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SEQUENCE VARIABILITY AT THE INTERNAL RIBOSOME ENTRY SITE OF THE HCV GENOME IN RELATION TO THERAPY OUTCOME

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Abstract — Different types of interferon are widely used to treat hepatitis C virus (HCV) infection. Results obtained *in vitro* suggest that interferon inhibits internal ribosome entry site (IRES)-mediated translation of the HCV genome. To elucidate the possible effect of the nucleotide sequence of IRES on therapy outcome, we compared HCV isolates from patients with sustained response and non-response to interferon/ribavirin combination therapy. In 56 analyzed HCV isolates, nucleotide changes appeared strictly in the stem-loop IIIb region, the stem part from 243 nt to 248 nt, and the polypyrimidine-II region. The natural sequence variability of IRES in isolates of genotype 3a was significantly higher than in isolates of genotype 1b ($p < 0.05$). The average number of nucleotide changes in genotype 3a correlated with response to therapy ($p < 0.05$).

Key words: Hepatitis C virus, interferon, internal ribosome entry site, genotypes 1b and 3a

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INTRODUCTION

Hepatitis C virus (HCV) causes persistent chronic infection in ≈ 80% of cases, often leading to liver cirrhosis and hepatocellular carcinoma. The chronicity of HCV infection is mainly a consequence of its considerable genetic heterogeneity (Pawlotsky, 2003). Hepatitis C virus has an enveloped single-strand positive-sense RNA genome of approximately 9600 nucleotides in length. Phylogenetic analysis of different HCV genomes has identified six major types with about 30% of nucleotide differences (named 1, 2, 3…) and many different subtypes with about 20% of nucleotide differences (named a, b, c…). Like other RNA viruses, HCV exists in a single individual as a genetically heterogeneous pool of distinct yet closely related variants referred to as quasi-species. Isolates from one infected person contain many different quasi-species with one or two predominant variants and several minor variants present in very low frequencies (Soler et al., 2002; Pawlotsky, 2003).

It is known that this HCV diversity is reflected in the range of responses to interferon (IFN) therapy. The genotype is one of the predictive parameters currently used to define the antiviral treatment strategy and the chance of therapeutic success (Zein, 2000; Wohnsland et al., 2007). Application of the presently most effective combined therapy, pegilated interferon α-2a (PEG-INF)/ribavirin, revealed that patients with genotypes 2 and 3 respond more promptly, require shorter duration of treatment, and have twice the frequency of sustained virological response as patients with genotypes 1 and 4 (Zein, 2000). In addition, numerous results indicate the potential impact of viral genetic polymorphism on the outcome of antiviral therapy in patients infected by the same HCV genotype (Soler et al., 2002).

Variability of the HCV genome is not uniform, due to the presence of conservative and hypervariable regions. The most conserved parts, the 5' and 3' non-translated regions (NTR), flank the open reading frame at each end and contain signals required for replication (Zein, 2000; Pawlotsky, 2003). Moreover, translation of the HCV is regulated by the highly conserved region, named the "internal ribosome entry site" or IRES, which encompasses the 5' NTR and the first part of the core gene, spanning the genome sequence 40-370 nt. The particular structure of the IRES enables initiation of translation in HCV in a cap-independent manner. The IRES sequence folds into a complex structure grouped in four distinct stem-loop domains: SLI to SLIV (Kieft et al., 2001). The HCV IRES is unique among the viral IRESs, since it is able to bind to the 40S ribosomal subunit without involving most canonical translation initiation factors and requires only the eIF2-GTP-Met-tRNA complex for interaction (Kieft et al., 2001).

