Interferon resistance of *hepatitis C virus* genotypes 1a/1b: relationship to structural E2 gene quasispecies mutations

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Abstract

Hepatitis C virus (HCV) envelope glycoprotein-2 (E2) inhibits the interferon (IFN)–induced, double –stranded RNA activated protein kinase (PKR) *via* PKR eukaryotic initiation factor-2α phosphorylation homology domain (PePHD). Present study examined the genetic variability of the PePHD in patients receiving interferon therapy. The PePHD region from HCV genotype 1a/1b infected patients receiving IFN was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and analyzed using bidirectionaly sequencing. The PePHD sequence was different in pretreatment isolates from three months treated patients. It was shown that the major PePHD quasispecies could change after three months IFN therapy and in one patient; the major PePHD quasispecies could change after six months IFN therapy. These mutations were occurred at codons 665, 666 and 667 of followed-up samples and at codons 660, 661, 666 and 670 of randomly treated patients. Some of these mutations were similar to those reported in previous studies. Other mutations were also detected in upstream and downstream regions of PePHD which may have influenced the structure, conformation and configuration of this region and thereby suppressing PePHD inhibitory properties. In conclusion our data suggested that *HCV* E2 PePHD may play an important role in determining the interferon response among Iranian *HCV* infected patients.

Keywords: Hepatitis C virus; E2 glycoprotein; PePHD region; IFN therapy; Treatment resistance.

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INTRODUCTION

Hepatitis C infection was first recognized as a separate disease entity in 1975 when the majority of cases of transfusion-associated hepatitis were found not to be caused by the only two hepatitis viruses recognized at the time*, hepatitis A virus* (*HAV*) and *hepatitis B virus* (*HBV*). The disease was called "non-A non-B hepatitis" and it was demonstrated to be transmissible to chimpanzees (Alexopoulou *et al*., 2005). It was not until 1989, however, the cloning and sequencing of the viral genome of the non-A non-B hepatitis virus was first reported and the virus was renamed "Hepatitis C virus" (HCV) (Choo *et al*., 1989).

HCV is one of the leading pathogens of chronic hepatitis (Egusa *et al*., 2005; Zein. 2000 ; Hnatyszyn *et al*., 2003) often results in liver cirrhosis or hepatocellular carcinoma (HCC) (Egusa *et al*., 2005 and De Mitri *et al*., 2000). Treatment of chronic hepatitis is an important clinical problem. Currently IFN-α alone or in combination with ribavirin and sometimes amantadin is the only available approved therapy for chronic hepatitis C (Helbling *et al*., 2002). Determination of the HCV genotype has become accepted as the standard procedure in laboratory practice. Genotype assignment helps in disease prognosis and assists in establishing the appropriate duration of treatment (Hofmann *et al*., 2005). Due to heterogenicity of *HCV* genome, more than 10 genotypes and 70 subtypes of *HCV* have been described so far (Simmonds, 2001).

Based on the chronic nature of *HCV* infection and the tremendous burden on healthcare resources, clinicians and laboratorians have looked for key epidemiological, pathological and viral characteristics that may provide insights into disease progression, severity and response to therapy for better administration of effective therapeutic regimens as well as long-term management of infected individuals. Determination of viral genotype has been identified as one parameter that could provide direction in the clinical management of patients with chronic *HCV* infection (Hnatyszyn *et al*., 2003; Hofmann *et al*., 2005). Such information has provided important clues for mechanism of IFN resistance of *HCV*.

Recently, Taylor *et al*., have reported that the *hepatitis C virus* envelope glycoprotein-2 inhibits the interferon (IFN)-induced, double-stranded RNA-activated protein kinase (PKR) via the PKR eukaryotic initiation factor-2 alpha phosphorylation homology domain (PePHD) in cell culture system (Polyak *et al*., 2000; Taylor *et al*., 1999 and 2000). This region (aa 659-670 in genotype 1) of glycoprotein E2 contains a stretch of amino acids that share a high degree of homology with the autophosphorylation site of PKR and phosphorylation site of its substrate eIF2α and can inhibits PKR activation and prevents translation shutoff mediated by IFN (Kato *et al*., 1990; Hosseini-Moghaddam *et al*., 2006; Voisset 2004; Uedaa *et al*., 2004). Greater homology was found between PKR and E2 GenBank sequences from *HCV* genotypes 1a and 1b than genotypes 2a, 2b and 3a. This finding is noteworthy, because *HCV*-1a and *HCV*-1b infections are typically more resistant to IFN therapy than *HCV*-2 and *HCV*-3 genotypes (Ralston *et al*., 1993). On the other hand, some groups have suggested negative results in relationship between PePHD and IFN efficacy in studied patients (Yang *et al*., 2003; Watanabe *et al*., 2003).

