Iranian Journal of Virology 2008; 2(1&2)
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GENETIC DRIFTS IN PKR-EIF2A PHOSPHORYLATION HOMOLOGY DOMAIN REGION IN IRANIAN HCV POSITIVE PATIENTS DURING INTERFERON THERAPY

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Abstract: Hepatitis C virus (HCV) envelope glycoprotein-2 could block eIF2 phosphorylation with PKR suppression and in this way stops IFN pathway activation via homology domain with phosphorylation site of eIF2-PKR (PePHD) in its C- terminal. In some studies, mutations in PePHD region have been shown to be associated with sustained response to IFN. Present study examined the genetic variability of the PePHD in patients receiving IFN therapy.The PePHD from HCV genotype 1a/1b infected patients receiving IFN was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and analyzed using bidirectional sequencing. The PePHD sequence was different in pretreatment isolates from that of three months treated patients. The sequencing data were analyzed by Clustal X, MEGA and Bio Edit soft wares.

The finding indicated 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 in codons 665, 666, 667 of followed-up samples and in codons 660, 661, 666, 670 of randomly treated patients. Some of these mutations were similar to those reported by prior studies. Other mutations were also detected at up and downstream regions of PePHD which could have influenced the structure, conformation and configuration of this region and thereby suppressing PePHD inhibitory properties. Thus our data suggested that HCV E2 PePHD may play an important role in determining the interferon response.

Keywords: • HCV • PePHD • IFN therapy • Treatment resistance

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 and hepatitis B virus. The disease was called "non-A non-B hepatitis," and it was demonstrated to be transmissible to chimpanzees [1]. It was not until 1989, however, that 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*)* [2].

HCV is one of the leading pathogens of chronic hepatitis [3, 4, 5], and often results in liver cirrhosis or hepatocellular carcinoma [5, 6]. Treatment of chronic hepatitis is an important clinical problem. Currently IFN-α alone or in combination with ribavirin and some times amantadin is the only available approved therapy for chronic hepatitis C [7]. 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 [8]. Due to heterogenicity of HCV genome, more than 10 genotypes and 70 subtypes of HCV have been described so far [9]. Based on the chronic nature of HCV infection and the

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[DOI: 10.21859/isv.2.1.2.6]

tremendous burden on healthcare resources, scientists have looked for key epidemiological, pathological and viral characteristics that may provide insights into disease progression, severity and response to therapy for a 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 [3, 8]. 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 [10,11,12]. 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 thus prevents translation shutoff mediated by IFN [13,14,15,16]. 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 [17]. On the other hand, some groups have found no correlation between PePHD and the effectiveness of IFN therapies [18, 19].

In this study, the PePHD sequence in the specimens of 23 randomly selected patients infected with HCV genotype 1a/1b was analyzed to assess the mutations observed in PePHD. The patients were all treated with IFN-α.

MATERIALS AND METHODS

Patients

Thirty-six HCV infected Iranian patients were enrolled in this survey. The participqnts consisted of 25 males and 11 females with the mean age of 33.5 (ranging 12-67 years). All patients were checked for HCV, human immunodeficiency virus (HIV) and hepatitis B virus (HBV) by commercial available ELISA kits (Organon Technica, Turnhout, Belgium) and genotyped by RFLP. All patients were positive for HCV antibody and negative for HBsAg and HIV antibodies. Thirty patients were studied cross-sectionally. Five patients were chosen to be monitored for 3 to 6 months after antiviral treatment. These patients received 6 MU of recombinant IFN-alpha 2b and ribavirin three times weekly. Other 25 patients received this treatment with out follow up monitiring.