Even though it is the most highly conserved part of the virus genome, the 5' NTR shows quasi-species distribution with minor variants in its sequence (Buratti et al., 1997; Soler et al., 2002; van Leeuwen et al., 2004). Nucleotide changes in the IRES are single nucleotide polymorphisms, including substitution and insertion/deletion, and occur in the 5'NTR in a small percentage of nucleotides and in the core gene in about 15% of nucleotides (Pawlotsky, 2003; van Leeuwen et al., 2004). *In vitro* experiments and *in silico* predicted IRES RNA structures suggest that some of these small nucleotide changes could have dramatic effects on translation efficiency (Buratti et al., 1997; van Leeuwen et al., 2004). Additionally, these nucleotide changes can influence IRES interactions with some non-canonical trans-acting eukaryotic cell factors included in the internal initiation of HCV RNA translation, like La auto antigen, polypyrimidine (Py) tract binding protein (PTB), and poly(rC)-binding protein 2 (Spangberg and Schwart, 1999; Kieft et al., 2001).

Some new results indicate that INFs inhibit HCV translation not only over double-stranded RNA-activated protein kinase and oligoadenylate synthetase, but also by suppression of the HCV IRES function, even though selective inhibition of IRES-mediated translation of viral polyprotein is a general mechanism by which IFNs inhibit HCV replication (Hazari et al., 2005). In view of these data, it is proposed that some differences in HCV IRES sequences could influence the efficacy of INF therapy, but consistent results have not yet been obtained (Yamamoto et al., 1997; Suzuki et al., 1998; Thélu et al., 2004). In this investigation, we examined nucleotide changes in the SLII and SLIII part of the IRES in relation to PEG-INF/ribavirin therapy response. Concerning the influence of other factors on the response to therapy, we included in the analyses the main clinical characteristics of chronic infection in our patients (age, sex, ALT level, stage of fibrosis, and HCV RNA level).

MATERIAL AND METHODS

Sixty patients with chronic HCV infection were registered randomly in the Clinical Center of Serbia and the Military Medical Academy in Belgrade between January 2003 and June 2005. All patients received PEG-INF (180 μg/week) plus ribavirin (800 mg/d) for 24 weeks (HCV genotype 2 and 3) or PEG-INF (180 μg/week) plus ribavirin (1000 mg/d, if their body weight was < 75 kg, or 1200 mg/d, if it was ≥ 75 kg) for 48 weeks in patients with HCV genotypes 1 or 4. Approval from the Ethical Review Committee of Clinical Center of Serbia was obtained to conduct the study, and written informed consent was obtained from all patients. Serum RNA-HCV was detected and the concentration determined with the Amplicor HCV Monitor 2.0 according to the manufacturer's instructions (Roche Diagnostics, Germany). Histological findings were evaluated according to the stage of fibrosis (F0-F3) and cirrhosis 4 by METAVIR score. A sustained virological response (SR) was defined as the absence of HCV RNA in serum as determined by the COBAS Amplicor HCV qualitative test 2.0 (Roche Diagnostics, Germany) 6 months after the end of treatment. Non-response (NR) was defined as the presence of serum HCV RNA 6 months after the end of treatment.

Isolates of the HCV genome were prepared from the patients a month before starting therapy.

Ribonucleic acid was extracted from 100 µL of serum using a TRIzol kit (BRL Life Technologies, Gaithersburg, MD) and dissolved in 25 µL of RNase-free water. The selected part of the IRES sequence was amplified by RT-PCR (QIAGEN One Step RT-PCR Kit, Germany) with primers IR-F (*ggcgacactccaccatagat*) and IR-R (*cacggtctacgagacctccc*), which covered 319 nt. We carried out RT-PCR for 35 cycles (reverse transcription at 50°C for 30 min and 95°C for 15 min), followed by amplification at 94°C for 45 s, 56°C for 45 s, and 72°C for 60 s. In the second PCR, the same primers were used as in RT-PCR, with 0.5 U/reaction Platinum *Taq* polymerase (Invitrogene, Brazil), and the reaction ran for 28 cycles at 95°C for 3 min, 94°C for 45 s, 58°C for 30 s, and 72°C for 60 s (Applied Biosystems Gene Amp® PCR System 2700). Before starting the second PCR, uracil N-glycosylase (UNG, Fermentas, Lithuania) treatment was performed to eliminate carry-over contamination. This was done by incubating the PCR mix with 15 U of UNG/reaction for 15 min at 37°C, followed by inhibition of UNG for 5 min at 95°C. The final PCR products were analyzed electrophoretically by polyacrylamide gel electrophoresis and visualized with silver nitrate. The total PCR product was directly sequenced in both directions using the BigDye Terminator v. 3.1 Cycle sequencing kit with an ABI 3730 DNA automatic sequencer (Applied Biosystems and MWG-Biotech AG, Ebersberg, Germany). By this means, we obtained the sequence of the most frequent or predominant quasi-species in each isolate. In order to screen for nucleotide changes in the complete SLII and SLIII regions of the IRES, the nucleotide sequence of 278 nt was analyzed (39 nt to 317 nt, excluding the primer regions).

