Molecular characterization and fluorescence analysis of HCV non-structural proteins NS3, NS3-4A and NS4A of genotype 3a

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Summary. – Hepatitis C virus (HCV) chronically infects almost 2% of world's population. Chronic infection can lead to liver failure and hepatocellular carcinoma (HCC). Approximately 10% of the Pakistani population is infected with HCV and type 3 is the most prevalent genotype with 75–90% prevalence. In this study we have developed transiently expressing cell culture based system for the expression of HCV non-structural NS3, NS3-4A and NS4A proteins of genotype 3a. HCV non-structural genes NS3, NS3-4A and NS4A were cloned in to pFLAG-CMV2 and pEGFP-C1vectors. All vectors were transfected separately to Huh-7 cells and their protein expression was analyzed by Western blot and immunofluorescence. All proteins were expressed correctly and in the transfection we have obtained 42–70% efficiency for all clones. This system can be used for the development of novel antiviral strategies to inhibit the viral replication, to study apoptosis pathways induced by HCV, for the evaluation of vaccine candidates and also to study the role of HCV different signaling pathways.

Keywords: HCV; non-structural proteins NS3; NS3-4A, NS4A; Huh-7; in vitro model; genotype 3a

Introduction

Hepatitis C virus (HCV) infection is the major cause of viral hepatitis, steatosis, cirrhosis and liver cancer (Moradpour *et al.*, 2007). Before 1989, HCV has been referred to as "non A, non B hepatitis" and after about 25 years from its identification, World Health Organization (WHO) declared that 3% of world's population is suffering from this deadly virus (Scheel and Rice, 2013). Each year about 3 to 4 million new cases of HCV occurred. In Pakistan, approximately 10% of the population is infected chronically with HCV (Idrees and Riazuddin, 2008).

Complete vaccine protection of HCV and its therapeutic possibilities (Idrees and Riazuddin, 2008) are quite restricted

(Williams, 2006). The only therapeutics available against this deadly virus is combination of pegylated interferon α and ribavirin, which is difficult to tolerate and effective only in 50% of patients due to the lack of continuous virological response (Moradpour *et al.*, 2007; Joyce and Tyrrell, 2010). The outcome of the treatment basically depends on a variety of factors including viral disease stage, patient immune response and most importantly the genotype of the virus (El-Khattib *et al.*, 2012). Lack of cheap and efficient *in vitro* cell culturing technique, lack of animal model for study of HCV pathogenesis, molecular pathways and screening of candidate antiviral drugs are the main restrictive factors in the study of HCV (Couto and Kolykhalov, 2006; Butt *et al.*, 2011; Tariq *et al.*, 2012).

HCV (the family *Flaviviridae*, the genus *Hepacivirus*) is an enveloped virus with single stranded RNA with positive polarity and genome size of 9.6 kb (Alter *et al.*, 1992). HCV genome contains a single large open-reading frame (ORF) flanked by non-coding regions at 5' and 3' ends (Tang and Grise, 2009). Due to significant genetic heterogeneity, the

virus can be classified into six genotypes, 52 subtypes and within each HCV infected individual diverse quasi-species populations are present. Source of this variation is that HCV genome has highly error prone RNA polymerase with high mutation rate (Timm and Roggendorf, 2007). Single poly-protein encoded by HCV genome, after processing gives NS2, NS3, NS4A, NS4B, NS5A, NS5B, core, E1, E2, and p7, ten mature non-structural and structural proteins with diversity in their functions (Ivanov *et al.*, 2013).

NS3 is a 69 K multifunctional protein and is indispensable for HCV replication. This protein has protease and helicase activities which are essential replicative components of HCV (Kolykhalov *et al.*, 2000; Chevaliez and Pawlotsky, 2006; Lam and Frick, 2006). NS3 protein's N-terminal domain is an inte-gral part of NS2-NS3 proteinase, has a serine protease activity with assistance of cofactor NS4A which permits its stabiliza-tion and localization at the ER membrane (Bartenschlager *et al.*, 1995). Another important function of NS3-4A serine protease is to cleave its own NS2-NS3 junction together with all other (NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B) intersections (Brass, Moradpour *et al.*, 2006; Tang and Grise, 2009). Whereas on the other hand C-terminal domain of NS3 is part of a superfamily 2 RNA helicase/ NTPase, which unwinds RNA-RNA substrates, resolving secondary structures during RNA replication and also takes part in assembly of viral particles (Ma *et al.*, 2008; Tang and Grise, 2009).

