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Prevalence of NS3 Mutations Inducing Resistance to Protease Inhibitors in Chronically Infected Hepatitis C Virus Genotype 4 Patients in Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. Author OK designed the study. Authors AG and IN managed all practical experiments. Author AG managed the analyses of the study and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background and Objectives: Directly acting antiviral agents, like NS3 protease inhibitors, are representing the backbone for the current therapy of Hepatitis C virus infection.
Given the heterogeneities in HCV genome, not all protease inhibitors will be equally effective against all HCV genotypes and subtypes. Data on HCV-4 mainly Egyptian strains are still inadequate.
Here we investigated the prevalence of natural NS3 mutations and Resistance-associated substitutions (RAS) among HCV-4 Egyptian isolates.
Methods: The NS5b and NS3 domains from 40 HCV Egyptian patients (20 therapy-naïve and 20 pegylated interferon-experienced patients, who failed to achieve sustained viral response) were sequenced.
Results: Using NS5b nucleotide sequences, our 40 cases were genotyped as HCV-4 of which 38 (95%) were HCV-4a, and 2 (5%) were HCV-4o.
NS3 amino acid sequence analysis showed that substitutions C16T and V36L were detected in all

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cases while D168H/E and T54A were found in only 3 and 1 cases, respectively. No difference could be detected between naïve and experienced patients in NS3 polymorphism.
Conclusion: Our results confirm the high genetic diversity of NS3 in HCV-4 that could impair the use of some protease inhibitors to treat HCV-4 infections. The use of cocktail therapy for HCV-4 is indispensable.

Keywords: Directly acting antiviral agents DAAs; protease inhibitors; NS3 polymorphism; resistance associated variants.

1. INTRODUCTION

Hepatitis C virus (HCV) infection remains one of the profoundly pressing health emergencies worldwide, with an estimated global prevalence of 130-150 million chronically infected patients [1,2]. Egypt reports the highest incidence of HCV in the world, with an average of 14.7% [2].

HCV is characterised by genetic heterogeneity. At least seven main HCV genotypes are recognised [3]. Egypt shows the highest prevalence of genotype 4 (HCV-4), which is responsible for more than 90% of infections [2]. However, HCV-4 has significant genetic heterogeneity with more subtypes than other genotypes. To date, 18 HCV-4 subtypes have been characterized [4,5].

Up to 2011, the only available treatment for HCV infections was a standard protocol consisting of combination therapy of pegylated-interferon (PEG-IFN) and ribavirin [6]. However, according to the European Association for the Study of Liver (EASL) [7] and American Association for the Study of Liver Disease (AASLD) [8], directly acting agents (DAA), like inhibitors of the NS3 protease and NS5b polymerase, with or without PEG-IFN and ribavirin therapies are representing the backbone for the current therapy.

The NS3 protease is responsible for cleavage of the HCV polyprotein, a crucial step in HCV replication. Therefore, inhibiting the NS3 protease activity represents an attractive mechanism for blocking the release of non-structural proteins necessary for HCV replication process [9].

Protease inhibitors (PIs) are potent but have a low genetic barrier in a way that few changes in the amino acid sequence of the NS3 protease domain affect greatly its activity [10]. Considering the HCV genetic heterogeneity, not all PIs will show the same efficiency in all HCV genotypes, subtypes and strains. The first generation PIs, Telaprevir and Boceprevir, are effective only against HCV-1 genotype. The second wave inhibitors, like Simeprevir, Danoprevir, Asunaprevir, have a relatively higher genetic barrier and better activity against more genotypes [11]. The second generation PIs have a pan-genotypic activity that includes viral isolates carrying resistance against first generation inhibitors [10].

Indeed, HCV exists as a population of diverse quasispecies rather than a genetically homogenous virus. Therefore, monitoring HCV treatment with protease inhibitors will concentrate on detection of resistant variants which have not been a matter of PEG-IFN and ribavirin therapy. Additionally, several HCV mutations have been defined to be associated with reduced susceptibility to protease inhibitors [12]. This makes sequence analysis of NS3 domain an integral part of management of patients who are receiving or candidates to receive protease inhibitors.

Studies on NS3 protease polymorphism focus mainly on HCV-1, the most common genotype worldwide [13-16]. Few data are available on HCV-4 [9,17], and none on Egyptian HCV-4 strains.

In this study, the NS5b domain sequencing was used to determine the HCV genotype and subtype among chronically infected Egyptian patients. Nucleotide sequence analysis of NS3 protease domain was used to investigate the protein polymorphism and to detect the prevalence of possible mutations expected to induce resistance to PIs.