RESULTS AND DISCUSSION

Within the analyzed group, 34 patients were infected with genotype 1b (19 patients were SR and 15 patients were NR) and 22 patients with genotype 3a (17 patients were SR and five patients were NR). Three patients infected with genotype 4 and one patient with genotype 2a were excluded from the analysis due to the small number for comparison. Variability of the IRES nucleotide sequence was evaluated in relation to the consensus isolate, which was the most frequent in our isolates and in the GenBank database (Fig. 1).

In our analyzed group, 26 (46%) isolates showed variations. Thus, 15 isolates exhibited one nucleotide change, while nine isolates had two or three nucleotide changes (Fig. 1). Genotype 3a had double the average number of nucleotide changes per isolate compared with genotype 1b (1.05 ± 1.0 *vs.* 0.4 ± 0.7, P = 0.01). This difference could result from slower genetic evolution of HCV genotype 1b, as indicated by a significant decrease in its transmission due to systematic blood and blood product screening. In contrast, the current lack of control of HCV epidemics among intravenous drug users is associated with rapid ongoing genetic evolution of HCV genotypes 1a and 3a, related to their spread (Pawlotsky, 2003; Morice et al., 2006).

Nucleotide substitutions in the right-hand part of sub-domain IIIb were the most frequent in our isolates (19/56). Nucleotide changes G203A and C204T in genotype 3a appeared in 14 out of 22 isolates, and variability in these sites contributed considerably to the general genotype 3a variability (Fig. 1). The A203 change appeared in 66% of sequences and T204 in 16% of sequences of genotype 3a in the GenBank database (first 100 BLAST hits). In genotype 1b in five isolates, sub-domain IIIb showed substitutions C204T and C204A. In the GenBank database of genotype 1b, T204 occurred in 35% of cases and A204 was very rare (2%, first 100 BLAST hits). The sub-domain IIIb regulates the recruitment of eIF3 and Met-tRNS-eIF2, so the substitutions in this sub-domain may have an effect on viral translation efficiency (Buratti et al., 1997; Kieft et al., 2001).

Considering that initiation of protein translation may be one factor affecting the level of HCV-RNA, we compared this clinical characteristic in the groups with and without nucleotide changes in HCV isolates. In the group with genotype 1b, the HCV RNA level was 16.9 ± 31.3 x 10^5 IU/mL (without nucleotide changes) and 9.4 ± 8.7 x 10^5 IU/mL (with nucleotide changes). In the group with genotype 3a, the HCV RNA level was 9.2 ± 12.9 x 10^5

