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ORIGINAL ARTICLE

Molecular Characterization of NS5A Resistance Associated Substitutions after Failure of Sofosbuvir/Daclatasvir Combination Therapy in Chronic Hepatitis C genotype 4a Infected Egyptian Patients Compared to Direct-acting Antiviral Agent (DAA)-naïve Sequences

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ABSTRACT

Background: *Direct-acting antiviral agents (DAAs) for treatment of chronic HCV had proven an outstanding efficacy with low rates of virologic failures. However, the pre-existence and on-treatment selection of resistant subpopulations to the DAA in use can contribute to treatment failure.* ***Objectives:*** *To investigate the pattern of NS5A resistance associated substitutions (RASs) related to failure of Sofosbuvir/ Daclatasvir combination therapy in HCV genotype 4a infected Egyptian patients in comparison to baseline sequences from DAA-naïve patients, infected with HCV genotype 4a deposited in the GenBank.* ***Methods:*** *A 100 chronic HCV genotype 4a patients who achieved an end of treatment response to a 12 weeks course of Sofosbuvir/Daclatasvir combination therapy were tested for achieving sustained virologic response (SVR). In patients with relapse, characterization of RASs in NS5A domain I was done by direct sequencing technique after amplification by reverse transcriptase nested PCR. Baseline mutations prevalent in NS5A region of HCV genotype (GT) 4a were determined by studying similar sequences from DAA inexperienced GT4a patients deposited into the GenBank.* ***Results:*** *Seven out of 100 chronic HCV patients showed a relapse. 75% of patients experiencing a relapse showed NS5A RASs; 2 patients (50%) showed 2RASs and 1(25%) showed 3 RASs. Meanwhile, 17% of the studied GT4a sequences from the GenBank showed baseline NS5A RASs; 1sequence (2.86%) showed 2 baseline RASs and 5 (14.29%) show 1 baseline RAS.* ***Conclusion****: NS5A RASs were detected at higher rates in chronic GT4a patients with relapse. Their role in DAA treatment failure in presence of other negative predictors should be thoroughly investigated.*

INTRODUCTION

In Egypt, the country with the highest HCV prevalence worldwide, HCV and its related complications are a pronounced health and economic problem.[1,2] The Demographic Health Survey (DHS) of 2015 showed that the total seroprevalence in those aged <60 years is 6.3% and the viremic prevalence is 4.4%. In Egypt, genotype (GT) 4 accounts for approximately 90% of infections, with subtype 4a predominating.[3]

The combination of pegylated interferon and ribavirin had been the mainstay of treatment with a sustained virologic response (SVR) that did not exceed 60% in genotype 4 infected patients.[4] In addition, side effects of IFN and ribavirin and multiple contraindications were major problems.[5]

The introduction of direct-acting antiviral agents (DAAs) for the treatment of chronic hepatitis C virus (HCV) has represented a substantial shift in HCV treatment. They are highly effective, with rates of SVR of around 90%-95%, well tolerated and safe [6-8].

Currently, four classes of DAAs have been approved for the treatment of HCV. They act on three therapeutic targets: non-structural NS3/4A protease inhibitors (telaprevir, boceprevir, simeprevir, paritaprevir, and grazoprevir), non-structural 5A (NS5A) replication complex inhibitors (daclatasvir, ledipasvir, ombitasvir and elbasvir) and nucleoside (sofosbuvir) and non-nucleoside (dasabuvir) NS5B RNA dependent polymerase inhibitors [9,10].

In clinical practice the rate of therapeutic failure with these DAAs is estimated at 10%-15%[11], mainly presenting as relapses and occasionally as viral breakthrough during treatment in non-adherent patients[12]. Most cases are associated with the presence of drug resistant variants which are viral variants with amino acid polymorphisms in a viral protein targeted by a DAA that reduce viral susceptibility to the DAA or

DAA class. These polymorphisms are known as "resistance associated substitutions" or "RASs". [12-15]

The great genetic variability of HCV due to the high turnover rate of the virus and the lack of proof reading function of HCV RNA polymerase, results in presence of natural or baseline RASs. These can be present in major, highly fit viral populations but more often, they are present in minor viral populations with reduced fitness relative to wild-type viruses. These viral populations usually exist in equilibrium with proportions relevant to their replication fitness. However, the administration of DAAs alters this equilibrium, inhibits the sensitive wild-type variants and selects for the resistant variants that predominate at the time of virologic failure [16].