NS4A is a 7 K, small sized multifunctional protein with 54 amino acid residues that work as an essential co-factor for the NS3 protease enzyme (Tang and Grise 2009; Joyce and Tyrrell 2010). It is a three domain protein with membrane anchor, hydrophobic N terminal domain that directs NS3-4A complex to the mitochondrial outer membrane and endo-plasmic reticulum, hydrophobic central domain involved in the activation of NS3 that works as a cofactor peptide, and its acidic C terminal domain that promotes helicase directed ATP hydrolysis at the time of RNA replication (Wolk *et al.*, 2000; Beran, Lindenbach *et al.*, 2009; Zaidi *et al.*, 2012).

As a result of high genetic mutability among all HCV genotypes they differ in their biology, transmission dynamics, persistence, disease development and sensitivity to therapeu-tics (Simmonds, 2005; Feld and Hoofnagle, 2005; Gottwein *et al.*, 2010). High prevalence of genotype 3a is reported world-wide, especially in several countries of South America and Asia. In Pakistan, type 3 is the prominent genotype with the prevalence between 75–90% (Qureshi *et al.*, 2009). As HCV *in vitro* culturing is not possible, it is very essential to discover a research technique for all major genotypes to study viral rep-lication and different pathways responsible for inducing HCV pathogenesis in host (Moriishi and Matsuura, 2007; Tariq *et al.*, 2012). Some experimental models such as robust replica-tion model of HCV genotype 2a has been implicated to study HCV pathogenesis. Complete genome and HCV 1a genotype subgenomic replicons, intragenotypic and intergenotypic

HCV genotype 1a, 1b, 2a, 2b and 3a chimeras are effective but 2a/2a chimera between J6CF and JFH-1 derived sequences are most effective, as these genotypes are most prevalent in developed countries (Kato *et al.*, 2003; Pietschmann *et al.*, 2006, 2009). In current study, we have selected non-structural proteins NS3, NS3-4A and NS4A of HCV genotype 3a strain as it is most prevalent genotype in Pakistan. In this study we have successfully cloned HCV NS3, NS3-4A and NS4A genes into pFLAG-CMV2 and pEGFP-C1 expression vectors and analyzed in florescence analysis. These expression vectors have the potential to be useful tool to study HCV replication and various pathways involved in HCV induced pathogenesis in cell culture system.

Material and Methods

Amplification and construction of recombinant vectors of NS3, NS3-4A and NS4A genes. HCV genotype 3a pS52 strain (Acc. No. GU814263) cDNA was used for the amplification of NS3, NS3-4A and NS4A genes. Primers for amplification and sequencing of recombinant clones were designed according to mammalian expres-sion vectors pFLAG-CMV2 and pEGFP-C1 (IDT-Integrated DNA Technologies, USA). Primer sequences are given in Table 1. HCV non-structural NS3, NS3-4A and NS4A genes were amplified using Platinum® Taq DNA polymerase high fidelity (Invitrogen). Ampli-fied DNA products and above mentioned particular vectors were digested with *Bam*HI and *Bgl*II restriction endonucleases (Biolabs), gel purified and ligated using T4 DNA ligase (BioLabs). Ligated vec-tors were transformed to Z-competent™ *Escherichia coli* bacterium strain (Zymo Research, USA) and positive clones were screened, firstly through restriction digestion (*Bam*HI and *Bgl*II) and then by sequencing (data not shown) (Eton Biosciences, USA).