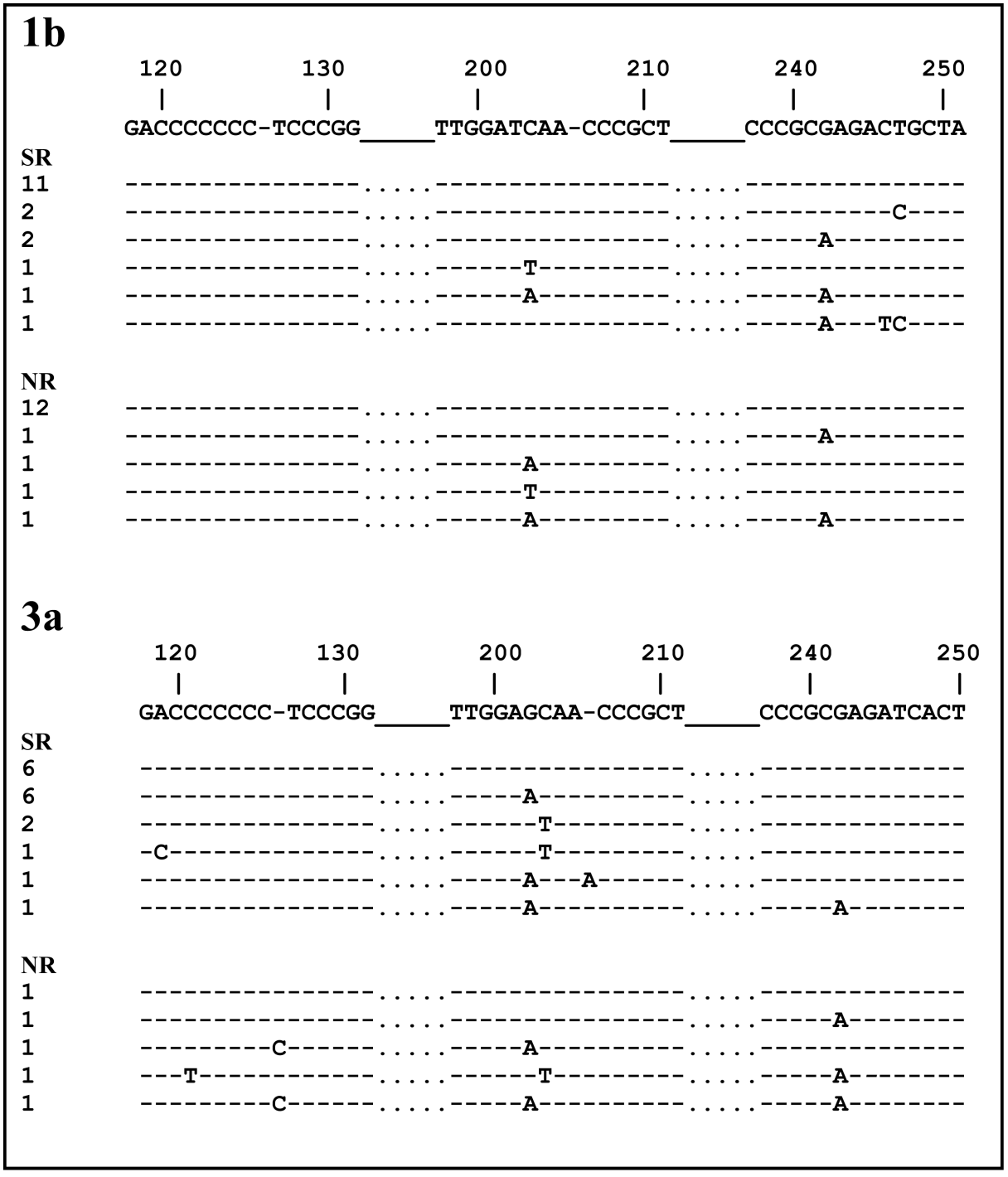

Fig. 1. Alignment of examined IRES HCV sequences from 56 isolates. The sequences are compared to prototype HC-J4 (AF054250) for genotype 1b and NZL1 (D17763) for genotype 3a (www.ncbi.nlm.nih.gov/Genbank). The short lines indicate identity with the prototype HCV sequences. Dots indicate larger regions without any nucleotide changes (130-200 nt and 210-240 nt). The abbreviation SR stands for sustained responder, NR for non-responder. Numbers below SR or NR indicate overall numbers of detected isolates with the given sequence. The shown nucleotide sequences are deposited in the GenBank database (www.ncbi.nlm.nih.gov/Genbank) under accession numbers for genotype 1b: EU164929, EU164966, and EU164931 to EU 164964; for genotype 3a: EU164930, EU164965, and EF193864 to EF193883.