DAA therapy for HCV aims at complete elimination of the virus in a biphasic course; first: a rapid decline in HCV RNA by the direct inhibitory effect of the drug(s) on viral replication followed by a slower decline where the host innate immunity degrades non-replicating viral RNAs from infected cells. In this perspective, a number of host, viral and treatment-related factors have been implicated in treatment failure. Host factors include genetic characters (e.g., Interferon lambda 3 (IFNL3), formerly IL28B, gene polymorphism) and cirrhosis. Viral factors like HCV genotype or subtype and baseline RASs. Factors related to the treatment regimen include previous treatment, duration of treatment, drug potency and genetic barrier to resistance and the addition of ribavirin. [15]

NS5A inhibitors target domain I of NS5A protein, a multifunctional phosphoprotien that regulates viral replication and assembly. They have pan genotypic efficacy in a single daily dose [17]. First-generation NS5A inhibitors (daclatasvir, ledipasvir and velpatasvir), currently approved for treatment of GT4 infected patients, posses both a low genetic barrier to resistance and wide cross resistance. Therefore they are used in combination therapy with other DAAs

NS5A RASs have been detected in the N terminus of NS5A domain I. The most common mutations are associated with substitutions in Met 28, Gln 30, Leu 31, Pro 32, and Tyr 93 [18,19]. These positions mostly represent the sites of NS5A/NS5A inhibitors interaction. RASs at other positions also exist and are believed to be compensatory mutations selected to restore replication fitness altered by the primary RASs. Unlike treatment emergent NS3/4A RASs which disappear within a few weeks to months after treatment with NS3/4A containing regimens and are replaced by wild-type DAA-sensitive virus, NS5A RASs can persist for years after treatment failure, either because they are naturally more fit or because they are unable to revert to wild-type virus [20-24]. This fact has implications when deciding a re-treatment strategy after DAA failure.

Sofosbuvir, the only commercially available NS5B nucleoside inhibitor, has a high genetic barrier to resistance and is thus used in combination with NS5A inhibitors to provide all-oral interferon-free regimens for treatment of GT4 infection in Egypt [3]. The aim of this study is to investigate pattern of NS5A RASs related to failure of Sofosbuvir/Daclatasvir combination therapy in HCV GT4a infected Egyptian patients using direct sequencing technique of NS5a domain I in comparison to baseline sequences from DAA-naïve patients, infected with HCV GT4a deposited in the GenBank.

METHODOLOGY

Patients and study setting*:*

This cross-sectional study was conducted in Microbiology and Immunology and Tropical Medicine Departments in collaboration with The Scientific and Medical Research Center, Faculty of Medicine Zagazig University on 100 chronic HCV patients that received DAA therapy, they were chosen by systematic random sampling and tested for achieving a SVR 12 weeks after completing a 12 weeks course of Sofosbuvir/Daclatasvir combination therapy received according to the 'National Committee for Control of Viral Hepatitis' (NCCVH) HCV treatment program. All patients had undetectable serum virus level at the end of treatment.

Patients with history of hepatocellular carcinoma, co-infection with hepatitis B virus or human immunodeficiency virus, other chronic liver diseases, or evidence of hepatic decompensation were excluded.

Institutional approval was obtained from the Institutional Review Board, Faculty of Medicine, Zagazig University. A written informed consent was obtained from each patient.

Data collection:

HCV infection data were collected retrospectively from patients' medical records including HCV genotype, initial and follow-up viral loads, previous HCV treatment, date of starting treatment and duration.

