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Assessment of Hepatitis C Virus Protein Sequences with Regard to Interferon/Ribavirin Combination Therapy Response in Patients with HCV Genotype 1b

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Abstract Hepatitis C virus (HCV) infection is a major and rising global health problem, affecting about 170 million people worldwide. The current standard of care treatment with interferon alpha and ribavirin in patients with the genotype 1 infection, the most frequent genotype in the USA and Western Europe, leads to a successful outcome in only about 50% of individuals. Accurate prediction of hepatitis C treatment response is of great benefit to patients and clinicians. The informational spectrum method, a virtual spectroscopy method for structure/function analysis of nucleotide and protein sequences, is applied here for the identification of the conserved information of the HCV proteins that correlate with the combination therapy outcome. Among the HCV proteins that we have analyzed the informational property of the p7 of HCV genotype 1b was best related to the therapy outcome. On the basis of these results, a simple bioinformatics criterion that could be useful in assessment of the response of HCV-infected patients to the combination therapy has been proposed.

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Keywords Hepatitis C virus · Bioinformatics · Informational spectrum method · Protein sequence · *In silico* HCV treatment response assessment

Abbreviations

CIS	Consensus informational spectrum
DFT	Discrete Fourier transform
EIIP	Electron-ion interaction potential
FFT	Fast Fourier transform
HCV	Hepatitis C virus
IS	Informational spectrum
ISM	Informational spectrum method
MR	Marked-responder
NR	Non-responder
PEG-IFN	Pegylated interferon
PR	Poor-responder
SVR	Sustained viral responder

1 Introduction

Chronic hepatitis C virus (HCV) infection with the estimated worldwide prevalence of 2.2% is a global health problem, affecting 170 million people worldwide with 3–4 million persons newly infected each year [4]. HCV has been reported as the main cause of chronic liver disease, cirrhosis, and liver cancer [16]. At present, the standard-of-care treatment for chronic HCV is pegylated interferon (PEG-IFN) and ribavirin [25]. Response to combination interferon/ribavirin therapy is genotype specific and the genotype 1, the most frequent genotype in the USA and Western Europe, is more resistant to treatment than other genotypes, with a sustained response rate of 50%, [13, 20, 44]. In addition, this long and costly therapy is associated with serious adverse effects [13, 20, 25]. Regardless of the progress being made toward

the development of less-toxic and more effective alternatives to the current standard-of-care therapy for chronic HCV, the high rate of HCV replication and the error-prone nature of the HCV polymerase leading to the emergence of drug resistant HCV variants, continue to be the key challenge to drug development [32, 44]. Therefore PEG-IFN is likely to remain the mainstay of treatment in the foreseeable future, whose limitations necessitate an accurate and simple pre-treatment prediction [32].

Multiple factors including clinical and epidemiological, host genetic and viral ones are connected with a response to the peginterferon and ribavirin combination therapy [12]. Genome viral sequence variation in the ORF and within the NS5A and the specific regions of the NS5A PKR-BD, ISDR, IRRDR and V3 has been reported to be related to the treatment response in patients with genotype 1 [8, 10, 11, 24, 26, 30, 31, 33, 45]. Several other studies have identified the correlation between diversity in the core and the outcome of the combination therapy [1, 8, 9]. Different genetic association with therapy response in the genotypes 1a and 1b provided evidence that a prognostic test needs to be subtype specific [8]. HCV variability has been documented across the entire genome [21]. Current molecular techniques allow fast and inexpensive detection of viral sequence variations and a simple *in silico* criterion for assessment of the effect of variations on biological function of proteins could be useful for analyzing HCV sequences.

In the present study, we carried out a bioinformatics analysis based on the informational spectrum method (ISM) of the pretreatment HCV genotype 1b sequences from the HCV infected patients enrolled in the viral resistance to antiviral therapy of chronic hepatitis C (Virahep-C) clinical study. The Virahep-C study has investigated the efficacy of the pegylated IFN-α plus ribavirin for treating the genotype 1 HCV [8]. The results of our analysis showed that the primary protein structure of the HCV 1b p7 encodes the specific and highly conserved information related to the response to IFN/ribavirin-based therapy. Based on these results, we propose a simple and efficient bioinformatics criterion that could be useful for the assessment of the response to the interferon/ribavirin HCV therapy in patients infected with HCV genotype 1b.