Table 1. Clinical characteristics of patients and nucleotide variation of IRES in relation to therapy response. Categorical data were compared by the χ^2 and Fisher's exact probability test. a) Data expressed as mean ± SD; b) Stage of fibrosis expressed by METAVIR score (fibrosis 0, 1, 2, 3, and cirrhosis 4); c) expressed HCV RNA level x 105 IU/mL; NC - nucleotide changes; ns - not statistically significant.

	Genotype 1b (N = 34)				Genotype 3a (N = 22)				
Clinical characteristics of patients	Sustained responders (N = 18)	Non-responders (N = 16)	MEAN±SD	p	Sustained responders (N = 17)	Non-responders (N = 5)	MEAN±SD	p	P (1b/3a)
Age (years) [a]	45.39±9.80	45.88±13.72	45.62±11.62	ns	34.71±10.32	43.6±8.14	36.73±10.41	ns	0.005
Sex (male/female)	12/7	8/7	-	ns	13/4	5/0	-	ns	ns
ALT level [a]	84.6±34.8	101.2±47.4	92.4±41.4	ns	89.1±55.7	109.0±42.6	93.6±52.8	ns	ns
Stage of fibrosis [a, b]	2.4±1.4	2.4±1.6	2.4±1.5	ns	1.5±1.0	3.0±1.0	1.9±1.2	0.011	ns
HCV RNA level [a, c]	5.3±2.8	23.9±34.9	14.2±25.5	ns	8.1±10.1	9.9±13.4	8.5±10.5	ns	ns
Number of NC	0.6±0.9	0.3±0.6	0.4±0.7	ns	0.8±0.7	1.8±1.3	1.0±1.0	0.040	0.010

IU/mL (without nucleotide changes) and 7.0 ± 9.2 x 10^5 IU/mL (with nucleotide changes). Although we found large differences in the mean level of HCV-RNA between the two groups of patients, statistical significance was absent. Similarly, there were great differences in the HCV RNA level in relation to therapy response, but without statistical significance (Table 1). The very high variances within tested groups indicate that these analyses should be done in a larger group of patients.

We found that some parts of the IRES were conservative, suggesting great importance of these structures in its function. First of all, the entire SLII of the IRES sequence was monomorphic, which agrees with earlier results for prevalent quasi-species variants (Yamamoto et al., 1997; Suzuki et al., 1998). Also, we detected some absolutely conservative regions in the SLIII domain, which are contact points with eIF3 (160-167 nt), one of the major PTB protein-binding sites (Py-III region from 191-199 nt), and the four-way junction (SLIIIabc) structure. These are simultaneously involved in the recruitment of eIF3 and the 40S ribosomal subunit (Kieft et al., 2001). In addition, our findings showed that IRES contact points with the 40S ribosomal subunit were unaltered (128-138 nt, 145-156 nt, and 237-250 nt). Finally, these regions were conserved across all the standard genotypes of HCV (Buratti et al., 1997; Hazari et al., 2005). *In silico* results showed that the rare nucleotide changes which we detected in this region (G243A, CT247/8TC) did not have a particular effect on stem-loop III folding (Moratorio et al., 2007). Moreover, all other nucleotide changes detected in our isolates were located in single-stranded parts of the IRES (Py-II, sub-domain IIIb, and unpaired nucleotide G243). These results are consistent with other investigations concerning sequence variability of IRES, suggesting that maintaining the predicted secondary structures of IRES is an important prerequisite for survival advantage of the prevalent quasi-species in host cells (Suzuki et al., 1998; Thélu et al., 2004).

