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Research Article

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Impact of HCV Genome Structure on Responder to Pegylated Interferon Therapy in Egyptian Patients

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ABSTRACT

This study aimed to investigate relationship of the HCV genome structure and treatment with Pegylated Interferon-α/Ribavirin (peg-IFNα/RBV) Egyptian patient. Mutations in two sites of HCV genome; the internal ribosome entry site (IRES) and the interferon sensitivity determining region (ISDR) of HCV genotype 4a were studied in details including DNA sequences and mutations detection in response to treatment. Ninety patients, responders and non-responders, to treatment with peg-IFN α /RBV were included in this study. IRES and ISDR regions were amplified by RT-PCR using specific designed primers, and amplified regions were sequenced. The data obtained were aligned with published sequences in GenBank using BLAST program. Results of this study have revealed that there are different mutations in the studied sequences in both ISDR and IRES regions. The predicted amino acids sequences in the ISDR region showed significant differences ranging from one up to more than eight mutations in the HCV Genome sequences. Although there was a significant difference between sequences of HCV RNA isolated from responders and non-responders, these data were not able to give an absolute answer whether response to interferon therapy is directly/relates to the structure of the HCV genome.

Key words: *Hepatitis C virus, Pegylated Interferon-α, IRES, ISDR.*

INTRODUCTION

Inflammation of the liver (Hepatitis) may have many different causes, including viral infections, alcohol, fat accumulation in the liver, an incorrectly functioning immune system, exposure to chemicals and other toxins, and some drugs [1]. Hepatitis C virus (HCV) is a member of the Hepacivirus genus (Flaviviridae family) [2]. Chronic Hepatitis C Virus infection remains a serious burden to public health worldwide and is considered the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma [3]. Recently, the Egyptian Demographic Health Survey (EDHS) conducted in 2016, has reported that the overall prevalence who was positive for the antibody to HCV was 12% [4].

About 50-60% of chronically infected with HCV patients are achieve a sustained virological response to the antiviral therapy, which consists of peg-IFNα in combination with Ribavirin (RBV) [5]. In patients infected with HCV, the viral RNA genome and its proteins are of importance for response to interferon-based treatment [6].

Mutations in the HCV proteins functional regions may be correlated with the response to interferon therapy have indicated by many studies, such as amino acid substitution at core amino acid 70, two or more amino acid mutations in the ISDR of the NS5A region in HCV genotype [7]. Although ISDR is necessary but not sufficient for the interaction between the PKR enzyme (that is very important for the activation of IFN) and NS5A, with an additional 26 amino acids distal to the ISDR being required [8]. This region is termed the PKR binding domain (PKRBD). The multiple mutations introduction with the region of PKR-binding, including those with

the ISDR, abrogated the ability of NS5A to bind to PKR. HCV type 1 mutations within the PKRBD are associated with a long term sustained response to IFN-α and IFN- α /RBV therapy was found by Macquillan [9].

MATERIALS AND METHODS

This study was conducted in Biochemistry and Molecular Biology Department at Theodor Bilharz Research Institute (TBRI), in the period from September 2013 to October 2015.Ninetypatients participated in the present study; were with chronic HCV infection. The patients treated for 48 weeks, as a standard protocol for treatment of Egyptian patients. Two peg-IFNs are available; peg-IFN α-2b (PEG-Intron®, Merck, Germany) and peg-IFN α-2a (PEGASYS®, Roche, Germany) [10, 11]. RBV administered orally according to the bodyweight of the patient HCV-RNA analysis was performed at 4, 12, 24 and 48 weeks. The Patients were classified into two groups, the responders and non-responders. The responders are defined as not detectable virus at week 48. Non-responders HCV RNA titer was over 12 IU/ml at week 48.

Quantification of HCV RNA inPlasma of patients:

HCV RNA was extracted from 200 µL of each patient serum using a commercially available RNA extraction kit (Abbott Molecular Inc., USA) following the manufacturer's protocol. The HCV-RNA was quantitatively determined in serum before and after treatment using Abbott Real Time HCV kit (Abbott Molecular Inc., USA) following the manufacturer's protocol.