2 Materials and Methods

2.1 Sequences

The pretreatment HCV genotype 1b sequences, employed in this study, derived from the patients from the Virahep-C viral genetics study. The patients enrolled in the the Virahep-C study were treated with the peginterferon plus ribavirin for a maximum of 48 weeks. The therapy was discontinued for patients with a detectable viremia at 24 weeks due to the virological non response (NR). The patients with sustained virological response (SVR) had undetectable viremia 6 months post-treatment [8, 22].

The HCV genotype 1b sequences from the study by Donlin et al. [8], that were used as the training set, were retrieved from the GenBank database with the following accession numbers: [EF407458] to [EF407472]—pre-therapy serum samples with a marked response to therapy at day 28; [EF407473] to [EF407488]—pre-therapy serum samples with a poor response to therapy at day 28. Marked responders (MR) had a decline in HCV titers greater than 3.5 log10 or to undetectable between baseline and day 28 of therapy and poor responders (PR) had decline less than 1.4 log10 [2, 8]. The training set was created by extracting the following HCV 1b protein sequences: C; E1; E2; p7; NS2; NS3; NS4A; NS4B; NS5A, NS5B and ISDR and IRRDR in NS5A from the listed GenBank polyprotein sequences. The two discrete subsets for the responder and the non-responder group were created from all the investigated sequences from the Virahep-C data set. The pretreatment HCV sequences from day 28 were also re-grouped by treatment outcome (SVR or NR) [9]. Unpublished data about the therapy outcome for the sequences of patients were provided by Tavis JE, personal communication.

The HCV p7 sequences from the study of Mihm et al. [22] with the known outcome to pegylated IFN+ ribavirin treatment, that were used as the test set, were retrieved from the GenBank database with the following accession numbers: [AM263232], [AM263233], [AM263235], [AM263239], [AM263240], [AM263241], [AM263245], [AM263246], [AM263247], [AM263248], [AM263249], [AM263250], [AM263260], [AM263261], [AM263262], [AM263264], [AM263269], [AM263274], [AM263276], [AM263277], [AM263278], [AM263287], [AM263288], [AM263289].

2.2 Informational Spectrum Method (ISM)

The base of ISM was described in details elsewhere [7, 17, 36, 37], and here we will only in brief present this bioinformatics method. A sequence of N residues is represented as a linear array of N terms, with each term given a weight. The weight assigned to a residue is EIIP, determining electronic properties of amino acids and nucleotides, which are responsible for their intermolecular interactions. The EIIP can be determined for organic molecules by the following simple equation derived from the "general model pseudopotential" [34, 35]:

$$W = 0.25Z^* \sin(1.04\pi Z^*)/2\pi \quad (1)$$

where Z^* is the average quasi valence number (AQVN) determined by

$$Z^* = \sum {}^m n_i Z_i / N \quad (2)$$

where Z_i is the valence number of the *i*-th atomic component, n_i is the number of atoms of the *i*-th component, *m* is the number of atomic components in the molecule, and Nis the total number of atoms. The EIIP values calculated according to equations (1) and (2) are in Rydbergs (Ry). In this way the alphabetic code is transformed into a sequence of numbers. The obtained numerical sequence, representing the primary structure of protein, is then subjected to a discrete Fourier transformation, which is defined as follows:

$$X(n) = \sum x(m) e^{-j(2/N)nm}, n = 1, 2, \ldots, N/2 \quad (3)$$

where x(m) is the m-th member of a given numerical series, N is the total number of points in this series, and X(n) are discrete Fourier transformation coefficients. These coefficients describe the amplitude, phase and frequency of sinusoids, which comprised the original signal. The absolute value of a complex discrete Fourier transformation defines the amplitude spectrum and the phase spectrum. The complete information about the original sequence is contained in both spectral functions. However, in the case of protein analysis, relevant information is presented in an energy density spectrum [36, 37], which is defined as follows:

$$S(n) = X(n)X^*(n) = |X(n)|^2, n = 1, 2, \ldots, N/2. \quad (4)$$

In this way, sequences are analyzed as discrete signals. It is assumed that their points are equidistant with the distance d = 1. The maximal frequency in a spectrum defined as above is F = 1/2d = 0.5. The frequency range is independent of the total number of points in the sequence. The total number of points in a sequence influences only the resolution of the spectrum. The resolution of the N-point sequence is 1/n. The n-th point in the spectral function corresponds to a frequency f(n) = nf = n/N. Thus, the initial information defined by the sequence of amino acids can now be presented in the form of the informational spectrum (IS), representing the series of frequencies and their amplitudes.

The IS frequencies correspond to the distribution of structural motifs with defined physicochemical properties determining a biological function of a protein. When comparing proteins, which share the same biological or biochemical function, the ISM technique allows detection of code/frequency pairs which are specific for their common biological properties, or which correlate with their specific interaction. These common informational characteristics of sequences are determined by cross-spectrum or consensus informational spectrum (CIS). A CIS of N spectra is obtained by the following equation:

$$C(j) = \Pi S(i, j) \quad (5)$$

where Π(i,j) is the j-th element of the i-th power spectrum and C(j) is the j-th element of CIS. Thus, CIS is the Fourier transform of the correlation function for the spectrum. Thus, any spectral component (frequency) not present in all compared informational spectra is eliminated. Peak frequencies in CIS are common frequency components for the analyzed sequences. A measure of similarity for each peak is a signal-to-noise ratio (S/N), which represents a ratio between signal intensity at one particular IS frequency and the main value of the whole spectrum. If one calculates a CIS for a group of proteins, and finds strictly defined peak frequencies, it means that the primary structures of the analyzed proteins encode the common information which corresponds to their mutual interaction or to interaction with the common interactor [41].

For rapid calculation of the informational spectra the fast Fourier transform (FFT) instead the discrete Fourier transform (DFT) is used. The difference between FFT and DFT is that FFT can be applied only to the signals with an exact power of 2. Consequently, for cross-spectral analysis of sequences of different length the shorter sequences are extended with zeros to the length of the longest sequence before FFT computing. This pending operation may cause error in some peak frequencies. In order to avoid this problem the Blackman–Tukey method combined with the Hanning window was proposed [7]. This approach involves three steps: (1) calculation of the autocorrelation function of the original sequence, (2) Fourier transformation of this autocorrelation function, which gives the energy density spectrum of the original data, and (3) windowing to smooth the edges at the end of the sequence and to decrease problems arising from the zero pending. In our analysis we used FFT because each of analyzed groups of HCV proteins contains sequences of the identical length.

The ISM was successfully applied in structure–function analysis of different protein and DNA sequences, prediction of biological function of novel proteins, de novo design of biologically active peptides, assessment of biological effects of mutations, and identification of new therapeutic targets [40, 41].

2.3 Computational Peptide Scanning

Computer assisted scanning survey of the p7 primary structure with overlapping windows of different length revealed that the domain encompassing residues 42–63 is essential for the information represented by the frequency 0.0625.

2.4 Statistical Analysis

The significance of the difference between the amplitude values for MR and PR and SVR and NR was calculated by

the non-parametrical Mann–Whitney test [19], as sample sizes were relatively small. For the each comparison, the level of significance P is given. The test was two-tailed and P values <0.05 were considered significant.

The Mann–Whitney test investigates the difference between two datasets. Like any non parametric test, it does not depend on assumptions on the distribution of data; moreover, The Mann–Whitney test is more powerful comparing to t test in cases when the sample size is small. It can be considered the method of choice when analyzing small datasets, since it can save time, effort, and cost of having to increase the sample size [3].