Cells and transfection. Huh-7 cells were maintained in Dulbec-co's modified eagle medium (DMEM) supplemented with 100 μg/ml penicillin; streptomycin, 1% non-essential amino acids (MEM) and 10% fetal bovine serum (Gibco, USA) and incubated at 37°C with 5% CO_2. Cells were seeded in 60 mm plates and cultured until 40% confluence. One-two μg of constructed vectors pEGFP-C1 (non-expressing control/empty vector), pEGFP-C1/NS3, pEGFP-C1/ NS3-4A, pEGFP-C1/NS4A, pFLAG-CMV2 (non-expressing con-trol/empty vector), pFLAG-CMV2/NS3, pFLAG-CMV2/NS3-4A and pFLAG-CMV2/NS4A were separately transfected into cells with trans-LT1 transfection reagent (Mirus Bio LLC, USA). Six hr post transfection, transfection media was replaced for fresh media and cells were further incubated at 37°C with 5% CO_2.

SDS polyacrylamide gel electrophoresis and Western blotting. Trans-fected cells were collected 72 hr post transfection separately, washed twice with PBS, scrapped and collected in 1.5 ml micro-centrifuge tubes. Cells were lyzed using RIPA buffer (1 mol/l TRIS pH 7.4, 2.5 mol/l NaCl, 10% NP40, 10% DOC, 10% SDS, 10 mmol/l PMSF and proteinase inhibitor), incubated on ice for 30 min and sonicated with 6–8 pulses. After sonication, extracts were centrifuged at 12,000 rpm

Table 1. Nucleotide sequences of primers for sequencing and PCR

The sequences were designed from reported sequences of HCV genomes (NZL and pS52 of genotype 3a) available at NCBI. Sequences of the restriction enzymes used for each primer are italicized while stop codons in reverse primers are underlined.

No.	Primer ID	Primer sequence 5' to 3'	Restriction site	Product size
PCR PRIMERS				
1.	NS3/GFP F	ATCTAAAA*GATCT*GGCCGTGAGGTGTTG	*Bgl*II	NS3 (1.9 kb)
2.	NS3/FLAG F	ATCTAAAA*GATCT*CGGCCGTGAGGTGTTG	*Bgl*II	NS3-4A (2.1 kb)
3.	NS3 R	TTAATT*GGATCC*TCAGGTGGTTACTTCCAG	*Bam*HI	
4.	NS4A/GFP F	ATCTAAAA*GATCT*GGCCGTGAGGTGTTG	*Bgl*II	
5.	NS4A/FLAG F	ATCTAAAA*GATCT*CAGCACCTGGGTGTTG	*Bgl*II	NS4A (165 bp)
6.	NS4A R	TTAATT*GGATCC*TCAGCACTCCTCCATC	*Bam*HI	
SEQUENCING PRIMERS				
1.	pFLAG-CMV2 F	ATAACCCCCCGTTG		
2.	pFLAG-CMV2 R	TTAGGACAAGGCTGGTGG		
3.	pEGFP-C1 F	AGCACCCAGTCCGCCCTGAGC		
4.	pEGFP-C1 R	GAAATTTGTGATGCTATTGC		

for 20 min at 4°C and supernatants were replaced to new ice chilled 1.5 ml micro-centrifuge tube and stored at -80°C. Extracts were mixed with 5x loading buffer and boiled for 5 min at 100°C. Fifty μg of each sample was loaded to 12% SDS gel.