Amplification of the IRES region in the HCV RNA Genome

The full length of IRES region was amplified by RT-PCR. PCR was performed in a 50 µL reaction volume containing: 25 µL RNA,20 pmol of each of the following specific primers (IRES Forward primer: 5'TTGGGGGCGACACTCCAC3' and IRES Reverse primer: 5' CTTTGAGGTTTAGGAATTCGTGCTC 3'), 5 µL 10x PCR buffer, 25 mM MgCl2, 200 µM dNTPs, 1.25 unit Taq DNA polymerase (Gotaq Flexi DNA, M8305, Promega, Inc, USA), 15 units of reverse transcriptase (AMV reverse transcriptase, M5108, Promega, Inc. USA) and sterile distilled water was added to a final volume of 50 µL.

The reaction was done in a PTC90TM Thermal Cycler (MJ, USA), programmed as follows: reverse transcription was performed at 42°C for 30 min, at 95°C for 5 min.The PCRamplification was as follows: 37 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, and final extension at 72°C for 5 minutes.

Electrophoresis through agarose gel is the standard method used to separate, identify and purify DNA fragments (Sambrook and Russell, 2001), The fragment of amplified IRES DNA 341 bp was excised from the gel using the DNA extraction kit (Fermentas, Canada).

Sequencing of IRES PCR amplified products

The automated sequencing using high throughput Applied Biosystems 3730XL sequence (Macrogen, Korea) sequenced the amplified fragment of HCV IRES. Sequence reactions on PCR products were performed using BigDye terminators and IRES forward and reverse primers, according to Sanger method [12].

Analysis of IRES Sequences and Genotype Determination.

Genotype of each sample was determined after sequence analysis. Vector NT1 advance11.5 program (http://www.invitrogen.com) was used to perform the multiple sequence alignment of all available 5'UTR sequences at NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy/ taxonomy home.htm) to reveal the subtype-specific consensus sequences of 4, 4a, 4a/4d, 4m, 4a/f, 4d and 1g.

Mutations Detection in the IRES Sequences data.

The IRES sequence was compared with the HCV sequences in the NCBI database. The DNA sequences were aligned using BioEdite software version 7.0.5.3 and online NCBI Blast site. These sequences of the amplified fragments were aligned against Hepatitis C virus subtype 4a isolate (GenBank: AB795432.1) and Hepatitis C virus replicon 4a ED43-Neo (R+I), complete sequence (GenBank: JX865252.1) from NCBI.

Amplification of the ISDR region in the HCV-RNA Genome

The protocol for amplification of the ISDR region of HCV RNA was performed as has been described for amplification of the IRES region, with the exception of using primers: ISDR forward: 5'-ATC TTG GCT ATG GGA GGT ATG GG-3' and ISDR reverse: 5'-TGT CAC AGC AGA ACG GGT GGT C-3'for first PCR run).

The fragment of amplified ISDR DNA was excised from the agarose gel and DNA eluted was pure and subjected for direct sequencing.

Sequencing of ISDR PCR products

The amplified fragment of HCV ISDR was sequenced similar to the HCV IRES using the automated sequencing using high throughput ABI 3730XL sequencer (Macrogen, Korea). The amino acid sequences were deduced and aligned using BioEdite software version 7.0.5.3 and online NCBI Blast site. These sequences of the amplified fragments were aligned against Hepatitis C virus subtype 4a isolate L835 polyprotein gene, complete cds (GenBank: DQ418789.1) for DNA, and polyprotein [Hepatitis C virus subtype 4a] (GenBank: ABD75831.1) for protein (designated wild type in this study). [The sequence data of HCV ISDR has been deposited in the GenBank nucleotide sequence databases with the accession number KF263686].

Statistical Analysis

Comparison between groups was made by the Students t-test. The P values were determined between the two groups with regard to age, gender, amino acid mutations in HCV ISDR and nucleotides substitution in HCV IRES. P< 0.05 was considered statistically significant.