In this work, the PePHD sequence in randomly-selected 23 patient samples with *HCV* genotype 1a/1b infection whom treated with IFN-α were studied in order to asses the mutations variation in the PePHD sequence and analyz their effects on IFN therapy.

MATERIALS AND METHODS

Patients: Thirty-six *HCV* Iranian infected patients were enrolled in this survey whom originated from Iran. This gallery consisted of 25 males and 11 females with the mean age of 33.5 (ranging 12-67 years). Samples were collected in tubes containing EDTA, centrifuged at the collect site (Public Health Central Laboratory, Porto Alegre, RS) and sent immediately in a refrigerated container to the Amplicon Laboratory, Porto Alegre for HCV genotyping. All samples were stored at -70ºC until use. To avoid RNA degradation, aliquots were not thawed more than once prior to analysis. All patients were checked for *HCV*, human immunodeficiency virus (*HIV*) and *hepatitis B virus (HBV)* by commercial available ELISA kits (Organon Technica, Turnhout, Belgium). All patients were positive for anti-*HCV* and negative for HBsAg and *HIV* antibodies. Thirty patients were randomly opted for and studied cross-sectionaly. This sample size was more or less similar to other studies. Five patients were chosen to be monitored for 3 to 6 months after antiviral regime treatment. These patients received 6 MU of recombinant IFN-α 2b and ribavirin three times weekly. Other 25 patients received this treatment regime without following.

HCV genotype determination based on 5´-UTR region: *HCV* positive sera were collected and then kept frozen immediately at -70°C. HCV RNA was extracted by High Pure Viral RNA extraction Kit (Roche, Mannheim, Germany) in biosafety level II Laboratory and practice using biosafty level II biological cabinet. A First Strand cDNA (complementary deoxyribonucleic acid) Synthesis Kit (Fermentas, Vilnius, Lithuania) was utilized for cDNA synthesis using random hexamer primers according to manufacturer procedure. *HCV* 5´-UTR region was amplified through a nested-PCR strategy and subjected to determination of *HCV* genotypes using restriction fragment length polymorphism (RFLP) method (Phjanpelto *et al*., 1996; Pérez-Ruiz *et al*., 1997 and MH Ahmadi Pour *et al*., 2006). Each batch of samples was processed with negative controls (Healthy non-smokers individuals for HCV, HBV and HIV) and positive control containing plasmids with HCV 5´-UTR region in all steps.

Amplification and sequencing of the PePHD region of HCV E2 glycoprotein: Based on *HCV* PCR-RLFP genotyping results, two different sets of nested PCR primers for HCV genotypes 1a and 1b were designed in the conserved sequences of the PePHD region based on data available from the Los Alamos *HCV* Sequence

Table 1. Sequence, polarity and position of primer sets used for amplification and sequencing analysis.

Designation	Sequence 5´-3´	Polarity	Positions on genome
genotype 1a			
MP1	ACATACTCTCGGTGCGGCTC	Outer and sense	2121-2140
MP2	TGGTGGAGGTGGATGAGGC	Outer and antisense	2407-2425
MP3	GGATTACGCCCAGGTGCC	nner and sense	2149-2199
MP4	AGAACACGGAAGGACCTGCC	nner and antisense	2359-2378
genotype 1b			
HS1	AACACCTTGACCTGCCCCACGG	Outer and sense	2079-2100
HS2	CAGGGTGGTGAAGGAACAGGG	Outer and antisense	2376-2397
HS3	CCTGCCCCACGGATTGCTTC	Inner and sense	2089-2108
HS4	GATCGTGGACAGCAGCAGCG	nner and antisense	2341-2360

Primer positions were referred to accession number AF009606 and AB016785 for HCV 1a and 1b.

