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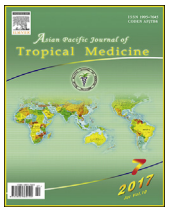

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In vitro inhibitory analysis of consensus siRNAs against *NS3* gene of hepatitis C virus 1a genotype

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ABSTRACT

Objective: To explore inhibitory effects of genome-specific, chemically synthesized siRNAs (small interference RNA) against *NS3* gene of hepatitis C virus (HCV) 1a genotype in stable Huh-7 (human hepatoma) cells as well as against viral replication in serum-inoculated Huh-7 cells.

Methods: Stable Huh-7 cells persistently expressing *NS3* gene were produced under antibiotic gentamycin (G418) selection. The cell clones resistant to 1000 μg antibiotic concentration (G418) were picked as stable cell clones. The *NS3* gene expression in stable cell clone was confirmed by RT-PCR and Western blotting. siRNA cell cytotoxicity was determined by MTT cell proliferation assay. Stable cell lines were transfected with sequence specific siRNAs and their inhibitory effects were determined by RT-PCR, real-time PCR and Western blotting. The viral replication inhibition by siRNAs in serum inoculated Huh-7 cells was determined by real-time PCR.

Results: RT-PCR and Western blot analysis confirmed *NS3* gene and protein expression in stable cell lines on day 10, 20 and 30 post transfection. MTT cell proliferation assay revealed that at most concentrated dose tested (50 nmol/L), siRNA had no cytotoxic effects on Huh-7 cells and cell proliferation remained unaffected. As demonstrated by the siRNA time-dependent inhibitory analysis, siRNA *NS3*-is44 showed maximum inhibition of *NS3* gene in stable Huh-7 cell clones at 24 (80%, $P = 0.013$) and 48 h (75%, $P = 0.002$) post transfection. The impact of siRNAs on virus replication in serum inoculated Huh-7 cells also demonstrated significant decrease in viral copy number, where siRNA *NS3*-is44 exhibited 70% ($P < 0.05$) viral RNA reduction as compared to *NS3*-is33, which showed a 64% ($P < 0.05$) decrease in viral copy number. siRNA synergism (*NS3*-is33 + *NS3*-is44) decreased viral load by 84% ($P < 0.05$) as compared to individual inhibition by each siRNA (i.e., 64%–70% ($P < 0.05$)) in serum-inoculated cells. Synthetic siRNAs mixture (*NS5B*-is88 + *NS3*-is33) targeting different region of HCV genome (*NS5B* and *NS3*) also decreased HCV viral load by 85% ($P < 0.05$) as compared to siRNA inhibitory effects alone (70% and 64% respectively, $P < 0.05$).

Conclusions: siRNAs directed against *NS3* gene significantly decreased mRNA and protein expression in stable cell clones. Viral replication was also vividly decreased in

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serum infected Huh-7 cells. Stable Huh-7 cells expressing *NS3* gene is helpful to develop anti-hepatitis C drug screening assays. siRNA therapeutic potential along with other anti-HCV agents can be considered against hepatitis C.

1. Introduction

Chronic hepatitis C virus (HCV) infection is challenging and represents a disease of significant global impact while infecting almost 170 million people around the world [1]. Chronic infection in 40%–60% of the infected individuals, if not managed properly, leads to hepatic fibrosis and cirrhosis which ultimately propagates to hepatocellular carcinoma [2]. The morbidity and mortality rates are increasing day-by-day due to persistent HCV infection and associated hepatic diseases [1]. Therapeutic efficacy of anti-HCV regimens based on pegylated interferon alpha (PEG-IFNα) and weight-based nucleoside analog ribavirin (RBV) is dismally poor in chronically infected HCV patients [3]. Therapeutic options are improving with time in the form of novel direct-acting antivirals; however, therapy cost and treatment access to low and middle-income countries and even to resource-replete nations is a potential pitfall associated with these regimens [1]. Consequently, there is a desperate need to work out cost effective, well-tolerated and less toxic alternative therapeutic regimen against hepatitis C.

HCV is a positive sense single strand RNA virus, and its genome comprises a single open reading frame which upon translation is proteolytically cleaved into four mature structural (C, E1, E2 & p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [4,5]. Among HCV nonstructural proteins, NS3 acts as a serine protease as well as an RNA helicase/NTPase during HCV polyprotein processing and virus replication [6]. The protease activity involves a "catalytic triad" (Ser-139, His-57, and Asp-81) and an oxyanion hole comprising backbone amides of Gly-137 and Ser-139 [7]. NS3 in combination with NS4A cleaves the downstream non-structural proteins at four junctions, which are very critical in establishing an HCV replication complex [8]. Besides its pivotal role in polyprotein processing, NS3 unwinds viral RNA by integrating into the HCV replication complex [9]. Some studies also predict the role of NS3 in persistent HCV infection by inhibiting the host innate immune mechanisms via blocking RIG-I (Retinoic acid-inducible gene I) and Toll-like cell signaling receptors (i.e., TRIF & Cardiff) [10]. Therefore, inhibition of NS3 protein expression may reinstate the host innate immune mechanisms against HCV replication and translation in the host cells [7,9].

Several studies have described that siRNAs can significantly inhibit virus replication and translation in Huh-7 and derived cell lines [11–16]. HCV replication in the cytoplasm of hepatocytes makes RNAi work technically better than other anti-mRNA-based therapeutics which tends to silence gene functions at the nuclear level [17]. The phenomenon can be triggered by small exogenous chemically-synthesized double-strand RNA (i.e., siRNA or short hairpin RNA (shRNA)), which target the desired mRNA in the cytoplasm of host cells [18,19]. Furthermore, natural degradation of siRNA and transient induction of targeted gene silencing without affecting host genome also minimize their side effects and cytotoxic effects as compared to IFN-α and ribavirin-based therapeutic regimens [17].