RESULTS

Ninety Egyptian patients were included in this study. The 90 included patients had median age of 45 years ranging from 20 years to 65 years old. 30% of them were female and 70% were male.

Quantification of HCV-RNA viral load by Real Time PCR.

Abbott Real Time HCV assay has advantages of the detection limit is 12 IU/ml.

Patients were selected properly since no significate difference between viral load in responders and non-responders patients.

Patients were divided to two groups according their response to standard regimen of treatment with pegylated interferon plus ribavirin.

In addition, no significant difference was observed in age or gender in response to treatment.

DNA Sequencing of HCV-RNA IRES:

RT-PCR was done using the specific primers for amplification of the total RNA extracted.

Extraction of PCR product of amplified IRES from agarose gel:

The amplification products, after separation on agarose gel, were excised from the gel; purified fragment was sequenced.

Direct sequencing of PCR products of HCV IRES.

The amplified fragment of HCV IRES was sequenced by the automated sequencing, and the full sequence of the PCR product was obtained.

Sequence analysis was performing using NCBI Blast program.

The 5'UTR sequences of sample under this study were aligned and compared to published GCV genotypes, and results of genotypes are shown in table 1.

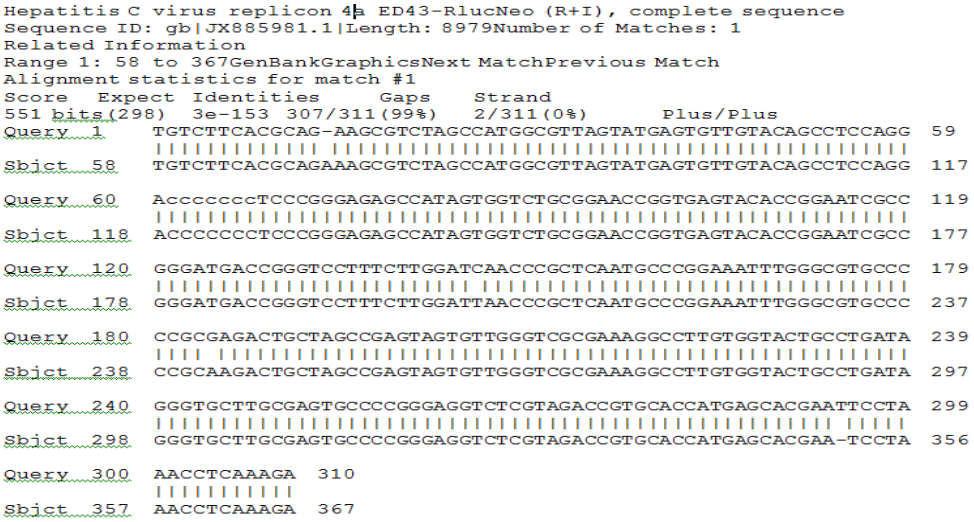

Figure 1. Alignment of sample (1) as Query sequence with the available HCV genotype (4a) sequences from the gene data bank. The genotype assignments of this sample was confirmed by the blast search at (http://www.ncbi. nlm.nih.gov/Taxonomy/ taxonomy home.html).

Table 1. The HCV genotypes and subtypes of the ninety samples.

HCV Genotype	Subtype	Percentage %
4	a	85.6
4	d	4.4
4	-	3.3
1	a	1.1
1	g	3.3
3	a	2.2

IRES region sequence of HCV RNA:

Using Clustalw2 online alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and online NCBI Blast site sequences were analyzed.

Comparative analysis of the aligned sequences of the all samples has shown different nucleotide substitution. Detected nucleotide substitution shown in Figure 3,

The substitution detected were classified into three types; type 1 (1-3 bp), type 2 (4-6 bp) and type 3 (7-12) as shown Table 2.

ISDR region of HCV RNA isolated from patient's samples:

RT-PCR for ISDR region of HCV RNA isolated from patient's samples:

The HCV ISDR contain 40 amino acids located within the nonstructural 5A (NS5A) protein. NS5A is known to be potentially important for antiviral therapy outcome.