HCV Molecular workup:

Detection of virologic relapse and characterization of RASs in NS5A domain I at failure:

- ***Blood samples*** were collected from patients 3-6 months after the end of the treatment course. Sera were separated and stored at -20˚C**.**
- ***RNA extraction*** was performed using (QIAamp Viral RNA Mini kit, Netherlands).
- ***HCV RNA detection and quantification*** was determined using Artus® HCV RG RT-PCR Kit on Rotor-Gene Q real time cycler (Qiagen, Hilden, Germany) as per manufacturer's instructions.

Detection of Resistant associated substitutions in NS5A region in sera of patients experiencing virologic relapse:

HCV NS5A domain I (213aa) gene:

This was amplified from serum at time of virologic failure by reverse transcriptase nested PCR using genotype 4 specific primers previously reported by Plaza et al.[25] with few modifications as follows:

- Reverse transcription of RNA extract and first round of amplification were performed using One-step RT-PCR kit (QIAGEN, INC., Netherlands). Each reaction mixture contained 20 µl of the extracted RNA, 10 µl QIAGEN One-step RT-PCR Buffer, 400 µM of each dNTP, 2 µl QIAGEN One-step RT-PCR Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotstarTaq DNA Polymerase), 0.4 µM of each of external pair of GT 4-specific primers; NS5A EF4 5'-GGC AAY CAC GTG KCT CCC AC-3' , NS5A ER4 5'-CTG RCT MGC CGA GGA -3' (Invitrogen, Thermo Fisher Scientific, USA). Finally, RNAase free water was added to reach a total volume of 50µL.
- Cycling conditions were as follows: 50°C for 30 min for cDNA synthesis by the action of reverse transcriptases then 95°C for 15 min to inactivate reverse transcriptase enzyme and activate HotStarTaq DNA polymerase. This was followed by 45 cycles of 94°C for 35 sec, 53°C for 30 sec and 72°C for 1min and 30 sec. A final extension step at 72 °C for 10 min was done.
- A second round of amplification was performed using 3 µl from the product of the first round PCR, 25 µl of Taq PCR Master Mix (Qiagen GmbH, Hilden, Germany) and 0.4 µM of each genotype 4 specific internal primers NS5A IF4 5'-CAC AAG TGG AYC AAT GAR GA- 3', NS5A IR4 5'-GAG GGT SGT GAC CC-3' and water for the remaining of the 50 µl volume. Cycling conditions were as follows: 94°C for 3min followed by 45 cycles of 94°C for 35 sec, 50°C for 30 sec and 72°C for 1min and 30 sec. followed by final extension at 72 °C for 10 min.
- 10 µl of the amplified PCR products were run on 2% agarose gel electrophoresis and the specific products of length 696 bp, were purified using (Wizard SV Gel and PCR clean-up system, Promega USA) purification kit from the remaining PCR product volume.

Direct sequencing of the amplified HCV NS5A domain 1:

It was done using the dideoxynucleotide chain termination method and sequence analysis was performed with the ABITM3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The NS5A IF4 primer was used as a sequencing primer. The chromatogram sequencing files were inspected using Chromas 2.6.5 (Technelysium, Helensvale, Australia). Each obtained sequence was compared with those of NS5a prototypes obtained from GenBank, followed by further genetic analysis. All obtained sequences were retrieved and aligned using Mega software version 7.0.21.

Detection of baseline RASs prevalent in NS5A region of HCV genotype 4a:

This was done by studying similar sequences in HCV genomes, obtained from DAA inexperienced GT4a patients, deposited into the GenBank. The used GenBank search strategy was as follows; HCV genomic sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) in August 2017 using the key words "hepatitis C virus" or "HCV." After the initial search, near full-length HCV sequences with custom range from 8500-9600 and a date range from 2000 to end of 2012 to exclude sequences which were typed from relapsers, were screened for GT 4a and any duplicate sequences were discarded, GenBank Accession Number, and the geographic data was recorded. The obtained baseline sequences were aligned with those of NS5A sequences of patients with relapse. The sequence alignment was performed with Bio Edit sequence alignment editor (Bio Edit 7.1) software followed by sequence editing, exclusion of sequences with missing data, and translation of the nucleic acids sequences into amino acids.