Database (Kuiken *et al*., 2005). The conserved PePHD primer sets were identified based on E2 genes of various *HCV* genotypes and subtypes by multiple alignments using ClustalX program, version 1.81 (Thompson *et al*., 1997). The list of primers and their characteristics are shown in Table 1. The nested-PCR was done using *Tag* DNA polymerase (Fermentas, Vilnius, Lithuania) then second-round amplicons were extracted and purified from agarose gel (CoreBio™, Seoul, South Korea) and sequenced in both directions by inner primers using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A) and ABI Prism® 3700 DNA analyzer sequencer (Applied Biosystems) at Sequence Laboratories Göttingen GmbH (SEQLAB) Germany.

Reference sequences and phylogenetic analysis: One hundred-twenty-four *HCV* PePHD sequences (genotypes 1 to 6) were used in this study as our references (Kuiken *et al*., 2005) and then the Iranian *HCV* PePHD sequences were compared to 124 defined HCV isolates. Alignment was performed using CLUSTAL X software, version 1.81 (Thompson *et al*., 1997). Genetic distance was estimated by the Kimura two-parameter matrix (Kimura, 1980). Phylogenetic tree was constructed by the neighbor-joining method (Saitou *et al*., 1987). Bootstrap re-sampling and reconstruction was performed for 1000 times to confirm the reliability of phylogenetic trees (Felsenstein, 1988). Phylogenetic and molecular evolutionary analysis and nucleotide differences within and between the isolate sequences were calculated by MEGA program, version 2.1 (Kumar *et al*., 2001).

RESULTS

HCV genotyping and phylogenetic tree: The 5´-UTR of 30 *HCV* positive serum samples out of 36 were successfully amplified and digested by appropriate restriction enzymes for genotype determination. The RFLP results revealed 84.4% subtype 1a (25/30) and 16.6% subtype 1b (5/30) in studied Iranian *HCV* infected individuals. The PePHD region of the known genotype samples was amplified thoroughly using designed subtype specific primers. Twenty-three out of 30 samples were sequenced and compared with PePHD reference gene sequences. The nucleotide sequences determined in this work were deposited in the GenBank database under the accession numbers DQ463136-DQ463152 and DQ463155-DQ463159 (genotype 1a), DQ463153 - DQ463154 (genotype 1b). The phylogenetic tree analysis based on twenty-three samples (232 bp) of the PePHD amplicon revealed that most of the Iranian *HCV* infected patients have segregated in the branch of the subtype 1a that supported by 100% bootstrap values (1,000 replicates). Two isolates, 209 and 222, were grouped in the HCV-1b branch with 97% bootstrap values. Interestingly, isolate 221 that showed to be *HCV*-1b by RFLP method, clustered in the 1a branch (Fig. 1). The mean percentage of the inter-genotypic distance for the Iranian isolates with other *HCV* genotypes and subtypes are shown in Table 2. Genetic divergenicity in samples from patients who did not receive treatment was 1.5% but in treated patients was 7% (Fig. 2).

