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Association of multiple mutations in *NS5A* and *NS5B* genes and resistance to direct-acting antivirals in chronically infected Egyptian patients with Hepatitis C virus Genotype 4a

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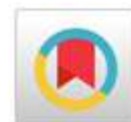

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Abstract

Approximately 71 million people worldwide are supposed to have chronic hepatitis C virus (CHCV). New direct-acting antiviral (DAA) medications have been used, which helped successfully in complete treatment of CHCV and achieved a sustained virological response (SVR). However, some patients may acquire HCV resistance to DAAs, which may result in treatments failure. The aim of the present study was to compare among the patients that were experiencing virologic relapse (VR) and SVR in relation to the patterns of *NS5A* and *NS5B* genes resistance associated substitutions (RASs) in the chronic HCV infected Egyptian patients, who received Sofosbuvir (SOF) and Daclatasvir (DCV) combination therapy. All patients that were infected by chronic HCV had completed treatment with SOF and DCV combination therapy and were followed up after the end of this treatment. A total of 10 out of 100 serum specimens were collected from the enrolled patients and analyzed, where two and eight specimens were representatives for VR and SVR, respectively. These samples had undergone reverse transcriptase-polymerase chain reaction amplification (RT-PCR) of *NS5A* and *NS5B* genes, partially sequenced by the Sanger method, and then analyzed phylogenetically to determine their genetic subtypes and RASs. Finally, SVR was gained in all but two patients who were experiencing VR that carried natural *NS5A*-RASs at positions L31M and Y93H. They were considered as significant for DCV resistance as well as natural *NS5B*-RASs (T282S), which represented the main polymorphism for SOF resistance. In this study, a number of mutational combinations in the analyzed *NS5A* and *NS5B* genes were identified, which may increase the risk of treatments failure in the patients administered regimens including multiple DAA, compared to the baseline sequences of those patients that were experiencing SVR.

Keywords: HCV, Drug resistance, *NS5A*, *NS5B*, Sofosbuvir, Daclatasvir

1. Introduction

Egypt is considered as one of the main countries that have the highest rates of hepatitis C virus (HCV) infection in the world. Approximately 58 million people worldwide have chronic HCV infection, with 1.5 million new cases reported each year. There are an estimated 3.2 million chronic HCV infections among the adolescents and the children (Abdel-Gawad *et al*., 2023). HCV is an enveloped +ve sense ssRNA virus belonging to genus *Hepacivirus*, order Amarillovirales, and family *Flaviviridae* (Simmonds *et al*., 2017).

The HCV genome is an RNA molecule that is roughly 9.6 kb nucleotides long and codes for a nearly 3,000 amino acid poly-protein precursors. At least ten distinct proteins, including three structural proteins (*i.e*., core, and envelope E1 and E2) are produced by cleaving the precursor, and seven of these proteins are non-structural ones (*i.e*., *p7*, *NS2*, *NS3*, *NS4A*, *NS4B*, *NS5A*, and *NS5B*) (Ahmad *et al*., 2011). HCV exhibits considerable genetic variability and is currently classified into major 8 genotypes that are designated as 1-8, and into a large number of subtypes (around 86) with different geographic prevalence's (Simmonds *et al*., 2005). The most common genotype in Egypt is genotype 4, which is present in almost 90 % of the Egyptian patients (Abdel-Aty *et al*., 2017). However, genotype 4 that has 18 subtypes; the most common is subtype 4a, due to its high level of genetic diversity, is thus regarded as an extremely heterogeneous genotype (Simmonds *et al*., 2005). Prior to 2011, depending on the HCV genotype and for a considerable period of time, the most commonly used therapy was interferon-alpha and ribavirin (INF/ RBV) for 24-48 weeks. Low SVR, prolonged treatment duration, and significant side effects, were among this therapy's shortcomings; particularly for genotype 4 patients (Abdel-Razek and Waked, 2015). Within the previous eight years, the advent of the innovative direct-acting antiviral drugs (DAAs) used for treatment of the chronic HCV has resulted in a substantial change in the course of HCV treatment. According to Geddawy *et al*., (2017), these medications have shown to be safe, efficient, and represent brief therapies with high rates of sustained viral remission (SVRs), in which HCV RNA disappears 12 to 24 weeks after the therapy ends. In late 2018, Egypt launched the largest mass screening and treatment campaign for HCV infection, with the potential to screen 50 million people for HCV infection as a step towards disease eradication (Waked *et al*., 2020). Over the past ten years, Egypt has continued its HCV management efforts and is working towards the World Health Organization's (WHO's) global target of eliminating the viral hepatitis. With the development of DAAs, the HCV treatment becomes available to everyone that led to a standard shift in HCV management (Naguib *et al*., 2021). Worldwide, the 12-week antiviral therapy regimens, such as Sofosbuvir/ Velpatasvir, Sofosbuvir/ Ledipasvir, and Sofosbuvir/ Daclatasvir, were used for the goal of targeting the *NS3-4A* protease, *NS5A* region, and *NS5B* polymerase (Kliemann *et al*., 2016). Although DAAs have a high success rate of 90-95 %; a sizable minority of the patients (5-10 %) has not been able to completely eradicate HCV infection (Munir *et al*., 2022). In the viral regions that DAAs target *NS3-4A*, *NS5A*, and *NS5B*, several polymorphisms known as resistance-associated substitutions (RASs) have been associated with treatment failures (Izhari, 2023). Since all DAA regimens now contain the available *NS5A* and *NS5B* inhibitors, RAS in *NS5A* and *NS5B* may raise the possibility that resistance-associated mutations will emerge during therapy (Gömer *et al*., 2023).

In addition, Krishnan *et al*., (2015) reported that daclatasvir is the first *NS5A* inhibitor that is now approved for treating HCV-4 infected patients who have widespread cross resistance and a low genetic barrier to resistance. Furthermore, sofosbuvir is also the only *NS5B* nucleoside inhibitor that's commercially accessible, which incorporates a great security profile and has a high hereditary obstruction to resistance

(Kamal, 2007). Subsequently, the objectives of this study were to examine the design of *NS5A* and *NS5B* RASs that were related to disappointment of the sofosbuvir/ daclatasvir combination treatment for 12 weeks in HCV-infected Egyptian patients, and utilizing the coordinate sequencing procedures and phylogenetic examination of *NS5A* and *NS5B*.