The resulting protein sequences were analyzed to identify mutations in the following NS5A domain I positions; L28, L30, L31, P58, E62 and Y93**,** which had been previously reported to be associated with treatment failure and/or conferred a reduction in NS5A inhibitors susceptibility of more than two folds in comparison with a wild-type strain in *in-vitro* replicon assays of HCV GT 4.[24, 26]

RESULTS

Seven out of 100 chronic HCV patients, who received a 12 weeks course of Sofosbuvir/Daclatasvir oral combination, showed a relapse with detectable serum HCV 3-6 months after achieving an end-of treatment response to therapy. Patients' characteristics are presented in table 1

Table 1: Characteristics of patients with treatment failure

Pt. No	Gender	Age (ys)	GT	Previous treatment*	virus load IU/ml	Liver cirrhosis	DAA regimen
1	Male	64	4a	No	1,614,439	Yes	SOF/DAC
2	Male	59	4a	No	3, 496, 913	No	SOF/DAC
3	Male	48	4a	Yes	411, 192	No	SOF/DAC/RBV
4	Male	41	4a	Yes	917,114	Yes	SOF/DAC/RBV
5	Female	56	4a	No	1, 076,963	Yes	SOF/DAC
6	Male	35	4a	No	300, 741	No	SOF/DAC
7	female	61	4a	No	2, 251, 578	No	SOF/DAC

*Abbreviations: SOF; Sofosbvir, DAC; Daclatasvir, RBV; Ribavirin, GT; genotype. *Pegylated interferon /Ribavirin*

NS5A domain I was successfully amplified from the 7 patients with relapse (figure 1)

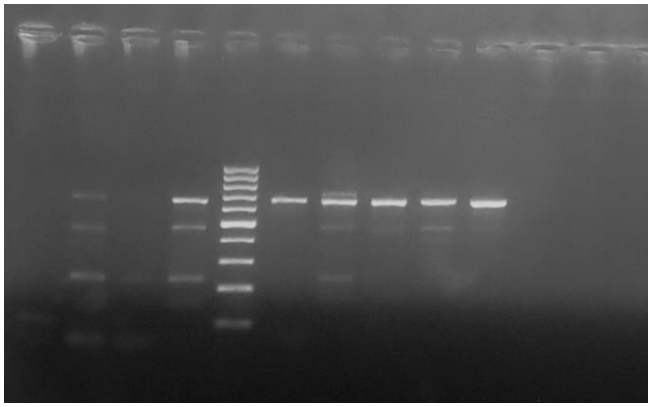

Fig. 1: Agarose gel electrophoresis for the amplified NS5A in 7 patients with relapse, showing a band of length 696 bp.

Out of the seven amplified products, 4 samples only were successfully sequenced and the NS5A RASs were detected in Daclatasvir binding sites as shown in Table 2. The frequency of RASs was higher in patients with relapses than the studied GT4a baseline sequences particularly at positions L28, L30, P58 and E62 (fig. 2, Fig. 3). Apart of L31M present in all studied sequences; 75% of patients experiencing a relapse after 12 weeks DAA course showed NS5A RASs: 2(50%) showed 2RASs and 1(25%) showed 3 RASs (Table 2). Meanwhile, 17% (6 of 35) of the studied GT4a sequences from GenBank showed baseline NS5A RASs: 1(2.86%) shows 2 baseline RASs and 5 (14.29%) show 1 baseline RAS table 3.

Table 2: RASs in NS5A domain I detected at relapse in 4 sequenced patients

Patient	RAS type at relapse
Patient (1)	L28M, L31M, E62D
Patient (2)	L31M only
Patient (3)	L28M, L30S, L31M, P58S
Patient (4)	L28M, L30S, L31M

Table 3: Baseline NS5A RASs in studied GT4a sequences from GenBank

Amino acid position	Base line RAS
L28	L28M (5/35), L28V (1/35)
L30	L30S (0/35)
L31	L31M (35/35)
P58	P58S (0/35)
E62	E62D (1/35)
Y93	(0/35)

