



Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Consensus small interfering RNA targeted to stem-loops II and III of IRES structure of 5' UTR effectively inhibits virus replication and translation of HCV sub-genotype 4a isolates from Saudi Arabia

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ARTICLE INFO

Article history:

Received 9 September 2020

Revised 6 November 2020

Accepted 8 November 2020

Available online 17 November 2020

Keywords:

HCV

5' Untranslated region

Internal ribosome entry sites

Replication

Translation

Small interference RNA

Anti-mRNA based treatment strategies

HCV therapeutics

ABSTRACT

Being the most conserved region of all hepatitis C virus (HCV) genotypes and sub-genotypes, the 5' untranslated region (5' UTR) of HCV genome signifies its importance as a potential target for anti-mRNA based treatment strategies like RNA interference. The advent and approval of first small interference RNA (siRNA)-based treatment of hereditary transthyretin-mediated amyloidosis for clinical use has raised the hopes to test this approach against highly susceptible viruses like HCV. We investigated the antiviral potential of consensus siRNAs targeted to stem-loops (SLs) II and III nucleotide motifs of internal ribosome entry site (IRES) structure within 5' UTR of HCV sub-genotype 4a isolates from the Saudi population. siRNA inhibitory effects on viral replication and translation of full-length HCV genome were determined in a competent, persistent, and reproducible Huh-7 cell culture system maintained for one month. Maximal inhibition of RNA transcript levels of HCV-IRES clones and silencing of viral replication and translation of full-length virus genome was demonstrated by siRNAs targeted to SL-III nucleotide motifs of IRES in Huh-7 cells. siRNA Usi-169 decreased 5' UTR RNA transcript levels of HCV-IRES clones up to 75% ($P < 0.001$) at 24 h post-transfection and 80% ($P < 0.001$) at 48 h treatment in Huh-7 cells. 5' UTR-tagged GFP protein expression was significantly decreased from 70 to 80% in Huh-7 cells co-transfected with constructed vectors (i.e. pCR3.1/GFP/5' UTR) and siRNA Usi-169 at 24 h and 48 h time-span. Viral replication was inhibited by more than 90% ($P < 0.001$) and HCV core (C) and hypervariable envelope glycoproteins (E1 and E2) expression was also significantly degraded by intracytoplasmic siRNA Usi-169 activity in persistent Huh-7 cell culture system. The findings unveil that siRNAs targeted to 5' UTR-IRES of HCV sub-genotype 4a Saudi isolates show potent silencing of HCV replication and blocking of viral translation in a persistent *in-vitro* Huh-7 tissue culture system. Furthermore, we also elucidated that siRNA silencing of viral mRNA not only inhibits viral replication but also blocks viral translation. The results suggest that siRNA potent antiviral activity should be considered as an effective anti-mRNA based treatment strategies for further *in-vivo* investigations against less studied and harder-to-treat HCV sub-genotype 4a isolates in Saudi Arabia.

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Peer review under responsibility of King Saud University.



1. Introduction

HCV is a positive-sense single-strand RNA virus of approximately 9600 nucleotides in length (Tabata et al. 2020). The viral genome flanks by highly conserved 5' and 3' untranslated regions that abut a single open reading frame of 3010 to 3037 amino acids polyprotein (Neufeldt et al. 2018). Both 5' and 3' UTRs are prerequisite for virus replication and protein translation and signals required for both these phenomena are coordinated in a highly

<https://doi.org/10.1016/j.sjbs.2020.11.041>

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orchestrated manner with viral and cellular proteins and between these two regions of the HCV genome (e.g., molecular interaction of IRES domain III_d, stem-loop (SL) -II and SL-III of 3' UTR and *cis*-acting replication elements (CRE)/region at 3' end of NS5B is key for genome replication)) (Strating and van Kuppeveld 2017; Anjum et al. 2013).

The HCV 5' UTR spans from 332 to 343 nucleotides and contains up to 5 AUG codons depending on HCV genotypes (GTs) and sub-genotypes (Vopalensky et al. 2018). It is a highly conserved sequence among all HCV GTs (>90% conservation) which folds into a complex of secondary and tertiary structures encompassing multiple SLs and two RNA pseudoknots of IRES structures (Floden et al. 2016; el-Awady et al. 2006). The region can be divided into 5' and 3' parts, where the 5' part covers almost a fully single-strand structure and covers domain I of 5' UTR (el-Awady et al. 2006). However; 3' part is highly structured and folds into three additional domains (II-IV) and key to HCV IRES activity (el-Awady et al. 2006). Almost all 5' UTR and an adjacent part of the core (C) gene are occupied by several SLs of IRES which spans a region of ~341 nucleotides (Fig. 1) (Floden et al. 2016; el-Awady et al. 2006). IRES covers domain II to IV of 5' UTR but also spans to core coding region (Floden et al. 2016). Small SL I of IRES comprises upstream nucleotides 5–20 of domain I of 5' UTR and directly not involved in IRES activity (Floden et al. 2016; el-Awady et al. 2006; Friebe et al. 2001). However; its deletion suggests a regulatory role while enhancing RNA translation (Tang et al. 1999; Perard et al. 2013). From 43 nucleotide downstream, canonical representation deciphers SL-II, which is followed by SL-III encompassing subdomains or SLs III-a, b, c, d, e, and f of IRES (Honda et al. 1999; Fricke et al. 2015; Reynolds et al. 1995). Like SL-I, the role of SL-II in IRES function is also not known and some studies document enhanced translation activity in its presence (Friebe et al. 2001; Honda et al. 1999; Fricke et al. 2015; Reynolds et al. 1995). The SL-III segment starting from 141 to 279 residues (i.e. covering SL III-a to III-d) form the core of IRES structure and are highly conserved at the primary nucleotide level as well as at secondary and tertiary structure levels (Friebe et al. 2001). Interestingly, the 5' UTR role in HCV translation regulation is studied in detail, however; its significance for RNA replication so far is not fully elucidated (Anjum et al. 2013; Tang et al. 1999).

In addition to 5' UTR, HCV core (C) protein is the first protein to be synthesized during viral translation and is considered to be the most conserved part of HCV polyprotein, albeit; not directly involved in viral translation and viral replication mechanisms (Khaliq et al., 2011a). It packages the viral RNA in interaction with the two envelope glycoproteins (i.e. E1 and E2). The core protein amino acid composition is highly conserved among different HCV genotypes as compared to other HCV proteins, however; sequence variations have been reported within different domains of the protein in different HCV-induced pathological states. The key function of core protein is from RNA binding to encapsidation and associating membrane and lipid droplets (LDs) to produce virus-like particles and infectious virion progeny. Core protein also directly or indirectly interacts with host cellular factors to play an essential role in virus-mediated pathogenesis (i.e. oxidative stress, steatosis, insulin resistance, and hepatocarcinogenesis). Several studies document the down regulation of core protein expression by small interference RNA (siRNA) and short hairpin RNA (shRNA) in human hepatoma cell lines (Huh-7) (Aljowaie et al. 2020; Khaliq et al. 2010). HCV envelope glycoproteins are transmembrane proteins and are actively involved in HCV entry, fusion, and defense against neutralization by envelop-specific host antibodies (Khaliq et al. 2011). These exhibit the highest degree of genetic heterogeneity as compared to other HCV proteins, where E2 contains a hypervariable region 1 (HVR1) (Khaliq et al. 2011). An important correlation has been found between E2 HVR1 sequence variation and the

intensity and cross-reactivity of humoral immune responses (Khaliq et al. 2011). Furthermore, E2 glycoprotein also interacts with major host cellular receptors (i.e., CD81, SR-BI and CLDN1) during virus entry into the host cells (Khaliq et al. 2011). This interaction is a potential target site to prevent viral entry into host cells by suppressing E2 protein expression by siRNAs (Khaliq et al. 2011). Several studies also demonstrate the inhibition of E2 and host cellular receptors interaction by targeting E2 protein with siRNAs and shRNAs (Khaliq et al. 2011; Jahan et al. 2011). Despite all these plausible claims and justifications, HCV structural proteins (i.e. core, E1, and E2) are very much involved in viral progeny formation and progression of infection severity instead of viral replication and translation, thus HCV 5' UTR is the core target site of anti-RNA based treatment strategies like RNAi to inhibit viral replication and translation in cell culture system.

Since its discovery as a potential defense mechanism in plants by some elegant experiments of Andrew Fire and Craig Mello in 1999, the phenomenon of RNA interference has been investigated for most of the genetic disorders in plants, animals, and humans (Jarczak et al. 2005; Brummelkamp et al. 2002; Caplen et al. 2001; Cullen 2002; Kapadia et al. 2003; Seo et al. 2003). It is one of the most powerful antiviral approaches in basic and applied sciences to keep jumping genes under control and to regulate gene expression participates in defense against viral infections (Cullen 2002; Kapadia et al. 2003). siRNA specificity to silence gene expression of certain hard-to-cure diseases like HIV, cancers, and certain genetic disorders have been validated by numerous reported studies (Brummelkamp et al. 2002; Caplen et al. 2001; Seo et al. 2003; Kim et al. 2006). Investigators have already been documented siRNA based efficient gene silencing of certain animal viruses like HIV-1, flock house virus (FVH), and poliovirus (Caplen et al. 2001; Cullen 2002). However; it is the most investigated therapeutic weapon and a laboratory tool against HCV than any other human disease and infection in the world. Hundreds of thousand previous studies including our own experiences suggest that it is the most powerful tool to inhibit virus replication and silence protein expression up to 100-fold (Aljowaie et al. 2020; Yokota et al. 2003; Randall et al. 2003; Prabhu et al. 2006; Prabhu et al. 2005; Zekri et al. 2009; Shahid et al. 2018; Shahid et al. 2017). Previous studies report inhibition of full-length HCV sub-genotype 1a and 1b clones by sequence-specific siRNAs (Kim et al. 2006; Korf et al. 2007; Kronke et al. 2004; Wilson et al. 2003; Wilson and Richardson 2005). Similarly, some investigators reported replication inhibition of all HCV GTs and certain sub-genotypes by targeting the highly conserved region of the HCV genome and some demonstrated siRNA silencing impact on viral mRNA transcript and viral protein expression by targeting HCV variable genome regions (Aljowaie et al. 2020; Shahid et al. 2017; Shahid et al. 2018; Prabhu et al. 2005; Prabhu et al. 2006; Zekri et al. 2009). The bottom line message from all previous studies is evident that RNA interference efficiently works against viral infections to inhibit gene expression in mammalian cells (Shahid et al. 2018). Recently, the approval of the first small interference RNA (siRNA) based treatment; ONPATTRO[®] (patisiran) for the treatment of hereditary transthyretin-mediated amyloidosis has sparked the researchers to refocus RNA interference (RNAi) against the highly susceptible RNA viruses like HCV (Hoy 2018; Qureshi et al. 2018).