2. Materials and methods

2.1. Patients and study settings

One hundred chronic HCV patients receiving DAA medication had participated in the current study. Achievement of SVR was assessed 12 weeks following the completion of a 3-month regimen, consisting of sofosbuvir (400 mg) and daclatasvir (60 mg), in accordance with the HCV treatment protocol recommended by the 'National Committee for Control of Viral Hepatitis' (NCCVH). Enrollment in this study took place from February 2023 to December 2023. Individuals with a history of hepatocellular carcinoma, co-infection with hepatitis B or HIV, other persistent liver diseases, and/ or evidence of hepatic de-compensation were excluded from the treatment.

2.2. Data collection

Information about HCV disease were gathered retrospectively from patients' healthcare records, including starting and follow-up viral levels, and previous HCV therapy, in addition to the start and duration of the treatment dates. The patients were between the ages of 25 and 66; where 58 being males and 42 being females, and were living in the different areas of Sharkia governorate, Egypt. Using a COBAS® AmpliPrep/Quantitative Test (v2.0) and according to the manufacturer's instructions, the baseline HCV-RNA levels were measured and varied from 250,000 to 5,410,000 IU/ ml. The liver's cirrhosis was verified using fibroscan, while the fibrosis-4 (FIB4) score was computed using Sterling's formula (Sterling *et al*., 2006), which was as follows:

Age (years) × aspartate transaminase (AST) (U/ l)/ platelets (10^9/ l) × √ (alanine transaminase (ALT) (U/ l)

2.3. Collection of specimens

The specimens were collected as a routine part of the Al-Ahrar Teaching Hospital's activities in Zagazig, Sharkia, Egypt. Three months after completing the 12-week course of sofosbuvir/ daclatasvir combination therapy, the blood samples were taken from one hundred patients with chronic HCV. For every patient, 5 ml blood sample were taken. After blood clotting, the serum was separated, labeled, and kept at -20 °C for further examinations.

2.4. Extraction of viral RNA and synthesizing cDNAs

By referring to the spin protocol described in the previous study conducted by Murphy *et al*., (2007), the HCV-RNA was extracted from 2 serum samples obtained from non-respondent patients (VR) and 8 samples from respondent ones (SVR), using Gene JET Viral DNA/ RNA Purification Kit (Qiagen, Germany). Briefly 200 µl of each serum sample were mixed with 200 µl of a lysis solution and 50 µl of Proteinase K, incubated at 56 °C for 15 min., and then centrifuged for 3-5 s at 16000×g. Approximately 300 µl of ethanol (99 %) were added to each supernatant, mixed, and then centrifuged again for 3-5 s. at 16000×g. Afterwards, the lysate was transferred to a prepared Spin Column, and the column was centrifuged for 1 min. at 6000×g. Place the Spin Column in a new 2-ml wash Tube. 700 µl of wash buffer 1 supplemented with ethanol were added to the spin column, and the column was centrifuged for 1 min. at 6000×g. This step was repeated 3 times. Finally, 50 µl of AVE buffer were added, equilibrated to room temperature for 3 min., and then centrifuged for 1 min. at 13000×g. The extracted purified HCV RNA was collected in sterile vials for cDNA synthesis. Subsequently, HCV RNA was reverse transcribed using a Quantitect Reverse Transcription kit (Qiagen, Germany) according to

the manufacturer's instructions. The reaction conditions were 42 °C for 2 min., 42 °C for 15 min., and 95 °C for 3 min. The resulting cDNA was stored at -20 °C until use.

2.5. Polymerase chain reaction amplification of the partial HCV-*NS5B* region

Polymerase Chain Reaction (PCR) amplification of 389 bp from NS5B gene was performed using two primers: The sense 5'-TTC TCR TAT GAY ACC CGC TGY TTT GA -3' and the antisense 5'-TAC CTV GTC ATA GCC TCC GTG AA-3'(Invitrogen, Thermo Fisher Scientific, USA), as described by the previous study conducted by Murphy *et al*., (2007). The amplification process was carried out using Dream TaqTM Green PCR Master Mix (2X) (Fermentas, USA) under the following conditions: PCRs began with heating at 95 °C for 2 min. for primary denaturation of dsDNA, followed by 40 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min.), and a cycle of final extension at 72 °C for 10 min. The amplified products were visualized under an ultraviolet light transilluminator (Spectroline, Germany). After the amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific, USA) in accordance with the manufacturer's instructions, they were stored at -20 °C until sequencing.

2.6. Partial HCV-*NS5A* gene amplification

The 698 bp of the *NS5A* gene was amplified in compliance with Plaza *et al*., (2012). The first round of amplification of HCV-*NS5A* was carried out using external pair of GT 4-specific primers: NS5A_F 5'-GGC AAY CAC GTG KCT CCC AC-3', and NS5A_R 5'-CTG RCT MGC CGA GGA -3'. The second round of amplification was performed using 3 µl from the amplicon of the first PCR round, 25 µl of Dream Taq Green PCR Master Mix (2×), 0.1 µM of each genotype 4 specific internal primers; NS5A IF4 5'-CAC AAG TGG AYC AAT GAR GA- 3' and NS5A IR4 5'-GAG GGT SGT GAC CC-3', 17 µl of water, and nuclease-free to complete the volume to 50 µl. The cycling conditions were run as follows: 94 °C for 2 min., followed by 40 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1min., and a final extension at 72 °C for 10 min. Approximately 10 µl of the amplified PCR products were run on 1% agarose gel electrophoresis and the resulting specific product lengths were purified using GeneJET PCR Purification Kit (Thermo Scientific, USA) following the manufacturer's instructions, and kept at -20°C until sequencing.