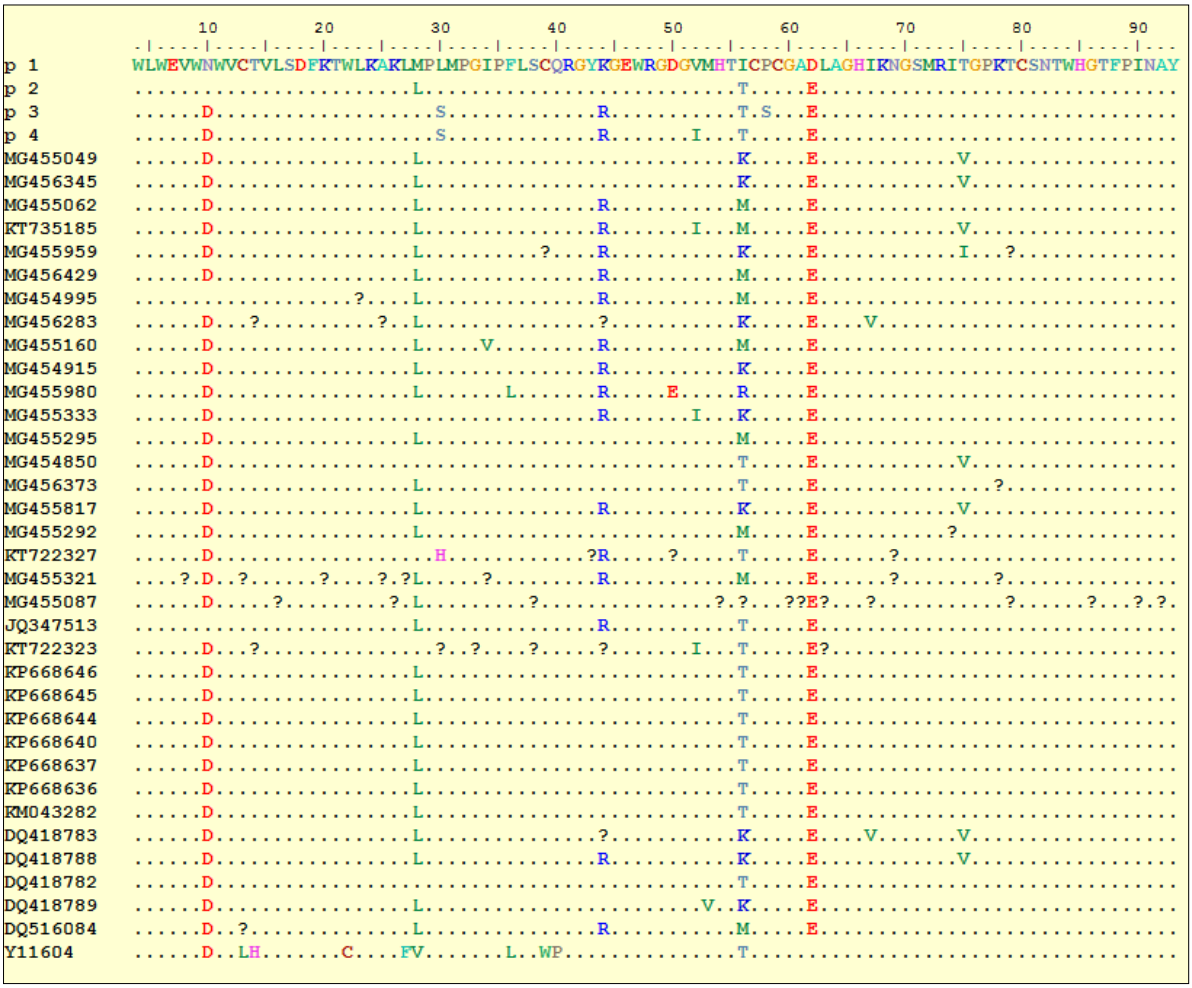

Fig. 2: Bio Edit sequence alignment of first 93 amino acids of the HCV NS5A domain I for the 4 patients with relapse (P1-4) followed by the 35 HCV-GT4a DAA- naïve sequences from the GenBank.