3 Results and Discussion

3.1 The ISM Criterion for Assessment of Interferon/Ribavirin Combination Therapy Response in Patients with HCV Genotype 1b

The primary structure of proteins encodes the information represented by the IS frequencies that correspond to the protein biological function [41]. This information in highly variable viral proteins remains conserved despite the high rate of mutation [41–43]. Here, we performed the ISM analysis of the pre-therapy HCV genotype 1b protein sequences from the marked responder and the poor responder group in order to identify the conserved information which is related to the interferon/ribavirin combination therapy response.

To determine the IS frequencies characteristic for each of the ten HCV proteins and two NS5A domains, the multiple cross-spectral analysis of all the particular HCV molecules: C; E1; E2; p7; NS2; NS3; NS4A; NS4B; NS5A, NS5B and ISDR and IRRDR domains in the NS5A was performed. To study whether the biological function of the HCV proteins, represented by the characteristic frequencies, is related to the therapeutic outcome of the combined therapy, we compared the amplitude values of the dominant frequencies in the CIS of all the HCV proteins and the protein domains collected from the marked and the poor responders. The analysis revealed that the CIS of the analyzed C; E1; E2; p7; NS2; NS3; NS4A; NS4B; NS5A, NS5B and ISDR and IRRDR in NS5A molecules contains the dominant peak corresponding to the major common frequency components (F) 0.01953, 0.27734, 0.23047, 0.0625, 0.26172, 0.19043, 0.34375, 0.21094, 0.25781, 0.14160, 0.10938, 0.31250, respectively. This analysis revealed that the amplitude values corresponding to the characteristic frequency F(0.0625) in IS of p7 from HCV genotype 1b isolated from responders and non-responders are significantly different (P values < 0.05). CIS of the analyzed p7 molecules is presented in Fig. 1.

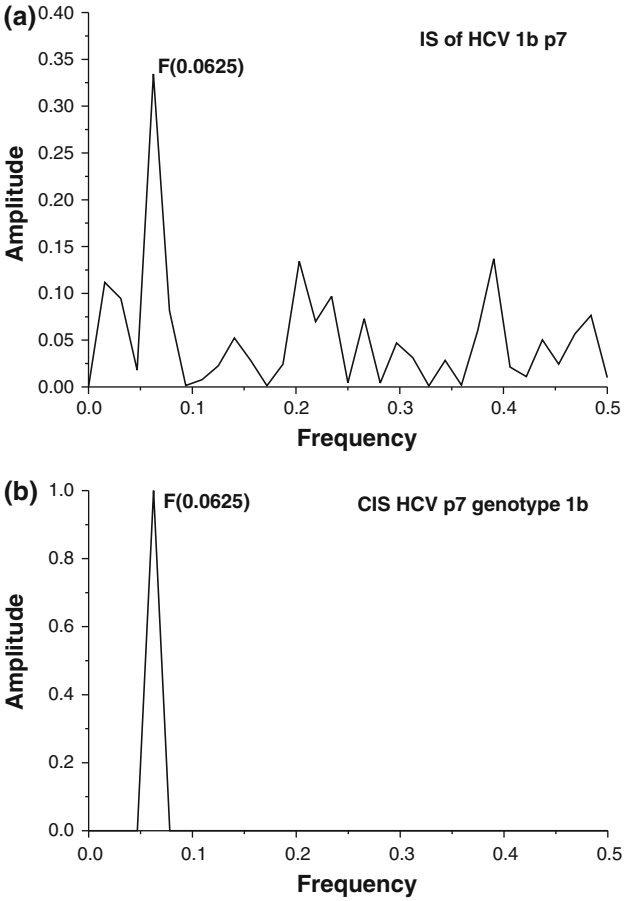

Fig. 1 **a** Informational spectrum of the HCV 1b p7 [EF407465], **b** Consensus spectrum of the HCV 1b p7. The prominent peak denote common frequency component. The abscissa represents ISM frequencies, the ordinates are normalized amplitudes corresponding to each of the frequency component