Two steps PCR were used to amplify ~750 bp of the ISDR region. The amplified product was purified from the agarose gel and sequenced by the Sanger's method. A full sequence of the PCR product of ~ 745 bp was obtained. Sequence analysis using NCBI Blast program, for one sample (Number5). The predicted amino acid sequence of the ISDR NDA region is shown in Figure 5.

According to the number of the amino acid mutations detected in ISDR sequence, it claddified into three types according to Enomoto 6. (Table 3)

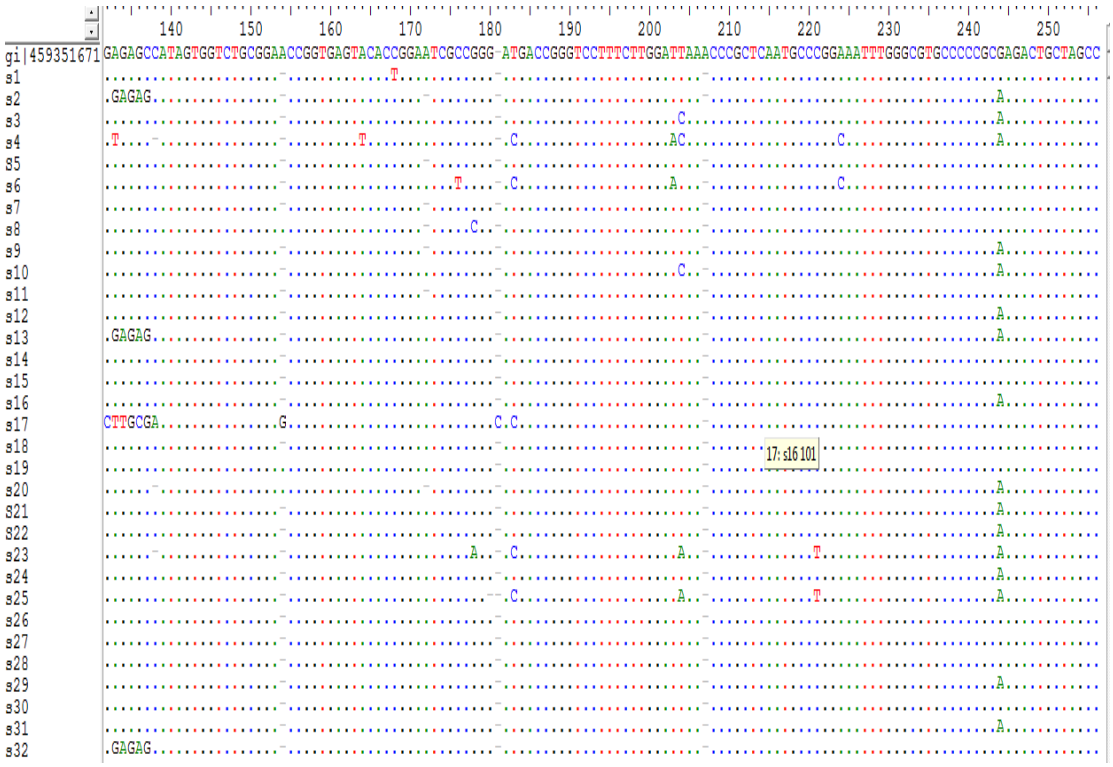

Figure 2. Multiple sequence alignment of the sequenced DNA and the reference Hepatitis C sequence. There are 341 nucleotide positions in this matrix data. The regions of similarity between the sequences are shown as dots, while the dispersed letters indicate the nucleotide variations. The gaps are represented in dashes. Black, yellow and red boxes indicate the 3 observed patterns of nucleotide diversity.

Table 2. Comparison of nucleotide differences of HCV-IRES sequences in responder and non-responder patients.