In this study, we characterized siRNA inhibitory effects against HCV replication and translation of sub-genotype 4a isolates from the Saudi population in a persistent Huh-7 cell culture system. For this purpose, consensus siRNA targeted to highly conserved nucleotide motifs of SL structures of IRES of 5' UTR were constructed, chemically synthesized, and co-transfected into Huh-7 cells along with 5' UTR-IRES reporter vector and Huh-7 cells inoculated with native viral particles of HCV sub-genotype 4a positive serum. 5' UTR RNA transcript level and GFP protein expression

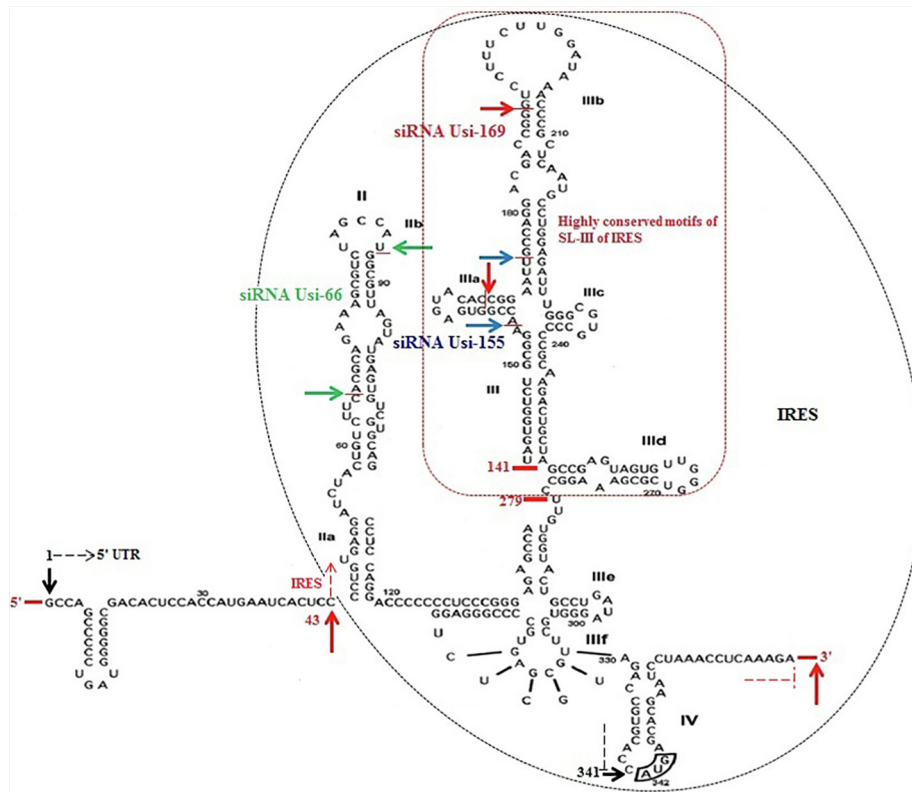


Fig. 1. HCV 5' UTR sequence and structure along with the location and position of siRNA targets. The predicted secondary structure shown here is that of sub-genotype 1a 5' UTR, reference prototype strain (H77), and structures based on previous studies (Friebe et al. 2001; Honda et al. 1999; Vopalensky et al. 2018). The HCV IRES borders are indicated with black dotted oval shape while the highly conserved IRES region within SL-IIIa to IIIId (starting from nucleotide 141 and ending to 279) is indicated by the red dotted oval shape. The solid black arrow starting from nucleotide 1 to nucleotide 341 spans 5' UTR of HCV. The red solid and dashed arrow marked from nucleotide position 43 to 3' end of the whole sequence represents the boundary of IRES structure within 5' UTR. The siRNA targeted positions for HCV sub-genotype 4a isolates discussed in this study are relative to the alignment of prototype strain and marked and labeled with colored arrows and text along with their aligned nucleotide positions.

were efficiently decreased by siRNA targeted to pCR3.1/5'UTR/GFP clones in Huh-7 cells at 48 h post-transfection as determined by semi-quantitative RT-PCR, real-time PCR, fluorometry, Western blot analysis, and flow cytometer analysis. siRNA potent silencing impact on HCV replication and translation in an *in vitro* serum inoculated Huh-7 tissue culture system with persistent viral replication support and consistent protein expression was measured on day 3 siRNA post-transfection by nested RT-PCR and real-time PCR of minus and plus-strand RNA synthesis and protein expression of HCV Core (C) antigen and hyper-variable glycoproteins (i.e. E1 and E2) expression by Western blot analysis and protein densitometry. This is the first study in its kind reported from Saudi Arabia to evaluate siRNA antiviral potential against the highly conserved region of the HCV genome because previous studies only decipher this phenomenon against less conserved and variable regions of the viral genome (Aljowaie et al. 2020). In the current report, we clearly showed that siRNAs targeted to SL-II and III nucleotide motifs of IRES significantly decreased RNA expression of 5' UTR and also downregulate protein expression of GFP in the Huh-7 cell culture system. We also unveiled that IRES suppression by consensus siRNAs, not only strongly inhibits viral replication but also suppress translation of full-length HCV genome in an *in vitro* Huh-7 cell culture system. Furthermore, siRNA antiviral potential to inhibit viral replication and translation in a persistent hepatic cell culture system revamps the efforts to re-evaluate/reconsider siRNA therapeutic potential against HCV despite the plausible qualms of siRNA stability in the cellular environment, deficiency of optimal siRNA delivery system, and the emergence of viral escape mutants.

2. Materials and methods

2.1. Patient ethics and consent statement

The patients' demographic data, blood, and sera samples were documented and provided by the Department of Pathology and Laboratory Medicine, Molecular Biology Unit, Ministry of National Guard health affairs, King Abdul Aziz Medical City, Jeddah, Saudi Arabia. The patients were also given informed consent for the collection of blood samples. The research project, data forms, and ethical consent were approved by the King Abdullah City for Science and Technology (KACST), Riyadh, Saudi Arabia, King Abdul Aziz Medical City, Jeddah, Saudi Arabia, and the research ethics committee of the College of Pharmacy, Umm Al-Qura University, Makkah, Saudi Arabia (REC/2480-19/CP/UQU-SA) respectively and were in total compliance with the Helsinki Declaration of 1975 as revised in 2008.

2.2. Clinical specimen and sample collection

Thirty serum samples both from male and female patients excluding children were collected and stored at -70°C before use. The estimated duration of infection varied from 6 months to 10 years. The patients below 18 and above 70 years of age, patients with HCV/HBV or HCV/HIV co-infection, and pregnant females were excluded from the study. HCV positive criteria was based on elevated serum SGPT (serum glutamic pyruvic transaminase) and SGOT (serum glutamic oxaloacetic transaminase) levels at least for six months, histological examination, and persistent detection of viral RNA load in serum samples of participating sub-

jects. Anti-HCV antibodies (performed by 3rd generation ELISA, (DIAsource Immunoassays[®], Nivelles, Belgium)) were present in all samples. All patients were negative for HAV, HBV, and HDV surface antigens.

2.3. HCV viral load and GTs/sub-genotypes identification

HCV viral load in sera samples was detected by using Real-TM Quant SC kit (Sacace[™] Biotechnologies, Como, Italy) and fluorescent reporter dye probes specific to the Real-Time PCR SmartCycler[®] (Cepheid, Sunnyvale, USA) following kit protocol and manufacturer's instructions. HCV viral titers in the range from 3×10^5 to 5×10^8 IU/mL were considered to inoculate Huh-7 cells for persistent viral replication and reproducible protein expression up to 30 days. For HCV GTs and sub-genotypes identification, we followed a recently reported diagnostic method of HCV genotyping based on a one-step PCR amplification method of 5' UTR and partial core region instead of the conventional methods of direct hybridization and probe assays directed against the 5' UTR (Virtanen et al. 2018). This protocol is consistent, robust, and advantageous to identify all HCV GTs/sub-genotypes in one thermal cycle reaction. Only the confirmed HCV sub-genotypes 4a isolates were further considered for 5' cloning, plasmid construction, siRNA designing, and to characterize siRNA antiviral potential in Huh-7 cells. The isolates of other HCV sub-genotypes (e.g., 1a, 1b, 2a, 2b, 3a, 3b, 4b, 4d, etc.), mixed, and undifferentiated sub-genotypes were excluded from the study.

2.4. Amplification of 5' UTR

Viral RNA was extracted from 140 μ L of all serum samples by using QIAamp Viral RNA Mini kit[®] (QIAGEN, California, USA) by following the kit protocol. The extracted RNA pellet was resuspended in 40 μ L of TE (Tris EDTA) buffer and purified by using PureLink[™] Viral RNA/DNA kit (Invitrogen[™], Carlsbad, CA, USA) for RNA concentration measurement in ng/ μ L by using NanoDrop spectrophotometer[®] (Thermo-Fisher Scientific[™], Delaware, USA). The RNA yield was calculated from 45 ng/ μ L up to 150 ng/ μ L. RevertAid H Minus First Strand cDNA synthesis kit[®] (Thermo-Fisher Scientific[™], Delaware, USA) was used for cDNA synthesis by using 5' UTR outer antisense primer of 4a sub-genotype in separate reaction mixture tubes by following the kit protocol (Table 1). The cDNA products were further used for 5' UTR amplification in 25 μ L reaction volume by using HCV sub-genotype 4a 5' UTR specific primers in thermal cycler 9700[®] (Applied Biosystems[™], CA, USA) (Table 1). The reaction mixture contained KCl buffer 2.5 μ L, 25 mM MgCl₂ 2.5 μ L, 10 mM dNTPs 2.0 μ L, 5' UTR inner sense primer (10 pmol/ μ L) 1.0 μ L, 5' UTR inner antisense primer (10 pmol/ μ L) 1.0 μ L, Taq DNA polymerase (1.25U/ μ L) 0.25 μ L, nucleic acid template (70–80 ng/ μ L) 2 μ L, and water nuclease-free up to final volume 25 μ L. The thermal cyclic profile for amplification was 95 °C for 2 min, 94 °C for 35 s, 58 °C for 30 s, 72 °C for 25 s, and final extension for 10 min at 72 °C. 1.8% agarose gel prepared in 1X TAE buffer (Tris-acetate-EDTA) and stained with ethidium bromide (2 μ L) was used to separate amplified PCR products and characterized under UV transilluminator. PCR fragments were purified by eluting the gel to eliminate unincorporated primers and dNTPs by using the QIAquick gel extraction kit (QIAGEN, California, USA) protocol.

