
	T110N	1/4 (25)

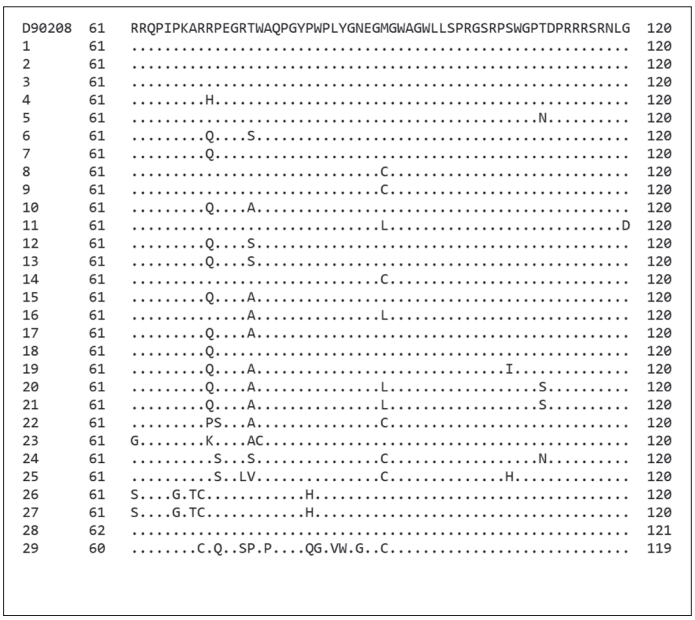

Fig. 1. The sequences of all isolates with the most frequent aa substitutions at specific positions. All sequences from the HCV core region were aligned to a reference sequence, which is HCV-J (GenBank accession number D90208) for genotype 1b and their nucleotide sequences were deposited in the GenBank database following accession numbers (OQ607636-OQ607663). The region between amino acids 61 and 120 is shown. Dots indicate residues identical to those in the reference sequence.

Association of amino acid substitutions in HCV core protein with clinicopathological parameters, *IL28B* genotypes, and methylation status of *RASSF1A* and *p16* genes

There was no correlation between the different aa substitutions in the core protein at positions 70, 75, 91, and 110 and the baseline characteristics of patients with genotype 1b CHC infection, including age and gender, stage of liver fibrosis, and IL28B genotypes, except that we found that the CC genotype was more frequent in a group of patients with aa 91 substitutions, while the CT/TT genotypes were more frequent in the group of patients without aa substitutions at position 91. However, this result was not statistically

significant (P=0.055, Table 2). In addition, there was no association between the aa substitutions and the methylation status of the *RASSF1A* and *p16* genes. Interestingly, all patients with R70Q substitution had an unmethylated *RASSF1A* gene. However, in the group of patients without R70Q aa substitution, 66.7% (2/3) of cases had a methylated *RASSF1A* gene (P=0.046, Fisher's exact test).

Table 2. Association of amino acid substitutions in HCV core protein with baseline patient characteristics, methylation status of *RASSF1A* and *p16* genes, *IL28B* genotype, and response to therapy in patients with genotype 1b chronic hepatitis C infection.

