NS5A Sequence Heterogeneity of Hepatitis C Virus Genotype 4a Predicts Clinical Outcome of Pegylated-Interferon–Ribavirin Therapy in Egyptian Patients

Ahmed El-Shamy,a,b* Ikuo Shoji,a Wafaa El-Akel,c Shymaa E. Bilasy,d Lin Deng,a Maissa El-Raziky,c Da-peng Jiang,a Gamal Esmat,c and Hak Hottaa

Hepatitis C virus genotype 4 (HCV-4) is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world. HCV-4 infection is common in the Middle East and Africa, with an extraordinarily high prevalence in Egypt. Viral genetic polymorphisms, especially within core and NS5A regions, have been implicated in influencing the response to pegylated-interferon and ribavirin (PEG-IFN/RBV) combination therapy in HCV-1 infection. However, this has not been confirmed in HCV-4 infection. Here, we investigated the impact of heterogeneity of NS5A and core proteins of HCV-4, mostly subtype HCV-4a, on the clinical outcomes of 43 Egyptian patients treated with PEG-IFN/RBV. Sliding window analysis over the carboxy terminus of NS5A protein identified the IFN/RBV resistance-determining region (IRRDR) as the most prominent region associated with sustained virological response (SVR). Indeed, 21 (84%) of 25 patients with SVR, but only 5 (28%) of 18 patients with non-SVR, were infected with HCV having IRRDR with 4 or more mutations (IRRDR ≥ 4) (P = 0.0004). Multivariate analysis identified IRRDR ≥ 4 as an independent SVR predictor. The positive predictive value of IRRDR ≥ 4 for SVR was 81% (21/26; P = 0.002), while its negative predictive value for non-SVR was 76% (13/17; P = 0.02). On the other hand, there was no significant correlation between core protein polymorphisms, either at residue 70 or at residue 91, and treatment outcome. In conclusion, the present results demonstrate for the first time that IRRDR ≥ 4, a viral genetic heterogeneity, would be a useful predictive marker for SVR in HCV-4 infection when treated with PEG-IFN/RBV.
In the present study, we aimed to investigate this issue in Egyptian patients infected with HCV-4.

**MATERIALS AND METHODS**

**Ethics statement.** The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Cairo University Hospital and in Kobe University, and written informed consent was obtained from each patient prior to the treatment.

**Patients.** A total of 43 previously untreated patients who were chronically infected with HCV-4a (34 patients), HCV-4m (3 patients), HCV-4n (3 patients), or HCV-4o (3 patients) were consecutively evaluated for antiviral treatment at Cairo University Hospital, Cairo, Egypt, between January 2008 and September 2010. The HCV subtype was determined according to the method of Okamoto et al. (31). The patients were treated with PEG-IFN α-2a (180 μg/week, subcutaneously) and RBV (1,000 to 1,200 mg daily, per os) for 48 weeks. The quantification of serum HCV RNA titers was performed as previously reported (14). To minimize the therapeutic burdens, including the high cost and possible side effects, therapy was discontinued if HCV RNA titers at week 12 did not drop by 2 log compared with baseline values or if HCV RNA was still detectable at week 24. These were considered a null response (see Results).

**Sequence analysis of the NS5A and core regions of the HCV genome.** Blood samples were collected using Vacutainer tubes. The sera were separated within 2 h of blood collection, transferred to sterile cryovials, and kept frozen at −80°C until use. HCV RNA was extracted from 140 μl of serum using a commercially available kit (QiAppl viral RNA kit; Qiagen, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for the HCV genome encoding a carboxy terminus of NS5A (amino acids [aa] 2193 to 2417) and the core protein (aa 1 to 191) using SuperScript III one-step RT-PCR Platinum Taq HiFi (Invitrogen, Tokyo, Japan). The resultant reverse transcription (RT)-PCR product was subjected to a second-round PCR by using Platinum Taq DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of the 3′ half of the NS5A region of HCV-4 were as follows: NSSA-4-F1 (5′-CTGAAATGTTCGTCRGGTTGGATC-3′; sense) and NSSA-4-R1 (5′-GAAAGGTCACCTTCTTCTCGCCG-3′; antisense) for one-step RT-PCR; and NSSA-4-F2 (5′-ATGGCGACCYYAGCCCGACGCT-3′; sense) and NSSA-4-R2 (5′-GCTCAGG GGGTATTTGGGACGCT-3′; antisense) for the second-round PCR. Primers for amplification of the core region of HCV-4 were 249-F (5′-CTAGCCGAGTATGGTTG-3′; sense) and 984-R (5′-GATGTGRTGRTGC GGCCCTC-3′; antisense) for one-step RT-PCR; and 249-F (5′-GGA TTCTCCTGTAAGCCGTCGC-3′; sense) and 984-R (5′-ATGGTACC CCCATGAGGTCGCC-3′; antisense) for the second-round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 90 s. The second-round PCR was performed under the same conditions. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan). The numbering of amino acid residues for HCV-4 isolates is according to the polyprotein of ED43 isolate (accession no. Y11604) (10). Consensus sequences of the carboxy terminus of NS5A of a given HCV-4 subtype were inferred by alignment of all sequences obtained in this study as well as all available NS5A sequences of HCV-4a (accession no. Y11604, DQ418782 to DQ418789, DQ516084, and DQ888073 to DQ888079), HCV-4m (FJ462433), HCV-4n (FJ462441), and HCV-4o (FJ462440) from the databases.