2.5. Sequencing of the 5' UTR amplicons

The purified PCR amplicons were sequenced by using the Big-Dye[™] Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Germany) by following the kit protocol. The sequencing reaction mixtures were transferred into a 96-well sequencing plate. Standard Sanger dideoxy sequencing method was followed and the samples

were analyzed by using ABI PRISM 3700 genetic analyzer (Applied Biosystems, Foster City, California, USA). All the samples were sequenced bidirectional (i.e. forward and reverse) to get consensus sequences and only HCV sub-genotypes 4a sequenced isolates were considered for plasmid construction and siRNA targeting.

2.6. Plasmid construction

pCR3.1 mammalian expression plasmid expressing 5' UTR along with reporter gene GFP (i.e. green fluorescent protein) was designed and constructed to express 5' UTR in the human hepatoma cell line (Huh-7; a well-differentiated hepatocyte-derived carcinoma cell line extracted from the liver tumor of a Japanese male in 1982). First, the GFP gene was isolated from the pUbC-GFP vector (Addgene[®], MA, USA) and tagged with 5' UTR into pCR3.1 plasmid. pCR3.1 plasmids were transformed into competent bacterial cell JM109 *E. coli*. (Agilent[®], CA, USA) and recombinant plasmids were purified from individual white bacterial cell colonies by using the plasmid isolation miniprep method (Promega, WI, USA). Insert DNA clones of 5' UTR products were digested by primers incorporated with *EcoRV* and *XbaI* restriction enzymes sites at 5' and 3' overhangs and were sequenced in both forward and reverse directions by using 5' UTR sequence-specific primers and T7 primers respectively. The obtained sequences representing independent isolate were aligned with comparable referenced sequences of prototype strains of HCV sub-genotype 4a for nucleotide identities.

2.7. siRNA design and synthesis

The highly conserved nucleotide sequences within SL-II and III of IRES of 5' UTR were selected for siRNA design and synthesis. For this purpose, the gene sequences of representative HCV isolates were aligned to prototype HCV sub-genotypes 4a strain (ED43; accession number Y11604) to correspond their relative alignment positions. Three consensus siRNAs targets were identified within SL-II and III nucleotide residues of IRES of 5' UTR from all representative isolates in this study based on 100% alignment with prototype HCV strains. No cross alignment of the selected target sequences were found with any other sequences on GenBank NCBI database and within IRES of the HCV 5' UTR. The siRNAs were designed by using the online Ambion[™] siRNA design tool at http://www.ambion.com/techlib/misc/siRNA_finder.html. siRNAs with 21 nucleotides in length targeted to selected consensus sequences within SL-II and III- of IRES started with AA nucleotide at 5' end followed by 19 nucleotides and 30–60% GC contents were selected. The designed siRNAs were chemically synthesized by using Ambion[™] Silencer[™] siRNA construction kit (Fisher Scientific[®], Goteborg, Sweden) by following the manufacturer's protocol. A scrambled siRNA (used as a negative control, to maintain cellular mRNA integrity during trials, and to make sure equal RNA and protein sample loading in all experiments) target sequence for GAPDH (i.e., glyceraldehyde phosphate dehydrogenase) was also selected, designed, and synthesized with the same specifications as of 5' UTR siRNAs but lacking homology to HCV and human genome. All the siRNAs were named according to their respective targeted positions within SL-II and III- of IRES of 5' UTR to prototype H77 HCV strain (Table 2 and Fig. 1). The siRNAs were HPLC purified and sterilized with ultra-filtration to remove toxic interfering impurities to the cell culture system.

2.8. Huh- 7 cell culturing and co-transfection with constructed vectors and siRNAs

Huh- 7 cell line was routinely grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum

Table 1

List of primers used for 5' UTR amplification, GTs/sub-genotypes identification, sequencing, and RNA expression of 5' UTR from HCV sub-genotype 4a Saudi isolates.

A. Primers for 5' UTR PCR amplification							
Sr. No	Primer name	Primer sequence	Primer length	Sr. No	Primer name	Primers for 5' UTR amplicon sequencing	Primer length
01	SA-IS/5'-1 (F)	ACCGAAAGCGTTAAGCCATGGGCC	24	03	SA-IS/5'-3 (F)	GCGAGCTTACCTGCCTCGTA	20
02	SA-IS/5'-2 (R)	GTTGCAAGCACGGTATCAGGCAGA	24	04	SA-IS/5'-4 (R)	CGAGGCAAGATGTCGTTGAA	20
Primers for GTs/sub-genotype identification							
05	5'UTR- 1F	GTCTAGCCATGGCGTTAGTATGAGTG	26				
06	5'UTR -1 R	ACAAGTAAACTCCACCAACGATCTG	25				
Primers for GAPDH and 5' UTR RNA expression by RT-PCR							
07	GAPDH-F07	ACCACAGTCCATGCCATCAC	20	09	5'-UTR-RT-F09	GAGAGCCATAGTGGTCTGGC	20
08	GAPDH-R08	TCCACCACCTGTGTCTGTA	20	10	5'-UTR-RT-R10	TTTCGCGACCAACTACT	20

Table 2

siRNA sequences directed against the SL-II and III of IRES structure of 5' UTR of HCV sub-genotype 4a isolates.

No.	siRNA name	RNA oligonucleotide sequences Antisense template (5'→3') Sense template (5'→3')
01	Usi-66-antisense	AATTAATCCAAGAAAGGACCCCTGTCTC
02	Usi-66-sense	AAGGGTCCTTTCTTGGATTAACCTGTCTC
03	Usi-155-antisense	AATATCAGGCAGTCCCACAAGCCTGTCTC
04	Usi-155-sense	AACTTGTGGGACTGCGTATACCTGTCTC
05	Usi-169-antisense	AATATCAGGCAGTACCACAAGCCTGTCTC
06	Usi-169-sense	AACTTGTGGTACTGCGTATACCTGTCTC
07	Scrambled-antisense	AACCTGCATACGGACTCGACCTGTCTC
08	Scrambled-sense	AAGTCGAGTCGCGTATGACGGCTGTCTC

(FBS) and 100 µg/mL antibiotics (penicillin/streptomycin) and were maintained at 37 °C in an atmosphere of 5% CO₂. Cell line passage were maintained by changing the complete cell growth medium (DMEM + 10% FBS, and 100 µg/mL penicillin/streptomycin) at every 2–3 days. Huh-7 cell viability was determined by trypan blue dye explosive method (5 g/L; Biochrom KG, Berlin, Germany) and MTT cell proliferation assay was performed to assess the cytotoxic effects of siRNAs by using Vybrant[®] MTT Cell Proliferation Assay Kit (Invitrogen life technologies[®], California, USA) by following the kit's protocol as previously described (Shahid et al. 2017; Shahid et al. 2018). siRNA transfection protocol was optimized empirically because of the varying capacity of different cell lines to cell plating density, optimal amount of transfection reagent, cell inoculation with serum-free or serum-containing medium, and an optimal amount of siRNAs.

For constructed vectors and siRNA co-transfection, Huh-7 cells were seeded in 24-well or 6- well cell culture plate at a density of 1×10^5 cells/well or 5×10^5 cells/well respectively until 40–60% confluent with complete cell growth medium. Huh-7 cells in 24-well plate were transfected with siRNAs in a dose-dependent manner (10, 20, and 40 nM/well dose) along with 0.5 µg of pCR3.1/GFP tagged-5' UTR vectors in a reaction mixture by using lipofectamine[™] 2000 (Invitrogen life technologies[®], California, USA) as a transfection reagent according to manufacturer's protocol. The scrambled siRNA (i.e. human GAPDH) was also transfected while following the same protocol and used as an internal control (i.e. housekeeping gene) for uniform sampling of HCV siRNA transfected and un-transfected Huh-7 cells and to maintain cellular mRNA integrity. The cells were maintained in 10% FBS free media and incubated at 37 °C in 5% CO₂. After 6 h, the complete cell growth medium was added to the cells and allowed to grow until harvested at 24 and 48 h post-transfection for total RNA analysis.

2.9. RNA extraction and 5' UTR RNA expression

Total cellular RNA was extracted from siRNA transfected and un-transfected Huh-7 cells by using TRIZOL[®] Reagent, (Invitrogen

life technologies[®], California, USA) at 24 h and 48 h post-transfection. 500 ng isolated RNA was used to synthesize cDNA by using RevertAid H Minus First Strand cDNA synthesis kit[®] (Thermo-Fisher Scientific[™], Delaware, USA) by following the kit protocol. To explore siRNA inhibitory effects on 5' UTR RNA expression, semi-quantitative reverse transcription PCR (RT-PCR) was performed by using QIAGEN one-step RT-PCR kit[®], (QIAGEN[™], Hilden, Germany) with 5' UTR specific primers (Table 1). The thermal cyclic profile was set as follows; 95 °C for 3 min, 30 cycles of 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min, and final extension for 10 min at 72 °C. 5' UTR expression was analyzed by using 1.8% agarose gel electrophoresis and visualized by using gel documentation apparatus GEL DOC XR[™] (Bio-Rad[®], Pennsylvania, USA). Quantitative viral RNA inhibition was performed by using real-time PCR ABI 7500 (Applied Biosystem Inc, USA) with SYBR-Green real-time PCR master mix (ThermoFisher Scientific, CA, USA) with 5' UTR specific primers. The real-time PCR profile for the reaction was set as follow; initial hold at 50 °C for 10 min, initial denaturation at 95 °C for 4 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 40 sec, and final extension for 7 min at 72 °C. SDS 3.1 software (Applied Biosystem Inc, USA) was used to analyze relative 5' UTR RNA expression. All the trials were performed in triplicate.

2.9.1. GAPDH mRNA and protein expression

GAPDH mRNA and protein expression was used as an internal control to infer that the RNA inhibition of 5' UTR and decreased protein expression of GFP in siRNA transfected cells are siRNA specific and unrelated siRNA (i.e. scrambled siRNA or negative control) have no inhibitory effects on RNA and protein expression in co-transfected cells. Furthermore, we want to establish that the integrity of cellular mRNA was maintained either in un-transfected Huh-7 cells or cells co-transfected with constructed vectors and siRNAs. By quantification of GAPDH mRNA level by real-time PCR with GAPDH specific primers and protein expression by Western blot in siRNA co-transfected and un-transfected Huh-7 cells at 24 and 48 h post-transfection, we ensured that siRNAs used in this study did not adversely impact the expression of a house-keeping gene from host cells. The real-time PCR protocol for the GAPDH reaction was the same as described above in 2.9.