Clinical and pathological characteristics of patients	**Amino acid substitution in viral core protein at position 70**		**P**	**Amino acid substitution in viral core protein at position 75**		***P***
	+	-		+	-	
Age (Years) †	45.667±12.985	40.941±12.060	0.331	42.308±14.291	43.375±11.194	0.828
Gender						
Male	5/16 (31.25)	11/16 (68.75)	0.396	7/16 (43.75)	9/16 (56.25)	0.806
Female	7/13 (53.75)	6/13 (46.15)		6/13 (46.15)	7/13 (53.75)	
Stage of fibrosis ‡						
F0 – F2	3/11 (27.3)	8/11 (72.7)	0.273	3/11 (27.3)	8/11 (72.7)	0.249
F3 – F4	9/18 (50)	9/18 (50)		10/18 (55.6)	8/18 (44.4)	
Methylation status of the *RASSF1A* gene ¶						
Methylated	2/10 (18.7)	8/10 (81.3)	0.230	2/10 (20)	8/10 (80)	0.124
Unmethylated	8/17 (33.3)	9/17 (63.7)		9/17 (52.9)	8/17 (47.1)	
Methylation status of the *p16* gene ¶						
Methylated	2/7 (18.7)	5/7 (81.3)	0.678	1/7 (14.3)	6/7 (85.7)	0.183
Unmethylated	8/20 (33.3)	12/20 (63.7)		10/20 (50)	10/20 (50)	
***IL28B* genotype** §						
CC	5/15 (33.3)	10/15 (66.7)	0.477	6/15 (40)	9/15(60)	0.724
CT/TT	7/13 (53.8)	6/13 (46.2)		7/13 (53.8)	6/13 (46.2)	
Therapy outcome						
Non-responders (NR)	8/18 (44.4)	10/18 (55.6)	0.717	10/18 (55.6)	8/18 (44.4)	0.249
Sustained virologic responders (SVR)	4/11(36.4)	7/11 (63.6)		3/11(27.3)	8/11 (72.7)	
	Amino acid substitution in viral core protein at position 91		**P**	**Amino acid substitution in viral core protein at position 110**		**P**
	+	-		+	-	
Age (Years) †	39.700±12.815	44.579±12.258	0.336	39.500±13.026	43.440±12.544	0.603
Gender						
Male	8/16 (50)	8/16 (50)	0.114	4/16 (25)	12/16 (75)	0.107
Female	2/13 (15.4)	11/13 (84.6)		0/13 (0)	13/13 (100)	
Stage of fibrosis‡						
F0 – F2	4/11 (36.4)	7/11 (63.6)	1.000	0/11 (0)	11/11 (100)	0.268
F3 – F4	6/18 (33.3)	12/18 (66.7)		4/18 (22.2)	14/18 (77.8)	
Methylation status of the *RASSF1A* gene ¶						
Methylated	2/10 (20)	8/10 (80)	0.219	1/10 (10)	9/10 (90)	1.000
Unmethylated	9/17 (52.9)	8/17 (47.7)		2/17 (11.8)	15/17 (88.2)	
Methylation status of the *p16* gene ¶						
Methylated	1/7 (14.3)	6/7 (85.7)	0.363	1/7 (14.3)	6/7 (85.7)	1.000
Unmethylated	10/20 (50)	10/20 (50)		2/20 (10)	18/20 (80)	
***IL28B* genotype** §						
CC	8/15 (53.3)	7/15 (46.7)	0.055	3/15 (20)	12/15(80)	0.600
CT/TT	2/13 (15.4)	11/13 (84.6)		1/13 (7.7)	12/13 (92.3)	
Therapy outcome						
Non-responders (NR)	4/18 (22.2)	14/18 (77.8)	0.114	1/18 (5.6)	17/18 (94.4)	0.139
Sustained virologic responders (SVR)	6/11(54.5)	5/11 (45.5)		3/11 (27.3)	8/11 (72.7)	

+ – presence of amino acid substitutions at the identified positions; - – absence of amino acid substitutions at the identified positions; † data expressed as the mean±SD; ‡ fibrosis stage expressed by the METAVIR score; ¶ data are missing on two patients for a given parameter; § data are missing on one patient for a given parameter; HCV – hepatitis C virus. Statistical tests that were used are Student's t-test, Pearson's $\chi 2$ test, Fisher's exact two-tailed test. In parentheses is the frequency in %.