Before the start of the interferon/ribavirin therapy, it is important to assess the sensitivity of the virus to this combination therapy, in order to avoid application of this toxic treatment in patients infected with resistant HCV strains. For the analyzed group of HCV patients the amplitude on the characteristic frequency F(0.0625) in IS of the HCV 1b p7 is higher in resistant $\left(A^{R}_{mean} = 0.356 \pm 0.068\right)$ than in sensitive viruses $\left(A^{S}_{mean} = 0.299 \pm 0.073\right)$ (see Table 1). For discrimination of the responders from non-responders, we used the amplitude value $A_{F(0.0625)} = 0.272$, representing $A^{R}_{mean} - Sd^{R} - DSd^{R}$, where ΔSd^{R} is the confidence interval which is equal to $0.25\Delta Sd^{R}$.

The amplitude value below this threshold has 9 out of 15 (60%) marked responders and above this threshold 14 out of 15 (93.3%) poor responders on day 28. Further, applying this criterion to the end treatment response (Table 1; Fig. 2) demonstrated that 14 out of 15 (93.3%) non-responders have an amplitude above the threshold and 10 out of 15 (66.7%) sustained viral responders below the threshold. The

Table 1 The amplitudes values at the frequency F(0.0625) in informational spectrum of the p7 MR, SVR, PR and NR

Genbank accession number	Day 28 response	Treatment outcome (TO)	A[F(0.0625)]
EF407458	MR	SVR	+
EF407459	MR	SVR	−
EF407460	MR	SVR	+
EF407461	MR	SVR	−
EF407462	MR	SVR	−
EF407463	MR	SVR	−
EF407464	MR	SVR	+
EF407465	MR	NR	+
EF407466	MR	SVR	−
EF407467	MR	SVR	−
EF407468	MR	SVR	+
EF407469	MR	NR	+
EF407470	MR	SVR	−
EF407471	MR	SVR	−
EF407472	MR	SVR	−
EF407473	PR	NR	+
EF407474	PR	NR	−
EF407475	PR	NR	+
EF407476	PR	NR	+
EF407477	PR	NR	+
EF407478	PR	NR	+
EF407479	PR	SVR	−
EF407480	PR	SVR	+
EF407481	PR	NR	+
EF407482	PR	NR	+
EF407483	PR	NR	+
EF407484	PR	NR	+
EF407485	PR	NR	+
EF407487	PR	NR	+
EF407488	PR	NR	+

−, value of the amplitude at characteristic frequency is below threshold

+, value of the amplitude at characteristic frequency is above threshold

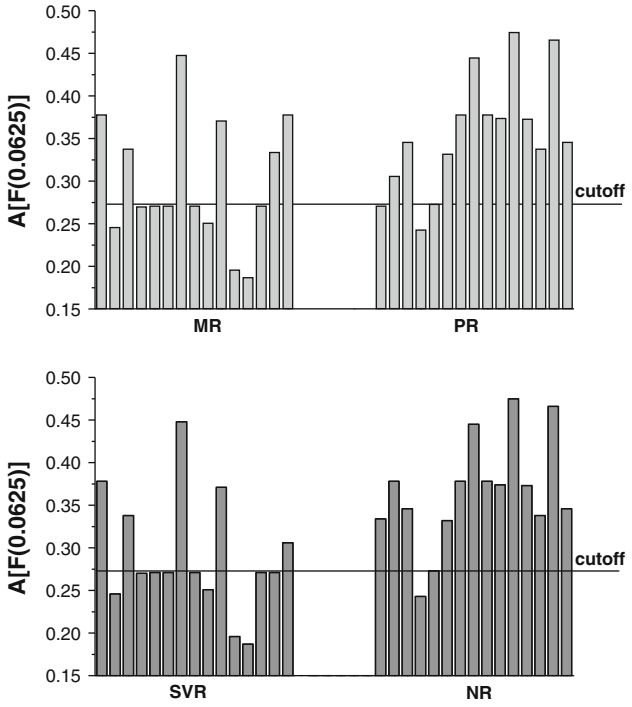

Fig. 2 HCV 1b p7 amplitude values at the F(0.0625) of the **a** MR and PR, **b** SVR and NR sequences

