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RASSF1A and *p16* promoter methylation and treatment response in chronic hepatitis C genotype 1b patients treated with pegylated interferon/ribavirin

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Abstract: Prevention of chronic hepatitis C (CHC) and its complications is based on antiviral therapy and early detection of reliable molecular markers in persons under risk. We investigated whether the methylation status of *RASSF1A* and *p16* genes, alone or in combination with host and viral factors, affects the response to therapy with pegylated interferon/ribavirin (PEG-IFN/RBV). Methylation-specific polymerase chain reaction (MSP) was used to determine the methylation status of the target promoter sequences of *RASSF1A* and *p16* in circulating-free DNA from the peripheral blood of 49 patients with CHC genotype 1b. The methylation status of the examined genes did not affect the response to therapy. However, the simultaneous presence of either *RASSF1A* or *p16* methylation and the CC genotype of *IL28B* was significantly related to a sustained virologic response (P=0.009 and P=0.032, respectively). After Bonferroni correction, only the result concerning the *RASSF1A* gene remained significant (P<0.0125). Methylation of *RASSF1A* was associated with the CC genotype of the *IL28B* gene (P=0.024) and a higher viral load (≥400 000 IU/mL, P=0.009). Our results suggest that combined analysis of *RASSF1A* gene methylation and *IL28B* rs12979860 polymorphism could potentially help in the prediction of therapy response in CHC genotype 1b patients.

Keywords: hepatitis C virus; DNA methylation; *RASSF1A*; *p16*; biomarkers

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

INTRODUCTION

Chronic hepatitis C infection (CHC) caused by hepatitis C virus (HCV) is a global health problem associated with progressive fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC) [1]. The risk of HCC development is higher in patients infected with HCV genotype 1b, which is the most prevalent genotype in Serbia, Western Europe and the United States [2,3]. Patients carrying this genotype require more intensive monitoring for the early detection and appropriate management of the disease.

Therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) is the standard option available for treating HCV infection in Serbia [3]. The best indicator of effective treatment is a sustained virologic response (SVR), defined as the absence of detectable HCV RNA in plasma 6 months after the end of treatment [4].

Compared with interferon/ribavirin therapy, current direct-acting antiviral combination regimens significantly increase the rate of SVR and are of shorter treatment duration, but are still limited by viral resistance, adverse effects and high cost, especially in countries with limited resources [5]. Finally, the cure for HCV infection does not eliminate the risk of developing HCC, especially in patients with advanced fibrosis [6].

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Different viral factors (HCV genotype and viral load) and host factors (age, gender, BMI, pre-treatment alanine aminotransferase levels, stage of liver fibrosis) can influence the response to therapy [7,8]. One of the strongest host genome predictors of the response to PEG-IFN/RBV therapy is the single-nucleotide polymorphism (SNP) rs12979860 near the *IL28B* gene that encodes interferon-λ [9,10]. The rs12979860 SNP is found upstream of the *IFN-λ 3* gene in the promoter sequence. This gene produces IL28A (IFN-λ 2), IL28B (IFN-λ 3) and IL29 (IFN-λ 1), cytokine members in the IFN-λ family [11]. However, additional factors may be required to accurately predict the treatment response and the course of the disease.

Previous research indicates that HCV-induced epigenetic changes in the host genome, especially changes in the pattern of DNA methylation, can affect the response to PEG-IFN/RBV therapy [8]. These changes are an early event in hepatocarcinogenesis, and they also occur during the early stages of fibrosis [12]. DNA methylation is an epigenetic modification that refers to the covalent attachment of a methyl group to the 5-position of the pyrimidine ring of cytosine within 5'-cytosine-phosphate-guanine-3' (CpG) islands in promoter regions, which leads to gene silencing [13].

Tumor suppressor genes *RASSF1A* and *p16* are often inactivated by methylation of the promoter region, not only in HCC but also in varying degrees of liver fibrosis and cirrhosis [8, 14-17]. RASSF1A is a multifunctional protein that prevents carcinogenesis through different cellular processes, including cell cycle arrest, inhibition of metastasis, microtubular stabilization and induction of apoptosis [18]. P16INK4a is a cyclin-dependent kinase inhibitor that prevents phosphorylation of the cell cycle regulator, retinoblastoma 1-encoded protein (pRb), inducing G1 phase arrest [19].