The current study was undertaken to establish a stable Huh-7 cell culture system that persistently expressed NS3 protein up to 30 d post transfection. Such a model could be helpful in understanding the molecular kinetics of infection and facilitate the development of anti-HCV drug screening assays. We also characterized the antiviral potential of siRNAs while silencing NS3 expression in stable Huh-7 cell clones. The therapeutic potential of siRNAs to block viral replication was also explored in Huh-7 cells inoculated by serum of HCV genotype 1a patients. siRNA synergism to decrease viral replication in serum inoculated Huh-7 cells while using a mixture of siRNA targeting different region of the viral genome was also demonstrated. The findings of the study reveal that the therapeutic potential of siRNA could be considered as an anti-HCV regimen either as an adjuvant or in combination therapies in future, provided that the safety and drug delivery issues related to siRNA can be resolved.

2. Materials and methods

2.1. Construction of mammalian expression vector expressing NS3 gene

The non-structural gene *NS3* was amplified by using 200 ng H/fL plasmid (expressing the full length genome of HCV 1a genotype and generously gifted by Dr. Hassan Hafeez, Hepatology and Gastroenterology Department, Fatima Memorial hospital and college of Medicine and Dentistry, Lahore, Pakistan) in a PCR reaction. PCR amplification was performed by using forward primer (5′-GCG ATA TCG CGC CCA TCA CGG CGT AC-3′ with *Eco*R V restriction enzyme sites and reverse primer (5′-AAT CTA GAT TAC GTG ACG ACC TCC AGG TC-3′) with *Xba* I restriction sites with 2× PCR master mix (Fermentas, Maryland, USA) by following the kit protocol. The PCR conditions were 94 °C for 5 min (initial denaturation), 35 cycles of 94 °C for 20 s (denaturation), 56 °C for 30 s (annealing), 72 °C for 2 min (extension) and 72 °C for 10 min as final extension. The PCR product was gel electrophoresed to confirm the desired amplified *NS3* PCR product. For *in vitro* expression of *NS3* gene in Huh-7 cells, pCR3.1/Flag-TAG mammalian expression vector expressing *NS3* gene was constructed by following the standard cloning procedures. First, the 2× double strand Flag-TAG (Flag-TAG sense; *Bam*H I-5′-GAT CCA TGG ACT ACA AGG ACG ACG ATG ACA AGG ACT ACA AGG ACG ATG ACA AGG T-3′-*Eco*R V) and Flag-TAG antisense; *Eco*R V-5′-ATC CTT GTC ATC GTC GTC CTT GTA GTC GTT GTC ATC GTC GTC CTG GTA GTC CAT G-3′-*Bam*H I) was tagged to the C-terminus of the promoter. The Flag-TAG cloning into pCR3.1 vector was confirmed by 1.0% TAE agarose gel electrophoresis, restriction digestion reaction and subsequent DNA sequencing of Flag-TAG. After that, the desired gene (*NS3*) was cloned into pCR3.1/Flag-TAG vector and confirmed by the same methods as those for Flag-TAG and

sequencing. The mammalian expression vector (pCR3.1/Flag-TAG/NS3) DNA was purified by performing maxi preparation with Qiagen plasmid purification kit (Qiagen, USA) by following the kit protocol. The purified, isolated vector DNA was transfected into Huh-7 cells for further experiments.

2.2. Huh-7 cell cultivation and transfection of pCR3.1/Flag-TAG/NS3 vector

The Huh-7 cell line (hepatocellular carcinoma cell line derived from the hepatic tissue of a 57-year-old Japanese male) was cultured in DMEM with addition of cell culture-tested 10% FBS as a supplement (Sigma–Aldrich, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin, and incubated at 37 °C and 5% CO_2. This cell line is fundamental to the study of hepatic associated diseases, establishing infectious replicon system, stable cell line production, expressing viral proteins and characterizing anti-mRNA-based therapeutics. Huh-7 cells were grown to 80% before being split. Approximately 2 × 103 and 3 × 105 cells/well were seeded into 96 and 6-well cell culture plates respectively and placed at 37 °C with 5% CO_2 for 24 h before use for vector transfection (6-well plate) or MTT (tetrazolium dye) cell proliferation assay to determine siRNA cytotoxicity (96-well plate).

After 24 h, cells grown to 80% confluency were transfected with constructed mammalian expression vector using transfection reagent lipofectamineTM 2000 (Invitrogen life technologies, CA) by following kit protocol. Prior to transfection, 1× PBS was used to wash the cells and 800 μL of fresh cell culture media added to each well. The cells were incubated at 37 °C with 5% CO_2 for 24 h after plasmid transfection. Cells transfected with pCR3.1/Flag-TAG vector only without *NS3* gene were considered as a negative control (i.e., Mock transfection).

2.3. Confirmation of NS3 gene expression in stable Huh-7 cell clones

Stable Huh-7 cells with consistent expression of *NS3* gene 30 d post transfection were produced by following the same protocol as previously reported [20]. RT-PCR and Western blotting were used to confirm NS3 expression at mRNA and protein levels, respectively.