IRES nucleotide substitution				
Patients	No.	(1-3 base)	(4-6 base)	(7-12 base)
Responders	46	50%	32.8%	17.2%
Non-responders	44	38.63%	38.63%	22.74%
All patients	90	44%	36%	20%

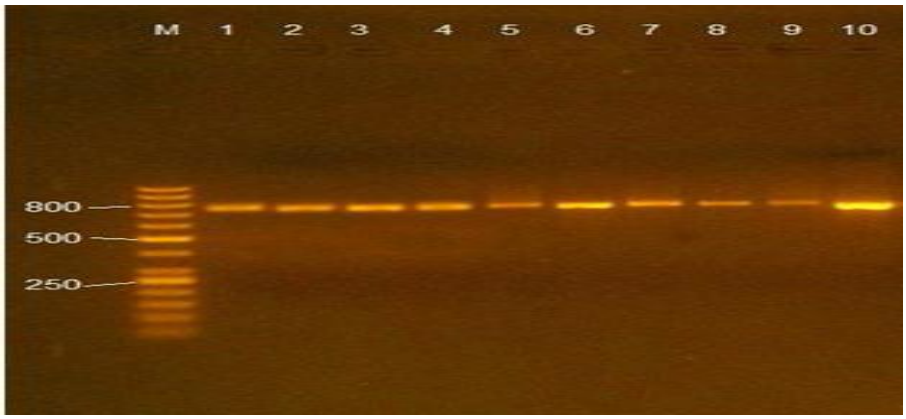

Figure 3. Shows agarose gel electrophoresis of the purified RT-PCR products of samples. M: DNA ladder (50 bp); lanes from 1 to 10: ten different samples of HCV-patients included in this study; the molecular size of the purified band (~ 745bp).

Polyprotein [Hepatitis C virus subtype 4a]
Sequence ID: gb|ABD75825.2|Length: 3008Number of Matches: 1
Related Information
Range 1: 2109 to 2313GenPeptGraphicsNext Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
363 bits(933)	2e-112	Compositional matrix adjust.	194/205(95%)	198/205(96%)	0/205(0%)	+3

```
Query  3     HIKCPCQVPAPEFFTEVDGIRLHRHAPKCKPLLRDEVSFSVGLNSFVVGSQLPCEPEPDV  182
             +IKCPCQVPAPEFFTEVDGIRLHRHAPKCKPLLRDEVSFSVGLNSFVVGSQLPCEPEPDV
Sbjct  2109  NIKCPCQVPAPEFFTEVDGIRLHRHAPKCKPLLRDEVSFSVGLNSFVVGSQLPCEPEPDV  2168

Query  183   AVLTSMLTDPSHITAETARRRLARGSPPSLASSSASQLSAPSLIKATCTARHDSLSADLLE  362
             AVLTSMLTDPSHITAETARRRLARGSPPSLASSSASQLSAPSLIKATCT RHDS DLLE
Sbjct2169  AVLTSMLTDPSHITAETARRRLARGSPPSLASSSASQLSAPSLIKATCTGRHDSPGTDLLE  2228

Query363   ANLLWGSSATRVETDEKVIILDSFEPCVAEPDDDREVSVAAEILRPAKKFPPALPIWARP  542
             ANLLWGS+ATRVETD+KVIILDSFEPCVAEPD+ DREVSVAAEILRP KKFPPALPIWARP
Sbjct2229  ANLLWGSTATRVETDDKVIILDSFEPCVAEPDNDREVSVAAEILRPTKKFPPALPIWARP  2288

Query 543   DYNPPLTEMWKQQDYKPPTVHGCAL  617
             DYNPPLTE WKQQDYKPPTVHGCAL
Sbjct 2289   DYNPPLTET WKQQDYKPPTVHGCAL  2313
```

Figure 4. Shows the alignment of one sequence of HCV-ISDR for patient Number 5, with sequence data of hepatitis C virus strain B-20-NRNS5A gene, from the GenBank nucleotide sequence databases with the accession number KF263686.

Table 3. Comparison of the subgroup patients as divided by the types of ISDR mutations into three types.