2.9.2. Western blotting

For protein expression of GFP and GAPDH under the influence of 5' UTR and siRNA, the same transfection protocol was repeated (as described in 2.8) in 6-well plates with 0.5 µg of constructed vector and 40 nM/well of each siRNA. Un-transfected and siRNA transfected Huh-7 cell lysates were subjected to 12% SDS-PAGE at 24 and 48 h post-transfection by following a standard Western blotting protocol as previously described (Shahid et al. 2017; Shahid et al. 2018). The protein expression incubated against primary monoclonal antibodies (Santa Cruz Biotechnology Inc, USA) specific to GFP and GAPDH, and secondary horseradish per-oxidase-

conjugated anti-goat anti-mouse antibodies (Sigma Aldrich, USA) were evaluated by using Chemiluminescence's detection kit (Sigma Aldrich, USA).

2.9.3. GFP fluorescence and flow cytometer analysis for positive GFP Huh-7 cells

For siRNA silencing impact on GFP expression of different pCR3.1/5'UTR/GFP reporter vectors in co-transfected Huh-7 cells, GFP expression was analyzed under a fluorescence microscope. Briefly, the cells were seeded in a 12-well tissue culture plate and were grown the day up to 60–80% confluence before transfection. After 24 h, the cells were transfected with 0.5 µg constructed vectors and incubated for 2 h. Then the cell growth medium was removed and transfected the cells with 40 nM concentration of each siRNA by using FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). Green fluorescence expression was recorded at 24 h and 48 h under a fluorescence microscope. The ratio of 5'UTR-GFP vector to each siRNA concentration for maximum inhibition of GFP expression at 24 h and 48 h was recorded. Each siRNA inhibitory effect on GFP expression from six different HCV 5' UTR sequences were quantitatively determined by flow cytometer analysis. Briefly, Huh-7 cells co-transfected with six different 5'UTR-GFP constructed vectors and siRNAs were harvested with trypsin-EDTA (Ethylendiamine tetraacetic acid) treatment at 24 h and 48 h post-transfection, and resuspended in PBS buffer (Phosphate buffer saline) for a while (Invitrogen Life Technology, Carlsbad, CA) and then subjected to flow-cytometric analysis (Becton Dickinson, BD Biosciences Clontech). The percentage of positive GFP Huh-7 cells after siRNA treatment for each siRNA was quantified and compared with control siRNA.

2.10. Huh-7 cells inoculation with HCV serum and co-transfection with siRNAs

To investigate *in vitro* siRNA antiviral potential against HCV replication and protein translation, Huh-7 cells with persistent high viral titer/load ($>1 \times 10^8$ IU/mL) were maintained by identifying and detecting HCV plus mRNA copy numbers in cell lysates for one-month post-viral inoculation with fresh sera of HCV 4a infected patients. We followed the same protocol for Huh-7 cells inoculation as we previously described in with slight modifications (Shahid et al. 2018; Shahid et al. 2017). Briefly, 3×10^5 cells/well were seeded into 6-well cell culture plates to semi-confluence (i.e., 50–70% adherent cells). Cells were washed with 1X PBS and 10% FBS-free growth medium three times and inoculated with 1×10^8 IU/mL/well sera of HCV infected patients. The inoculated Huh-7 cells were incubated at 37 °C in 5% CO₂ for 24 h. The next day, cells were washed again with 1X PBS three times and the old cell growth medium was replaced with fresh complete cell growth media and continued the incubation for the next 48 h. After 72 h post-inoculation, Huh-7 cells were harvested and evaluated for quantitative viral mRNA (viral titer) by using the Real-TM Quant SC kit (Sacace™ Biotechnologies, Como, Italy) and fluorescent reporter dye probes specific in the Real-Time PCR SmartCycler® (Cepheid, Sunnyvale, USA).

For siRNA transfection, Huh-7 cells with persistent viral load were seeded again into 24- and 6-well cell culture plates at a density of 1×10^5 cells/well and 5×10^5 cells/well respectively until 60% confluent. The cells were transfected with 40 nM/well and 100 nM/well of siRNAs respectively in both plates by using Lipofectamine™ 2000 (Invitrogen life technologies®, California, USA) at a period of 24 h or 48 h post-transfection and incubated at 37 °C in 5% CO₂. The cells were harvested at 72 h post-transfection both from 24-well and 6- well cell culture plates for quantitative viral load, plus and minus RNA strand detection, and protein expression analysis.

2.11. Viral replication determination by plus and minus-strand RNA detection

Persistent HCV replication in serum inoculated Huh-7 cells, and in serum inoculated Huh-7 cells transfected with siRNAs was determined by detection and quantification of plus and minus HCV RNA strand in intracellular Huh-7 cell lysates and cell supernatant of cultured cell medium by using real-time PCR (ABI 7500, Applied Biosystems, USA) following the protocol previously described by el-Awady et al and Zekri et al. (el-Awady et al. 2006; Zekri et al. 2009)

2.12. Quantitative detection of viral load

Total intracellular mRNA was extracted from Huh-7 cells inoculated with HCV serum and/or transfected with siRNAs at 72 h post-transfection by using the Purescript® RNA Isolation kit (Gentra System Pennsylvania, USA) according to the kit's protocol. Real-TM Quant SC kit (Sacace™ Biotechnologies, Como, Italy) based on the detection and quantification of 5' UTR of viral copy number detected by fluorescent reporter dye probes specific in the Real-Time PCR SmartCycler® (Cepheid, Sunnyvale, USA) was used for viral quantification (viral load). The reaction mixture preparation and PCR thermal cycle profile were followed according to the kit's protocol and manufacturer's instructions respectively.

The viral load was calculated in IU/ml by using the following equation;

$$\frac{\text{Cy3 STD/Res}}{\text{FAM Std/Res}} \times \text{coefficient IC}^* = \text{HCV viral load in IU/mL.}$$

*IC = internal control, which is specific for each kit's lot.

2.13. Western blotting and quantitative analysis of HCV core (C) antigen and hypervariable glycoproteins (E1 and E2) in serum inoculated Huh-7 cells

For viral protein expression of GFP, C, E1, and E2 at day 3 of viral inoculation in Huh-7 cells and/or transfection with siRNAs, the cells were washed three times with 1X PBS before harvesting, the protein was extracted and subjected to Western blotting by following a standard protocol established in our laboratory and previously described (Shahid et al. 2017; Shahid et al. 2013; Shahid et al. 2018). Quantitative measurement of C, E1, E2, and GAPDH protein expression was performed by densitometric analysis using image lab software (BioRad®, USA), and percentage protein expression to positive control was determined. The GAPDH protein expression was used to verify the equal amount of viral protein loading on each lane of SDS-PAGE gels.

2.14. Statistical analysis

Statistical analysis of the data was performed by using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software version 18. Data for MTT cell proliferation assay, relative 5' UTR RNA expression, HCV viral load quantification, viral copy numbers, plus-minus RNA strand quantification, GFP, GAPDH, C, E1, E2 protein expression in siRNA transfected and untransfected Huh-7 cells, in serum inoculated Huh-7 cells, and in cells co-transfected with siRNA plus 4a-serum were carried out in triplicates and were collected as mean ± standard deviation (S. D), and were presented as mean ± standard errors of the mean (S.E.M). Unpaired t-tests and one-way analysis of variance (ANOVA) were performed to compare variables among different groups. A statistically significant difference was assumed where

the p-values were less than 0.05, 0.01, and 0.001 among different groups where appropriate and marked with asterisks.

3. Results

3.1. Cytotoxic impact of siRNAs

The relative mRNA and protein expression of a housekeeping gene (i.e. GAPDH) from siRNA transfected Huh-7 cells qualitatively evaluate the integrity of cellular RNA and host cell viability. However; sometimes it becomes difficult to differentiate a decrease in viral mRNA and cell viability either due to siRNA silencing impact or cytotoxic concentration of siRNAs. MTT cell proliferation assay is a reliable quantitative method to determine siRNA potential cytotoxic effects on cellular mRNA and host cell viability before antiviral screening of siRNA against viral mRNA in transfected hepatic cells. In this study, before exploring siRNA antiviral potential, we assessed the cytotoxic impact of all siRNAs in a dose-dependent manner (i.e., from 10 nM to 50 nM). The results revealed that at 40 nM concentration, siRNA had no toxic effects on cells viability as determined by persistent viable cell count with formazan products (i.e. Yellow MTT (3–4, 5-Dimethylthiazol-2-yl) –2, 5-diphenyltetrazolium bromide, a tetrazole) is converted to purple formazan in the mitochondria of living cells) in viable cells by measuring absorbance at a test wavelength (570 nm) and reference wavelength (620 nm) through an ELISA plate reader. However; at 50 nM siRNA dose, the viable cell count was significantly decreased (i.e. 8–10% decrease in viable Huh-7 cells) as compared to the percentage of positive control viable cells and scrambled siRNA transfected Huh-7 cells (Fig. 2). MTT cell proliferation assay also provided a clue about effective concentrations of siRNAs to be tested against viral mRNA and protein expressions.

3.2. siRNA inhibitory effects against 5' UTR RNA and GFP protein expression in Huh-7 cells

To evaluate siRNA silencing potential against 5' UTR RNA expression and GFP protein expression in Huh-7 cells co-transfected with constructed vectors and 5' UTR-IRES-targeted siRNAs, three different siRNAs (Usi-66, Usi-155, Usi-169) targeting highly conserved nucleotide motifs of IRES structure within 5' UTR of sequenced isolates were selected. Their locations and positions representing the consensus RNAi target sites relative to the predicted secondary structure of HCV IRES of prototype strain are shown in Fig. 1. Huh-7 cells were co-transfected with constructed vector and each siRNA and after 24 h and 48 h incubation, cellular RNA and proteins were extracted to measure relative RNA and protein expression analysis. The following findings were elucidated;

3.2.1. Persistent and reproducible expression of 5' UTR RNA and GFP protein in Huh-7 cells

First, we determined siRNA silencing impact on 5' UTR RNA expression and GFP protein expression in Huh-7 cells transfected with constructed vector (i.e. pCR3.1/5UTR/GFP) that express GFP under the control of 5' UTR in the presence or absence of 5' UTR specific siRNAs. For this purpose, we qualitatively measured persistent, efficient, and reproducible RNA expression of 5' UTR by semi-quantitative RT-PCR in Huh-7 cells for three consecutive days and further quantified by real-time PCR. No apparent decline in 5' UTR RNA expression was noticed in qualitative RT-PCR analysis and 5' UTR RNA transcript levels were almost similar from day 1 to day 3 as quantitatively measured (in folds) and shown in Fig. 3A and 3B. Continuous protein expression of GFP was also evaluated from day 1 to day 3 by Western blot analysis in Huh-7 cells to further analyze siRNA inhibitory effects on GFP expression in transfected

cells (Fig. 3C). GAPDH mRNA and protein expression was used as an internal control and housekeeping gene to intact cellular mRNA integrity and equal load of RNA and protein samples during RT-PCR, real-time PCR, and Western blot analysis.