Association of amino acid substitutions in the HCV core protein with response to therapy

In general, in our group of patients, SVR was associated only with the presence of the *IL28B* genotype CC (P=0.005, Fisher's exact test). We did not detect any association between individual core aa substitutions and the response to therapy. However, we obtained the following results after more comprehensive analyses, which included IL28B polymorphism and methylation status of RASSF1A and p16 genes. In the group of patients with a core aa 70 substitution, no difference in response to therapy was observed depending on *IL28B* polymorphism and *RASSF1A/p16* methylation status. However, as many as 85.7% of patients with aa70 substitution and CT/TT *IL28B* genotypes (aa70$^+$/CT/TT) were

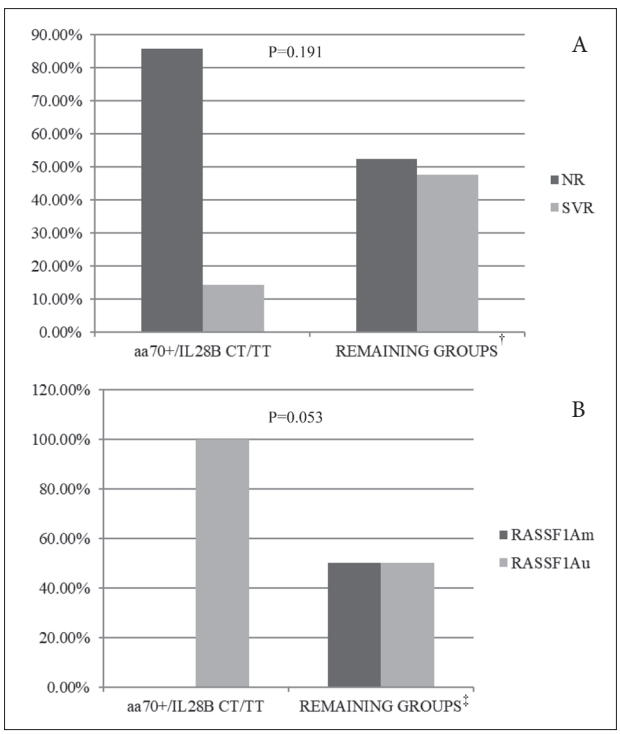

Fig. 2. Association between amino acid substitution at position 70 in the HCV core region and the IL28B genotype with therapy outcome (A) and methylation status of the *RASSF1A* gene (B). **A** – Effect of core aa 70 substitution and *IL28B* genotype on the response to therapy. † The remaining groups are aa70$^-$/CT/TT, aa70$^+$/CC, aa70$^-$/CC; aa70$^+$, core aa substitution at position 70 is present; aa70$^-$, core aa substitution at position 70 is absent; SVR – sustained virologic responders; NR – non-responders. **B** – Distribution of *RASSF1A* methylation in patients with different aa 70 status and *IL28B* genotype. ‡ The remaining groups are aa70$^-$/CT/TT, aa70$^+$/CC, aa70$^-$/CC; aa70$^+$ – core aa substitution at position 70 is present; aa70$^-$ – core aa substitution at position 70 is absent; m – methylated; u – unmethylated.