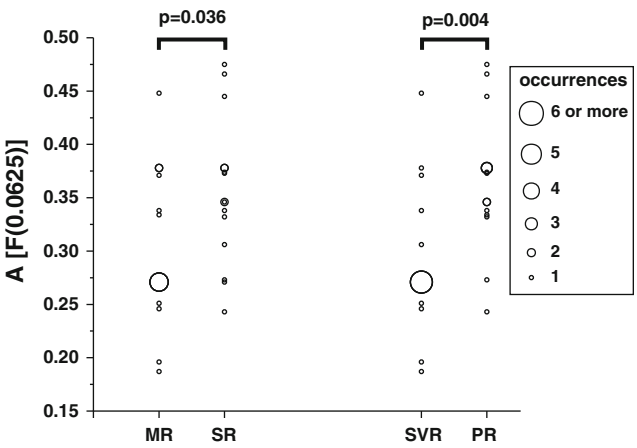

Fig. 3 HCV 1b p7 amplitude values at the F(0.0625) of MR, PR, SVR and NR sequences. Amplitude values were significantly higher in PR than in MR ($P = 0.036$) as well in NR compared to SVR sequences ($P = 0.004$)

comparison of the p7 amplitude values between MR and PR revealed that the difference between the analyzed groups were statistically significant ($P = 0.036$) as well as between SVR and NR subjects ($P = 0.004$), indicating a higher amplitude value in PR and NR subjects (Fig. 3).

Currently, there is no other openly available polyprotein sequence set with the known standard combination treatment outcome that could be used as a test set in order to affirm the findings of our study regarding all the HCV proteins. However, other sequence set of HCV p7 sequences from the study of Mihm et al. [22] was accessible. It is very important to stress that, thus far, these HCV 1b p7 sequences represent the only available other sequence set with the known standard combination of the IFN+ ribavirin treatment outcome.

To confirm our findings and postulate a bioinformatics criterion for evaluation pretreatment sequences, we analyzed a further 24 HCV 1b p7 pretreatment sequences of NR and SVR from the study of Mihm et al. [22] (Table 2). Analysis of the other set of the sequences showed that 8 out of 12 (66.7%) sequences from the non-responders have an

amplitude below the threshold and 8 out of 12 (66.7%) from the sustained viral responders below the threshold (Table 2). The differences between the amplitude values between the SVR and NR in the test set were not statistically significant.

Although the number of the tested sequences is not sufficient for absolute accuracy and validation of these observations in a larger cohort is required, the amplitude at frequency 0.0625 in IS of HCV p7 was useful for assessing the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b from the Virahep-C clinical study.

Actually, so far the p7 has not been documented as a significant viral determinant of resistance to interferon or combinatorial IFN/ribavirin treatment in HCV patients, although it was recognized that the non-responders with the HCV-1b infection showed higher numbers of non-conservative amino acid substitutions within the complete p7 protein and the TM2

Table 2 The amplitudes values at the frequency F(0.0625) in informational spectrum of the p7 from SVR and NR

Genbank accession number	Treatment outcome (TO)	A[F(0.0625)]
AM263232	SVR	+
AM263233	SVR	−
AM263235	SVR	+
AM263239	SVR	−
AM263240	SVR	−
AM263241	SVR	−
AM263245	SVR	+
AM263246	SVR	−
AM263247	SVR	−
AM263248	SVR	+
AM263249	SVR	−
AM263250	SVR	−
AM263260	NR	+
AM263261	NR	+
AM263262	NR	+
AM263264	NR	+
AM263269	NR	+
AM263274	NR	−
AM263276	NR	−
AM263277	NR	−
AM263278	NR	−
AM263287	NR	+
AM263288	NR	+
AM263289	NR	+

−, value of the amplitude at characteristic frequency is below threshold

+, value of the amplitude at characteristic frequency is above threshold

[22]. The p7 is essential for infection [29], although not necessary for RNA replication [18]. The p7 primarily localized to the endoplasmic reticulum as an integral membrane protein and displayed a topology with both the N- and the C-termini pointing toward the endoplasmic reticulum lumen [5]. The p7 comprised two trans-membrane domains separated by a short basic loop. The p7 homo-oligomerizes to form ion channels in cellular membranes and is also sensitive to several classes of inhibitor compounds [15, 27, 28]. Moreover, the p7 ion channel activity can be specifically inhibited by different drugs suggesting the p7 protein as a new target for future antiviral therapy.