2. PATIENTS AND METHODS

2.1 Patients

This study was carried out on randomly selected forty HCV-infected Egyptian patients; 20

treatment-naïve and 20 treatment-experienced patients, who failed to achieve a sustained viral response with combined PEG-IFN (1.5 µg/kg/week) and Ribavirin (1000-1200 mg/day) therapy. Patients were enrolled from the outpatient clinic of the Medical Research Institute, Alexandria University. The study was approved by the Ethics committee of the Medical Research Institute. Informed written consent was signed by each patient before enrollment in the study.

2.2 Viral RNA Extraction

Viral RNA was extracted from patients' sera using QIAamp viral RNA mini spin Kit (Qiagen®) according to manufacturer's instructions.

2.3 Determination of HCV Viral Load

The extracted viral RNA was used as a template for HCVho load determination using Artus HCV QS-RGQ-PCR Kit (Qiagen®) and real-time PCR machine MX3000 ^{TM} (Stratagene).

2.4 Preparation of cDNA

A cDNA strand was synthesized from HCV RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using random hexamer primers provided with the kit.

2.5 Polymerase Chain Reactions

The primers used for PCR amplifications and DNA sequencing are listed in Table 1. For amplification of NS5b domain, nested PCR was used. The first amplification was performed in 25 µL final reaction volume containing 2X DreamTaq Green PCR Master Mix (Thermo Scientific), five picomoles of each of the outer primers and two µL of copy DNA. The thermal cycling consisted of 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 seconds and preceded by one cycle of initial denaturation at 95°C for 5 minutes and followed by one cycle of final extension at 72°C for 5 minutes. The same reaction was used for the second amplification (nested) in 50 µL final reaction volume containing one µL of the first amplification product as a template.

For the amplification of NS3, 50 µL PCR reaction containing 2X DreamTaq Green PCR Master Mix (Thermo Scientific), ten picomoles of each primer and five µL of copy DNA. The thermal cycling consisted of 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds and preceded by one cycle of initial denaturation at 95°C for 5 minutes and followed by one cycle of final extension at 72°C for 5 minutes.

2.6 DNA Sequencing

The purified NS3 and NS5b PCR products were sequenced using the BigDye Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems) on the automated sequencer ABI prism 310 genetic analyzers (Applied Biosystems).

2.7 Sequencing Results Analysis

For determination of HCV genotypes and subtypes, NS5b as well as NS3 sequencing results were analyzed using a HCV BLAST free online tool from HCV sequence database (https://hcv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html).

BioEdit Sequence alignment editor version 7.2.5 and Clustal W multiple alignment tools from accessory application menu were used to align NS3 amino acid sequences to determine the protease polymorphism.

Table 1. Primers used for PCR amplification of NS3 and NS5b domains

Gene	Primer name	Sequence 5' →3'	Ta (ºC)	References
NS3	G4F1	ATCTTGCTCGGGCCGGCCGA	54	[9]
	G4F2	GCGACCTGRTAGGTCTGRGGCA		
NS5b	JA 230	CTACCATCATGGCTAA(A/G)AA(C/T)GAGGT	50	[18]
	JA 233	ATGATGTTATGAGCTCCA(A/G)GTC(A/G)TA		
	JA 231	TATGA(C/T)ACCCGCTG(C/T)TTTGAC	50	
	JA 232	CCTGGTCATAGCCTCCGTGAA		

Amino acid sequence variation and signature pattern were determined using Viral Epidemiology Signature Pattern Analysis (VESPA) online software from HCV sequence database (https://hcv.lanl.gov/content/sequence/VESPA/vespa.html) as previously described [19]. It was used with NS3 amino acid sequences of 40 HCV genotypes other than HCV-4 as background reference sequences and NS3 sequences of our cases as the query sequence.

3. RESULTS

Clinical and virological characteristics of patients included in this study are summarised in Table 2.

The present study included 40 HCV patients; 20 (50%) treatment-Naïve cases and 20(50%) patients who failed to achieve sustained viral response (SVR). Twenty (50%) out of the 40 HCV cases were males, and 20 (50%) were females. Their age ranged from 9 to 69 years.

Prothrombin activity and alanine transaminase (ALT) were normal in 85% and 62.5% respectively of cases included in the study. Additionally, all patients had normal platelet count.

The majority of the 40 HCV patients (80%) had a viral load ranging between $>10^5$ - $<10^7$ IU/ml. Only 3 cases (7.5%) had a viral load $< 10^5$ and 5 cases (12.5%) had a viral load $>10^7$ IU/ml.