Amplification and sequencing of the PePHD region of HCV E2 glycoprotein HCV positive sera were collected and then frozen immediately at -70°C. HCV RNA was extracted by High Pure Viral RNA extraction Kit (Roche, Mannheim, Germany). Based on HCV genotypes, 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 Database (Table 1) [20, 21, 22]. The conserved PePHD primer sets were identified based on E2 genes of various HCV genotypes and subtypes by multiple alignments using

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	Inner and sense	2149-2199
MP4	AGAACACGGAAGGACCTGCC	Inner 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	Inner and antisense	2341-2360

Primer positions were referred to accession number AF009606 and AB016785 for HCV

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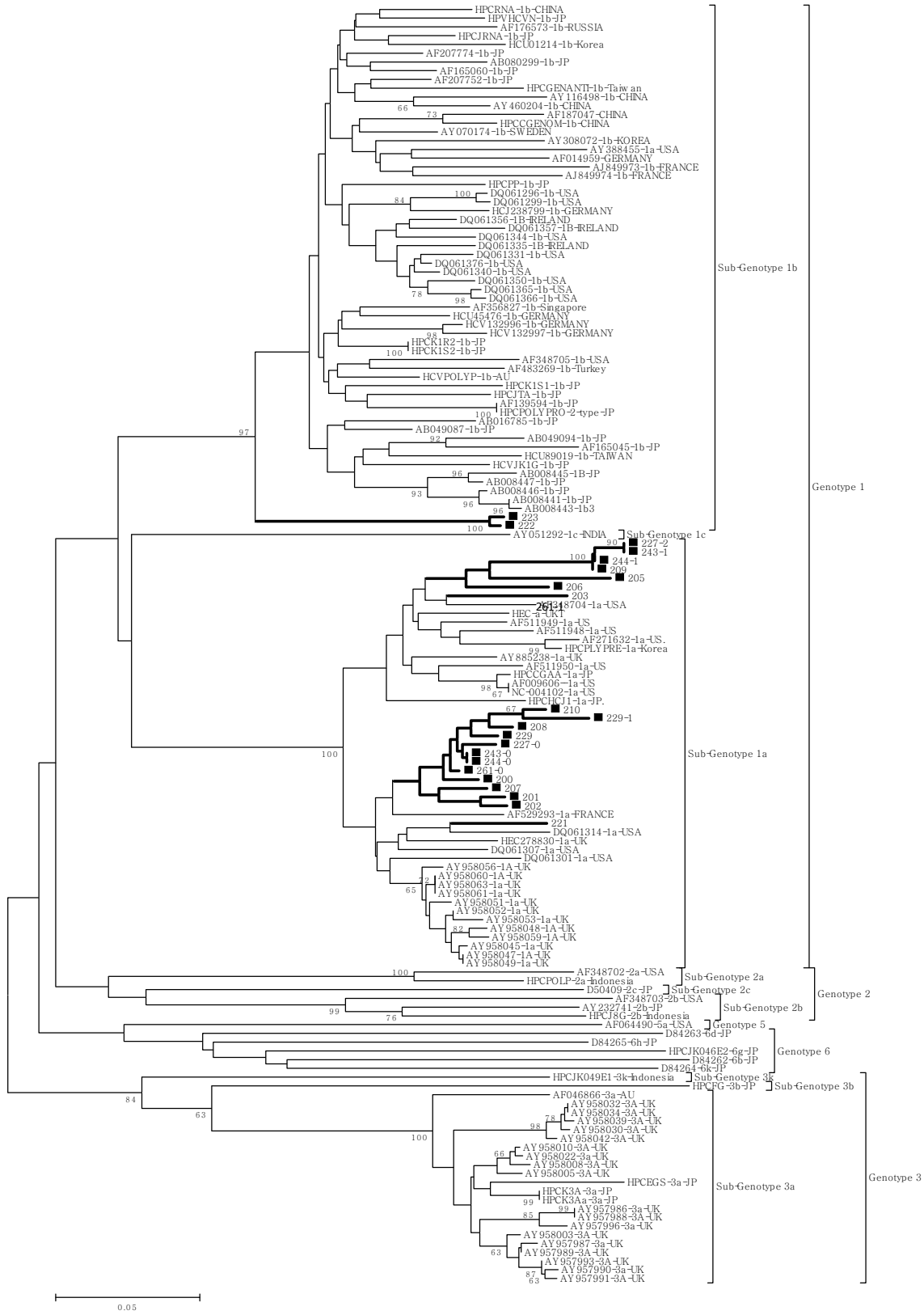


Fig. 1. Tree was created by phylogenetic analysis based on Kimura two-parameter distance method followed by Clustal X Neighbor Joining for part of E2 sequence containing PePHD. Samples with treatment history branched in separate cluster from those who did not received therapy.