The viral protein sequences that were analyzed in this bioinformatics study were identified in the Virahep C genetics study that was the part of the Virahep-C clinical study that besides clinical, immunological, host genetic, and interferon cell signaling factors examined the viral genetic parameters associated with the lack of response to treatment [6]. This study has found an association of low viral genetic diversity relative to the population-wide consensus sequence with a poor response to therapy [8, 9]. Furthermore, they have found that by applying covariance network analysis the result of the therapy could be predicted [2].

3.2 The Potential Functional Role of Information Represented by the Frequency (0.0625) in the IS of the HCV p7 Protein

The ISM, a bioinformatics approach has been previously used for the identification of the conserved information responsible for the interaction between the envelope glycoprotein gp120 of the human immunodeficiency virus type 1 (HIV-1) and their CD4, CCR5 and CXCR4 receptors, HA influenza/receptor interaction, LPL dimer formation [14, 38, 39, 43]. These findings suggest that the HCV p7 molecules also encode conserved information, important for its function.

The computer assisted peptide scanning of the HCV p7 was performed to identify regions of the p7 essential for information corresponding to the frequency F(0.0625). The p7 domain with maximal contribution to the IS frequency F(0.0625) is located in the transmembrane domain (TM2) of the p7 and the C terminal end of the p7 protein encompasses residues 42–63 of the p7 protein (Fig. 4). It was proposed that the residues present in the transmembrane alpha helices TM1 and TM2 are responsible for the stable formation of the oligomers [5]. In another study it was suggested that TM2 might be involved in the interaction of the p7 with other viral or cellular factors. In addition, it was proposed that the well-conserved polar segment 59–63 could also play a particular role in the p7 intermolecular interactions with other viral and/or cellular proteins [23].

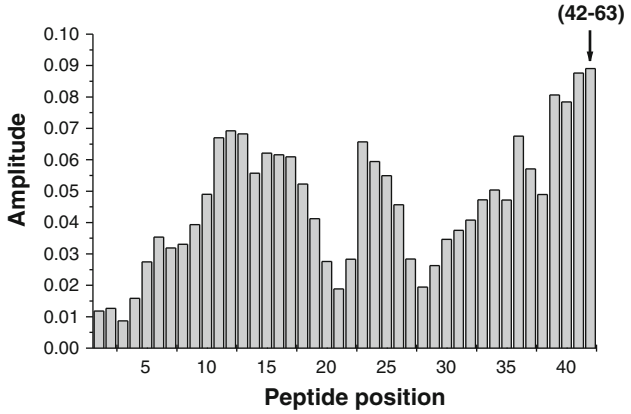

Fig. 4 Mapping of domains with maximal contribution to the frequency component F(0.0625) in the informational spectrum of HCV p7 1b

With respect to the assumption that the p7 amplitude is higher in NR sequences, it can be presumed that the conserved information, encoded in the primary structure, may have influence on the higher oligomer stability in NR and/or the likelihood of interaction of the p7 with the putative protein in molecular pathways linked with interferon/ribavirin treatment response.

The results presented here suggest further investigation of the role of p7 in the resistance to IFN based therapy.

4 Conclusion

In conclusion, the bioinformatics criterion presented here, the amplitude at the characteristic frequency F(0.0625) in the IS of the HCV p7 protein may be useful for assessment of the response of the HCV genotype 1b patients to the interferon/ribavirin combined therapy. Moreover, the ISM approach provides important predictive information regarding the response to therapy, complementary to other data of viral and host factors, recognized by other methods, and therefore it could improve accuracy of the combination therapy response prediction.

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

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