Methylation changes can be detected in the tissue as well as in the blood of patients at the stage of precancerous alterations [12,20,21]. Recent research has shown that circulating-free DNA, if used as a non-invasive "liquid biopsy", i.e., a blood test that enables molecular testing of liver diseases, could possibly alter the diagnosis, prognosis and prediction of treatment response in liver fibrosis and cirrhosis and HCC development [22,23].

The central aim of this study was to detect biological markers present in peripheral blood that could serve as potential predictive factors of response to antiviral treatment with an IFN-based cure in patients chronically infected with HCV genotype 1b. We analyzed promoter methylation of the *RASSF1A* and *p16* genes and SNP rs12979860 to identify their relationship with clinicopathological parameters, as well as their individual and simultaneous impact on response to therapy.

MATERIALS AND METHODS

Patients

All procedures were carried out with prior informed consent of patients. The study complies 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 49 patients (20 females, 29 males; median age 44.34, range 22-67 years) with CHC genotype 1b. All samples were collected before the start of PEG-IFN/RBV therapy. The virologic response was defined as a sustained virologic response (SVR) if there was an absence of HCV RNA in the plasma 6 months after the end of treatment, and non-response (NR) if the presence of HCV RNA was observed in plasma 6 months after of cessation of treatment. The METAVIR scoring system [24] was used for evaluation of histological activity grade and fibrosis as follows: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis with few septa), F3 (severe fibrosis with numerous septa without cirrhosis), F4 (cirrhosis).

HCV RNA extraction

Ribo-Sorb-100 (HCV Quant) RNA/DNA Extraction Kit (Sacace Biotechnologies, Como, Italy) was used for the extraction of total RNA from 100 μL of plasma according to the manufacturer's protocol. The concentration of HCV RNA was determined by real-time PCR (Applied Biosystems 7500, Foster City, USA) using the commercially available R-TMQ HCV Kit (Sacace Biotechnologies, Como, Italy) according to the manufacturer's instruction (sensitivity limit-250 IU/mL),

while genotyping of HCV was performed with a combination of type/specific primers as described previously [25,26].

Genotyping of *IL28B*

Genomic DNA was isolated from plasma using a commercially available kit QIAmp UltraSens (Qiagen, GmbH, Germany) according to the manufacturer's instructions. The *IL28B* single nucleotide polymorphism rs12979860 was analyzed using Custom® SNP Genotyping Assays (Applied Biosystems) with allele-specific TaqMan probes designed and reported previously [27]. Genotyping was performed on ABI-7500 real-time PCR (Applied Biosystems, Foster City, USA) in 25 μL of reaction volume containing 10 ng DNA, 12.5 μL TaqMan® Universal PCR Master Mix and 1.25 μL (40x) Custom® SNP Genotyping Assays.