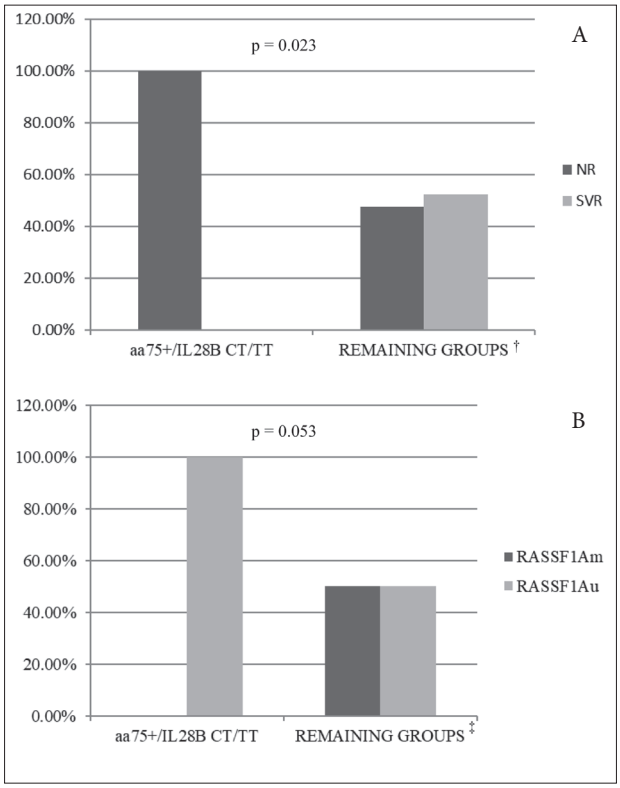

Fig. 3. Association between amino acid substitution at position 75 in the HCV core region and *IL28B* genotype with response to therapy (A) and the methylation status of the *RASSF1A* gene (B). **A** – Effect of core aa 75 substitution and *IL28B* genotype on the response to therapy. † The remaining groups are aa75$^-$/CT/TT, aa75$^+$/CC, aa75$^-$/CC; aa75$^+$– core aa substitution at position 75 is present; aa75$^-$ – core aa substitution on position 75 is absent; SVR – sustained virologic responders; NR – non-responders. **B** – Distribution of *RASSF1A* methylation in patients with different aa 75 status and *IL28B* genotype. ‡ The remaining groups are aa75$^-$/CT/TT, aa75$^+$/CC, aa75$^-$/CC; aa75$^+$ – core aa substitution at position 75 is present; aa75$^-$– core aa substitution at position 75 is absent; m – methylated; u – unmethylated.

NR, compared to the 52.4 % of NR patients who did not have the aa70$^+$/CT/TT combination (P=0.191, Fig. 2A). In addition, all patients in the aa70$^+$/CT/TT group had an unmethylated *RASSF1A* gene, whereas 50% of all other patients had an unmethylated *RASSF1A* gene (P=0.053, Fig. 2B). However, this combination did not affect the response to therapy. In addition, all patients with aa 75 substitutions and CT/TT *IL28B* genotypes were NR, which was statistically significant compared to all other patients who did not carry the aa75$^+$/CT/TT combination (P=0.023, Fig. 3A). Moreover, all NR patients with the aa substitution at position 75 and the CT/TT *IL28B* genotype had unmethylated *RASSF1A* compared to 50% of all other patients (P=0.053, Fig.