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BioEdit software version 5.0.9. The list of primers and their characteristics that were used in this study are shown in Table 1. The PCR products were extracted and purified from agarose gel (CoreBio™, Seoul, South Korea) and then sequenced in both directions by a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A) using an ABI Prism® 3700 DNA analyzer sequencer (Applied Biosystems, Foster City, CA, U.S.A) 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 the reference, [22] and the Iranian HCV PePHD sequences were compared to 124 defined HCV isolates. Alignment was performed using CLUSTAL X software, version 1.81 [23]. Genetic distance was estimated by the Kimura two-parameter matrix [24]. Phylogenetic tree was constructed by the neighbor-joining method [25]. Bootstrap re-sampling and reconstruction was performed for 1000 times to confirm the reliability of phylogenetic trees [26]. Phylogenetic and molecular evolutionary analysis and nucleotide differences within and between the isolate sequences were calculated by MEGA program, version 2.1 [27].

RESULTS

HCV phylogenetic tree analysis

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 phylogenetic tree analysis based on twenty-three samples (232 bp) of the PePHD amplicon and its flanked region 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). Genetic divergenicity among samples of patients who did not receive treatment was 1.5% however in the treatment patients was 7% (fig. 2).

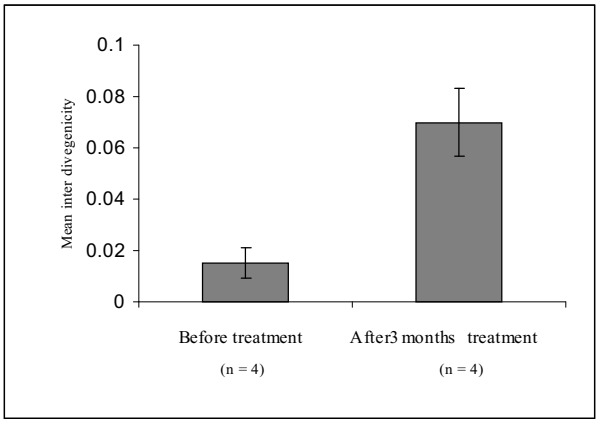


Fig. 2. Mean inter genotyping divergenicity within before and after treatment samples.

Amino acid variations in PePHD region

In order to 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 using samples obtained one week before and three months after IFN therapy (Fig. 3).



Fig. 3. Alignment of pre and after treatment of PePHD sequences from five HCV infected patients receiving interferon therapy. PePHD sequence changed during antiviral therapy.PePHd region is shown by fleshes.

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 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

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one case six months) in patients infected with HCV-1a. Four out of five had amino acid substitutions in their PePHD sequence. These four 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 two HCV-1b isolates, while seven out of 12 HCV-1a PePHD sequences form 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 illustrates the PePHD amino acids and the outcome of IFN therapy in HCV-1a/1b infected patients.

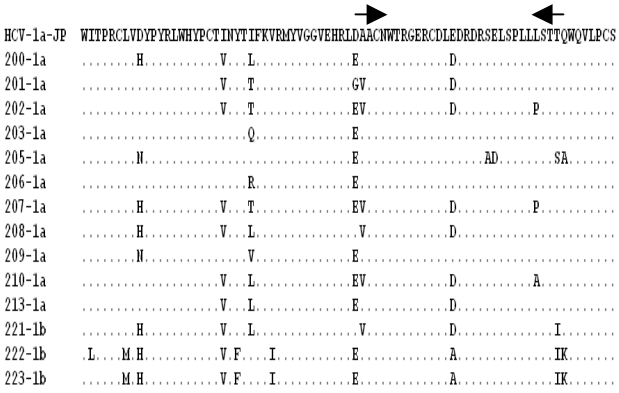


Fig. 4. Alignment of region of hepatitis C virus (HCV) envelope glycoprotein (E2) containing PePHD region in random samples. Sequence were translated and the deduced amino acid sequence were aligned relative to HCV genotype 1a ,accession number HPCHCJ1. The PePHD encompassing amino acid 276-287 (RSELSPLLLTTT) for HCV genotype 1a of the E2 protein, is shown by fleshes.