Amino acid variations in PePHD region: In order to

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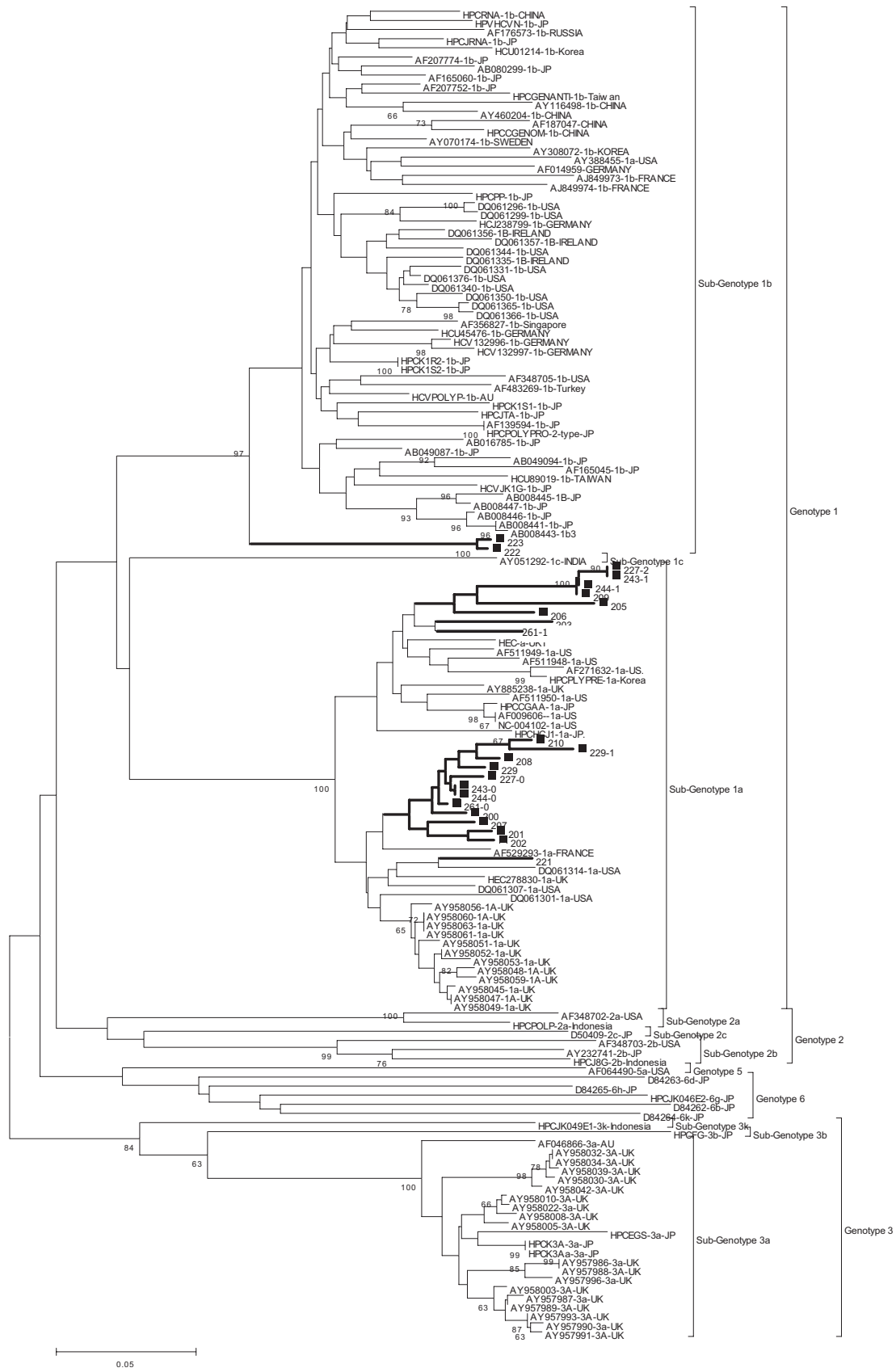
Figure 1. Tree was created by phylogenetic analysis based on Kimura two-parameter distance method followed by Clustal X Neighbor Joining for part of E2 sequence containing PePHD. Samples with treatment history branched in separate cluster from those who did not received therapy. The codes which contain (-0) at the end of themselves, belong to untreated samples.

Table 2. Comparative genetic divergenicity among HCV subtypes of a 230-nucleotide segment derived from the PePHD region of Iranian HCV isolates.