NS3 and N5b regions were successfully amplified and sequenced in all 40 cases included in this study. The forty HCV cases involved in this study were genotyped as HCV genotype 4 using both NS3 and NS5b regions sequencing. Both regions were in concordance in sub-genotyping 37 cases (92.5%) as 4a. The remaining 3 cases were differently sub-genotyped as 4a or 4o.

Analysis of NS3 amino acid sequences using VESPA program revealed 30 amino acid signatures (1) among our HCV genotype four isolates, of which 18 were common with those reported by Lopez-Labrador et al. in 2008 [19]. V36L amino acid substitution conferring resistance to Telaprevir and Boceprevir [19,20] was detected as a signature amino acid in 100% of our strains.

Table 2. Clinical and virological data of the patients

	Treatment Naïve (n=20)		Treatment experienced (n=20)	
Gender				
Male	11	55%	9	45%
Female	9	45%	11	55%
Age				
< 20	3	15%	1	5%
20-40	4	20%	2	10%
40-60	10	50%	13	65%
> 60	3	15%	4	20%
Viral Load IU/mL				
$< 10^4$	0	0	1	5%
$>10^4$-$<10^5$	1	5%	1	5%
$>10^5$-$<10^6$	8	40%	5	25%
$>10^6$-$<10^7$	9	45%	10	50%
$>10^7$	2	10%	3	15%
Alanine Transaminase (ALT)				
Normal	11	55%	14	70%
Abnormal	9	45%	6	30%
Prothrombin activity				
Normal	16	80%	18	90%
Abnormal	4	20%	2	10%
Platelet count				
Normal	20	100%	20	100%
Abnormal	0	0%	0	0%

Signature sequences

Query signature .....FS.....T..NC.....S....AV...M.....IS.....N.....V...A....A.....V.V....T.GA.....T.....L...M..RA.....V..V...S..

Background signature .....LG.....K..VE.....T....CI...L.....LA.....T.....T...T....S.....I.A.....S.AS.....Y.....V...S..VV.....L..I...N.....

Threshold query signature: .....

Signature AA Frequencies

Query signature	F	S	T	N	C	S	A	V	M	I	S	N	V	A	A	V
Frequency in query	0.951	0.927	0.927	1.000	0.927	1.000	0.951	0.927	0.951	1.000	0.951	0.951	1.000	0.537	0.829	0.829
Frequency in background	0.067	0.067	0.000	0.000	0.000	0.467	0.033	0.233	0.333	0.133	0.000	0.000	0.000	0.067	0.300	0.067
Background signature	L	G	K	V	E	T	C	I	L	L	A	T	T	T	S	I
frequency in query	0.049	0.049	0.024	0.000	0.000	0.000	0.000	0.073	0.024	0.000	0.000	0.000	0.000	0.268	0.171	0.146
frequency in background	0.800	0.933	0.967	0.500	0.567	0.533	0.400	0.767	0.367	0.867	1.000	0.333	0.400	0.500	0.700	0.867

Query signature	T	G	A	T	L	M	R	A	V	V	S
Frequency in query	1.000	1.000	0.976	0.951	1.000	0.951	0.537	0.854	0.976	0.854	0.976
Frequency in background	0.300	0.467	0.400	0.300	0.333	0.000	0.000	0.167	0.233	0.233	0.367
Background signature	S	A	S	Y	V	S	V	V	L	I	N
frequency in query	0.000	0.000	0.024	0.000	0.000	0.000	0.049	0.146	0.000	0.098	0.000
frequency in background	0.400	0.533	0.567	0.400	0.467	0.567	0.567	0.833	0.500	0.767	0.433

Fig. 1. HCV-4 NS3 protease signature pattern using VESPA online tool.