3.2.2. HCV 5' UTR RNA levels were down-regulated by siRNAs directed against HCV-IRES in Huh-7 cells

siRNA inhibitory effects against 5' UTR RNA expression was carried out in a dose-dependent manner (starting from 10 nM up to 40 nM) in Huh-7 cells co-transfected with constructed vectors and targeted siRNAs at 24 h and 48 h incubation. No significant and intermingled RNAi activity was observed for each siRNA at concentrations from 10 nM to 30 nM (data not shown), however; all siRNAs exhibited a significant decrease to 5' UTR RNA expression at 40 nM dose as compared to scrambled siRNA (i.e. control siRNA) which showed no apparent change in 5' UTR expression of transfected vectors to positive control cells. The siRNA Usi-169 targeted to SL-III of HCV IRES was the most efficient even at low concentrations to silence the expression of 5' UTR RNA in Huh-7 cells both at 24 h and 48 h treatment. siRNA Usi-155 was also targeted to SL-III of IRES, but was moderately effective to decrease 5' UTR RNA expression in treated cells at 48 h post-transfection than 24 h at 40 nM concentration. siRNA Usi-66 designed from SL-II of IRES was the least effective and expressed less apparent RNA silencing in 24 h treated Huh-7 cells than 48 h at 40 nM dose (Fig. 4A). GAPDH expression was not altered in any experiment lane with control siRNA (i.e. scrambled) at 24 h or 48 h post-transfection indicating that siRNA mediated gene silencing is highly specific and scrambled siRNA is irrelevant to any decrease in gene expression. The findings of relative quantitative analysis by real-time PCR unveiled that 5' UTR RNA transcript levels were decreased up to 76% ($P < 0.001$) by siRNA Usi-169 at 24 h post-transfection and 80% ($P < 0.001$) at 48 h treatment. For siRNA Usi-155, relative RNA transcript levels were noticed to decrease by up to 72% ($P < 0.01$) at 24 h and 70% ($P < 0.01$) at 48 h post-transfection. A less significant decrease in RNA transcript level was observed in Huh-7 cells incubated with siRNA Usi-66 at 24 h and 48 h period i.e. 68% ($P < 0.05$) and 64% ($P < 0.05$) respectively (Fig. 4B). A maximum decrease in HCV 5' UTR RNA transcript levels was noticed with siRNA Usi-169 from 75 to 80% relative to positive HCV 5' UTR RNA transcript levels. No apparent decrease in 5' UTR RNA transcript levels was noticed with scrambled siRNA.

3.2.3. 5-UTR tagged GFP protein expression was also silenced by siRNAs in Huh-7 cells

Likewise, 5' UTR RNA transcript levels, All three siRNAs Usi-66, Usi-155, and Usi-169 effectively decreased HCV 5-UTR tagged GFP protein expression in Huh-7 cells at 24 h and 48 h siRNA treatment. GFP expression was photographed under a fluorescent microscope from Huh-7 cells after 24 and 48 h siRNA post-transfection. The findings indicated that GFP fluorescence was decreased in siRNA treated cells when compared to control siRNA (i.e. scrambled) which showed no decrease of GFP fluorescence. Furthermore, GFP fluorescence in siRNA treated cells was also compared to positive clones of GFP (i.e. pUBC-GFP) and pCR3.1/GFP/5' UTR transfected vectors in Huh-7 cells which also indicated a significant decrease of GFP fluorescence in siRNA treated Huh-7 cells (Fig. 4C). The quantitative measure of GFP expression in Huh-7 cells after siRNA treatment was performed by flow cytometer analysis. It was found that siRNA Usi-169 decreased GFP expression in approximately 82% of siRNA transfected Huh-7 cells at 24 h count and almost in 90% Huh-7 cells at 48 h siRNA post-transfection. Other siRNAs like Usi-155 decreased 5'UTR-tagged GFP expression in approximately 65–70% of transfected Huh-7 cells at 24 and 48 count, while the GFP expression was the least decreased by siRNA Usi-66 which was determined from 40 to 50% of Huh-7 cells at 24

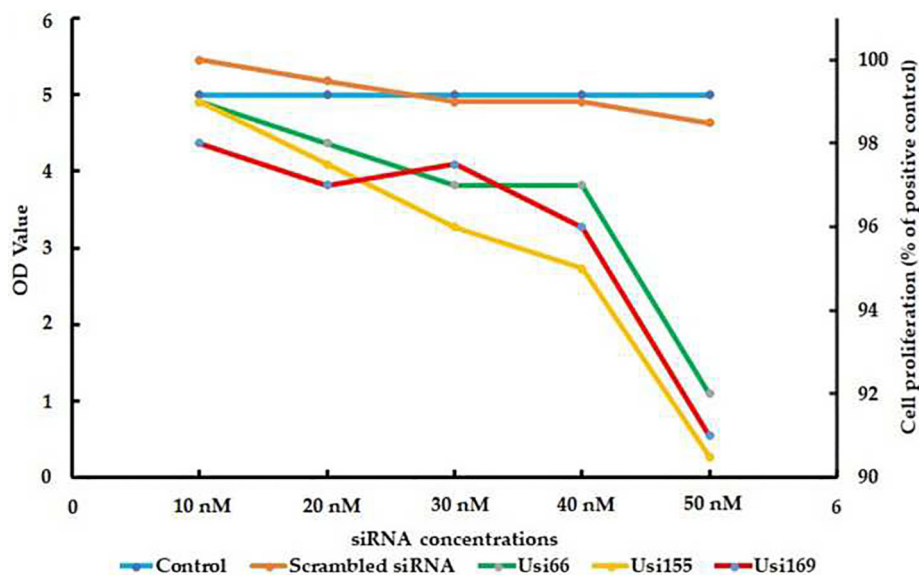


Fig. 2. MTT cell proliferation assay for cytotoxic impact of siRNAs on Huh-7 cells. For every siRNA concentration, MTT assay was carried out in triplicate presenting the data mean ($n = 3$) \pm S.D. A relative decrease in cell proliferation was noticed for all siRNAs at 50 nM concentration as compared to the percentage of positive control cells. Control siRNA (i.e. scrambled siRNA) did not show any significant decrease in cell proliferation as compared to positive control cells.

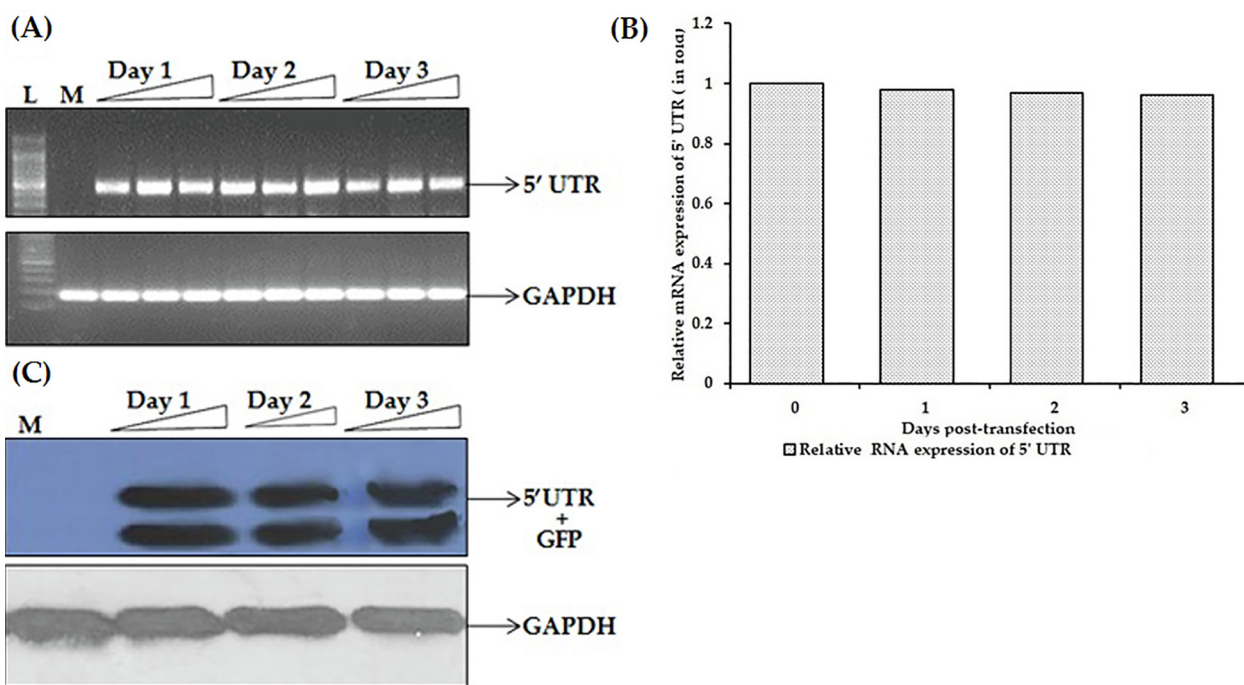


Fig. 3. 5' UTR RNA and protein expression in Huh-7 cells. Huh-7 cells were transfected with 0.5 μ g constructed vector (i.e. pCR3.1/5'UTR/GFP) and mock (i.e. pCR3.1 empty vector) treatment for day 1, 2, and 3 respectively. Intracellular mRNA and protein were extracted from harvested cells for relative 5' UTR RNA and GFP protein determinants by using RT-PCR and Western blot analysis respectively. **(A)** 5' UTR RNA expression was determined by semi-quantitative RT-PCR for three consecutive days. Three independent experiments were performed for each determinant with triplicate samples. L = 50 bp DNA marker, M = mock-treated (i.e. pCR3.1 empty vector) cells. GAPDH mRNA expression was used as an internal control. **(B)** Quantitative analysis of HCV 5' UTR RNA expression in Huh-7 cells at days 1, 2, and 3 respectively by real-time PCR relative to infold increase or decrease. **(C)** 5' UTR tagged GFP protein expression at three consecutive days incubated with primary monoclonal antibodies specific for GFP. M = mock-treated cells. GAPDH protein expression was used as an internal control to normalize each sample.

and 48 h siRNA post-transfection (Fig. 4E). No significant change in cell count with decreased GFP protein expression was noticed in Huh-7 cells transfected with control siRNA at any period.