2.3.1. RT-PCR

RNA was extracted by using TRIzol® reagent (Invitrogen Life Sciences, CA) in accordance with the kit's procedure and stored at −80 °C prior to use. RNA was quantified using spectrophotometer (Nanodrop ND-1000, Optiplex, USA) and the first-strand cDNA was prepared by following the protocol of first strand H minus cDNA synthesis kit (Fermentas, Maryland, USA). Briefly, 100–500 ng template RNA and 1 μL of oligodT primers were added to a clean, sterile microfuge tube on ice and further added DEPC-treated water to make reaction volume up to 12 μL. The reaction mixture was incubated at 65 °C for 5 min and immediately transferred to ice and added the remaining components of cDNA synthesis kit as directed. The tubes were briefly spun and incubated at 42 °C for 60 min and later for inactivation of the enzyme at 70 °C for 10 min. Sequence-specific RT-PCR primers were designed to analyze cellular gene *GAPDH* (Glyceraldehyde phosphate dehydrogenase; used as an internal control) and HCV genotype 1a *NS3* gene expression by RT-PCR. Expression of the target gene was normalized to the internal control (housekeeping genes (e.g., *GAPDH*, *β-actin*)). Expression levels of the internal control gene remained constant in all experimental and control cells under consideration. PCR amplification was performed by using 1 μL cDNA with forward primer (5′-GTA CGC CCA GCA GAC GAG-3′) and reverse primer (5′-CCT CGT GAC CAG GTA AAG GT-3′) for *NS3* gene with optimized PCR conditions (95 °C for 4 min (initial denaturation), 35 cycles of 95 °C for 1 min (denaturation), 56 °C for 45 s (annealing), 72 °C for 1 min (extension) and 72 °C for 10 min as final extension) by using 2× PCR master mix by following the kit protocol. Similarly, the RT-PCR was set using 1 μL cDNA of internal control cellular mRNA (i.e., *GAPDH*) with gene specific forward primer (5′-ACC ACA GTC CAT GCC ATC AC-3′) and reverse primer (5′-TCC ACC ACC CTG TTG CTG TA-3′) with optimized PCR conditions (95 °C for 2 min (initial denaturation), 35 cycles of 95 °C for 1 mine (denaturation), 58 °C for 50 s (annealing), 72 °C for 1 min (extension) and 72 °C for 10 min as final extension) by using *Taq* DNA polymerase (Fermentas, Maryland, USA) by following the kit protocol. The PCR amplified product was electrophoresed on 1.8% TAE agarose gel to confirm GAPDH and NS3 expression in stable cell clones.

2.3.2. Western blotting assay

A standard Western blotting protocol was used for the protein expression of internal control and NS3 in the stable cell line. Briefly, total protein was extracted from stable cell line after day 30 post transfection, and the protein concentration in each sample was measured using Pierce Coomassie (Bradford Assay®) protein assay kit (Thermo Scientific, USA). Eighty to 100 μg of total protein was electrophoresed to 12% SDS-PAGE gel electrophoresis at 100 mV for 2 h in the separate cathode (100 mmol/L Tris, 100 mmol/L Tricine, 0.1% SDS, pH 8.3) and anode (0.2 mol/L Tris, pH 8.8) running buffers. The proteins were transferred onto nitrocellulose membranes by electroblotting in transfer buffer (35 mmol/L glycine, 48 mmol/L Tris, pH 8.8 and 20% methanol) for 70 min at 16 mV. The membranes were blocked overnight in 5% skim milk at 4 °C to prevent non-specific binding of antibodies. The primary antibody staining was done by using NS3 and GAPDH monoclonal antibodies (Santa Cruz Biotechnology Inc, USA) at 1:1 000 dilution in TBS solution (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.6) containing 1% skim milk for 3–4 h at room temperature. Anti-goat anti-mouse horseradish peroxidase-conjugated antibody (Sigma–Aldrich, USA) were used as secondary antibody at 1:10 000 dilution in TBS (Tris-buffered saline) containing 1% skim milk. The proteins were detected by a chemiluminescence detection kit (Sigma–Aldrich, St. Louis, USA) in accordance with kit's procedure.

2.4. siRNA designing, transfection to stable Huh-7 cells and gene suppression analysis

The "siRNA Target Finder" (Ambion, USA), a web-based tool (http://www.thermofisher.com/sa/en/home/references/ambion-tech-support/rnai-sirna/general-articles/-sirna-design-guidelines.html) was used to determine the highly conserved sequences of *NS3* genome for siRNA designing. According to the program, siRNAs starting with AA nucleotides at the 5′ end followed by 19 nucleotides of the target gene (30%–50% GC

enriched) were selected. A leader sequence eight nucleotides in length (5′-CCT GTC TC-3′) complementary to T7 RNA polymerase promoter was added at the 5′-end of selected siRNA sequence. The siRNAs named *NS3*-is33 and *NS3*-is44 were designed in this manner (Table 1). Similarly, *NS5B* protein targeted siRNAs *NS5B*-is88 and *NS5B*-is99 (Table 1) were designed in a similar way. The designed siRNAs were chemically synthesized by using the *Silencer* siRNA construction kit (Ambion, USA) by following the kit protocol. The cellular toxicity of siRNAs was determined by colorimetric MTT cell proliferation assay by following the same protocol as reported earlier [21]. Huh-7 cells were transfected with siRNAs in a dose-dependent manner (from 10 nmol/L to 50 nmol/L) or with scrambled siRNA and DMSO as a control. After 24 h, MTT assay was conducted.

To characterize siRNA inhibitory effects, siRNAs were transfected to stable Huh-7 cell clones against *NS3* gene by using lipofectamineTM 2000 as described for transfection of mammalian expression vector in Huh-7 cells. Total cellular mRNA and protein were extracted after 24 and 48 h to analyze differential expression of *NS3* gene against sequence-specific siRNAs by RT-PCR and Western blotting.

The siRNA inhibitory effects against *NS3* gene at the transcript level was determined by using *NS3* specific RT-PCR primers, SYBR Green mix (Fermentas, Maryland, USA) following kit's protocol and qPCR (ABI 7500 Applied Biosystem, USA) at optimized PCR conditions, i.e., 50 °C for 10 min (initial hold), 95 °C for 4 min (initial denaturation), 35 cycles of 95 °C for 30 s (denaturation), 56 °C for 30 s (annealing), 72 °C for 40 s (extension) and 72 °C for 7 min as final extension. All experiments for qPCR were performed in triplicate, and for data normalization, the *GAPDH* expression was used as an internal control. Data acquisition was performed during the extension step. The quantitative siRNA inhibitory impact on *NS3* gene was studied using SDS3.1 software.

2.5. Characterization of siRNA inhibitory impact on viral replication in serum inoculated Huh-7 cells

To evaluate *NS3* sequence specific siRNA inhibitory effects in an *in vitro* viral replication system, we used HCV Real-TM Quant SC kit (Cepheid Sunnyvale, USA) and the SmartCycler® (Cepheid). For *in vitro* viral replication in Huh-7 cells, the viral inoculation procedures published earlier were used [5,11–13,22]. An informed consent was obtained from the patients whose sera were used for *in vitro* viral replication assay.