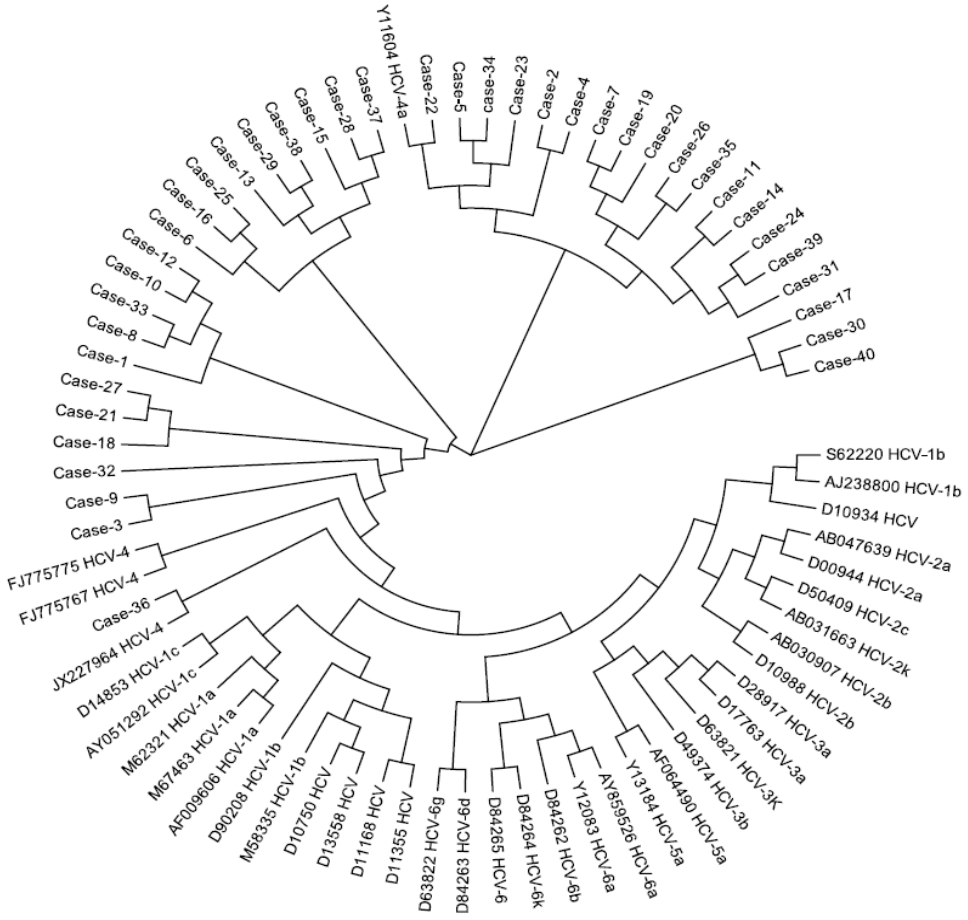

Fig. 2. Phylogenetic tree obtained from the analysis of the hepatitis C virus (HCV) NS3 gene sequences collected during the current study and other HCV genotypes found in the GenBank database

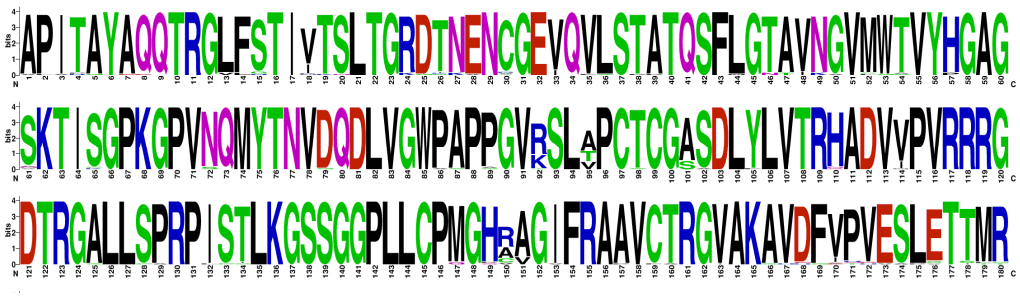

Fig. 3. Alignment of sequence logo showing Amino acid sequence diversity in NS3 protease among 40 HCV-4 Egyptian isolates

Table 3. Number of NS3 amino acid mutations per case

Number of mutations/case	Naïve Patients		Treatment experienced		Total	
	No.	%	No.	%	No.	%
<5 mutations	3	15	1	5	4	10
>5-<10 mutations	14	70	15	75	29	72.5
>10-<15 mutations	-	-	3	15	3	7.5
>15-<20 mutations	2	10	1	5	3	7.5
>20 mutations	1	5	-	-	1	2.5
Total	20	100	20	100	40	100

Phylogenetic analysis and amino acid sequence logo alignment of NS3 sequences are shown in (Figs. 2 and 3).

Table 4. Mutations associated with resistance to protease inhibitors

Mutations	Naïve		Treatment-experienced	
	No.	%	No.	%
A39P	1	2.5	-	-
T54A	1	2.5	-	-
D79G	1	2.5	-	-
H149P/Q	1	2.5	1	2.5
D168H/E	1	2.5	1	2.5
V170A/E/I	2	5	2	5

4. DISCUSSION

HCV is characterized by genetic heterogeneity. At least seven major HCV genotypes are identified, of them, HCV genotype 4 has the highest prevalence among Egyptians. Defining the natural variation of the NS3 protease among Egyptian patients is crucial for the identification of future resistance to currently available and newly introduced PIs.