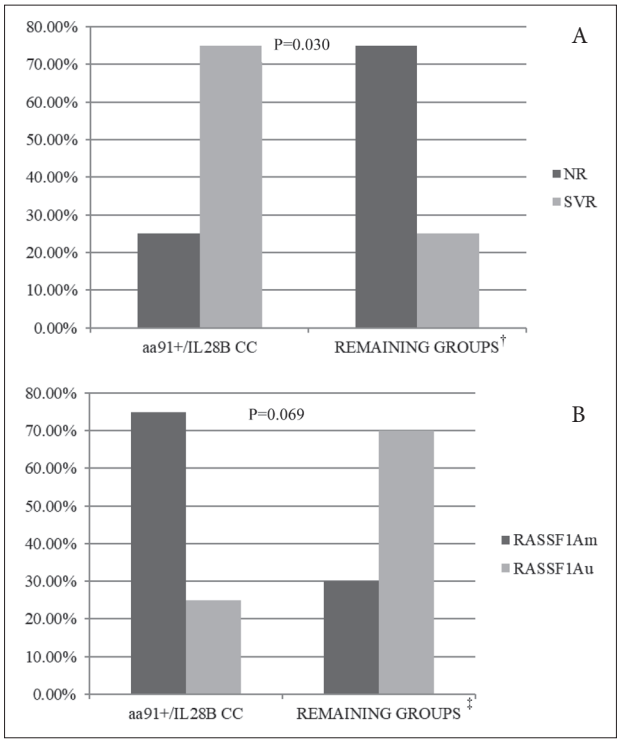

Fig. 4. Association between amino acid substitution at position 91 in the HCV core region, and IL28B genotype with response to therapy (A) and the methylation status of the *RASSF1A* gene (B). **A –** Effect of core aa 91 substitution and *IL28B* genotype on the response to therapy. † Remaining groups are aa91$^+$/CT/TT, aa91$^-$/ CT/TT, aa91$^-$/CC; aa91$^+$ – core aa substitution at position 91 is present; aa91$^-$ – core aa substitution at position 91 is absent; SVR – sustained virologic responders; NR – non-responders. **B –** Distribution of *RASSF1A* methylation in patients with different aa 91 status and *IL28B* genotype. ‡ Remaining groups are aa91$^+$/ CT/TT, aa91$^-$/CC, aa91$^-$/ CT/TT; aa91$^+$ – core aa substitution at position 91 is present; aa91$^-$ – core aa substitution at position 91 is absent; m – methylated; u – unmethylated.

3B). In the subgroup of patients with a core aa 91 substitution and the CC *IL28B* genotype (aa91$^+$/CC), SVR was achieved in 75% of cases, compared to only 25% of all other patients (P=0.030, Fig. 3A). Although not significant, the methylated *RASSF1A* gene was detected more frequently in the subgroup of aa91$^+$/ CC patients (71.4%, P=0.069, Fig. 3B). Associations regarding core aa 110 substitution and the response to therapy were not found. There was no evident association between the methylation status of the *p16* gene and aa substitutions in the HCV core region.

DISCUSSION

The first studies on the presence of specific polymorphisms in the HCV genome and the development of HCC were published in the early 2000s [40,41]. Thereafter it was also shown that amino acid substitutions of the HCV core protein have an impact on the response of chronically HCV-infected patients to combined antiviral therapy with PEG-IFN/RBV [6,29,42]. The core protein can inactivate different genes in the host genome by DNA methylation of their promoters, leading to liver damage and carcinogenesis [6,14-16]. Two genes whose methylation status is affected by the core protein are the tumor suppressor genes *RASSF1A* and *p16* [14,24,25]. Because of this, we investigated the possible association between the most common core aa substitutions and the response to PEG-IFN/RBV therapy, both alone and in combination with the *IL28B* gene polymorphism and the methylation status of the *RASSF1A* and *p16* genes.

The impact of single or combined mutations in the HCV core region and the response to therapy and the progression of liver fibrosis is not entirely clear. In our study, the most common aa substitution in the HCV core protein was R70Q, followed by T75A and M91C, which is largely consistent with previous studies [6,34,43,44]. In terms of sequence variability of the HCV core region, we found no association between aa substitutions in the viral core protein at positions 70, 75, 91, and 110, and baseline characteristics such as patient age and gender, the genotype of *IL28B,* and the methylation status of the *RASSF1A* and *p16* genes. We also found no association between aa substitutions in HCV core protein and the stage of liver fibrosis, which is in line with previous research [45]. In contrast, other research has reported an association between aa substitutions at positions 70, 75, and 91 in the HCV core protein and disease progression or hepatocarcinogenesis [30,31,35-37,42]. Moreover, we found no association between single aa substitutions in the HCV core protein and the response to therapy. On the other hand, some have authors reported different results [29,32,33,43,46]. For example, the aa core substitution at position 70 was associated with NR in patients with the 1b genotype [29,43], while the absence of aa core substitution at position 70 was related to SVR in HCV 1b genotype [32]. In addition, the absence of aa substitutions at core positions 70 and 91 was related to SVR in patients infected with

HCV 1b genotype [33], while no association between aa core substitutions and the response to therapy in HCV genotype 1a and 3a was detected [36,37].

In our study, there was no statistically significant association between aa substitutions at position 70 and CT/TT *IL28B* genotypes, but this group of patients presented a worse response to therapy and an unmethylated *RASSF1A* gene. In addition, we found that the subgroup of patients with the aa substitution at position 75 and CT/TT *IL28B* genotypes had a worse response to therapy, which was statistically significant, and that this subgroup had an unmethylated *RASSF1A* gene. Our results are consistent with the observation that core 70 substitutions were associated with a worse response to therapy in patients with the CT *IL28B* genotype [43]. On the other hand, the subgroup of patients with the aa substitution at position 91 and the CC *IL28B* genotype had SVR more frequently, which was statistically significant. As regards the methylation status and substitutions at positions 70 and 75, we detected that more NR had the CT/TT *IL28B* genotypes and unmethylated *RASSF1A,* which is in agreement with our previous findings [19].