2.7. Direct DNA sequencing and phylogenetic analysis of the amplified HCV *NS5A* and *NS5B* genes

The purified amplicons of 10 *NS5B* and 10 *NS5A* genes were prepared by using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions contained 2 µl of 2 × reaction premix, 3 µl of each primer (10 µM), 6 µl of a buffer, 5 µl of purified amplicon, and nuclease-free water to complete the final volume to 20 µl. The tubes were placed in a thermocycler, and the following thermal cycling profiles were carried out for the PCR: for a total of 25 cycles, 96 °C for 30 s, 50 °C for 15 s, and 60 °C for 2 min. were run, followed by a rapid thermal cooling to 4 °C. The complete reaction mixtures were spined down the tubes in a microcentrifuge (Sigma1-14k, Sigma, Germany), and the purified reaction mixtures were vacuum dried (Di Maio *et al*., 2018). Direct sequencing of *NS5A* and *NS5B* was performed by Sanger sequencing of PCR products from both directions using an automatic sequencer (ABI3730XL, DNA analyzer, Macrogen Inc., Korea). The DNA sequences were analyzed phylogenetically using the BLAST software of the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

Each obtained sequence was compared to reference sequences retrieved from GenBank, and then an additional genetic analysis was performed. The software Clustal W Multiple alignment of Bio Edit Version 7.0 was used to perform comparative

amino acid alignment, in reference to Hall, (1999). Phylogenetic trees were constructed *via* the neighbor-joining method with bootstrapping 1000 in the MEGA 6.06 software (Tamura *et al*., 2013).

2.8. Statistical analysis

Statistical analyses were performed using SPSS 26 software. Basic descriptive statistics were carried out, including the means and standard deviations. In univariate analysis, the quantitative data were compared using the t-test student's and/ or the chi-square test to compare the qualitative data among the groups. In multivariate analysis, the binary logistic regression was used for each group to identify the variables that showed statistical significance. The p value was considered significant if it was less than 0.05.

3. Results

3.1. Clinical and virological patterns of the involved patients

The laboratory results and profiles of the 100 patients who started and finished the course of treatment are summarized in Table (1). In the current trial, 100 patients received a 12-week treatment of sofosbuvir (400 mg/ d) and daclatasvir (60 mg/ d). Variable clinical characteristics were noted with respect to the baseline characteristics of the tested patients. The mean patient age at the start of treatment was 55 years. At baseline, the mean recorded haemoglobin, serum albumin, serum bilirubin, platelet counts, and serum creatinine levels were 14.3 g/ dl, 4.1 g/ dl, 0.69 mg/ dl, $202.4\times10^3/mm^3$, and 0.92 ml/ min. Meanwhile, the mean alanine transaminase (ALT) and aspartate transaminase (AST) levels were 47.9 IU/ l and 44.9 IU/ l, respectively. The observed existence of diabetes mellitus was 75 %.

3.2. Patients' virological responses to the combined therapy with sofosbuvir and daclatasvir

A review of the virological responses was conducted on all the tested 100 chronic HCV patients. The responses to treatment, age, liver enzyme levels (ALT and AST), and viremia level were all significantly correlated. Only 2 out of 100 patients (2 %) were classified as having an NVR (non-virological response), whereas 98 patients (98 %) had obtained SVR (Table 2). There were notable distinctions detected between SVR and NVR in terms of the patient's age, ALT, AST, and viremia level. Concerning the remaining laboratory results such as serum bilirubin, haemoglobin, platelet count, and serum albumin level, no significant differences were recorded between both groups.

3.3. Predictive factors associated with SVR in the multivariate analysis

All data of 100 patients were analyzed to determine the predictors of non-virological response. The univariate analyses identified the levels of viremia (p = 0.005), ALT (p = 0.001), AST (p = 0.0001), and patient's age (p = 0.004), which displayed statistical significance. On the other hand, the multivariate logistic regression analysis was performed among the patients who achieved SVR and NVR, where NVR was regarded as the dependent variable. The recorded age, ALT, AST, and viremia level were significant predictors of the non-virological response (Table 3).

3.4. Phylogenetic study and partial length amplification of *NS5B* region

The current investigation verified that all the examined and chosen specimens had successfully amplified *NS5B* fragments (Fig. 1). According to the gene sequencing studies, the ten HCV isolates were all related to HCV genotype 4a. Two specimens were from the patients representing virological relapse, while the remaining eight ones were from the respondent patients to SOF and DCV combination therapy. They were 8 males and 2 females and their ages ranged about 32-66 years. In addition, they had a high viral load of 1.080.000 to 5.000.000 IU/ ml.

Table 1: Profiles and laboratory data of patients infected with HCV before starting the treatment program

Demographic data	**Mean± SD**
Number of patients	100
Age, years	55 ± 9.5
Sex, M/F	58 (58 %)
BMI	27.61 ± 4.47
Laboratory data	
Hemoglobin (g/ dl)	14.30 ± 1.41
Platelets Count (Thousands/ mm^3)	202.44 ± 58.99
WBC	6.63 ± 2.17
Creatinine	0.92 ± 0.25
HbA1c	4.41 ± 2.69
Serum albumin (g/ dl)	4.13 ± 0.45
Bilirubin (mg/ dl)	0.69 ± 0.35
ALT (IU/ l)	47.98 ± 33.63
AST (IU/ l)	44.99 ± 26.17
Level of viremia (IU/ ml)	2.1 ± 0.8
Fib-4 Score	2.02 ± 0.97

Where; Data are expressed as Mean ± Standard deviation (SD), BMI (Body mass index), WBC (white blood cell count), ALT (alanine transaminase), AST (aspartate transaminase), Fib-4 Score: Fibrosis-4 score

Table 2: Profiles and laboratory data of respondents (SVR) and non-respondents (NVR) patients after sofosbuvir and daclatasvir combination therapy for 12 weeks in the 100 HCV-infected patients