To explore the siRNA inhibitory impact on viral load, serum inoculated Huh-7 cells were seeded up to 60%–80% confluency in 24-well tissue culture plate and transfected with 50 nmol/L siRNA/well as described earlier [5,11–13,22]. Total RNA was extracted from cells after 72 h incubation with siRNAs by using TRIzol® reagent in accordance with the kit's procedure. Quantitative detection of viral load in serum inoculated cells after siRNA treatment was performed by using HCV Real-TM Quant SC kit following the kit protocol and Real-Time PCR (SmartCycler, Cepheid) according to the program outlined. The viral load in each trial was calculated by using the following equation.

$$\text{HCV load(IU/mL)} = (\text{Cy3 STD/Res/FAM STD/Res}) \times \text{Coefficient IC.}$$

where, CY3 STD = Cyanine Standart; FAM STD = 6-carboxyfluorescein Standart; IC = Internal Control.

2.6. Statistical analysis

Relative gene expression of *NS3* as compared to internal control (*GAPDH*) and siRNA gene silencing impact relative to control/scrambled siRNA was analyzed by SPSS software (version 16.0). For this purpose, the data were collected as mean ± standard deviation and analyzed for standard error and level of significance. A *P*-value less than 0.05 was considered statistically significant where applicable.

3. Results

3.1. NS3 expression at mRNA and protein levels in stable Huh-7 cell clones

The cloned *NS3* gene was transfected into Huh-7 cells by using lipofectamine and stable cell clones were generated under the selection pressure of antibiotic gentamycin (G418). Total cellular RNA and protein were extracted from Huh-7 cells on day 10, 20 and 30 post transfection to evaluate *NS3* gene and protein expression. Viral RNA was transcribed to cDNA for the RT-PCR and protein was used for Western blot analysis (Figure 1). GAPDH expression was used as an internal control both in vector transfected stable cell clones and negative control cells. The RT-PCR gel and Western blot show that there was no difference in the expression of *GAPDH* in stable Huh-7 cells expressing *NS3* gene as compared to non-transfected Huh-7 cells (Figure 1A and C). The RT-PCR product on a gel and Western blot showed a persistent and constitutive expression of *NS3* gene and protein in stable cell clones on day 10, 20, and 30 post transfection (Figure 1B and D).

3.2. NS3 gene silencing in stable Huh-7 cell clones by consensus siRNAs

The characterization of gene silencing effects of selected conserved siRNAs directed against *NS3* gene was performed in a dose-dependent manner. We used 10 nmol/L to 50 nmol/L doses of synthesized siRNAs to investigate their gene silencing

Table 1
The siRNAs used to *NS3* gene suppression analysis in stable Huh-7 cell clones and serum-inoculated Huh-7 cells.

siRNA name	siRNA sequences (5′-3′)
NS3-is33 antisense	5′-AAT GTG GAC CAA GAC CTT GTG CCT GTC TC-3′
NS3-is33 sense	5′-AAC ACA AGG TCT TGG TCC ACA CCT GTC TC-3′
NS3-is44 antisense	5′-AAT AAT TTG TGA CGA GTG CCA CCT GTC TC-3′
NS3-is44 sense	5′-AAT GGC ACT CGT CAC AAA TTA CCT GTC TC-3′
NS5B-is88 antisense	5′-AAC CAG AAT ACG ACT TGG AGC CCT GTC TC-3′
NS5B-is88 sense	5′-AAG CTC CAA GTC GTA TTC TGG CCT GTC TC-3′
NS5B-is99 antisense	5′-AAT CAT TCA AAG ACT CCA TGG CCT GTC TC-3′
NS5B-is99 sense	5′-AAC CAT GGA GTC TTT GAA TGA CCT GTC TC-3′

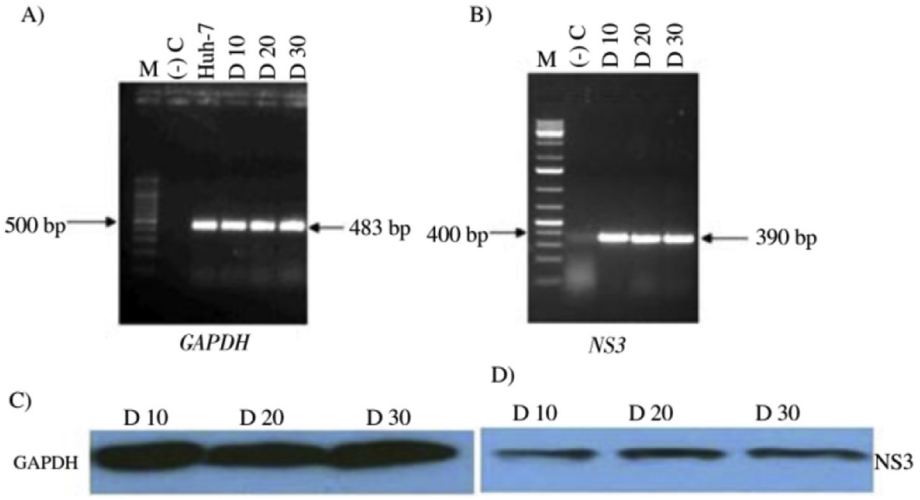

Figure 1. GAPDH and NS3 expression in stable Huh-7 cells.
A): *GAPDH* expression (used as an internal control) for the confirmation of cDNA and data normalization. The gel electrophoresis showed that there was no difference in the expression of GAPDH in mock transfected (empty vector pCR3.1/FlagTAG) Huh-7 cells and pCR3.1/FlagTAG/NS3-transfected stable cell clones. M: 100 bp DNA ladder, (−) C: negative PCR control without Huh-7 cells, Huh-7: Huh-7 cells with Mock transfection, D10, D20, and D30: stable cell clones on Day 10, 20 and 30 after transfection with pCR3.1/FlagTAG/NS3 vector. B): *NS3* mRNA expression in stable cell clones. M: 100 bp DNA ladder, (−) C: Huh-7 cells with mock transfection, D10, D 20, and D30: *NS3* gene expression on Day 10, 20 and 30 post transfection respectively. C & D): The GAPDH and NS3 protein expression on Day 10, 20 and 30 post transfection confirmed by Western blotting with GAPDH and NS3 specific antibodies.