**Statistical analysis.** Numerical data were analyzed by Student’s t test and categorical data by Fisher’s exact probability test. To evaluate the optimal threshold of the number of amino acid mutations in IRRDR for prediction of treatment outcomes, the receiver operating characteristic (ROC) curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P value of <0.05 was considered statistically significant.

**Nucleotide sequence accession numbers.** The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB725987 through AB726066.

**RESULTS**

**Patients’ responses to PEG-IFN/RBV combination therapy.** Among 43 patients enrolled in this study, 30 (70%) patients completed the entire course of PEG-IFN/RBV treatment for 48 weeks and follow-up for 24 weeks. On the other hand, the treatment was discontinued for 13 (30%) patients due to poor virological responses at 12 or 24 weeks after initiation of the therapy. Overall, 25 (58%) patients achieved SVR while 18 (42%) patients had non-SVR (Table 1). When analyzed on the basis of the subtype classification, SVR was achieved by 56% (19/34), 100% (3/3), 33% (1/3), and 67% (2/3) of patients infected with HCV-4a, -4m, -4n, and -4o, respectively.

**Non-SVR patients are classified into two groups:** (i) patients with null response, who did not achieve >2-log reduction of the initial viral load at week 12 or who had detectable viremia at week 24 of the treatment period; and (ii) patients with relapse, who were negative for HCV-RNA at the end of the treatment period (week 48) followed by a rebound viremia at a certain time point during the follow-up period of 24 weeks. Patients with null response represented 30% (13/43) of all the HCV-4-infected subjects analyzed, while those with relapse represented 12% (5/43). A similar tendency was observed for subtype HCV-4a.

Among various patients’ demographic characteristics, SVR patients had a significantly lower average age than that of non-SVR patients (Table 2). Furthermore, a tendency for SVR patients to have a lower average titer of initial viral load than that of non-SVR was noted, although the difference was not statistically significant, due possibly to the small number of patients analyzed (P = 0.07).

**Table 1. Virological responses of HCV-4-infected patients treated with PEG-IFN/RBV**

<table>
<thead>
<tr>
<th>Virological response</th>
<th>Proportion (% of patients with indicated response (no. of patients/total no.:)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVR</td>
<td>58 (25/43) 56 (19/34) 100 (3/3) 33 (1/3) 67 (2/3)</td>
</tr>
<tr>
<td>Non-SVR</td>
<td>42 (18/43) 44 (15/34) 0 (0/3) 67 (2/3) 33 (1/3)</td>
</tr>
<tr>
<td>Null response</td>
<td>30 (13/34) 32 (11/34) 0 (0/3) 67 (2/3) 0 (0/3)</td>
</tr>
<tr>
<td>Relapse</td>
<td>12 (5/43) 12 (4/34) 0 (0/3) 0 (0/3) 33 (1/3)</td>
</tr>
</tbody>
</table>

*Includes all 43 cases with HCV-4 infection (34 cases with HCV-4a and 3 cases each with HCV-4m, -4n, and -4o).
TABLE 2 Demographic characteristics of HCV-4-infected patients with SVR and non-SVR