Genotype	IR-1a	IR-1b	1a	1b	1c	2	6	5	3a	3b	3k
IR-1a		[0.033]	[0.013]	[0.033]	[0.037]	[0.038]	[0.038]	[0.039]	[0.045]	[0.048]	[0.049]
IR-1b	0.772		[0.035]	[0.023]	[0.036]	[0.039]	[0.042]	[0.047]	[0.050]	[0.055]	[0.048]
1a	0.903	0.759		[0.031]	[0.036]	[0.037]	[0.036]	[0.038]	[0.044]	[0.049]	[0.048]
1b	0.725	0.836	0.743		[0.032]	[0.034]	[0.038]	[0.042]	[0.039]	[0.049]	[0.040]
1c	0.735	0.743	0.746	0.739		[0.037]	[0.039]	[0.041]	[0.043]	[0.052]	[0.043]
2	0.632	0.645	0.646	0.671	0.659		[0.037]	[0.043]	[0.043]	[0.043]	[0.039]
6	0.634	0.613	0.653	0.628	0.664	0.603		[0.037]	[0.043]	[0.043]	[0.043]
5	0.686	0.649	0.705	0.650	0.641	0.634	0.664		[0.053]	[0.052]	[0.057]
3a	0.596	0.376	0.615	0. 660	0.649	0.396	0.585	0.568		[0.039]	[0.041]
3b	0.563	0.563	0.590	0.534	0.589	0.392	0.590	0.594	0.717		[0.043]
3k	0.592	0.623	0.600	0.676	0.676	0.602	0.602	0.566	0.693	0.687	

analyze the relationship between the modification of the viral quasispecies and response to antiviral treatment, the pattern of *HCV* quasispecies was explored by sequence and amino acid alignment in samples obtained one week before and three months after IFN therapy (Fig. 3). The majority of three months treated samples showed replacement of amino acids in the C-terminal of PePHD region. In this study, five patients that showed *HCV*-1a genotype were followed before and after IFN therapy. Results of the analysis demonstrated that most of *HCV* E2 PePHD sequences of the followed patients (4/5) had amino acid variation(s). Figure 3 shows the relationship between PePHD amino acids before and during IFN plus ribavirin therapy (three months and in one case six months) in patients infected with *HCV*-1a. Four out of five had amino acid substitutions in their PePHD sequence. These 4 patients had one or two substitutions in the PePHD. One isolate (code 229) had substitutions at

L666P, L667A and three isolates (codes 227, 243 and 261) had a substitution at L665S.

Phylogenetic analysis results revealed 12 HCV-1a isolates and 2 HCV-1b isolates, while 7 out of 12 HCV-1a PePHD sequences from Iranian patients had the same sequence as in HCV-1a wild type reference isolates (Fig. 4). Five *HCV*-1a patient samples had substitution at S660A, E661D, L667A (in one sample), L667P (in two samples) and T670I. Two *HCV*-1b patient samples had substitution at T670I. Figure 2 depicts the PePHD amino acids and the outcome of IFN therapy in *HCV*-1a/1b infected patients.

DISCUSSION

Recent studies showed that genotype 1a, 1b, and 3a are predominant in Iran (Samimi Rad *et al*., 2004; Zali *et al*., 2000). In Pakistan, genotypes 3b and 3a, in Turkey genotype 1b, in Uzbekistan 1a, 1b, 2a, 2k and 3a, in Lebanon 1g are reported to be the dominant genotypes (Pavio *et al*., 2003). Genotype 4 is the main genotype circulating in most Arabic countries. In Bahrain 4a and in Saudi Arabia *HCV* genotype 4 were detected in 50% of patients and genotype 1b was found in nearly 40% of patients (Elahi *et al*., 2003; Samimi Rad *et al*., 2004). In the present study, our data showed the same result as those demonstrated by Zali and Samimi Rad (Samimi-Rad *et al*., 2004; Zali *et al*., 2000).

Phylogenetic tree analysis explained that the pattern of Iranian 1a subtypes is similar to those of France isolates, but different from other isolates detected in Middle-East countries. This may be related to the fact
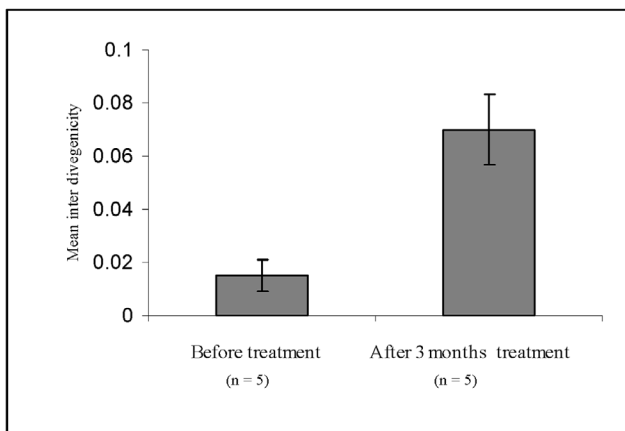
Figure 2. Mean inter genotyping divergenicity within before and after treatment samples.