Data	Respondents (SVR)	Non-Respondents (NVR)	*P* value
Demographic data:			
Number of patients	98 (98 %)	2 (2 %)	
Age (year)	54.9 ± 9.5	66 ± 9.8	0.03*
BMI	27.6 ± 4.3	26.8 ± 2.4	0.65
Laboratory data:			
Hemoglobin (g/ dl)	12.8 ± 1.6	12.4 ± 1.4	0.56
Platelets Count (Thousands/ mm^3)	197.1 ± 52.8	194.1± 52.1	0.89
WBC	6.6 ± 2.1	6.4 ± 1.8	0.82
Creatinine (ml/ min.)	1.01 ± 0.27	0.97 ± 0.24	0.77
HbA1c	4.5 ± 4.2	5.1 ± 3.1	0.6
Serum albumin (g/ dl)	4.2 ± 1.7	4.3 ± 0.50	0.89
Bilirubin (mg/ dl)	0.86 ± 0.44	0.86 ± 0.29	0.98
ALT (IU/ l)	46.3 ± 30.1	121.1 ± 56.22	0.0001***
AST (IU/ l)	51.6 ± 26.8	99.3 ± 28.7	0.0002***
Level of viremia (IU/ ml)	2.8 ± 0.9	5.4 ± 0.8	0.02

Where; Data are expressed as Mean ± Standard deviation (SD), BMI Body mass index, WBC white blood cell count, ALT alanine transaminase, AST aspartate transaminase. Results were statistically significant at $p < 0.05$

Table 3: Multivariate regression analysis, where non-response is the dependent variable

Factor	OR	*P* value	95 % (CI-OR)
Age > 60	0.99	0.04**	0.99-1
ALT (IU/ l)	0.99	0.0001***	0.99-1.00
AST (IU /l)	1.00	0.05*	1-1.02
Level of viremia (IU/ ml)	1.001	0.001	0.83-1.20

Where; Only the variables that achieved statistical significance ($p < 0.05$) on the multivariate logistic regression are presented. CI: Confidence interval, OR: Odds ratio. *: Indicates statistical significance at $p < 0.05$

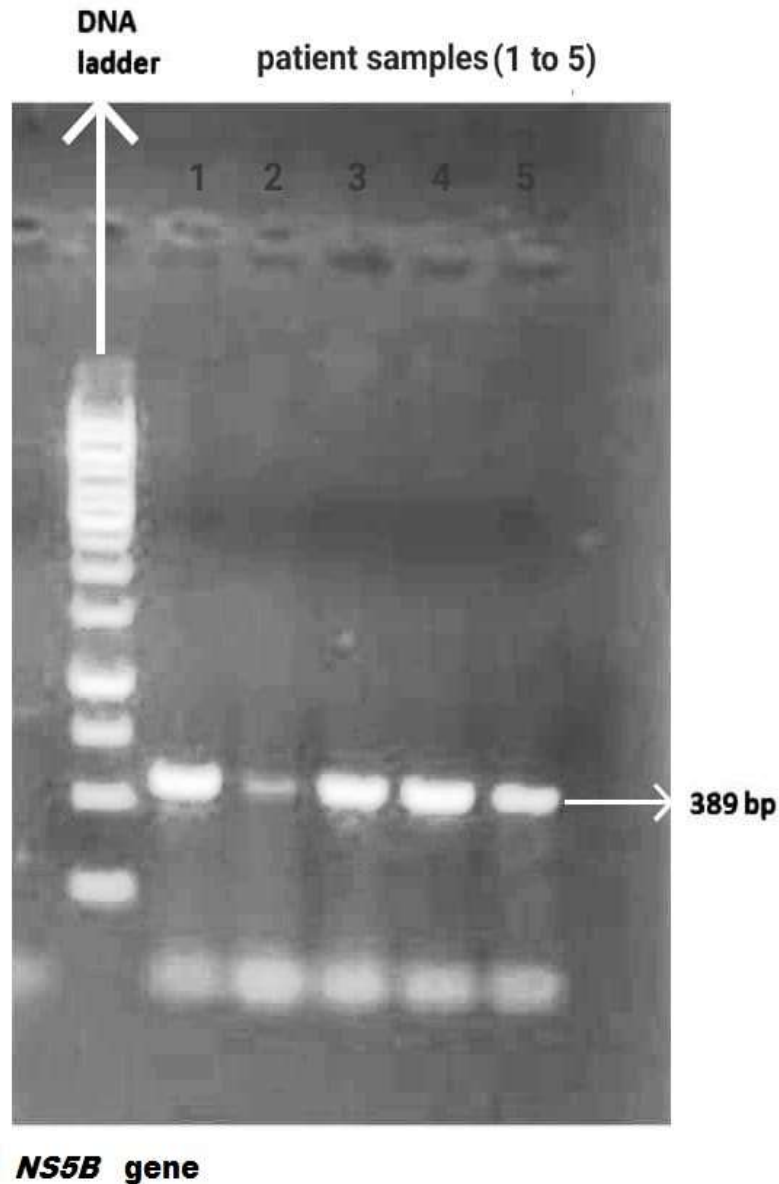

Fig 1: Agarose gel electrophoresis patterns for HCV *NS5B* isolated from Egyptian patients infected with HCV-4a. The HCV amplified PCR products was detected as a single band at 389 bp visualized using ultra violet illumination on 2 % agarose gel stained with ethidium bromide

Sequences of the partial length of *NS5B* region (389 bp) of the ten sequenced specimens were aligned with the reference strains obtained from NCBI database (Fig. 2). The truncated sequences (345 nucleotides) were presented to the GenBank database under the accession numbers OR265503-OR265512.