Naturally occurring polymorphisms may lead to a decreased viral response to specific therapy. However, selected viruses exhibiting resistance to some inhibitors may show reduced fitness. Genotypic-specific naturally occurring variations also affect the efficacy of PIs [21-23].

In the present study, nucleotide and amino acid sequences of the NS3 region were aligned with Clustal W and Bioedit version 7.2.5 software and analysed for the presence of previously identified substitutions in HCV genotype-4 conferring resistance to NS3 protease inhibitors and for polymorphism.

The amino acids at positions described to be involved in important catalytic activities of NS3, mainly those of the triad (H57, D81 and S139), zinc-binding residues (C97, C99, C154 and H149) and substrate binding region (L135, F154, R161 and K165) [9,19] were conserved in all HCV genotypes. In the present study, an H57P substitution in the catalytic triad and a second substitution in the zinc-binding domain H149P were detected in 1 (2.5%) and 2 (5%) cases respectively. Residues involved in substrate binding were totally conserved among all our cases.

Polymorphism at positions not central to NS3 protease catalytic activity and substrate binding may contribute to clinical resistance to HCV protease inhibitor therapy [9].

Lin et al. in 2004 [23] and Seiwert et al. in 2007 [24] stated that polymorphism at position

D168H/E could be associated with high-level resistance to Danoprevir, Ciluprevir and Simeprevir PIs. Paolucci et al[17] also observed D168E in 13% of their genotype-4 patients. This mutation was detected in only 2 (5%) of our cases.

Amino acid substitutions D168 to T/I/A/V were reported in association with Vaniprevir resistance [9], while D168 substitution to A/E/N/T/V/Y were reported with Asunaprevir (withdrawn from FDA approval) resistance [25]. Wu et al. [26] detected a D168N mutation in 6.8% of their 88 cases. Amino acid substitutions associated with Vaniprevir resistance were not detected among our cases, while a D168E associated with Asunaprevir resistance was found in only one the(2.5%) case.

Several studies [9,16,26,27] reported that a T54A/S substitution was associated with low-level resistance to Telaprevir and Boceprevir. T54A mutation was detected in only 1 (2.5%) case in our study.

Lopez- Labrador et al. [19], Susser et al. [16] and Tong et al. [28] reported that V170A/T is a major resistance mutation for Boceprevir. In the present study, V170A substitution was detected in 2 (5%) cases. Vallet et al. [9] detected V170T substitution, which confers a low level of resistance to Faldaprevir (to be discontinued). This substitution was not found among our cases.

A conservative substitution V170I was detected in 45% of genotype-1and in almost 100% of genotype-2 and -3 isolates in a study conducted by Lopez-Labrador et al. in 2008 [19]. This was detected in only 1 (2.5%) case in this study. Sarrazin et al. in 2007 [27], demonstrated that this substitution did not affect Telaprevir efficacy.

The substitution V170E was detected before by Morsica et al. [29], who found it in a patient co-infected with HIV receiving antiretroviral therapy. This mutation was detected in only 1 (2.5%) case in this study in the absence of HIV co-infection.

V158I substitution previously reported [9] to be associated with low-level resistance to Boceprevir was not detected in all 40 cases.

Halfon et al. [30] and Fonseca-Coronado et al. [31] reported that A156T and R155K mutations are known to confer a high level of resistance to all PIs. Paolucci et al. [17] reported that these mutations were not detected in their study and they were not detected in our study as well.

Substitutions Q80K/R responsible for resistance to Simeprevir is common among genotype-1 patients; who were also advised to check for this mutation before the start of any PI. Lenz et al[32] and Vallet et al[9] detected this substitution in the majority of their cases. This mutation was not detected among our cases since they were all of genotype-4.

Our findings agree with Lopez-Labrador et al. [19] who reported that amino acid variants at sites associated with resistance to Danoprevir (F43S, Q41R, S138T and A156S/V) were not detected.

Vallet et al. [9] in 2011 stated that position 47, at the N-terminal extremity of the third β-strand, was the most polymorphic. However this was not observed in this study, where only 2 (5%) cases showed A47T mutation.

Moreover, more mutations were detected in our cases that were not yet published and should be further evaluated.

5. CONCLUSION

In summary, our results ascertain the high genetic diversity of NS3 in genotype 4. This could impair the use of protease inhibitors as well as hinder the development of new NS3 targeted drugs effective against genotype-4 infected patients. Polymorphism in NS3 is not affected by previous treatment with PEG-IFN/Ribavirin combined therapy. The clinical consequences of this naturally occurring polymorphism in HCV genotype-4 strains are, to date, unknown, and they will remain so until large therapeutic trials in HCV genotype-4 infected patients will be conducted on all new PIs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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