specificity while evaluating NS3 inhibition in stable cell clones at 24 and 48 h at mRNA and protein levels by RT-PCR, qPCR, and Western blotting, respectively (Figure 2). This time-dependent siRNA inhibitory analysis was in accordance with the studies of Khaliq *et al.*, who demonstrated *in vitro* siRNA gene silencing impact against core (C) gene of HCV genotype 1a up to 3 d post transfection [11,12]. The relative percentage inhibition of *NS3* RNA in siRNA co-transfected cells at 50 nmol/L dose over

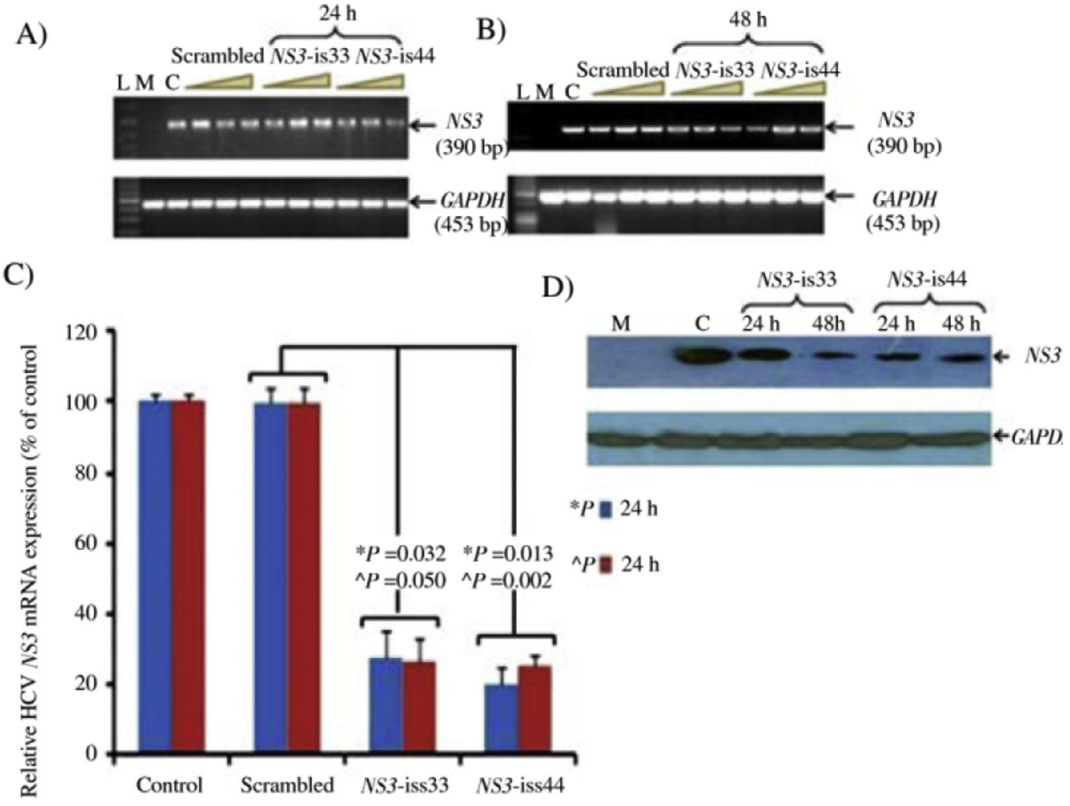

Figure 2. Gene silencing effects of siRNAs against *NS3* in stable Huh-7 cell clones.
The silencing effects of siRNA targeting *NS3* gene were analyzed by RT-PCR, Western blotting, and real-time PCR. A & B) mRNA expression of *NS3* gene at 50 nmol/L siRNAs after 24 and 48 h post transfection compared to mock (pCR3.1/FlagTAG), positive control (pCR3.1/FlagTAG/NS3) and scrambled siRNA-treated cells. All experiments were performed in triplicate. mRNA expression of *GAPDH* was used as positive control both in siRNA-treated and untreated cells. L: 100 bp DNA ladder, M: Huh-7 cells with mock transfection, C: Positive control cells. C) Quantitative real-time PCR (qPCR) showed a relative decrease in mRNA expression of *NS3* gene in stable cell clones treated with siRNAs as compared to controlled expression. Each sample was tested in triplicates. The error bars indicate mean (n = 3) ± S.D whereas $P < 0.05$ considered significantly. D) siRNA gene silencing effect at protein level as determined by Western blotting. M: Huh-7 cells with mock transfection, C: Positive control cells.

control were calculated while normalizing it with *GAPDH* in qPCR. *NS3* mRNA decreased by 72% ($P = 0.032$) in stable cells treated with siRNA *NS3*-is33 and 80% ($P = 0.013$) with *NS3*-is44 after 24 h post-transfection, while *NS3* mRNA was decreased by 73% ($P = 0.050$) with *NS3*-is33 and 75% ($P = 0.002$) with *NS3*-is44 siRNAs at 48 h post transfection. No mRNA inhibition was reported in cells treated with scrambled siRNA (Figure 2C). Thus, siRNA *NS3*-is44 showed maximum inhibition of *NS3* gene (75%–80%) in stable Huh-7 cell clones at 24 and 48 h post transfection (Figure 2C and D).

The siRNA inhibitory effects were also confirmed at the protein level using NS3-specific antibodies by Western blotting. Stable Huh-7 cells treated with siRNAs showed reduced NS3 protein expression at 24 and 48 h post transfection as compared to control expression (Figure 2D). The immunoblot results also showed relatively more reduced expression of viral NS3 protein with *NS3*-is44 siRNA than *NS3*-is33 at 24 h post transfection. However, the relative NS3 protein inhibition was almost equal at 48 h post transfection with both siRNAs (Figure 2D).