Methylation analysis of the *RASSF1A* and *p16* genes

Circulating-free DNA was extracted from plasma using the commercially available kits QIAmp UltraSens (Qiagen, GmbH, Germany) and Ribo-Sorb-100 (HCV Quant) RNA/DNA Extraction Kit (Sacace Biotechnologies, Como, Italy) according to the manufacturers' instructions. The methylation status of the *RASS1A* and *p16* genes was determined by chemical bisulfite modification of DNA and subsequent PCR, using primers specific for either methylated or unmethylated DNA. DNA methylation patterns in the promoter CpG islands of the *RASS1A* and *p16* genes were determined by methylation-specific PCR (MSP). For each sample, we performed two PCR reactions, one with specific primers for unmethylated DNA and one with specific primers for methylated DNA. The EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA) was used for sodium bisulfite conversion of circulating-free DNA (100-500 ng), according to the manufacturer's instruction. For MSP reactions, 1 μL of 10 μL of bisulfite modified DNA was used. The PCR mixture for the MSP reaction for the *p16* gene contained 10 × PCR buffer (16 mmol/L ammonium sulfate, 67 mmol/L Tris-HCL, pH 8.8, 10 mmol/L 2-mercaptoethanol), 6.7 mmol/L $MgCl_2$, dNTP (each at 1.25 mmol/L), primers (300 ng each per reaction), 5% dimethylsulfoxide (DMSO) and 0.4 mg/ mL of bovine serum albumin (BSA) in a final volume of 25 μL. The PCR mixture for the MSP reaction for *RASSF1A* contained the same components except DMSO and BSA. The PCR reaction mixtures were hot-started at 95°C for 5 min before the addition of 1 U of Taq polymerase (Thermo Scientific, USA). Amplification was carried out in an Applied Biosystems 2720 temperature cycler for 40 cycles: 45 s at 95°C, 45 s at the annealing temperature being specific for each primer set (primer sets for *RASSF1A* methylated at 60°C, primer set for *RASSF1A* unmethylated at 55°C and primer sets for *p16* unmethylated at 60°C, primer sets for *p16* methylated at 60°C), and for 60 s at 72°, followed by final extension for 5 min at 72°C. The primer sequences are listed in Supplementary Table S1 [28,29]. As a negative control for the methylated alleles, DNA isolated from peripheral blood lymphocytes of 10 healthy volunteers was used. The same lymphocyte DNA served as a positive control for all genes after it was methylated *in vitro* with excess M.SssI methyltransferase (New England Biolabs, USA), which generate completely methylated DNA at all CpG sites. The PCR products were separated by electrophoresis on 6% acrylamide gels, stained with silver nitrate and sodium carbonate.

Statistical analysis

The results are presented as the mean±standard deviation or number (percentage). Differences in frequency distribution between two categorical variables were evaluated by Pearson's χ^2 test or Fisher's exact two-tailed test, when 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. We performed the Bonferroni correction for multiple testing where the appropriate and considered P-value cut-off <0.0125 was significant. For all other analyses, a P-value <0.05 were considered statistically significant. All statistical analyses were performed using the Sigma Plot 14.0 licensed statistical analysis software package.

RESULTS

Concerning HCV infection, clinical and pathological characteristics of the patients, the following results were obtained. The mean age of all our patients was

Table 1. *IL28B* rs12979860 polymorphism distribution according to response to therapy and baseline characteristics of patients with chronic HCV infection genotype 1b

Clinical and pathological characteristics of patients	*IL28B* rs12979860 polymorphism		P
	CT/TT	CC	
Age (Years) †	48.857 ± 10.101	41.923 ± 11.883	0.039*
Gender			
Male	13/29 (44.8%)	16/29 (55.2%)	0.782
Female	8/18 (44.4%)	10/18 (55.6%)	
Stages of fibrosis ‡			
F0 - F2	11/26 (42.3%)	15/26 (57.7%)	0.945
F3 - F4	10/21 (47.6%)	11/21(52.4%)	
Therapy outcome			
Non-responders (NR)	20/27 (74.1%)	7/27 (25.9%)	<0.001*
Sustained virologic responders (SVR)	1/20 (5%)	19/20 (95%)	

† Data expressed as the mean±SD; ‡ Stage of fibrosis expressed by the METAVIR score (fibrosis 0-2 and 3-4); HCV – hepatitis C virus; * Statistically significant. Statistical tests used were Student's t-test, Pearson's χ^2 test, and Fisher;s exact two-tailed test when the expected frequencies were lower than five. The analysis failed in two patients.