<table>
<thead>
<tr>
<th>Factor</th>
<th>SVR</th>
<th>Non-SVR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>38.47 ± 9.51</td>
<td>45.80 ± 5.65</td>
<td>0.014</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>18/7</td>
<td>15/3</td>
<td>0.48</td>
</tr>
<tr>
<td>BMI</td>
<td>27.36 ± 3.65</td>
<td>27.67 ± 5.28</td>
<td>0.85</td>
</tr>
<tr>
<td>Platelets (×10^4/µL)</td>
<td>204.4 ± 40.63</td>
<td>216.7 ± 87.25</td>
<td>0.59</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.4 ± 1.38</td>
<td>15.08 ± 1.39</td>
<td>0.25</td>
</tr>
<tr>
<td>WBC count</td>
<td>7,041 ± 1,876</td>
<td>7,078 ± 2,977</td>
<td>0.96</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.12 ± 0.36</td>
<td>4.328 ± 0.41</td>
<td>0.11</td>
</tr>
<tr>
<td>ALT (IU/liter)</td>
<td>78.72 ± 59.68</td>
<td>82.39 ± 41.80</td>
<td>0.83</td>
</tr>
<tr>
<td>AST (IU/liter)</td>
<td>64.94 ± 27.63</td>
<td>58.17 ± 23.98</td>
<td>0.44</td>
</tr>
<tr>
<td>HCV-RNA (IU/ml)</td>
<td>84,290 ± 186,300</td>
<td>501,800 ± 816,700</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*a* Values are means ± standard deviations. SVR, sustained virological response; BMI, body mass index; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

**Correlation between NS5A sequence heterogeneity and SVR in HCV-4 infection.** We and other researchers reported significant correlation between sequence polymorphisms within the C-terminal half of NS5A, including that in ISDR and IRRDR, and PEG-IFN/RBV treatment outcome in HCV-1 and HCV-2 infections (13, 15, 18, 30). However, this information is quite limited in HCV-4 infection. To clarify this issue, part of the HCV-4 genome encoding a carboxy terminus (aa 2193 to 2417) of NS5A in pretreatment sera was amplified and sequenced, and amino acid sequences were deduced. The sequences obtained as well as all available NS5A sequences of HCV-4a, -4m, -4n, and -4o obtained from SVR and non-SVR patients. In this connection, we also tested the impact of a higher (≥5) and a lower (<3) degree of IRRDR mutations on treatment outcome. IRRDR[HCV-4]≥5 was significantly associated with SVR, though with a relatively lower sensitivity (64%) than that of IRRDR[HCV-4]≥4 (Table 3). On the other hand, there was no significant correlation between IRRDR[HCV-4]≥3 and SVR.

**Correlation between core protein sequence heterogeneity and SVR in HCV-4 infection.** A close correlation between core protein sequence patterns at positions 70 and 91 and treatment outcome has been proposed, especially in Japanese patients with HCV-1b infection (4–6). To examine this hypothesis in Egyptian patients infected with HCV-4, core sequences of the viral genome were amplified from the pretreated sera, and the amino acid sequences were deduced. Due to a high degree of sequence homology among core sequences of various HCV-4 subtypes, all sequences obtained were aligned with the prototype sequence, ED43 (10). The residues at positions 70 and 91 were well conserved among the sequences analyzed, and therefore, no correlation with treatment outcome was observed for these residues (Fig. 4). All but two isolates had arginine at position 70 (Arg70), the residue that has been associated with an IFN-sensitive phenotype as far as the core protein of HCV-1b is concerned (4–6). On the other hand, Pro at position 71 showed a tendency to be more frequent in SVR than in non-SVR patients; however, the frequency was not statistically different between the two groups.

**Identification of independent predictive factors for SVR in HCV-4 infection.** In order to identify significant independent (referred to as IRRDR[HCV-4]≥4), with the difference between the two groups being statistically significant (P = 0.0004) (Table 3). It should be noted that 4 (31%) of 13 patients with null response and only 1 (20%) of 5 patients with relapse had HCV with IRRDR[HCV-4]≥4. These results collectively suggest that IRRDR[HCV-4]≥4 is significantly associated with SVR. In this connection, we also tested the impact of a higher (≥5) and a lower (<3) degree of IRRDR mutations on treatment outcome, IRRDR[HCV-4]≥5 was significantly associated with SVR, though with a relatively lower sensitivity (64%) than that of IRRDR[HCV-4]≥4 (Table 3). On the other hand, there was no significant correlation between IRRDR[HCV-4]≥3 and SVR.
predictive factors of SVR for PEG-IFN/RBV treatment outcome in HCV-4 infection, first, all available data of baseline patients’ parameters and IRRDR[HCV-4] polymorphism were entered in a univariate logistic analysis. This analysis yielded 3 factors that were correlated or nearly correlated with SVR: IRRDR[HCV-4]/H11350(0.0004), patient’s age (42 years; P = 0.03), and HCV RNA titer (5,200 IU/ml; P = 0.08). Subsequently, these 3 factors were entered in multivariate logistic regression analysis. This anal-