As in other investigated populations, some nucleotide changes in the IRES were exclusively observed in NR or SR patients (Magrin et al., 1996; Yamamoto et al., 1997; Suzuki et al., 1998; Yasmeen et al., 2003; Thélu et al., 2004; Moratorio et al., 2007). For example, we detected a single nucleotide polymorphism in the Py-II region in isolates of genotype 3a from NR patients (Fig. 1). Also, the transition TC247/8CT was found in genotype 1b and SR patients, while in other populations it occurred in solitary cases with a different response to therapy (Magrin et al., 1996; Yamamoto et al., 1997; Suzuki et al., 1998; Thélu et al., 2004). It is clear that the prevalence of these substitutions is too small to indicate any correlation with response to interferon therapy.

On the other hand, with regard to the average number of nucleotide changes between patient groups SR and NR, a significant difference appeared for genotype 3a (Table 1). *In vitro* results in the cell culture model suggest that selective inhibition of IRES-mediated translation of viral polyprotein is a general mechanism by which INF inhibits HCV replication. However, the mode of this inhibition and the influence of particular IRES primary and secondary structures is unknown so far (Buratti et al., 1997; Kieft et al., 2001; Kato et al., 2002; Hazari et al., 2005). Beyond that, some new results have shown different HCV quasi-species distribution during INF therapy (Thélu et al., 2007; Zekri et al., 2007). Thus, we can conclude that more nucleotide changes in NR than in SR patients found in genotype 3a could be important for the mechanism of INF action, but comparison of the prevalent quasi-species before and after therapy would be necessary in this kind of investigation.

Besides viral features, many factors regarding biochemical and histological characteristics of chronic HCV infection can influence the effects of antiviral therapy (Craxì et al., 1996; Magrin et al., 1996; Zein, 2000; Pawlotsky, 2003). When we divided the patients into SR and NR groups, no significant differences in the tested clinical parameters were obtained, except for stage of fibrosis in the group with genotype 3a (Table 1). However, in this type of investigation, we should consider that the clinical characteristics of HCV infection in some instances depend on the duration of infection. Since, the precise duration of HCV infection in most patients is not assessed, some authors use age as a surrogate for disease duration (Craxì et al., 1996; Magrin et al., 1996). In our population, the patients with genotype 1b were significantly older than those with genotype 3a ($p < 0.01$). This finding confirms previous results for patients from Serbia and other European countries, which indicates changes in the prevalence of HCV types according to age (Svirtlih et al., 2007). In addition, SR and NR patients with HCV 3a isolates differed in average age by approximately 10 years, but the range of ages was wide, from 17 to 55 years. These results suggest that the influence of viral determinants on INF action would be clearer in a more homogenous population of patients in relation to the main features of chronic inflammatory processes important for therapy success (Craxì et al., 1996; Magrin et al., 1996; Buratti et al., 1997; Wohnsland et al., 2007). This examination is in progress.

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ВАРИЈАБИЛНОСТ НУКЛЕОТИДНЕ СЕКВЕНЦЕ „УНУТРАШЊЕГ РИБОЗОМАЛНОГ УЛАЗНОГ МЕСТА“ У ГЕНОМУ ВИРУСА ХЕПАТИТИСА ТИПА С У ОДНОСУ НА УСПЕШНОСТ ТЕРАПИЈЕ

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Интерферонска терапија се данас најчешће користи у лечењу инфекције вирусом хепатитиса типа С (HCV). *Ин витро* резултати су показали да интерферон инхибира транслацију код овог вируса преко интеракције са делом генома који учествује у иницијацији транслације тзв. „унутрашње рибозомално улазно место“ (IRES). У овом раду смо испитивали нуклеотидне измене у IRES-у код изолата HCV-а

добијених из серума особа које су примале комбиновану терапију интерферон/рибавирин. У анализираној групи од 56 HCV изолата, нуклеотидне измене су утврђене у: IIIб петљи, регији између 243 нт и 248 нт и полипиримидин-II регији. Утврђена варијабилност IRES-а код изолата генотипа 3а значајно је већа у поређењу са изолатима генотипа 1б ($p < 0.05$). Просечан број нуклеотидних измена код изолата генотипа 3а је у корелацији са одговором на примењену терапију ($p < 0.05$).