Notes: L31M conferring low to moderate resistance is present in all GT4a sequences, E62D is present in patient (P1) and GenBank sequence (y11604), L30S is unique to patients (P3, 4) and P58S is unique to patient (P 3)

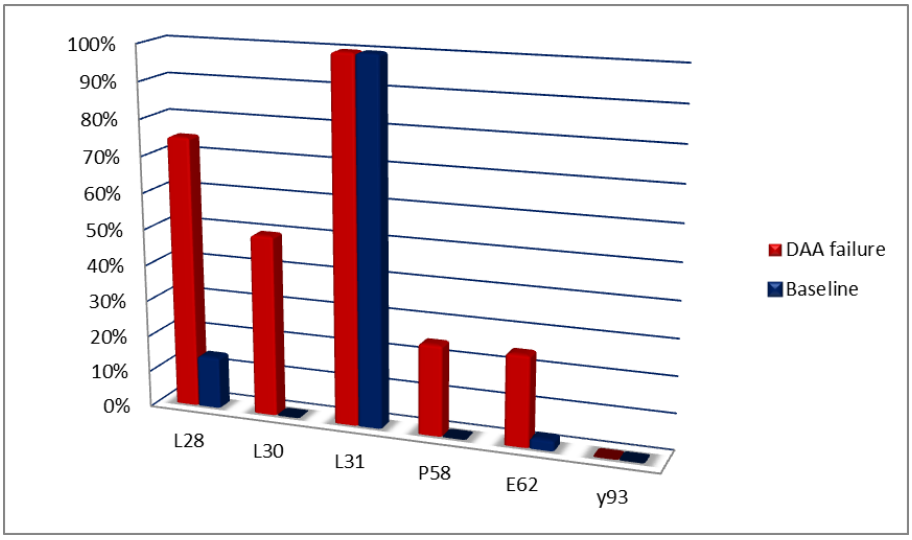

Fig. 3: Frequency of NS5A RAS in the studied sequences

DISCUSSION

In Egypt, since early 2016 the national guidelines for HCV treatment, as set by The National Committee for Control of Viral Hepatitis (NCCVH) established by the Ministry of Health in 2006, were modified to be exclusively IFN free, by giving Sofosbuvir/Daclatasvir with or without ribavirin for 12 weeks to all patients, justified by the low cost, the high response rate and the safety of this combination [3].

Despite the high SVR rates of DAA therapy, the small percentage experiencing treatment failure is actually a considerable number of patients due to the huge number of patients receiving DAA. Different factors have been proposed to act simultaneously in DAA treatment failure. Of these factors, the role of RASs has been thoroughly elucidated. Viral variants with these RASs present at baseline in low proportions of 1%-15% (i.e., below the lower limit of detection of direct sequencing techniques) do not appear to significantly influence the response while those present at higher rates seem to contribute to DAA treatment failure [10].

Several studies focused on the prevalence of baseline NS5A RASs in treatment inexperienced patients from different countries. Others studied the effect of each RAS on the activity of NS5A inhibitors by *in-vitro* replicon assays. Most of the previous studies were on GT 1 and 2 and only a few included GT4 sequences.

The prevalence of resistant variants to NS5A inhibitors is highly dependent on viral subtype due to several positions having different baseline amino acids in each subtype [27]. Therefore, the current study focuses on the pattern of NS5A RASs in GT4a infected Egyptian patients experiencing a relapse 12 weeks after achieving an end-of-treatment response to a 12 weeks course of Sofosbuvir/Daclatasvir treatment. For the purpose of comparison to the baseline (pretreatment) prevalence of such variants and due to unavailability of pretreatment data on such prevalence for the enrolled patients, 35 HCV GT4a sequences from DAA-inexperienced patients deposited in the GenBank were studied.

The SVR rate was found to be (93%) in the studied patients. this is in accordance with other Egyptian studies in Minia Upper Egypt (91%), in Cairo and Tanta (92.67%) and in a cohort of patients from a village in Sharkia (96%) [28,29,30].