![Graph](image-url)

**FIG 2** Correlation between IRRDR[HCV-4] sequence variations and treatment outcome. (A) Average number of amino acid mutations in IRRDR[HCV-4] obtained from SVR and non-SVR patients. (B) Alignment of IRRDR[HCV-4] sequences obtained from SVR and non-SVR patients with HCV-4a, -4m, -4n, and -4o. The consensus sequence (Cons) of each subtype is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in each IRRDR (4a, 4m, 4n, or 4o) are shown on the right.
yssis revealed that the IRRDR[HCV-4] ≥ 4 was the only independent predictive factor for SVR in HCV-4 infection (Table 4). We then assessed SVR predictability by means of IRRDR[HCV-4] ≥ 4. As shown in Table 5, IRRDR[HCV-4] ≥ 4 would predict SVR with a positive predictive value (PPV) of 81% (P = 0.002) and sensitivity of 84%. On the other hand, IRRDR[HCV-4] ≤ 3 would predict non-SVR with a negative predictive value (NPV) of 76% (P = 0.02) and specificity of 72%. Thus, the degree of sequence variation in IRRDR[HCV-4] would yield useful positive and negative predictive markers for PEG-IFN/RBV therapy outcome in HCV-4-infected patients.

**DISCUSSION**

Both host and viral genetic factors have been implicated in influencing the clinical response to PEG-IFN/RBV therapy for HCV infection (22). It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1 (20, 37, 39). As for the viral factor(s), polymorphisms of NS5A and core regions of a given HCV genotype have been linked to a difference in SVR rates (3, 4, 13, 18, 30). This hypothesis was mostly inferred from studies carried out with Asian populations, in particular Japanese, with HCV-1b infection. However, whether it can be applied to non-Asian populations infected with non-HCV-1 is still unknown. To the best of our knowledge, this is the first study that specifically examines the relationship between HCV genome heterogeneity, in particular in NSSA and core regions, and PEG-IFN/RBV treatment outcome in Egyptian patients infected with HCV-4. In analogy with our previous studies that identified IRRDR as a significant determinant for PEG-IFN/RBV treatment outcome in Japanese patients infected with HCV-1b, -2a, and -2b (12–16), we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely associated with the ultimate treatment outcome in Egyptian patients infected with HCV-4. A high degree of sequence variation in IRRDR[HCV-4], i.e., more than 4 (IRRDR ≥ 4), significantly correlated with SVR, while a low degree of sequence variation in this region (IRRDR ≤ 3) correlated with non-SVR, null response, and relapse. The majority of patients with SVR (84%) had HCV with IRRDR of ≥ 4. In contrast, nearly two-thirds (72%) of the patients with non-SVR had HCV with IRRDR ≤ 3 (P = 0.0004) (Table 3). Notably, 21 of the 26 patients infected with HCV with IRRDR[HCV-4] ≥ 4 achieved SVR. Accordingly, the PPV and NPV of IRRDR[HCV-4] ≥ 4 for SVR and non-SVR patients were 81% (P = 0.002) and 76% (P = 0.02), respectively (Table 5). Our present results thus strongly suggest that the degree of sequence heterogeneity within IRRDR[HCV-4] would be a useful marker for prediction of treatment outcome in HCV-4 infection.