DISCUSSION

Recent studies showed that genotype 1a, 1b, and 3a are predominant in Iran [28, 30]. In the present study, our data showed the same result as those demonstrated by Zali and Samimi Rad [28, 29, 30]. Phylogenetic tree analysis explained that the pattern of our 1a subtypes is similar to those of France but different from other Middle-East countries. This could be related to the fact that Iranian patients are infected with European isolates.

Virological response to antiviral therapy in HCV-infected patients is influenced by host-and/or virus-related factors [31, 32, 33, 17]. 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 PKR function **in vitro** through PePHD region [11].

In the current study, the mutational pattern within a part of carboxy-terminal of HCV E2 protein comprising the PePHD sequences was investigated in twenty-two Iranian HCV-infected patients. Five of them were followed one week before and three months after IFN therapy. In previous studies, Sarrazin et al showed that mutation in codons 668 and 669 in 3a sustain responders (SR) and codons 663 and 662 in 1b SR [34] and Lin and Lo indicated that HCV-1b/2a/2b PePHD mutations in codons 659, 660, 661, 662 and 665 have positive roles in IFN resistance [35]. This is consistence with the finding that HCV E2 protein plays important role to repress the function of PKR through mutations in PePHD region [11] but accordance with some of the former studies, the PePHD and its flanked within HCV-E2 was found to be highly conserved in SR, ETR, and NR patients [36]. In this research, it was observed that this region has mutations after therapy. 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 appearance of mutations in this region.

1a and 1b.

In code 229-1 mutations at L667A, L666P, in code 227-2, mutations at L665S and in code 243-1 mutation at L665S have occurred. Code 244-1 did not show any mutation during therapy. Codons 665, 666 and 667, are 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, up and downstream mutations and their effects on conformation and configuration of E2 and PePHD region are important. E2 is a protein and like other proteins every change in amino acid sequence could affected its functional properties [37]. In code 229-1 mutations at D629H*and A625V have occurred of which D610H is important because of changing an acidic amino acid to a non polar one. In code 227-2, mutations at L649V, L645V, L641I,

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D610N*, L655E and Q650E were shown of which D610N*is the most important for the reason that shifting an acidic amino acid to a polar one. Alteration at L655E because of shifting a non polar to a polar and Q650E as a consequence of changing a polar to an acidic amino acid are important. 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 previous 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 up and downstream shifted acidic phase to basic and polar phases and redundant may influence the charge of the peptide and the conformational and configurational changes in the product of the PePHD region. Mutation pattern in random samples was the same and 7 out of 13 patients had PePHD sequence variation. Mutations in code 210 at L667A, in codes 202 and 207 at L666P, in codes 221, 222 and 223 at T670I and in code 205 at S660A and E661D were observed in PePHD region. Codons 666, 667, 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 [38].Variation at positions I603L, D610H, D610N, V622I, L626T, L626V, L626R, L626I, L626Q, L640V, E641Q, E641D, E641G, A642V, D655E, D655A, Q671A and Q671K showed that Polarity shifts in the peptide region suspected of affecting binding of E2/PKR due to stereo conformational changes may be explored in an expression profile system that characterizes these changes on generated mutants.

On the other hand, our finding indicates that 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 this study, results revealed that three months treatment can cause mutation in PePHD and these mutations can 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.

Acknowledgment

We would like to thank Dr Hanieh Zaer-rezaee and Parisa Porsa from DDRC and Mr. Reihani from Bahar laboratory for sample collection and Mr. Malek Hossein Ahmadipour for his assistance.

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