We further validated that change in GFP fluorescence in siRNA transfected Huh-7 cells was directly correlating with mRNA suppression of GFP under the influence of RNAi activity against 5' UTR. For this purpose, the protein content of cellular extracts

was determined for GAPDH expression at 24 h and 48 h siRNA post-transfection in all experiments and compared to positive clone and control siRNA expressed GAPDH expression in Huh-7 cells. It was found that GAPDH expression was almost similar in all experiment lanes both at 24 h and 48 h period. Western blot analysis showed that GFP protein expression was efficiently decreased from 70 to 80% in Huh-7 cells co-transfected with con-

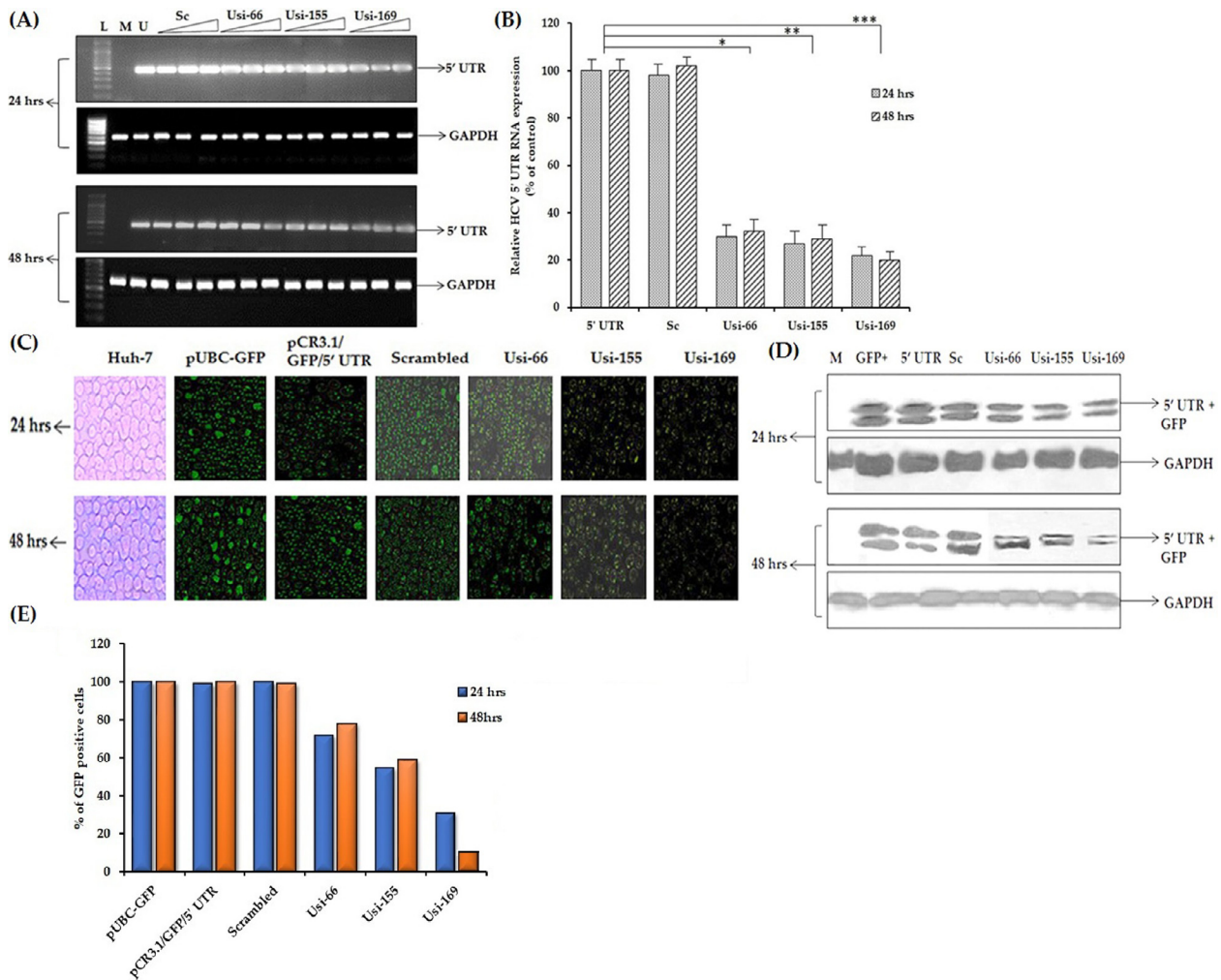


Fig. 4. Intracellular siRNA inhibits 5' UTR RNA transcript levels and 5'-UTR tagged GFP protein expression. (A) siRNA antiviral effect was demonstrated in a dose-dependent manner at 24 h and 48 h post-transfection. Each siRNA trial was performed in triplicate and RNA expression was noticed at 40 nM siRNA dose. 5' UTR RNA expression for mock (M), 5' UTR positive clone (U), and control siRNA (Sc) is also shown both for 24 h and 48 h siRNA post-transfection. L = 50 bp DNA ladder. (B) Quantitative real-time PCR analysis for relative 5' UTR RNA expression in Huh-7 cells. Triplicate samples were used to perform each experiment and each determinant. Bar graphs represent mean \pm S.D and error bars indicate mean \pm S.E.M. One way ANOVA and Unpaired t-tests were performed to compare the relative decrease in 5' UTR RNA transcript levels with control siRNA and versus 5' UTR positive clone. *P*-values were considered statistically significant where **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. (C) GFP fluorescence in Huh-7 cells co-transfected with positive GFP clone and with or without siRNAs were observed under a fluorescence microscope with 360–400 nm illumination light that excites GFP fluorescence up to 20 \times magnification. Control siRNA (i.e. scrambled) was irrelevant to make any change in GFP expression. (D) Western blot analysis unveiled that decrease in GFP protein expression was specific to siRNA activity in Huh-7 cells as quantitatively measured by protein densitometric analysis and compared to mock (M), GFP+, positive 5' UTR clone, and scrambled siRNA GFP protein expression. GAPDH protein expression is also shown as an internal control. (E) Quantitative measurement of GFP protein-positive Huh-7 cells by flow cytometer after siRNA transfection. Cells with positive GFP after siRNA treatment were quantified by using cell quest computer software. The findings were expressed as a percentage of control.

structed vectors (i.e. pCR3.1/GFP/5' UTR) and siRNA Usi-169 at 24 h and 48 h incubation, as compared to scrambled siRNA treated cells and positive clone cells. (Fig. 4D) The decrease in GFP protein expression was intermediate for siRNA-155 (~40 to 50%) and the lowest for siRNA-66 (~20 to 30%) at 24 h and 48 h post-transfection (Fig. 4D). We concluded that siRNA targeted to HCV-IRES not only silence 5' UTR RNA transcript levels but also suppress 5' UTR-tagged protein expression (e.g., in this report GFP).

3.2.4. Intracellular siRNA inhibits HCV replication and translation in an *in vitro* cell replication model

HCV replication in the cytoplasm of hepatocytes makes it an easier target of siRNA potent antiviral activity (Shahid et al. 2017; Shahid et al. 2013). As the above-mentioned results depicted that siRNAs targeted to 5' UTR-IRES clones of sub-genotype 4a isolates significantly silenced 5' UTR RNA transcript levels and efficiently suppressed 5' UTR-IRES directed GFP protein expression

in Huh-7 cells at 24 h and 48 h siRNA post-transfection, we tempted to evaluate siRNA antiviral potential against full-length HCV mRNA replication and translation in a persistent hepatic cell culture system. First, we maintained full-length HCV replication in Huh-7 cells from day 1 to day 30 post-viral inoculation.

At the time of analysis, Total cellular mRNA was extracted from Huh-7 cells inoculated with high viral titer of 4a-positive patient sera and 5' UTR-IRES directed siRNA to detect viral load by using real-time PCR with 5' UTR specific primers. As previously reported, chemically synthesized siRNA tends to degrade and metabolize in cell cytoplasm after exhibiting maximum inhibitory effects from 48 to 72 h (Khaliq et al. 2010; Khaliq et al. 2011; Zekri et al. 2011), we quantified relative viral load in percent and viral copy numbers in millions/mL in siRNA transfected Huh-7 cells as compared to positive control at day 1, day 2 and day 3 post-transfection (Fig. 5A). All siRNAs sufficiently down-regulated *in vitro* HCV RNA replication in Huh-7 cells as expressed in Fig. 5A

by decreased viral copy numbers at day 1, day 2, and day 3, albeit most prominent at day 2 and 3 respectively. However; maximal inhibition of HCV mRNA transcript level was noticed on day 3 where siRNA Usi-169 decreased more than 90% ($P < 0.001$) of viral copy numbers in serum inoculated and siRNA co-transfected Huh-7 cells as compared to the positive control (Fig. 5A). Viral load was also significantly decreased in serum-inoculated Huh-7 cells transfected with siRNA Usi-155 at day 3, where an 80% ($P < 0.01$) decrease in HCV viral load was noticed (Fig. 5B). siRNA Usi-66 also blocked HCV RNA replication up to 75% ($P < 0.01$) which was not so significant but prominent. Viral copy number was persistent with control siRNA transfection (i.e. scrambled siRNA) at any period and viral load was almost equal to control cells as shown in Fig. 5A-5B. siRNA inhibitory effects on viral replication was noticed to decrease from day 4 due to intracellular siRNA degradation and intracellular HCV RNA level was escalating in transfected Huh-7 cells from day 4 to onward as shown by increased viral copy numbers in Fig. 5A.

HCV replication and translation occur simultaneously in hepatocytes which means that signals for both phenomena overlap each other specifically for 5' UTR- IRES driven core protein translation of all HCV GTs/sub-genotypes (Friebe et al. 2001; Prabhu et al. 2006). We further elaborated that whether siRNAs used to block HCV replication could be effective to inhibit HCV protein translation by silencing HCV antigen (i.e. Core protein) and other hypervariable enveloped glycoproteins (i.e. E1 and E2) expression in serum inoculated and siRNA co-transfected Huh-7 cells. Total cellular protein lysates were prepared and were blocked with HCV Core, E1, E2, and GAPDH specific mouse monoclonal antibodies (mAbs). Viral protein expression was noticed to decrease for C, E1, and E2 proteins at 24 hr post-transfection and this decrease continued for the next 2 days (data not shown), however; maximal inhibition of protein expression was observed on day 3 with two siRNAs Usi-155 and Usi-169 (Fig. 6A). Densitometric analysis revealed the 70% ($P < 0.001$) decrease in core protein expression in serum inoculated Huh-7 cells transfected with siRNA Usi-169 at 72 hrs post-transfection (Fig. 6B). Envelope glycoproteins E1 and E2 expression were also suppressed 60% ($P < 0.01$) and 50% ($P < 0.05$) respectively with siRNA Usi-169 at 72 h post-transfection. Other siRNAs also silenced HCV antigen and envelope glycoproteins expression but not so much significant. Quantitative protein analysis in Fig. 6B showed that the relative decrease in HCV C, E1, and E2 protein expression with siRNA-155 was noticed from 40 to 60% ($P < 0.01$) as compared to positive control protein expression. Likewise, for siRNA Usi-66, the relative decrease in C, E1, and E2 protein expressions was measured from 30 to 50% ($P < 0.05$). No inhibition of core antigen and envelope glycoprotein E1 and E2 was noticed in cells transfected with control siRNA (i.e. scrambled siRNA) at day 3 post-transfection (Fig. 6A and 6B).