The molecular mechanism underlying the possible involvement of this region in IFN responsiveness of the virus is still unknown. The significant difference among IRRDR sequence patterns may suggest genetic flexibility of this region. Indeed, the C-terminal portion of NSSA was shown to tolerate sequence insertions and deletions (29). This flexibility might play an important role in modulating the interaction with various host systems, including IFN-induced antiviral machineries. It is also possible that the genetic flexibility of IRRDR is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN-based therapy (8, 29, 41). Also, it is worth noting that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV (25) whereas its upstream and downstream sequences show a higher degree of sequence conservation (15). This may suggest that whereas the upstream and downstream sequences have a conserved function(s) across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent function(s).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment out-

**TABLE 3** Correlation between NSSA sequence heterogeneity and virological responses in HCV-4 infection

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of isolates/total no. (%)</th>
<th>P value for SVR versus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVR</td>
<td>Non-SVR</td>
</tr>
<tr>
<td>IRRDR ≥ 4</td>
<td>21/25 (84)*</td>
<td>5/18 (28)</td>
</tr>
<tr>
<td>IRRDR ≤ 3</td>
<td>4/25 (16)</td>
<td>13/18 (72)*</td>
</tr>
<tr>
<td>IRRDR ≥ 5</td>
<td>16/25 (64)*</td>
<td>2/18 (11)</td>
</tr>
<tr>
<td>IRRDR ≤ 4</td>
<td>9/25 (36)</td>
<td>16/18 (89)*</td>
</tr>
<tr>
<td>IRRDR ≥ 3</td>
<td>22/25 (88)*</td>
<td>11/18 (61)</td>
</tr>
<tr>
<td>IRRDR ≤ 2</td>
<td>3/25 (12)</td>
<td>7/18 (39)*</td>
</tr>
</tbody>
</table>

* Sensitivity (proportion of SVR patients with the favorable factor).

* Specificity (proportion of non-SVR patients with the unfavorable factor).
In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection. The residue at position 70 of the core protein of all but two HCV-4 isolates analyzed in this study was Arg (Fig. 4), which is known to be associated with SVR in HCV-1b infection (4, 12). This high degree of sequence conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection.

Single nucleotide polymorphisms (SNPs) near the IL28B region have been identified as the strongest baseline predictors of SVR to PEG-IFN/RBV in patients with HCV-1 infection. More recently, in two major studies that were carried out exclusively with HCV-4-infected patients (9, 11), the CC genotype of rs12979860 IL28B SNP was also strongly associated with SVR. It is worth noting that although the SVR rate was more than 80% among the patients with the CC genotype, these patients represented only around 40% of total SVR cases in both studies. Furthermore, the CC genotype was found in only 34% of all Egyptian patients analyzed (9). Taken together, those observations support the idea that in addition to IL28B polymorphism, there should be an additional factor(s) that influences SVR. In this context, an interplay between IRRDR and IL28B polymorphisms might explain why some patients with undesirable IL28B genotype achieve SVR and why some patients infected with HCV isolates with IRRDR[HCV-4]/H11350 do not achieve SVR. Further comprehensive study is needed to validate the importance of IRRDR and IL28B polymorphisms in predicting the treatment outcome of HCV-4-infected patients.

In conclusion, the present study emphasizes the importance of IRRDR sequence heterogeneity in the prediction of PEG-IFN/RBV treatment outcome for different HCV genotype infections in

![Sequence alignment of the core protein of HCV-4 isolates.](image)

**TABLE 4** Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-4-infected patients treated with PEG-IFN/RBV therapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>Odds ratio (95% CI)</td>
</tr>
<tr>
<td>IRRDR mutations (IRRDR ≥ 4)</td>
<td>0.0004</td>
<td>10.5 (1.12–98.91)</td>
</tr>
<tr>
<td>versus IRRDR ≤ 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (&lt;42 years)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>HCV-RNA (&lt;5,200 IU/ml)</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 5** PPV, NPV, sensitivity, and specificity of IRRDR sequence heterogeneity on the likelihood of achieving SVR and non-SVR in HCV-4 infection

<table>
<thead>
<tr>
<th>Factor</th>
<th>PPV</th>
<th>NPV</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRRDR ≥ 4</td>
<td>81% (21/26)</td>
<td>84% (21/25)</td>
<td>76% (13/17)</td>
<td>72% (13/18)</td>
</tr>
<tr>
<td>IRRDR ≤ 3</td>
<td>72% (13/18)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a P = 0.002.
b P = 0.02.
c Proportion of SVR patients who were infected with HCV isolates with IRRDR of ≥4.
d Proportion of non-SVR patients who were infected with HCV isolates with IRRDR of ≤3.
different ethnic groups, including Egyptian patients infected with HCV-4.

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No conflicts of interest exist.

REFERENCES