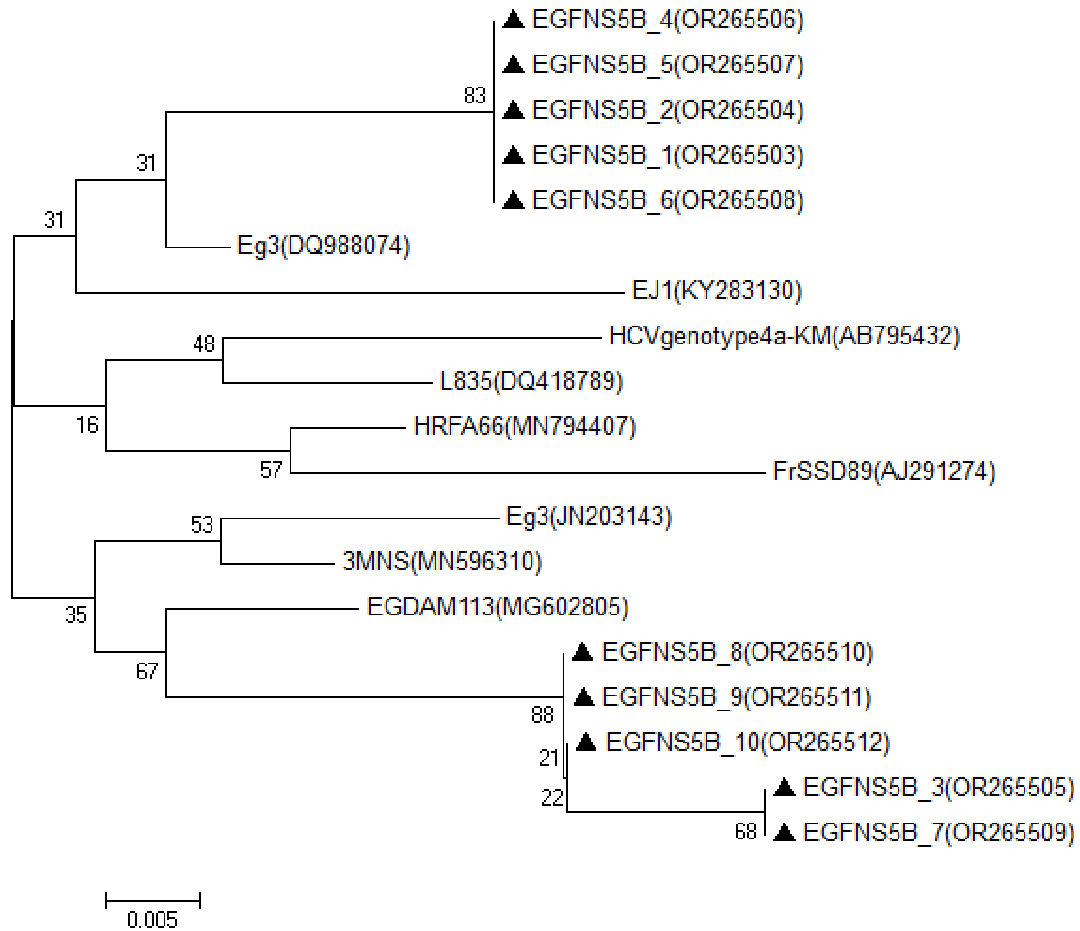

Fig. 2: Phylogenetic tree constructed *via* the neighbor-joining method with bootstrapping 1000 in the MEGA 6.06 software. *NS5B* sequences obtained from the treated sera of respondent and non- respondent patients infected with HCV-4

3.5. Variability in the *NS5B* region's partial length

In the current study, to examine the modifications along the *NS5B* protein, the HCV amino acid sequences were aligned to the reference *NS5B* consensus sequences obtained from the GenBank database. Two non-respondent patients' amplified specimens contained the substantial S282T mutation, which was associated with a severe level of resistance to SOF (Fig. 3). Furthermore, the specimens of the non-respondent patients showed an aberrant level of ALT and AST, as well at the S282T mutation site.

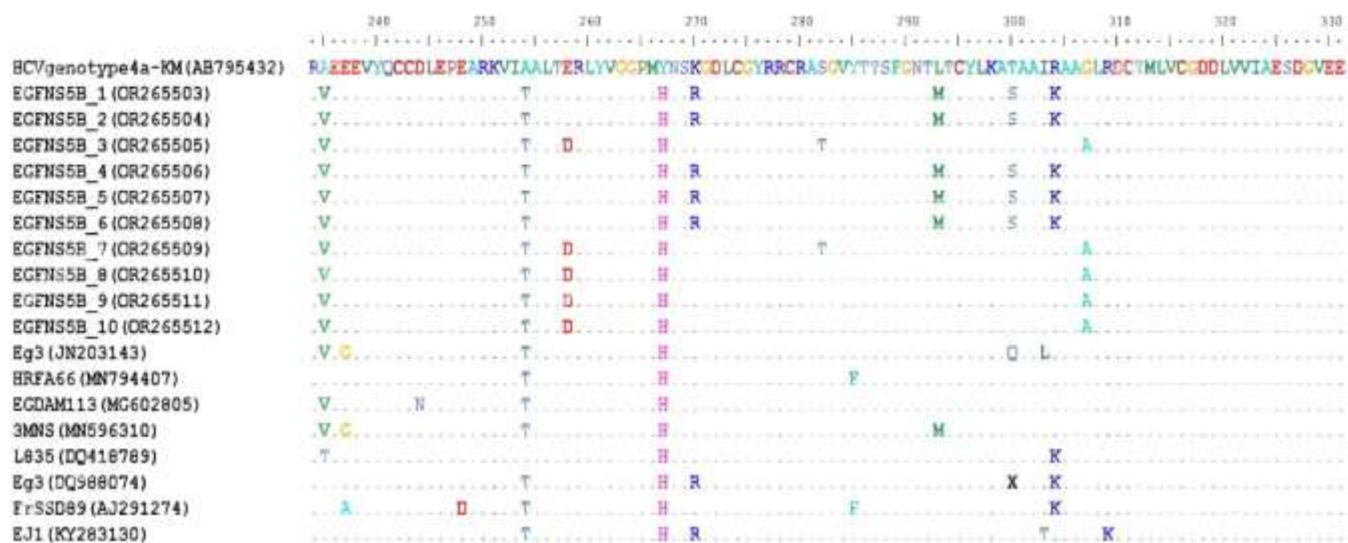

Fig. 3. BioEdit sequence alignment of the partial *NS5B* amino acids sequence for representative 19 HCV-4a strains in the treatment-naïve, respondent, and non-respondent patients with a reference strain (AB795432)

3.6. Phylogenetic analysis and amplification of the partial length of *NS5A* region

In all the tested specimens, the amplification of the *NS5A* fragments was confirmed (Fig. 4). Similar results were obtained as those of *NS5B* fragments. The ten specimens' partial length *NS5A* region sequences (200 bp) were aligned with reference strains from NCBI database (Fig. 5). The truncated sequences (197 bp) were presented to the GenBank database under the accession numbers OR265493 - OR265502.