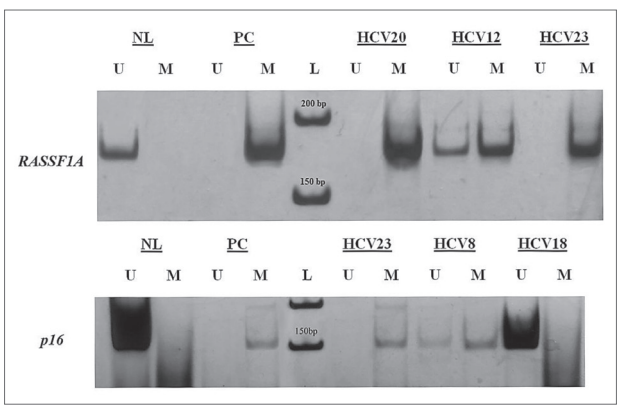

Fig. 1. Analysis of *RASSF1A* and *p16* gene methylation status by methylation-specific polymerase chain reaction (MSP). The presence of a visible PCR product in lanes U indicates the presence of unmethylated *RASSF1A* (169 bp) and *p16* (151 bp) genes; the presence of product in lanes M indicates the presence of methylated *RASSF1A* (169 bp) and *p16* (150 bp). Samples of plasma with HCV, 20 and 23 show *RASSF1A* promoter hypermethylation, while samples 23 and 8 show *p16* promoter hypermethylation; NL – normal lymphocytes as a positive control for unmethylated alleles; PC – *in vitro* methylated DNA from normal lymphocytes as a positive control for methylated alleles; L – molecular weight marker (50 bp); HCV – hepatitis C virus.

44.3±11.8; a sustained virologic response was achieved in 42.9% (21/49) of patients while 57.1% (28/49) were NR. *IL28B* typing showed that 55.3% (26/47) of the patients carried genotype CC, while 44.7% (21/47) had genotype CT/TT at rs12979860. Patients with the rs12979860 CC genotype were markedly younger than those with CT/TT genotypes (41.9±11.9 *vs* 48.9±10.1, P=0.039, Student's t-test; Table 1). Patients with the CC genotype of *IL28B* significantly more frequently had a SVR than the NR group of patients (P<0.001, Fisher's exact two-tailed test; Table 1).

RASSF1A and *p16* gene methylation status

Analysis of the *RASSF1A* and *p16* methylation status was successfully performed in all 49 patients. Aberrant methylation of the *RASSF1A* gene was detected in 32.6% (16/49), while aberrant methylation of *p16* was detected in 28.6% (14/49) of cases. Concomitant methylation of *RASSF1A* and *p16* was detected in 3 out of 49 (6.1%) cases, while 27 out of 49 patients (55.1%) had at least one methylated gene. Methylation of the analyzed genes was not observed in any of the control samples. Representative examples of the methylation analysis are shown in Fig. 1.

Association between *RASSF1A* and *p16* methylation status and clinicopathological parameters, *IL28B* genotype and the response to therapy

The results of analyses between the *RASSF1A* and *p16* methylation status and baseline characteristics of patients with CHC infection genotype 1b are summarized in Table 2. There was no correlation between the methylation statuses of either the *RASSF1A* or *p16* gene and the examined parameters, including patient age, gender and stage of liver fibrosis. However, a significant association between promoter methylation of *RASSF1A* and the CC genotype of *IL28B* ($p = 0.024$, Fisher's exact two-tailed test; Table 2) was observed. A significant association between promoter methylation of *RASSF1A* and a higher HCV RNA load (≥400 000 IU/ml, P=0.009, χ^2 test; Table 2) was noted. Such associations regarding the methylation status of the *p16* gene were not found.

The methylation status of *RASSF1A* and *p16* considered individually was not related to the response to PEG-IFN/RBV therapy. However, when we compared

Table 2. Methylation status of *RASSF1A* and *p16* genes according to *IL28B* rs12979860 polymorphism distribution and baseline characteristics of patients with chronic hepatitis C infection genotype 1b