Although our study did not establish a statistically significant association between IL28B genotypes, the methylation status of *RASF1A,* and liver fibrosis progression, previous data suggest a possible association between *RASSF1A* and *IL28B*, in part through the regulation of *IL6* gene expression [47,48]. It has been reported that *RASSF1A* induces IL-6 expression in A375 melanoma cells [48] and that increased IL-6 expression is related to unfavorable clinical outcomes in HCV patients and progression to HCC [49,50]. On the other hand, the R70Q substitution in the HCV core protein is related to the increased expression of IL-6, which may cause steatosis and HCC and inhibit interferon signaling, which is associated with a poorer therapeutic outcome [51]. Thus, we can assume that the unmethylated status of the *RASSF1A* gene and R70Q substitution may lead to a worse therapy outcome due to increased expression of IL-6, but this needs further investigation.

Previous studies have shown that core aa substitution at position 70 can be used as a predictor of the treatment outcome and as a pretreatment predictor of HCC after direct-acting antiviral (DAA) therapy [31,42,52]. Based on previous research as well as ours, the variability in the HCV core region could be a predictive factor of the therapy outcome with combined PEG-IFN/RBV therapy or DAA. The conflicting results could also be a consequence of the smaller sample size in our study.

To the best of our knowledge, this is the first study to analyze the concurrent effects of aa substitutions in the HCV core region, *IL28B* genotypes, and methylation status of the *RASSF1A* and *p16* genes. Core aa70 substitution in HCV-1b patients at the start of DAA therapy is an important predictor of hepatocarcinogenesis following the eradication of HCV RNA [31,52]. Therefore, our future research will focus on DAA therapy. It is important to detect aa substitution at position 70 in the HCV core region before initiating antiviral therapy, even if DAA therapy is continued after interferon (IFN)-based therapy. A larger number of studies are needed to confirm the potential use of aa substitution at position 70 in the HCV core region, *IL28B* rs12979860 polymorphism, and the methylation status of the *RASSF1A* gene as predictive factors related to treatment response, particularly with DAA therapy, where patients who have achieved SVR are expected to have a higher probability of developing HCC.

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Data availability: Data underlying the reported findings have been provided as part of the submitted article and can be accessed via the following link: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kokanov%20et%20al_Data%20Set.pdf

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Primers for amplification of the Core region of hepatitis C virus (HCV) genotype 1b.

Application	Direction	Primer	Sequence (5'-3')	Position†
RT and first PCR	Sense	G1+	CGCGCGACTAGG	478-487
	Antisense	G2	ATGTACCCCATGAGGTCGGC	720-739
Second PCR	Sense	F2	GGAGGTCTCGTAGACCGTGCA	307-327
	Antisense	G2	ATGTACCCCATGAGGTCGGC	720-739

† Nucleotide positions according to the HCV 1b prototype HCV-J (accession number D90208)

GenBank accession numbers for the nucleotide sequences:

https://www.ncbi.nlm.nih.gov/nuccore/OQ607636
https://www.ncbi.nlm.nih.gov/nuccore/OQ607637
https://www.ncbi.nlm.nih.gov/nuccore/OQ607638
https://www.ncbi.nlm.nih.gov/nuccore/OQ607639
https://www.ncbi.nlm.nih.gov/nuccore/OQ607640
https://www.ncbi.nlm.nih.gov/nuccore/OQ607641
https://www.ncbi.nlm.nih.gov/nuccore/OQ607642
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https://www.ncbi.nlm.nih.gov/nuccore/OQ607661
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https://www.ncbi.nlm.nih.gov/nuccore/OQ607663