3.7. Variability of the partial length of *NS5A* region

In the current investigation, the *NS5A* region of the ten HCV GT4a specimens was amplified, sequenced, and then examined for *NS5A* resistance associated substitutions (RASs). The HCV amino acid sequences were matched to the reference *NS5A* consensus sequences obtained from the GenBank database in order to examine the substitutions along the *NS5A* protein. One specimen of a patient who had a virological relapse after receiving therapy with SOF/ DCV for three months had the substantial Y93H polymorphism, which was associated with an excessive level of resistance to DCV (Fig. 6). Furthermore, the non-respondent patient's Y93H mutant location was shown to have aberrant ALT and AST levels.

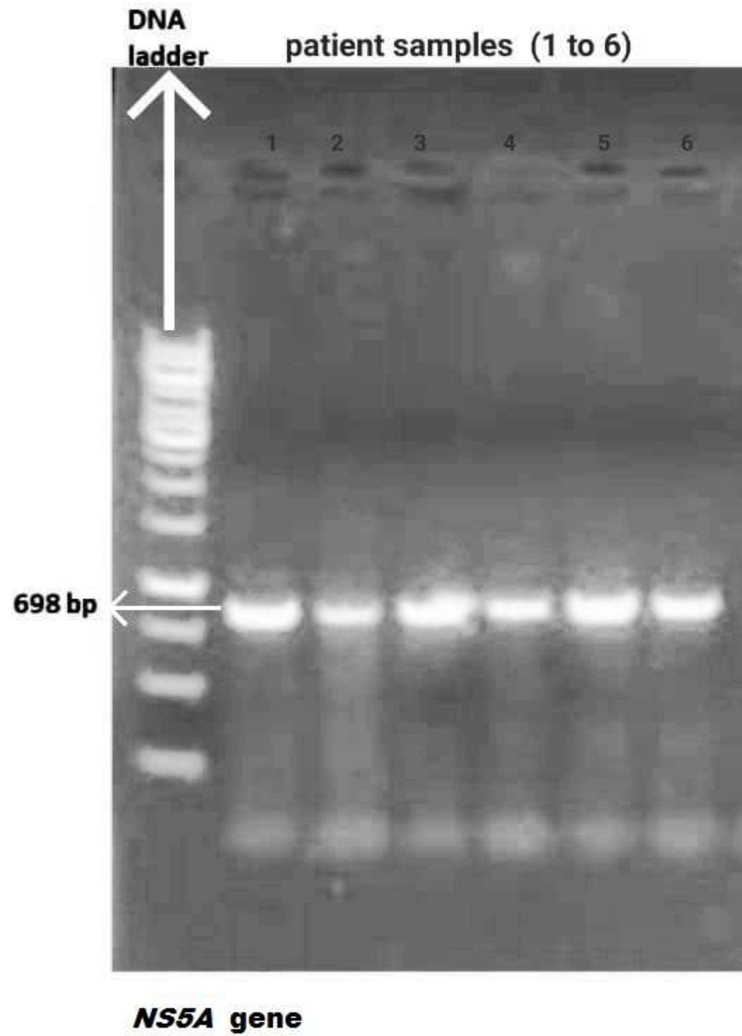

Fig. 4: Agarose gel electrophoresis patterns for HCV *NS5A* isolated from Egyptian patients infected with HCV-4a. The HCV amplified PCR products was detected as a single band at 698 bp visualized using ultra violet illumination on 2 % agarose gel stained with ethidium bromide