Clinical and pathological characteristics of patients	Methylation status of *RASSF1A* gene		P	Methylation status of *p16* gene			P
	Methylated	Unmethylated		Methylated	Unmethylated		
Age (Years) †	43.438 ± 13.059	44.788 ± 11.401	0.712	44.429 ± 10.646	44.314 ± 12.442		0.976
Gender							
Male	11/29 (37.9%)	18/29 (62.1%)	0.523	8/29 (27.6%)		21/29 (72.4%)	0.890
Female	5/20(25%)	15/20 (75%)		6/20 (30%)		14/20 (70%)	
Stages of fibrosis ‡							
F0 - F2	9/27 (33.3%)	18/27 (66.7%)	0.846	8/27 (29.6%)		19/27 (70.4%)	0.892
F3 - F4	7/22 (31.8%)	15/22 (68.2%)		6/22 (27.3%)		16/22 (72.7%)	
Therapy outcome							
Non-responders (NR)	6/28 (21.4%)	22/28 (78.6%)	0.104	7/28 (25%)		21/28 (75%)	0.749
Sustained virologic responders (SVR)	10/21 (47.6%)	11/21 (52.4%)		7/21(33.3%)		14/21 (66.7%)	
***IL28B* rs12979860 polymorphism**§							
CC	13/26 (50%)	13/26(50%)	0.024*	7/26 (26.9%)		19/26 (73.1%)	0.875
CT/TT	3/21 (14.3%)	18/21 (85.7%)		7/21 (33.3%)		14/21 (66.7%)	
HCV RNA load (IU/ml) ¶							
< 400,000	9/25 (36%)	16/25 (64%)	0.009*	8/25 (32%)		17/25 (68%)	0.607
≥ 400,000	15/20 (75%)	5/20 (25%)		5/20 (25%)		15/20 (75%)	

† Data expressed as the mean±SD; ‡ Stage of fibrosis expressed by the METAVIR score (fibrosis 0-2 and 3-4); § Data are missing for two patients for the given parameter; HCV – hepatitis C virus; * Statistically significant. Statistical tests that were used are Student's t-test, Mann-Whitney U test, Pearson's χ^2 test, Fisher's exact two-tailed test when the expected frequencies were lower than five.

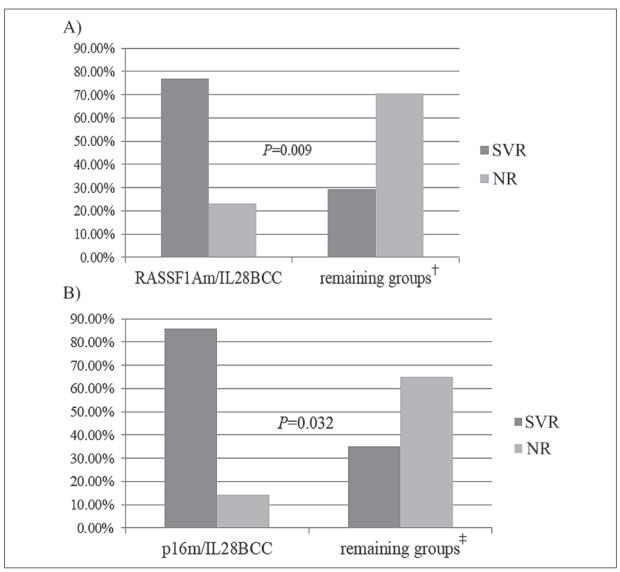

Fig. 2. Correlation of *RASSF1A* and *p16* methylation status, *IL28B* genotype and response to therapy. **A** – Impact of *RASSF1A* methylation and *IL28B* genotype on therapy response. † Remaining groups are *IL28B* CT,TT/*RASSF1A*n; *IL28B* CT,TT/*RASSF1A*m and *IL28B* CC/*RASSF1A*n. **B** – Impact of *p16* methylation and *IL28B* genotype on therapy response. SVR – sustained virologic responders; NR – non-responders. ‡ Remaining groups are IL28B CT,TT/*p16*n; IL28B CT,TT/*p16*m and IL28B CC/*p16*n. All P-values were obtained with Fisher's exact two-tailed test. After the Bonferroni correction, only the association of *IL28B* CC/*RASSF1A*m and therapy response remained significant (P<0.0125).

the groups of patients with both methylated (*p16m/RASSF1Am*) and both unmethylated genes (*p16u/RASSF1Au*), we noticed that all patients in the NR group carried *p16u/RASSF1Au* (15/15), while all three patients (3/3) with *p16m/RASSF1Am* belonged to the SVR group. Nevertheless, this result did not reach a statistically significant level (P= 0.052, Fisher's exact test).