Patient 229

```
PT   WITPRCLVDYPYRLWHYPCTVNYTLFKVRMYVGGVEHRLDAACNWTRGERCDLDDRDRSELSPLLLSTTQWQVLPCS
3MT  .......H.............................V...................PA................
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```
PT   WITPRCLVDYPYRLWHYPCTVNYTLFKVRMYVGGVEHRLQAACNWTRGERCDLDDRDRSELSPLLLSTTQWQVLPCS
6MT  .......N.......I..V.............VE...............E.......S.................
```

Patient 243

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PT   WITPRCLVDYPYRLWHYPCTVNYTLFKVRMYVGGVEHRLEAACNWTRGERCDLDDRDRSELSPLLLSTTQWQVLPCS
3MT  .......N.......I..V...........V..................E.......S.................
```

Patient 244

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PT   WITPRCLVDYPYRLWHYPCTVNYTLFKVRMYVGGVEHRLEAACNWTRGERCDLDDRDRSELSPLLLSTTQWQVLPCS
3MT  .......N.......I..V.............................E..........................
```

Patient 261

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PT   WITPRCLVDYPYRLWHYPCTVNYTLFKVRMYVGGVEHRLEAACNWTRGERCDLDDRDRSELSPLLLSTTQWQVLPCS
3MT  .......N.......I..V...........V..................E.......S.................
```

Figure 3. PT: HCV PePHD region amino acid before treatment, 3MT and 6MT: HCV E2 region amino acid after three and six month treatment with INF and Rivavidin, respectively. Amino acids were translated directly from sequences using BioEdit software. PePHD sequence changed during antiviral therapy. PePHD region is shown by fleshes.

that Iranian patients are infected with European isolates. Interestingly, the two *HCV*-1b isolates have shown no similarity to other 1b isolates and branched in separate cluster, but because of a low difference with other genotype 1 subtype it could not be confirmed as a new subtype (Zein, 2000). Genetic diver-

genicity among *HCV* subtypes showed that mutation rate of the studied region is the same as the whole genome (Table 2). The genetic sequence of different HCV genotype vary by as much as 35%. The degree of difference in nucleotide sequences among isolates vary from one genomic region to another genotype,

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HCV-1a-JP  WITPRCLVDYPYRLWHYPCTINYTIFKVRMYVGGVEHRLDAACNWTRGERCDLEDRDRSELSPLLLSTTQWQVLPCS
200-1a     .......H.............V...L.................E.................D.............
201-1a     ....................V...T.................GV................D.............
202-1a     ....................V...T.................EV................D.........P...
203-1a     .......................Q..................E...............................
205-1a     .......N..................................E...............AD.......SA.....
206-1a     ..........................R...............E...............................
207-1a     .......H............V...T.................EV................D.........P...
208-1a     .......H............V...L.................V................D.............
209-1a     .......N............V.....................E...............................
210-1a     ....................V...L.................EV................D.......A.....
213-1a     ....................V...L.................E.................D.............
221-1b     .......H............V...L.................V................D.........I...
222-1b     .L...M.H............V.F...I...............E................A.........IK...
223-1b     .....M.H............V.F...I...............E................A.........IK...
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Figure 4. Sequence alignment of hepatitis C virus (HCV) envelope glycoprotein (E2) containing PePHD region in random samples. The sequence was translated and the deduced amino acid sequence was aligned relative to HCV genotype 1a ,accession number HPCHCJ1. The PePHD encompassing amino acid 276-287 (RSELSPLLLTTT) for HCV genotype 1a of the E2 protein is shown by fleshes.

explained by Simmonds *et al*., (2001). Sequence similarities between members of the different genotypes of 230-bp segment of E2 region used in our laboratory had the same results with the percentage of nucleotide similarities when the full length sequence of the *HCV* genome was employed.