Collectively, the findings summarize that siRNAs targeted to 5' UTR-IRES of HCV sub-genotype 4a isolates show potent silencing of HCV replication and translation inhibition in a persistent *in-vitro* Huh-7 cell culture system. Furthermore, the inhibition of some viral protein expression like core (C) is important because it regulates apoptosis in hepatocytes which may lead CHC infection to hepatic fibrosis and hepatocellular carcinoma (HCC) (Zekri et al. 2011; Khaliq et al. 2010; Prabhu et al. 2004).

4. Discussion

We report siRNA antiviral potential against viral replication and translation in an *in-vitro* Huh-7 cell culture system by targeting one of the most conserved region of HCV genome (i.e. 5' UTR) of sub-genotype 4a isolates from the Saudi population. The isolates were sequenced for 5' UTR region to identify GTs and sub-

genotypes and representative nucleotide sequences were submitted to the NCBI GenBank database. Highly conserved regions of HCV 5' UTR from sequenced isolates were selected to design siRNA targets against viral RNA transcript. The 5' UTR in all HCV GTs and clinical strains is highly conserved and spans a length of 341–343 nucleotides (Tabata, Neufeldt, and Bartenschlager 2020). Sequenced isolates reported in this study covered SL-II and III regions of IRES structure within 5' UTR and are crucial for virus translation initiation and IRES activity (shown with red oval shape in Fig. 1 starting from nucleotide 141–279) (Friebe et al. 2001; Niepmann et al. 2018). Three siRNA targets were selected from the SL-II and III of IRES of 5' UTR (as shown in Fig. 1 with allocated positions). We showed that intracellular siRNA silences 5' UTR RNA expression and inhibits 5'UTR-tagged GFP expression from transfected clones of pCR3.1/5' UTR/GFP in Huh-7 cells. siRNA Usi-169 targeted to SL-III of IRES was found most effective to inhibit 5' UTR RNA transcript and protein expression of 5' UTR-tagged GFP clones in Huh-7 cells both at 24 h and 48 h siRNA post-transfection (Fig. 4). siRNA Usi-155 was moderately effective against HCV sub-genotype 4a IRES, and siRNA Usi-66 was the least effective (Fig. 4). Our findings are following with Prabhu et al. who demonstrated viral replication inhibition of six HCV GTs by targeting SL-II of 5' UTR (Prabhu et al. 2006). Similarly, Khaliq et al. showed viral replication inhibition of HCV sub-genotype 3a strains from the Pakistani population by targeting SL-II and III of 5' UTR (Khaliq et al. 2011). Ansar et al. reported inhibition of full-length genome replicon of HCV sub-genotype 1a in Huh-7 cells (Ansar et al. 2011). Zekri et al. demonstrated HCV GT-4 replication inhibition of Egyptian strains by consensus siRNA (Zekri et al. 2009). A difference in relative qualitative and quantitative expression of RNA and protein during siRNA screening could be due to nucleotide variations in the 5' UTR-IRES sequences among different isolates from different patients or even from the same patient (Fig. 4A to 4E). Many host cellular proteins bind to 5' UTR of HCV for translation of polyprotein, due to which some siRNA may not efficiently interact with targeted IRES sequences in transfected Huh-7 cells because of the complex secondary structure of 5' UTR (Zekri et al. 2009; Prabhu et al. 2006).

We further extended the study to elucidate siRNA induced silencing of HCV replication and translation in a persistent *in vitro* Huh-7 cell culture system inoculated with high viral titer serum of HCV sub-genotype 4a infected patients. We smoothly maintained this cell culture system to express high-level protein expression of HCV C, E1, and E2 proteins for one month as determined by Western blot analysis and densitometric analysis. Replication of the full-length HCV genome was supported by the consistent synthesis and measurement of minus- and plus-strand RNA by RT-PCR and real-time PCR. RNA replication inhibition was noticed very early after 24 h with all siRNAs in transfected hepatic cells. However, maximum inhibition was reached after 72 h post-transfection with siRNA Usi-169 (90% ($P < 0.001$) which targeted SL-III of IRES structure of 5' UTR. Our findings are in agreement with previous studies that demonstrated siRNA induced viral replication inhibition by targeting 5' UTR of HCV GT-4 strain in the Egyptian population (Zekri et al. 2009; el-Awady et al. 2006). Our results are also consistent with previous data that showed full-length viral genome replication inhibition of HCV sub-genotype 1a and 1b infectious clones in Huh-7 cells (Prabhu et al. 2005). As previously reported, viral translation and replication signals overlap (Friebe et al. 2001; Fricke et al. 2015; Dutkiewicz and Ciesiolka 2005), we were successful to show that inhibiting viral replication due to viral mRNA degradation by specific siRNA could also silence viral protein expression. HCV C, E1, and E2 expression were shown to efficiently decrease at 72 hrs post-transfection in Huh-7 cells transfected with siRNA Usi-169. Our findings comply with the previous studies which showed that siRNA induced

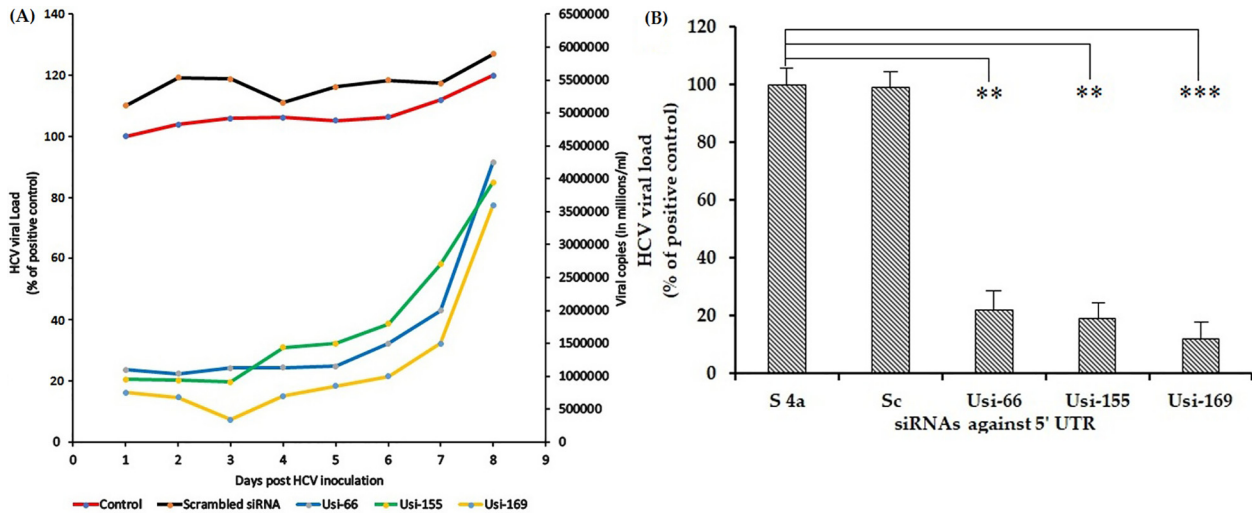


Fig. 5. HCV 5'UTR-IRES specific siRNAs downregulate viral replication in an *in-vitro* HCV replication model. (A) Relative HCV viral load in siRNA transfected Huh-7 cells percent to control cells was quantified by real-time PCR with 5' UTR specific primers from day 1 to day 8 siRNA post-transfection. Viral copies numbers in control cells, control siRNA, and HCV-IRES targeted siRNAs transfected cells from day 1 to day 8 are also shown on the secondary axis to maintain persistent and reproducible virus replication at the time of conducting trials. In each experiment, three triplicate samples were used. Straight lines with markers in scatter chart show the values of the determinant as mean \pm S.D. (B) Relative HCV viral load in siRNA transfected Huh-7 cells at day 3 compared with positive control Huh-7 cells inoculated with 4a serum. Each trial was performed with triplicate samples. Graph bars indicate determinant values as mean \pm S.D and error bars indicate mean \pm S.E.M. Unpaired t-tests were performed to compare the relative decrease of HCV viral load in siRNA transfected Huh-7 cells percent to the positive control (i.e. HCV 4a sera inoculated Huh-7 cells). *P*-values were considered statistically significant where ***p* < 0.01, and ****p* < 0.001 respectively. Control siRNA (i.e. scrambled siRNA) did not impact any change in viral titer as well as in viral copy number as compared to positive control in both figures.

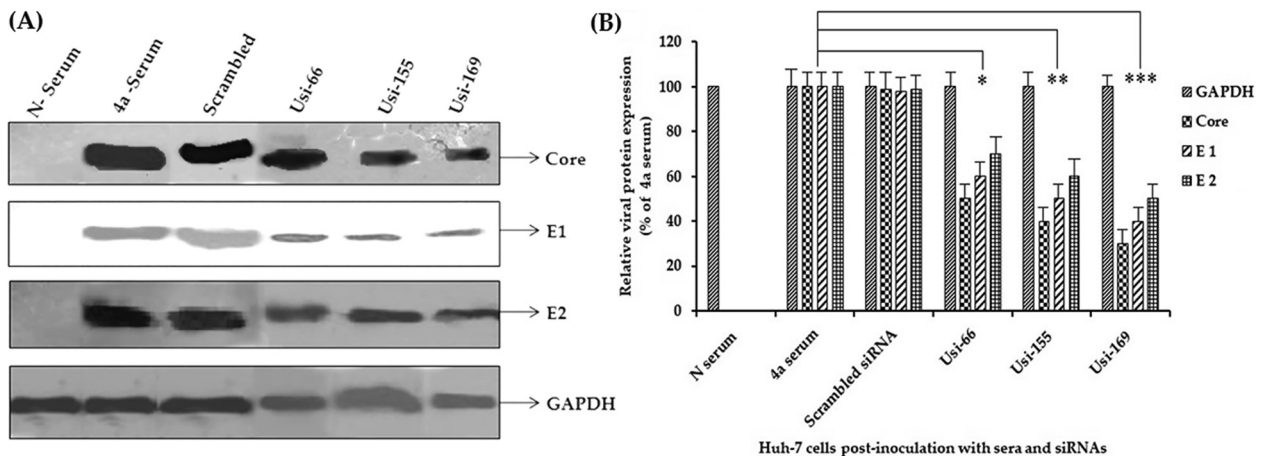


Fig. 6. siRNAs inhibit core (C), E1, and E2 protein expression in serum inoculated Huh-7 cells. (A) Western blot analysis showing the suppression of core antigen and envelope glycoproteins E1 and E2 expression from 4a serum inoculated Huh-7 cells transfected with HCV 5' UTR-IRES specific siRNAs. siRNA Usi-169 was most effective to suppress C protein and E1 and E2 expression at day 3 post-transfection. The figure shows Huh-7 cells inoculated with normal human serum (N-serum), HCV sub-genotype 4a positive serum (4a-serum), control siRNA (scrambled) transfection, and 5' UTR-IRES targeted siRNA treated cells. GAPDH protein level and expression was used as an internal control to make sure that an equal amount of proteins were present in protein extracts and lysates. (B) Quantitative measurement of Core, E1, and E2 protein expression in serum inoculated Huh-7 cells silenced by 5'UTR-IRES specific siRNAs. Densitometric analysis was performed by using Image Lab Software (BioRad®, USA) and relative protein expression to the positive control (i.e. 4a-serum) was determined. Each experiment was performed with triplicate samples. The graph bar represents determinant values as mean \pm S.D and error bars indicate mean \pm S.E.M. Unpaired t-tests were performed to compare the relative decrease in C, E1, and E2 protein expression to the positive control. *P*-values were assumed statistically significant where **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 respectively.