In further analysis, we explored if there was an association between the methylation status of each gene in combination with *IL28B* rs12979860 and the response to the antiviral therapy. We observed that in the group of patients with the simultaneous presence of methylated *RASSF1A* and *IL28B* CC genotype (*IL28BCC/RASSF1Am*), SVR had been achieved in 76.9% (10/13) of instances, and in all other patients in only 29.4% (10/34) of cases. Similarly, in the group of patients with a concurrent presence of *p16* methylation and the *IL28B* CC genotype (*IL28BCC/p16m*), SVR was observed in 85.7% (6/7) of the cases, and in 35% (14/40) of the remaining patients. The observed results were statistically significant for both genes (P=0.009 and P=0.032, respectively, Fisher's exact two-tailed test; Fig. 2). However, after performing the Bonferroni correction, only the result regarding the *RASSF1A* gene remained significant (P<0.0125).

In a more comprehensive analysis, we observed that in patients with advanced fibrosis who carried the CT/TT *IL28B* genotype, the *RASSF1A* gene was exclusively unmethylated (10/10, 100%; Supplementary Table S2) and 90% (9/10) of these patients were NR (Supplementary Table S3). On the other hand, the distribution of *RASSF1A* methylation among patients with the CC *IL28B* genotype and their response to the antiviral therapy was similar, irrespective of the stage of fibrosis. Thus, in the subgroup with mild or no fibrosis, 83.3% (5/6) of patients had SVR, while 71.4% (5/7) of the patients with severe fibrosis had SVR (Supplementary Table S3). These results were not statistically processed because of the small number of samples in each group.

DISCUSSION

In patients with CHC, both viral and host genetic and epigenetic factors can influence the response to therapy and progression of the liver disease [7,8]. One of the strongest host genome predictors of SVR for PEG-IFN/RBV treatment is the CC genotype of the *IL28B* gene, and our results are in agreement with the findings of other authors in different populations [9,10,30,31]. However, recent reports have shown that SVR does not eliminate the risk of HCC occurrence and that HCV-related epigenetic changes can persist even after treatment with antiviral therapy [6,32,33].

Previous research has revealed the role of HCV infection in accelerating promoter methylation of genes implicated in HCC development and response to treatment in CHC patients [8,12]. As methylation changes occur early in HCV infection and gradually increase during liver disease progression, they could also predict the risk of HCC.

RASSF1A and *p16* are two tumor suppressor genes often methylated in different stages of fibrosis, cirrhosis and HCC, but not in normal liver [8,14-17,19,34,35]. However, their clinical relevance is not fully understood. We assessed the methylation status of these genes in a group of patients with genotype 1b and their possible relation to host and viral factors, and the response to PEG-IFN/RBV therapy.

Aberrant methylation of *RASSF1A* and *p16* was detected in 32.6% and 28.6% of the cases, respectively. The observed frequencies of methylation in our study fit into the percentages reported in previous studies, which range from 16.2% to 68.4% for *RASSF1A* [17,36,37] and from 23.5% to 62% for the *p16* gene [34,37], depending on the stage of the disease. Existing literature data indicate that the methylation of *p16* and *RASSF1A* gradually increases with disease progression, with the highest levels detected in HCC [8,18,36-39]. The methylation status of *RASSF1A* or *p16* was not related to host factors, such as fibrosis, age and gender of patients. According to previous studies, it was reported that the methylation of both *RASSF1A* and *p16* gene is associated with mild fibrosis [37], while such an association was observed for *RASSF1A* promoter methylation only [8]. The discrepancies in our and these results could be attributed to the small sample size (as in our study), or to different HCV genotypes that were analyzed, since both research groups analyzed genotype 4.