NS5A domain I region was amplified from the 7 patients (Figure 1) but only 4 products were successfully sequenced (Figure 2). 75% of these patients revealed at least one RAS in their sequences, at the amino acids 28, 30, 31, 58 or 62, but not at amino acid 93 that has been reported to confer high level resistance to NS5A inhibitors. On the contrary, Only 6 (17%) of the DAA-naïve sequences from the GenBank showed baseline NS5A RASs; 2 baseline RASs in 1 patient (2.86%) and 1 baseline RAS in 5 (14.29%).

Interestingly, all studied NS5A regions from relapsing patients and sequences from untreated patients from the GenBank possessed L31M, which was found to be associated with high (>100 fold) resistance to Daclatasvir in GT1a replicon studies and low level (2-20 fold) resistance in GT1b, This is in accordance to other studies on GT4 that found L31M in all studied GT4 sequences [30-31]. RASs at position L28 was the second common (75%), all were L28M known to confer low level resistance (2-20 fold increase in Daclatasvir EC_{50}), followed by L30S (50%), then E62D and P58S (25% each) (fig. 3).

Recently, Deitz et al.[16] in a study on samples from DAA naïve patients and those experiencing DAA failure from different European countries found that low-level resistant L30R was detectable at relatively high frequencies in DAA-naïve GT4 infected patients, especially in those with subtype 4d. After DAA failure, RAS at position L28 were prominent (66%), which mainly was caused by an increase of low level resistant L28M. Moreover, Y93 variants appeared in (33%) with Y93C/H variants were mainly detected.

In a study on 166 chronic HCV patients in Spain, 16 (9.6%) had GT4 infection, all received NS5A inhibitor containing regimens, Baseline NS5A RASs were detected at a frequency of (36%) in DAA-naïve patients including M28A/G/T (5), Q30X (12), L31I/F/M/V (6), T58P/S (25), Q/E62D (1), A92 K (7) and Y93C/H[15]. In the same study, 10 (6%) patients failed DAA therapy; five (50%) of whom had baseline NS5A RASs. Upon failure, six patients showed treatment-emergent RASs, including Q30C/H/R (3), L31M (1), and Y93C/H (2). This study, however performed on patients with marked variability in infecting genotypes and treatment regimens, but had the advantage of studying the prevalence and types of RASs at baseline and on failure for each patient showing that the presence of two or more baseline NS5A RASs were significantly associated with failure to NS5A inhibitors-including regimens and were more frequent in GT4 (12.5%) [31].

There have been speculations regarding the impact of NS5A RASs on DAA treatment outcomes; viral eradication was reported in patients with baseline and treatment-selected RASs[32,33]. Meanwhile virologic failure, particularly relapses, was observed in patients without detectable RASs and with potent combinations[34].

Pretreatment testing for drug resistant variants is not currently recommended for GT4 patients, but the wide spread and prolonged use of DAA therapy makes the appreciation of the relevance of baseline resistant variants to the response to DAA therapy an important issue for the selection of the optimal regimen [35].

Moreover, the high rates of multiple RASs in DAA treatment failures, in addition to the high cost, make it useful to test for treatment-selected resistant variants to determine the most effective re-treatment option [26].

With the exception of NS5B nucleoside analogues, the current DAAs target the NS3/4A, as well as the allosteric sites of NS5B and NS5A; all have a low genetic barrier to resistance [18]. NS5B RASs were not investigated in our study, The NS5B RAS, S282T, known to convey resistance to Sofosbuvir in *in-vitro* assays was neither detected by a previous study on GT4 Egyptian patients treated with Sofosbuvir plus Ribavirin combination therapy [36] nor by other non-Egyptian studies [35,37], except for a single study that analyzed 1459 HCV sequences from GenBank that found this substitution in only one sequence [38]. Viral variants with this substitution have markedly reduced replication fitness and are thus rarely selected.

Detection of RASs in different DAAs targets by more sensitive deep sequencing methods in large numbers of HCV GT4 patients, and evaluating their exact role, in presence other negative predictors, in failure of DAA therapy are potential targets for future researches.

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