HCV-RNA cleavage not only inhibits viral replication but also silences protein expression (Zekri et al. 2009; Yokota et al. 2003; Prabhu et al. 2005). However; due to error-prone viral replication, HCV mRNA produces mutated viral structural and non-structural proteins to escape host immune-system defense mechanisms (Zekri et al. 2007; Zekri et al. 2009). These mutations are a predisposing factor to escape siRNA attack due to the emergence of viral escape mutants in certain HCV genome proteins and also because of viral off-target effects (Wilson and Richardson 2005; Korf et al. 2007; Shin et al. 2009). It was also evident from the previous studies that the protein coding-sequences targeted by siRNAs were dif-

ferent among different HCV-GTs and even among the strain of the same GT which might escape siRNA attacks due to the emergence of viral escape mutants (Yokota et al. 2003). Furthermore, poor-fidelity and lack of proof-reading ability of HCV RNA-dependent RNA polymerase (RdRp) enzyme, these silent mutations as siRNA escape mutants can emerge quickly in protein-coding sequences (Yokota et al. 2003; Zekri et al. 2009). In this report by selecting highly conserved domains of 5' UTR-IRES for siRNA targets, we were able to hypothesize that siRNA targets chosen for HCV sub-genotype 4a replication and translation inhibition were identical among all HCV isolates of this sub-genotype (i.e 4a). In addition

to that, the structural stability of 5' UTR isolated sequences in this study because of the highest nucleotide homologies/conservation of IRES SL-II and III motifs, would not allow to emerge escape mutations during 5' UTR directed cap-independent IRES-mediated HCV mRNA translation. This would be a future perspective of this study to test the antiviral potential of identified siRNA targets in this report against viral replication and translation of other HCV-GTs and derived sub-genotypes strains. Furthermore, viral escape mutants and off-target effects can be ameliorated by using multiplex siRNA, and short hairpin RNA (shRNA) against a particular target of the HCV genome (Wilson and Richardson 2005; Khaliq et al. 2010; Shin et al. 2009). We have also been previously reported efficient silencing potential of several different combinations of siRNA targeted to non-structural proteins of HCV sub-genotype 1a clones in an *in vitro* stable Huh-7 cell culture system and against full-length viral genome replication and translation in a transiently transfected cell culture system (Shahid et al. 2017; Shahid et al. 2018). Some studies also reported effective inhibition of virus translation and replication of HCV sub-genotype 3a strains in Huh-7 cells by using shRNAs (Kim et al. 2006).

It might be possible that all siRNA directed against 5' UTR would not be equally effective. Previous studies by Yokota et al. found that siRNA 331 directed against an upstream region of the translation initiation codon (i.e. AUG) was highly efficient in contrast to siRNA82 which was designed to target SL-II nucleotide motifs of 5' UTR-IRES with almost no effect on viral genome expression (Yokota et al. 2003). In-contrast, Prabhu et al. demonstrated that siRNA74 targeted to SL-II of 5' UTR-IRES was capable to inhibit IRES-mediated viral translation of all major HCV-GTs (Prabhu et al. 2006). We also faced the same scenario as Yokota et al. for siRNA Usi-66 in this study, which was designed within SL-II nucleotide motifs of IRES and was least effective to silence viral replication and block protein expression. Albeit the inhibitory response was not so much efficient with siRNA Usi-66, nevertheless it was prominent. The probable reason for lacking siRNA affectivity for this siRNA might be due to the highly folded structure of 5' UTR, which may leave a few single-stranded gaps that siRNAs can access. Furthermore, the previous studies also elucidate that siRNA inhibits more efficiently and potentially HCV replicon expression than 5'UTR-IRES reporter vector gene expression (Yokota et al. 2003; Zekri et al. 2009). Our results are also clearly validating the same findings where RNA transcript and protein expression of 5' UTR-tagged GFP protein in constructed vectors (i.e. pCR3.1/5'UTR/GFP) were less potently suppressed (in terms of relative 5' UTR RNA expression and relative GFP positive cells in siRNA transfected Huh-7 cells as shown in Fig. 4A to 4E) than full-length HCV genome replication and protein expression (in terms of relative viral load, viral copy numbers for RNA replication and densitometric analysis of protein expression for virus translation in serum inoculated Huh-7 cell culture system as shown in Figs. 5 and 6). A lot of factors behind this less suppressive effect of siRNA on reporter vector expression have been described by previous studies (Zekri et al. 2009; Yokota et al. 2003; McCaffrey et al. 2002). Degradation of HCV-IRES motifs by siRNA could evolve serious complications of virus protein translation (Yokota et al. 2003). IRES-mediated translation of HCV is negatively regulated by the most domains of 5' UTR as described in section 1 (i.e. in particular, 5' UTR domain I and II). It means that cleavage/deletion of some nucleotides from these 5' UTR domains leads to increased IRES-mediated translation (Fricke et al. 2015; Vopalensky et al. 2018). Yokota et al. explored that targeting domain I of 5' UTR by siRNA12 led to an enhanced IRES-mediated translation by inactivating *cis*- or *trans*-acting negative regulatory elements of the IRES (Yokota et al. 2003).

Our results first time demonstrate efficient viral replication and translation inhibition of HCV sub-genotype 4a Saudi isolates by

targeting the most conserved region of the HCV genome in an *in vitro* persistent and reproducible cell culture system. Although an elegant and safe siRNA transfection method to suppress viral replication and silencing protein expression to *in vivo* cells has not been established yet, chemically synthesized siRNAs are advantageous to deliver into cells on their own (Zekri et al. 2009; Khaliq et al. 2010; Shahid et al. 2018; Shahid et al. 2017). The approval of the first siRNA based treatment strategy by the FDA has raised the hopes to sort out efficient and robust siRNA delivery methods very soon that induce long-lasting RNAi activity to get rid of traditional methods of siRNA carriage by certain transfection reagents (e.g., Lipofectamine2000[®], FuGENE6) and cationic lipid carrier (Hoy 2018). In addition to that, careful selection of target sequence for siRNA activity matters much than only to achieve maximum efficiency (i.e. siRNA Usi-169 in this study) and the most concentrated effective dose (i.e. 40 nM/L in this study) to prevent adverse events in clinical applications (Zekri et al. 2009). The data also depict that targeting optimal site within the most conserved region of the HCV genome like 5' UTR in specific HCV strains could elicit an anti-HCV response and can be evaluated as an adjuvant therapy along with PEG-IFN/RBV or IFN-free DAAs to cure HCV patients.

5. Conclusions

We demonstrate that siRNA targeted to one of the most conserved regions of the HCV genome can efficiently inhibit viral replication and silence protein expression in an *in vitro* Huh-7 cell culture system. In this study, We tested first time siRNA potent silencing impact against HCV replication and translation by targeting SL structures of highly conserved 5' UTR region of HCV sub-genotype 4a Saudi strains. siRNA Usi-169 targeted to SL-III of IRES was the most effective to silence 5' UTR RNA expression and 5' UTR-tagged GFP protein expression of 5' UTR-IRES reporter vector in transfected Huh-7 cells. Full-length viral genome replication and viral translation was efficiently and significantly inhibited by Usi-169 in a consistent, reproducible Huh-7 cell culture system maintained for 30 days post-viral-inoculation. This potent approach of RNA interference can revolutionize anti-mRNA based treatment strategies against certain dreadful contagious and infectious viral infections like HCV, ebola virus disease (EVD), Zika virus (ZIKV) fever, dengue virus (DENV) fever, and COVID-19. HCV genetic heterogeneity and diversification of viral strains in infected populations hamper the attempts to design and develop effective prophylactic and protective anti-HCV vaccines. The approval of the first siRNA based treatment of hereditary transthyretin-mediated amyloidosis which shows its therapeutic effects by acting on hepatocytes has raised the hopes to explore, design, and develop anti-mRNA based treatment strategies against HCV very soon. The disease impacts conferred by the viral infections not only affect the humans but also have a negative blow on livestock and agriculture industry. However; human health has serious concern from the last two decades because of the emergence of fatal viral outbreaks as an epidemic, endemic, and pandemic including HCV, EVD, ZIKV fever, DENV fever, and the latest COVID-19 by SARS-CoV-2. It can be predicted that due to rapid development in molecular medicine and anti-mRNA based treatment strategies, siRNA could be available as a potential therapeutic agent as an adjuvant therapy or in combination with other DAAs against HCV.

Availability of data and material

The nucleotide sequences of HCV sub-genotype 4a isolates analyzed and discussed in this study are available on GenBank, NCBI database with their accession numbers from MT240924 to

MT240929 and can be retrieved by using an online tool; <https://www.ncbi.nlm.nih.gov/nucleotide>.

Funding

This work was supported by King Abdullah City for Science and Technology (KACST-STP), Riyadh, Saudi Arabia under the project ID: 13-MED944-10 to accomplish this research project.

Declaration of Competing Interest

The authors declare no potential conflict of interest by any means of financial, institutional, and organizational help to complete this research work and further its publication.

Acknowledgments

The authors cordially grateful Dr. Sajida Hassan, Viral hepatitis program, Laboratory of Medicine, University of Washington, Seattle, WA, USA to provide the pUBC-GFP vector and Huh-7 cells to accomplish this research work.

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