To ensure that the results observed were not due to the toxicity of siRNAs to Huh-7 cells, cell viability and cytotoxic activity of all siRNAs to be tested was determined by MTT cell proliferation assay in a dose-dependent manner (starting from 10 nmol/L up to 50 nmol/L) before transfection into stable Huh-7 cell clones. The cellular enzymes reduce MTT into its soluble formazan crystals form. Absorption of visible light at a certain wavelength from solubilized formazan crystals is directly proportional to the rate of cell proliferation, and the decrease in cell viability owing to apoptotic or necrotic metabolic events. The result of the MTT assay revealed that at the most concentrated dose tested (50 nmol/L dose/well), siRNAs had no cytotoxic effects on Huh-7 cells and cell proliferation remained unaffected (Figure 3). Huh-7 cells transfected with scrambled siRNAs and without any treatment were used as a control. The percentage cell viability was in a range of 97%–99% as compared to control (mock transfection, i.e., pCR3.1/Flag-TAG) and scrambled siRNAs treated cells (Figure 3).

3.3. Impact of siRNAs on virus replication in serum inoculated Huh-7 cells

The siRNAs specificity to silent gene expression was further speculated to be active in serum-inoculated Huh-7 cells containing replication-competent HCV, as described in many studies [11–14,23–27]. Thus, viral load was determined in siRNA-treated and serum-inoculated Huh-7 cells using sera from confirmed HCV-infected patients. A relative percentage decrease in viral load in siRNA-treated cells as compared to positive control was calculated by detecting 5′UTR (untranslated region; by using 5′UTR specific primers) of viral copy number through qPCR (Figure 4). The maximum decrease in viral load was noticed on day 3 post infection where siRNA *NS3*-is44 exhibited 70% ($P < 0.05$) viral RNA reduction as compared to *NS3*-is33, which demonstrated a 64% ($P < 0.05$) decrease in viral copy number (Figure 4A). These findings suggested that the targeting of *NS3* gene with consensus siRNAs had a significant inhibitory impact on HCV replication in Huh-7 cells infected with patient isolates. Consequently, the use of siRNAs for the downregulation of NS3 expression might be useful for the reduction of virus replication perhaps via NS3-related downstream cleavage of viral proteins.

3.4. Synergistic silencing impact of siRNAs against virus replication in serum inoculated Huh-7 cells

Several studies suggest that viral escape mutations and off-target effects often decrease the siRNA specificity to silence the expression of a particular gene *in vitro* or *in vivo* [17]. Such a caveat may be overcome by using an siRNA mixture comprising of a combination of siRNAs targeting different highly conserved regions of the viral genome [28–30]. Thus, the synergistic inhibitory potential of siRNAs when used in combination against NS3 in serum inoculated Huh-7 cells was tested by qPCR. The results showed that siRNA mixture containing *NS3* genome specific siRNAs (*NS3*-is33 + *NS3*-is44) decreased viral load by 84% ($P < 0.05$) as compared to individual inhibition of each siRNA (i.e., 64%–70% ($P < 0.05$) respectively) (Figure 4A). We also determined the inhibitory effects of a synthetic siRNAs mixture targeting a different conserved region of HCV genome (Figure 4B and C). The siRNAs directed against *NS5B* (i.e., an RNA-dependent RNA polymerase (RdRp) involved in HCV replication) and *NS3* genes (*NS5B*-is99 + *NS3*-is44) decreased HCV viral load by 82% ($P < 0.05$) as compared to siRNA inhibitory effects alone (68% & 70% respectively, $P < 0.05$) (Figure 4C). Similarly, siRNA mixture containing *NS3*-is33 and *NS5B*-is88 siRNAs also showed a marked reduction of viral load up to 85% ($P < 0.05$) than siRNA inhibitory effects alone (64% & 70% respectively, $P < 0.05$) (Figure 4B). This data suggests that the inhibitory impact of a combination siRNAs mixture could be a good

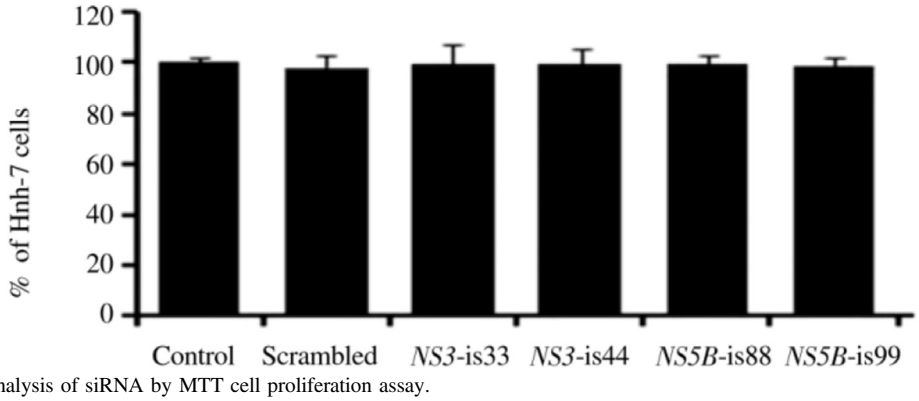

Figure 3. Cytotoxic analysis of siRNA by MTT cell proliferation assay.
Each experiment was conducted in triplicate presenting data as Mean ($n = 3$) ± standard deviation (SD) both for siRNA-treated and control cells. Huh-7 cells transfected with scrambled siRNAs and without any treatment were used as a control.