Virological response to antiviral therapy in *HCV*-infected patients is influenced by host-and/or virus-related factors (Layden-Almer *et al*., 2006; Pavio *et al*., 2003; Abid *et al*., 2000; Ralston *et al*., 1993). Because of highly different sustained response rates to antiviral therapy between *HCV* genotypes (30% in *HCV*-1 in comparison with 65% in *HCV* non-1 genotype), virus-related factors are apparently important. More recently, the *HCV* E2 protein of *HCV*-1a/b isolates was shown to bind PKR and inhibit its function *in vitro* through PePHD region (Taylor *et al*. 1999). In the current study, the mutational pattern within a part of C-terminal of *HCV* E2 protein comprising the PePHD sequences was investigated in 22 Iranian *HCV*-infected patients. Four of them were followed one week before and three months after IFN therapy. In previous studies, Sarrazin et al. showed mutations at codons 668 and 669 in 3a sustain responders (SR) and at codons 663 and 662 in 1b SR (Sarrazin *et al*., 2000) and others indicated that HCV-1b/2a/2b PePHD mutations at codons 659, 660, 661, 662 and 665 have positive roles in IFN resistance (Lo *et al*., 2001). This is consistence with the finding that HCV E2 protein plays important role to repress the function of PKR through mutations in PePHD region (Taylor *et al*., 1999) but accordance with some of the former studies, the PePHD and its flanked region within HCV-E2 was found to be highly conserved in SR, ETR, and NR patients (Sarrazin *et al*., 2000).

In the followed group, genetic divergenicity within samples who did not receive treatment was 1.5% but in treated patients was 7% which shows the occurrence of some mutations. It means that treatment could affect on mutations in this region.

In isolate 229-1 mutations at L667A and L666P, in code 227-2, mutations at L665S and in isolate 243-1 mutation at L665S have occurred. Code 244-1 did not show any mutation during therapy. Codons 665, 666 and 667 located in the homologous region to eIF2α and PKR, therefore the substitution at these positions seems to affect the binding of E2/PKR leading to IFN resistance. In addition, upstream and downstream mutations and their influence on conformation of E2

protein and PePHD region are important. E2 is a protein and like other proteins every change in amino acid sequence may affect its functional properties (Lewin *et al*. 1994). In isolate 229-1 mutations at D629H* and A625V have occurred which D629H* is important because of changing acidic amino acid to non polar. In isolate 227-2, mutations at L649V, L645V, L641I, D610N*, L655E and Q650E were shown in which D610N* is the most important one because of shifting acidic amino acid to the polar one. Alteration at L655E because of shifting non polar to polar and Q650E as a consequence of changing polar to acidic amino acid are important as well. In codes 243-1 and 261-1 mutations at L649V, L645V, L641I, D629N* and L655E have occurred which D629N* and L655E are important due to prior reasons and in codes 244-1, mutations at L641I, D629N*, L645V and L655E were shown that D629N* and L655E are repeated. All of these data indicate that PePHD region and its upstream and downstream shifted acidic phase to basic and polar phases and this change may influence the charge of peptide and conformational changes in the product of PePHD region. Mutation pattern in random samples was the same and 7 out of 13 patients had PePHD sequence variation. Mutations in isolate 210 at L667A, in isolate 202 and 207 at L666P, in isolate 221, 222 and 223 at T670I and in isolate 205 at S660A and E661D was observed in PePHD region. Codons 666, 667 and 670 that are located in the homologous region to eIF2α and PKR. Consequently the replacement at these positions seems to affect the binding of E2/PKR leading to IFN resistance (Taylor *et al*., 2001). Variation at positions I603L, D610H, D610N, V622I, L626T, L626V, L626R, L626I, L626Q, L640V, E641Q, E641D, E641G, A642V, D655E, D655A, Q671A and Q671K showed that acidic properties of this region changed to basic and polar which may affect the PePHD properties. On the other hand, amino acid variation in the PePHD consensus sequence of HCV-1a from the wild type could be detected more often in patients who were treated with IFN plus ribavirin.

In conclusion, these results revealed that three months treatment may cause mutation in PePHD and these mutations may influence its inhibitory properties and patients with these mutations may be treated completely. This suggests that mutations in wild type PePHD sequence could be correlated with complete response to the IFN treatment.

Acknowledgments

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