Response	Number	ISDR		
		Wild	Intermediate	Mutant
Responders	46	(39.13%)	(60.87%)	(0%)
Non-responders	44	(45.45%)	(34.1%)	(20.45%)
Total	90	(42.22%)	(47.78%)	(10%)

DISCUSSION

Egypt has a high HCV prevalence, wherever the results of blood screening and testing for the Egyptian blood donors showed 12% HCV antibodies positive [4]. HCV genotype 4 is that the commonest one in Egypt that represents more than 90% of the cases [13]. Over the past decades, HCV genotype 4 (HCV- G4) patients were treated by a pegylated interferon (peg-INF) and ribavirin (RBV) combination for 48 weeks [14]. The treatment efficiency of HCV-G4 patient with this combination was tiny. Solely 41% sustained virological response (SVR), defined as a viremia 24 weeks after completion of antiviral therapy for chronic hepatitis C virus was showed by a worldwide study containing 7163 HCV patient treated by peg-IFN/RBV [15]. It is believed that each host and viral factors, as well as many regions of viral genomic, are playing rollsin effective response to IFN therapy [16].

This study aimed to find a correlation, on molecular level of HCV structure and response to interferon therapy. The experimental style was to work out the factors that could be helpful to predict the effectiveness of peg-IFN-α/RBV combination therapy in chronically infected patients with genotype 4, the main genotype in Egyptian patients. Therefore, we have a tendency to analyze the link between the response to combination therapy and a variety of factors, mainly genetic factor of the HCV genome. Relationship between nucleotide variables in HCV IRES and amino acid mutations of HCV ISDR regions, and response topeg-IFN-α/RBV combination therapy, had been evaluated in this study. All Patients were treated with peg-IFN-α/RBV for 48 weeks consistent with the Egyptian protocol for patient's treatment [14].

Our data revealed that age and gender were not significantly associated with the combination therapy effectiveness. This finding suggests that age and gender, has no effect on the efficacy of peg-IFN-α/ RB combination therapy for HCV infection among Egyptian patients. These results are consistent with previous studies of Thelu [17].

The IRES sequence variability does not appear to correlate with difference in concentration of serum HCV-RNA that are expected to reflect the level of HCV replication in vivo, was observed by Yamamoto [18].

Most of the nucleotide substitutions found in IRES sequence had no significant correlation with the response to peg-IFN-α/RBV combination treatment was indicated by the present results. Even if the variability leads to changes in translation efficiency both in vitro and in various cell culture lines, no influence in the clinical context was found by Laporte [19]. Antiviral treatment might favor emergence of new 5′UTR variants was found by Iwona [20].

Previous studies have disclosed that age, gender and amino acid mutations in ISDR were considerably related to the combination therapy effectiveness [21-23]. This conflict within the results, demonstrates that gender, age, HCV RNA titer and ISDR mutations are not always precise helpful markers for prediction of interferon treatment outcome in patients infected with HCV [24]. This effect may be due to the pleiotropic of interferon activity, in addition to cellular and viral genetic factors that modulate the efficacy of peg-IFN-α/RBV therapy for chronic HCV infection [24, 25]. Conflicting knowledge are expressed for the link between amino acid mutations in the ISDR and response to the IFN-based therapy. Some studies show that the ISDR is a reliable predictor of the response to the IFN therapy, specifically in relevancy Japanese patients with a particular subtype of HCV 1b known as (HCV-J) [26-28]. Different correlations were detected in studies conducted in Europe, the United States, Turkey and one Korean study [29, 30].

The results of the studies carried out in Europe and USA, were in agreement with our study conducted mainly on HCV genotype 4, which is prevalent genotype in Egypt [31]. The ISDR 2209-2248 sequence revealed that no correlation of the mutation variety of amino acid, was observed between responders and non-responders to the combined peg-IFN-α/ RBV treatment.

CONCLUSION:

Our results were not able to find a direct correlation between neither nucleotide substitution in IRES region nor in the mutations in the amino acid sequence of the ISDR region of the HCV RNA genome, in relation to response to pegylated interferon alpha 2b/Ribavirin combined treatment in Egyptian patients infected mainly with HCV genotype 4.

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