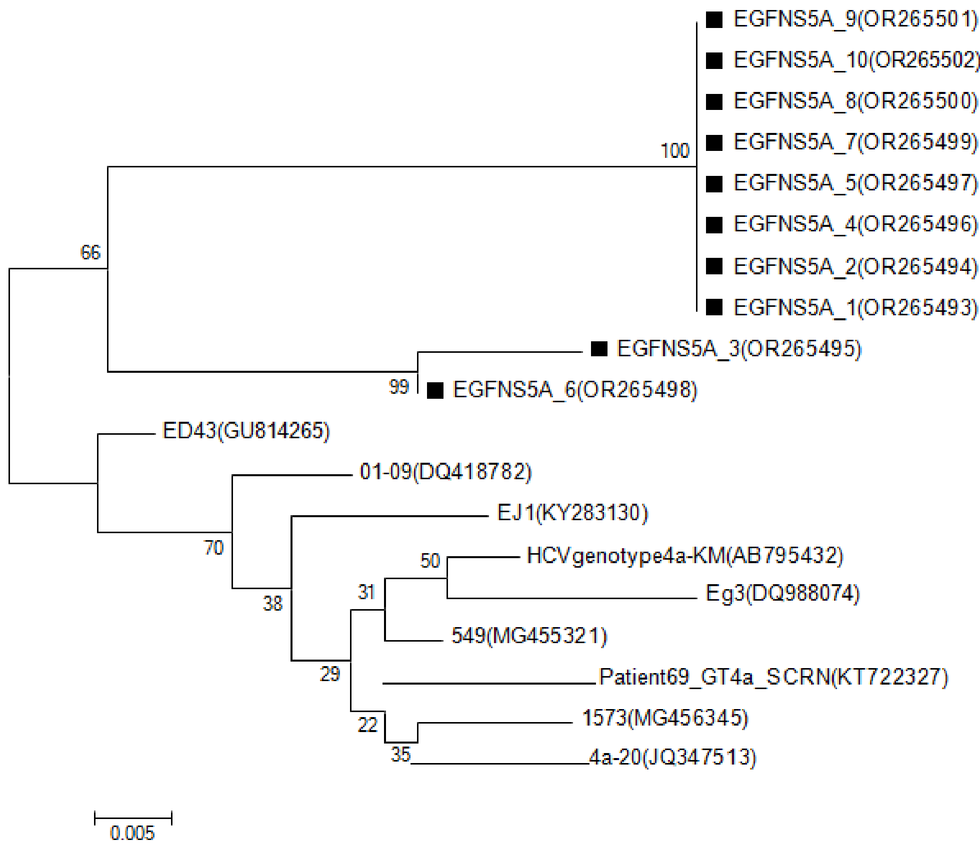

Fig. 5: Phylogenetic tree constructed *via* the neighbor-joining method with bootstrapping 1000 in the MEGA 6.06 software. *NS5A* sequences obtained from treated sera of respondent and non- respondent patients infected with HCV-4

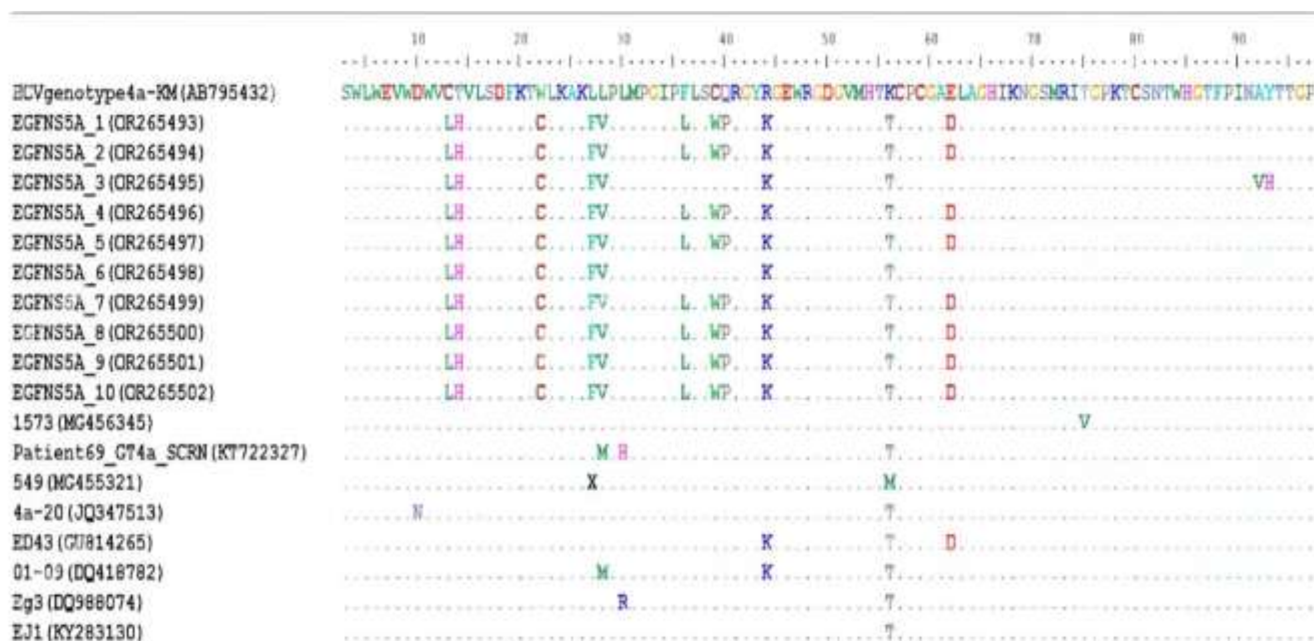

Fig. 6. BioEdit sequence alignment of the partial *NS5A* amino acids sequence for the representative 19 HCV-4a strains in treatment-naïve, respondent, and non-respondent patients with a reference strain (AB795432)

4. Discussion

One of the most disturbing blood-borne diseases is HCV with estimated infections of 2.8 % of the global population (Nygaard-Odeh *et al*., 2024). In Egypt, about 90 % of the people infected with HCV have genotype 4 (G4) with a predominant subtype 4a, while the remaining 10 % suffer from HCV genotype 1 (Ibrahim *et al*., 2022). The HCV quasispecies' ongoing evolution causes the virus to evade both the host immune system and the current antiviral treatments (Bukh, 2016), and no vaccine was created to control HCV infection (Fakhry *et al*., 2020). However, using the antiviral drugs is believed to be the only alternative option for controlling the HCV epidemic (Elgharably *et al*., 2017). Till 2011, the standard Peg-interferon (PEG-IFN) and ribavirin (RBV) were the only available treatments regimen for HCV. Unfortunately, this treatment course not only showed suboptimal efficacy, but also the treated patients suffered from severe side effects, such as anemia, neutropenia, and thrombocytopenia (Dusheiko, 1997). In order to treat HCV, the National Committee for Control of Viral Hepatitis (NCCVH) changed the national guidelines in the early 2016. These changes were justified by the fact that treatment with sofosbuvir/ daclatasvir; with or without ribavirin, is a low-cost treatment that requires little time to tolerate, has a high success rate in SVR, has few side effects, and has shown high efficacy and safety in treating the chronic HCV patients (Kamal, 2007). According to Said *et al*., (2020), these medications specifically targeted the viral proteins that are crucial in the process of viral replication. In addition to

daclatasvir (DCV), NCCVH has approved sofosbuvir as a potent and pan-genotypic inhibitor of HCV *NS5B* polymerase.