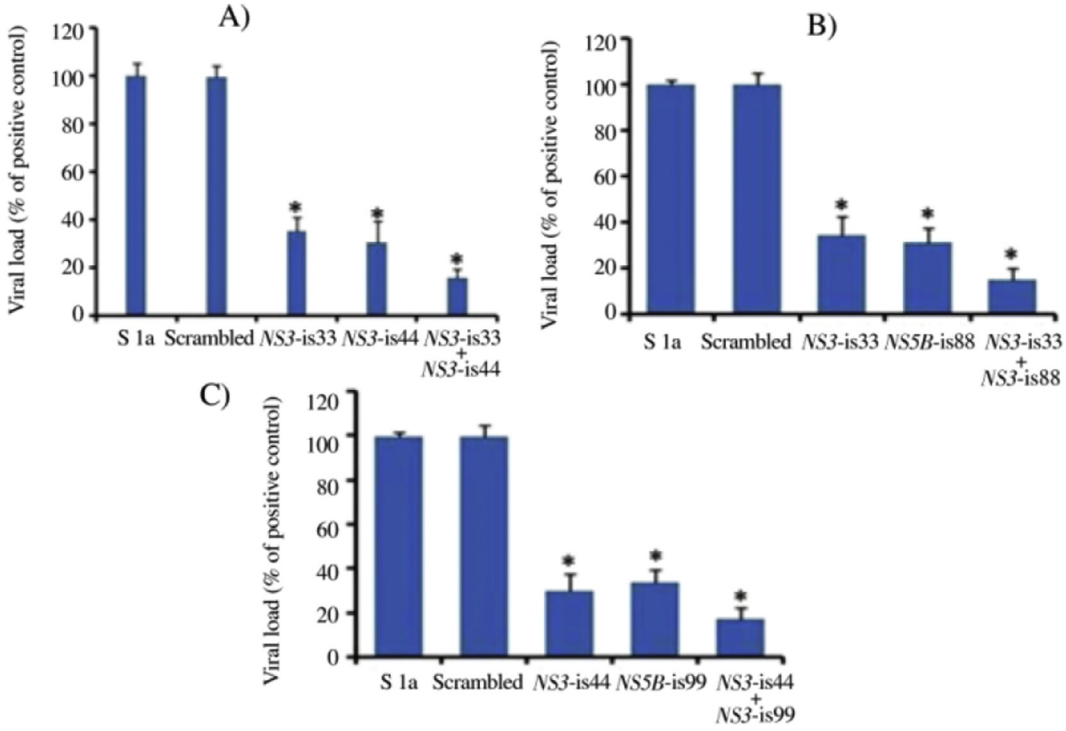

Figure 4. siRNAs inhibitory effects against viral replication in serum-inoculated Huh-7 cells.
A) qPCR analysis of viral load using 5′ UTR-specific primers. B & C) The inhibitory effects of siRNA mixtures on viral load containing different siRNAs targeting multiple regions of HCV genome. Each experiment was performed in triplicate, and the data were collected as the mean of relative percentage decrease in viral load in siRNA-treated cells versus positive control (i.e., S 1a). The error bars indicate mean ($n = 3$) ± S.D with a P value less than 0.05 being statistically significant.

strategy to decrease virus replication and minimizing the chances of viral off-target effects.

4. Discussion

In vitro stable cell culture system with persistent expression of HCV proteins may be very useful for viral pathogenesis studies at molecular levels and also to develop novel anti-HCV drugs screening assays [5,31]. Huh-7 and their derived cell lines are a standard in the study of HCV replication, development of an efficient replicon system, and for *in vitro* analysis of novel anti-HCV agents [32–34]. For example, efficient *in vitro* replication of HCV genotypes 1, 2, 3 and 4 was demonstrated in human primary hepatocytes [33–35]. Similarly, subgenomic replicons are widely used to investigate virus life cycle and HCV replication studies [34]. However, such infection systems do not produce enough viral particles for significant viral protein detection that limits the reliability of the assays [5,34]. Currently, stable cell culture systems have shown promise in studying functional genomics, recombinant proteins production and development of *in vitro* drug screening assays [20,31]. Furthermore, stable cell clones yield long-term expression of the inserted gene as compared to transient gene expression [20].

The study reported here presented the establishment of stable Huh-7 cell lines persistently expressing NS3 protein of HCV 1a genotype after 30 d post transfection and explored the gene silencing impact of siRNAs against NS3 protein. Reproducible expression of NS3 was apparent at mRNA and protein level at different time intervals (day 10, 20 and 30 respectively) post transfection by RT-PCR and Western blotting. The NS3 interaction with host cellular factors during downstream viral polyprotein processing in a stable cell line model could be used to understand *in vitro* molecular mechanisms of HCV-induced pathogenesis as well as to assay novel NS3 inhibitors for HCV treatment.

RNA interference (RNAi) has been documented as a gene silencing mechanism as well as a potential therapeutic tool for viral disease treatment and investigations of gene function studies [36–38]. Several studies have been demonstrated that HCV replication and expression of structural and non-structural genes is potentially inhibited by RNAi in replicon cell lines [23–25]. As the phenomenon is gene specific, the functional importance of targeted region and siRNA specificity to bind targeted RNA is vital for effective gene silencing [39]. In this study, our intention was to characterize the inhibitory effects of siRNAs against conserved regions of *NS3* gene in stable cell culture system and further apply their gene silencing impact on viral load in serum inoculated cells. We hypothesized that siRNA's gene suppression specificity could be used to silence *NS3* transcript at mRNA as well as protein level in a stable cell culture model. We showed that chemically synthesized siRNAs significantly decreased *NS3* mRNA and protein expression in stable Huh-7 cells. We used the unique sequence-specific siRNAs directed against the functional moieties of *NS3* gene which are conserved rather than other parts of *NS3* genome. These regions of *NS3* have not been studied earlier against siRNAs in a stable cell line model. It is also essential to design unique siRNAs by selecting a highly conserved sequence to show significant gene suppression, to minimize estimated non-specific effects and to reduce the chances of the emergence of viral escape mutations [40]. For this purpose, RNAi activity was induced in a dose-dependent manner in this study. It was similar to observations made by Liu *et al.*, and Kim *et al.*, who demonstrated siRNA inhibitory effects against the genome of HCV genotype 1a and 1b in a dose-dependent manner [15,40].