We observed that methylation of the *RASSF1A* gene was significantly more prevalent in patients with the CC genotype of the *IL28B* gene and associated with an HCV RNA load that was higher than 400,000 IU/mL. To the best of our knowledge, there are no other reports about such associations, hence our findings need further investigation. At the beginning of the research, 800,000 IU/mL of viremia represented a cut-off between a low and high HCV viral load [40]. However, subsequent research established that an initial HCV RNA concentration of 400,000 IU/mL is a cut-off point between low and high viremia in genotype 1b, which is also associated with the response to therapy [41,42], albeit not in ours. It is possible that the higher viral load induced higher methylation rates through dysregulation of the methylation machinery and indirectly, through proliferative stimuli associated with inflammation [36].

We did not observe the association of *RASSF1A* and *p16* promoter methylation in the response to PEG-IFN/RBV therapy when these two genes were analyzed separately. However, the simultaneous occurrence of either *RASSF1A* or *p16* methylation with the CC *IL28B* genotype was strongly associated with SVR, although only the result for *RASSF1A* remained significant after performing corrections for multiple comparisons. Therefore, the role of *p16* should be further examined on a larger sample size. The predictive role of DNA methylation profiles of specific genes in

CHC patients has not been well studied with only a few studies [8,12,37,43]. Existing literature data suggest that methylation of the *RASSF1A* and *p16* genes was significantly related to the NR group of patients [8,37]. This discrepancy in our results could be explained in part by the fact that both groups of authors examined patients with HCV genotype 4, and it is well known that virus genotype can affect the response to antiviral therapy. In addition, some studies have reported geographic variations and race characteristics in the methylation pattern of multiple genes involved in HCC, which should be also taken into account [44].

To the best of our knowledge, this is the first study to analyze the association between SNP rs12979860 and the methylation status of *RASSF1A* and *p16* and their simultaneous impact on therapy. Literature data suggest that *RASSF1A* and *IL28B* could be related, in part, through the regulation of interleukin-6 (*IL-6)* gene expression. IL-6 is a multifunctional cytokine implicated in the regulation of a wide range of activities, including inflammation, hematopoiesis and carcinogenesis [45]. Increased IL-6 expression is related to unfavorable clinical outcomes in HCV patients and is also implicated in the progression of different cancer types, including HCC [46,47]. While it was shown that CHC patients with the *IL28B* CC genotype have significantly increased concentration of IL6 [48], it was reported that *RASSF1A* induces IL-6 expression in A375 melanoma cells [49]. Thus, we can speculate that the absence or decreased *RASSF1A* expression because of *RASSF1A* promoter methylation could possibly lead to reduced IL6 expression as well. However, this assumption needs to be verified.

On the other hand, we demonstrated that in patients with advanced fibrosis who carried the CT/TT *IL28B* genotype, *RASSF1A* was exclusively unmethylated, while the distribution of *RASSF1A* methylation among patients with the CC *IL28B* genotype was similar, irrespective of the stage of fibrosis. This finding supports our hypothesis that there could be some association between the *IL28B* genotype and *RASSF1A* methylation. However, additional studies on a larger sample size are needed to confirm our findings.

Several studies have shown that methylation changes can be detected in tissue as well as in the blood [12,20,21]. Therefore, the methylation changes of circulating-free DNA isolated from serum or plasma could be preferably used as noninvasive prognostic or diagnostic biological markers. The limitation of the present study is that the assessment of the methylation status of *RASSF1A* and *p16* in liver tissue and its correlation with plasma levels was not available.

In conclusion, defining reliable molecular markers that will effectively predict the response to IFN-based therapy and the risk of HCC development would be of great clinical interest for patients chronically infected with HCV genotype 1b. According to the results of our study, the potential predictive role of *RASSF1A* and *p16* methylation deserves further investigation.

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Author contributions: Nikola Kokanov: experiments and statistical analysis, writing the manuscript; Milena Krajnović: conception and design of the study, data interpretation, writing and revision of the manuscript; Snežana Jovanović-Ćupić: conducting the experiments, data and sample acquisition, revision of the manuscript; Bojana Kožik: statistical analysis; Nina Petrović: conducting the experiments, Ana Božović: data analysis; Vesna Mandušić: revision of the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

Data availability: Data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kokanov%20et%20al_Data%20Report.pdf

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Supplementary Material

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kokanov%20et%20al_Supplementary%20Material.pdf