In a previous study, a sustained viral response was achieved at end of treatment with SVR in 96.6 % of the patients receiving 12 weeks of DCV + SOF treatment (Shiha *et al*., 2018). All of the specimens used in the current study were found to correspond to genotype 4a, with amino acid similarities of 90.9 % - 93.4 % with the reference strain that had an accession number of AB795432. This finding is consistent with the previous studies reported from different governorates in Egypt, including Damietta, Sharkia, Ismailia, and Alexandria (El-Tahan *et al*., 2018; Makhlouf *et al*., 2021; Shoun *et al*., 2022). Furthermore, following three months of treatment with sofosbuvir/ daclatasvir, a high SVR rate of 98 % was discovered in the infected patients (Fakhr *et al*., 2013), in agreement with the earlier Egyptian researches conducted in Cairo and Tanta (92.7 %), Minia (91 %), and Sharkia (96 %) (Abdel-Aziz *et al*., 2018; Ahmed *et al*., 2018a). Combination of sofosbuvir and daclatasvir was found to be a safe and efficient treatment regimen for the Egyptian patients suffering from HCV genotype 4 infections. By the 12th week of treatment, the patients' sustained a virological response that reached 96 % (Ahmed *et al*., 2018b). The increased SVR may be a sign that the Egyptian patients were responding to SOF and DCV treatments at higher rates.

Resistance associated substitutions (RASs), which are alterations that occur naturally and explain the different levels of resistance to DAAs, significantly impair the efficacy of HCV antiviral therapy (Chen *et al*., 2015; Makhlouf *et al*., 2021). Consequently, in order to compare GT4a-infected Egyptian patients experiencing virologic relapse with those patients who achieved SVR following a three-month course of sofosbuvir/ daclatasvir treatment, the current study further examined the patterns of *NS5A* and *NS5B* drug resistance-associated regions. The majority of the previous studies on HCV RAS have employed the Sanger method that is simpler to be used for sequencing, in order to identify the clinically significant polymorphisms (Sarrazin, 2016; Sharafi and Alavian, 2018). The HCV GT-4a reference Egyptian strain (HCV genotype 4a-KM) with an accession number of AB795432 from the NCBI was selected to be aligned with the sequenced specimens for the current study's alignment phase. Following alignment, we observed that the reference strains did not have the substitutions, while the tested specimens had, in agreement with Vermehren and Sarrazin, (2012). The RNA-dependent RNA polymerase (RdRp) was HCV *NS5B* (Koff, 2014). Since a few years ago, the Egyptian patients with HCV have been extensively treated with sofosbuvir, which targets the HCV *NS5B* region. Due to the fast pace of HCV replication and the inadequate proofreading of HCV polymerase, highly variable viruses are produced, which incorporate the genomic changes in order to lessen their vulnerability to the antiviral drugs (Vermehren and Sarrazin, 2012). The treatment outcomes (Chen *et al*., 2015) and HCV polymerase replication activity (Omar *et al*., 2018) have been impacted by several changes in the *NS5B* polymerase region's amino acid composition (Ahmed *et al*., 2021). In the current study, ten HCV specimens were sequenced for the partial length of their *NS5B* and were analyzed using phylogenetic analysis. Neighbor joining phylogenetic tree revealed that these ten sequenced specimens fall in subtype 4a clade, and cluster with each other and with HCV subtype 4a from Egypt and USA. In details, eight specimens of the respondent patients and two from the non-respondent ones were successfully amplified for the examined *NS5B* fragments. Significant S282T mutation that induces a high level of SOF resistance was recognized in two specimens of the non-respondents (Fig. 3).These results were consistent with several other previous studies, which showed that patients who did not respond well to SOF-based regimens had S282T substitution (Gane *et al*., 2017; Catalli *et al*., 2019). Additionally, *NS5B* (S282C/T) RASs were detected in 5 patients (38.5 %) who had therapy failure (Fourati *et al*., 2019). This conclusion is in line with the earlier studies, which reported that sofosbuvir and mericitabine resistances

are conferred by mutations in the active site. Among these alterations is S282T, which is present in every HCV genotype (Hetta *et al*., 2014; El-Ansary, 2023). Furthermore, the 10 patients' *NS5A* domain I region was effectively amplified and sequenced. One out of 10 patients showed the main polymorphism in its sequences at the amino acids 93 (Y93H), which conferred high level of resistance to *NS5A* inhibitors (daclatasvir) (Fig. 6). Moreover, L31M was shown to be associated with high daclatasvir resistance in all *NS5A* regions obtained from the relapsing patients that were analysed, sequences from patients with SVR, and reference strains from the GenBank. This is consistent with the other studies performed on GT4, which detected L31M in all the examined GT4 sequences (Carrasco *et al*., 2018; Ahmed *et al*., 2021). Y93H is one of the major signature resistance substitutions in genotype 1b, with an overall prevalence of approximately 8 % that was observed in a previous Japanese study reported by Wu *et al*., (2013). Overall, the resistance profile of DCV in genotype 4 infections seems to be closer to that previously observed in genotype 1 infection (Alves *et al*., 2013). Furthermore, for HCV GTs 2-4, daclatasvir has significantly higher *in vitro* potency compared to ledipasvir, with the previously recorded highest fold resistance values for variant Y93H in GT3 (Kjellin *et al*., 2019).

Conclusion

According to the current findings, the resistance to SOF as well as DCV is attributable to mutations in the *NS5B* and *NS5A* regions in genotype 4a, respectively. Meanwhile, patients with detectable RASs experienced virologic relapses. Finally, the high rates of multiple RASs in DAA treatment failures, in addition to the high costs; encourage designing a specific treatment protocol for HCV genotype 4a- Egyptian patients to avoid resistance.

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Conflict of interests

None of the authors have conflicts of interest to be declared.

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Ethical approval

An ethical approval code of the Regulation Survey Board (IBR) (10441-14-2-2023) was obtained from the regulation endorsement, Faculty of Medicine, Zagazig University, Sharkia, Egypt. Written informed consents were obtained from the participating patients before taking the specimens.

Author's Contributions

Conceptualization: F.A., A.B., and M.G.; Investigation: G.M.; Methodology: G.M.; Supervision: F.A., A.B., and M.G.; Roles/Writing - original draft: F.A., A.B., M.G. and G.M.; Writing, reviewing, and editing: F.A., A.B., M.G. and G.M.

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