NS3 is a vital nonstructural protein which plays a pivotal role in downstream proteolytic cleavage of hepatitis C viral genome at four different junctions in association with NS4A which acts as a cofactor for NS3 protein [41,42]. NS3 contains two functional domains: one acts as a protease domain located at the N-terminal one-third region and the other functions as helicase domain located at the C-terminal two third region although its exact function is still not clear [43]. The His-57, Asp-81 and Ser-139 residues of NS3 constitute the functional domain of NS3 serine protease [7,43]. siRNA *NS3*-is33 was designed from the regions that constitute the functional residues of NS3 serine protease, while *NS3*-is44 was designed from the helicase domain of NS3 protein. Lack of siRNA cell cytotoxicity was demonstrated by MTT cell proliferation assay in a dose-dependent manner. After performing cytotoxicity analysis, siRNAs were further tested for gene suppression analysis against NS3 in stable cell clones. The results showed that the tested siRNA could functionally inhibit *NS3* mRNA and protein expression. The most effective siRNA was *NS3*-is44 (80% mRNA inhibition, ($P = 0.013$, $P = 0.002$)) which was designed from the helicase domain of NS3, indicating it as a potent site for siRNA gene silencing. mRNA inhibition was also significantly observed by siRNA *NS3*-is33 (73%, $P = 0.032$, $P = 0.050$), which was directed against NS3 protease domain. These findings are in agreement with the previously reported studies which support potent RNAi activity against HCV in Huh-7 and derived cell lines [11–16].

In vitro cell culture models have been used to demonstrate efficient viral infection and have also been used to evaluate the anti-HCV drug activity [11–13,22]. Such studies have also been described for HCV in a subgenomic replicon format which supports HCV RNA replication and protein synthesis [23–25]. Several research groups have also used HCV replication models using serum-inoculated Huh-7 cells to study HCV infection kinetics in humans [26,44]. By adopting the same strategy with slight modifications, we authenticated the specificity of *NS3* directed siRNAs against viral replication after inoculating Huh-7 cells with high viral load serum from HCV genotype 1a patients. HCV replication was demonstrated by detection of HCV 5′UTR by qPCR in Huh-7 cells from day 3 post infection. A significant decline in HCV viral load was observed in the infected cells by the *NS3*-directed siRNA which validated the gene silencing impact of siRNAs. siRNA *NS3*-is44 showed a 70% ($P < 0.05$) decrease, while *NS3*-is33 showed a 64% decrease ($P < 0.05$) in viral load, respectively. The results are comparable with the findings of Jarczak *et al.*, who described hairpin ribozymes and siRNA as efficient inhibitors of HCV replication, while inhibiting 3′UTR and 5′UTR sequences respectively [30]. The findings are also in agreement with Khaliq *et al.*, studies who demonstrated viral replication inhibition by using core (C) gene-specific synthetic and vector-based siRNAs against HCV genotype 3a full length viral particles in Huh-7 cells [11,12]. Furthermore, our results are in agreement with Zekri *et al.*, who reported HCV genotype-4 replication inhibition in serum inoculated Huh-7 cells [13].

siRNA-based single therapies may fail due to the appearance of viral escape mutations and off-target effects while targeting conserved genome sequences [17]. For such circumstances, other possible approaches have been demonstrated by targeting different regions of HCV genome by combinatorial use of several siRNAs [29]. In 2009, Shin *et al.*, demonstrated the prevention of viral escape mutations while using multiplexed siRNAs against *E2* and *NS3* genes [29]. By following the same strategy, we also evaluated the synergistic/additive inhibitory effects of siRNAs combination on viral copy number in serum-inoculated Huh-7 cells. The results showed that both siRNAs (*NS3*-is33 + *NS3*-is44) in combination against the functional domains of NS3 decreased by 85% ($P < 0.05$) the HCV viral load in serum-inoculated Huh-7 cells. Similarly, the combination of siRNAs directed against different genome sequences also revealed the additive inhibitory effects of siRNAs on HCV viral load in serum-treated cells. siRNAs combination *NS3*-is44 + *NS5B*-is99 showed a 82% ($P < 0.05$) inhibition of viral load, while the maximum inhibitory effect of 85% ($P < 0.05$) was observed with the siRNAs combination *NS3*-is33 + *NS5B*-is88 in serum-inoculated Huh-7 cells. Our results support the previous findings that a significant decrease in NS3 protein levels than core (C) protein considering that more siRNA targeted sites are located on the upstream site of the viral genome [15,44]. One possible hypothesis might be due to the possible interactions between different regions of hepatitis C viral genome [17]. Our findings showed a much more significant decrease of viral load in serum-inoculated Huh-7 cells than those reported by Khaliq *et al.*, and Seo *et al.*, who used siRNA and shRNAs to silence HCV genome in serum-inoculated Huh-7 cells [11,12,15]. However, our findings are contradictory to observations made by Randall *et al.*, Wilson *et al.*, Lisowski *et al.*, and Kapadia *et al.*, who demonstrated relatively low siRNA efficacy against viral replication [16,23,24,45]. The siRNAs combination experiments are interesting and it will be valuable to confirm these observations in a HCV replication model in future perspective studies [27,29]. Similarly, the therapeutic potential of siRNAs in combination with other promising anti-hepatitis C agents could be elucidated in future as Pan *et al.*, who demonstrated additive inhibitory potential of siRNAs when used in combination with other anti-HCV agents [27].

Huh-7 cells with stable expression of HCV *NS3* gene might be helpful to study the intricate interplay between cellular and viral factors during polyprotein processing, virus replication and developing anti-HCV drug screening assays. siRNA inhibitory effects are effective against NS3 protein expression and virus replication, demonstrating its therapeutic potential or as an adjuvant therapy along with other promising anti-HCV regimens.

Conflict of interest statement

The authors report that no financial support was received for this study from any funding agencies in the public, commercial, or not-for-profit sectors and there was no conflict of interests.

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