Protein bands were electrophoretically transferred to Hybond-C extra nitrocellulose membrane using tank blotting apparatus (BioRad). Membrane was blocked with 5% skimmed milk for 1 hr at room temperature followed by one wash with TBS-T
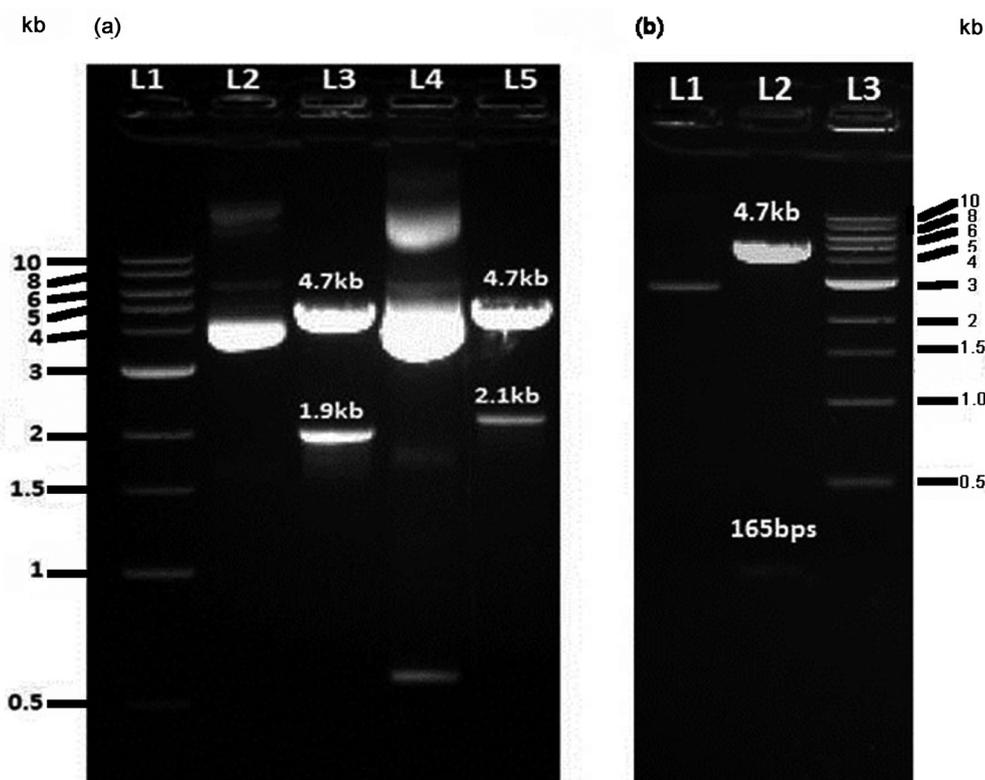
Fig. 1

Restriction digestion of recombinant pEGFP-C1 vectors

Double digestion of recombinant pEGFP-C1 vectors. (a) 1 kb ladder (lane 1), undigested recombinant vector pEGFP-C1/NS3 (lane 2), digested vector pEGFP-C1/NS3 (4.7 kb) releasing NS3 (1.9 kb) gene (lane 3), undigested recombinant vector pEGFP-C1/NS3-4A (lane 4), digested vector pEGFP-C1/NS3-4A (4.7 kb) releasing NS3-4A (2.1 kb) gene (lane 5) (b) undigested recombinant vector pEGFP-C1/NS4A (lane 1), digested vector (4.7 kb) releasing NS4A (165 bp) gene (lane 2), and 1 kb ladder (lane 3).

buffer (0.05% Tween 20). Further, membranes were incubated with appropriate primary antibody (1:1000) overnight at 4ºC. After being washed three times with TBS-T buffer membrane was incubated with particular horseradish peroxidase (HRP) labeled secondary antibody (1:10,000) at room temperature for 1 hr. After another wash with TBS-T buffer, the membranes were incubated with chemiluminescent HRP substrate for 1 min at room temperature to visualize the positive bands using Kodak image station (Digital science, 440) according to the manufacturer's instructions.

Immunofluorescence analysis of GFP-tagged NS3, NS3-4A and NS4A proteins. The cells expressing NS3, NS3-4A and NS4A proteins were grown on glass cover slips. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After fixation

cells were washed five times by PBS, mounted with ProLong® Gold antifade reagent with DAPI for nuclear staining and observed under a fluorescence microscope (Olympus).

Immunofluorescence analysis of FLAG-tagged NS3, NS3-4A and NS4A proteins. The cells expressing HCV non-structural NS3, NS3-4A and NS4A proteins were grown on glass cover slips. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were incubated with anti-FLAG mouse antibody (Cell Signaling Technology) overnight at 4ºC, followed by washing three times with PBS-T and stained with Alexa Fluor 488 anti-mouse secondary antibody (Life Technologies). The cells were washed again with PBS-T, mounted with ProLong® Gold antifade reagent with DAPI and observed under a fluorescence microscope.
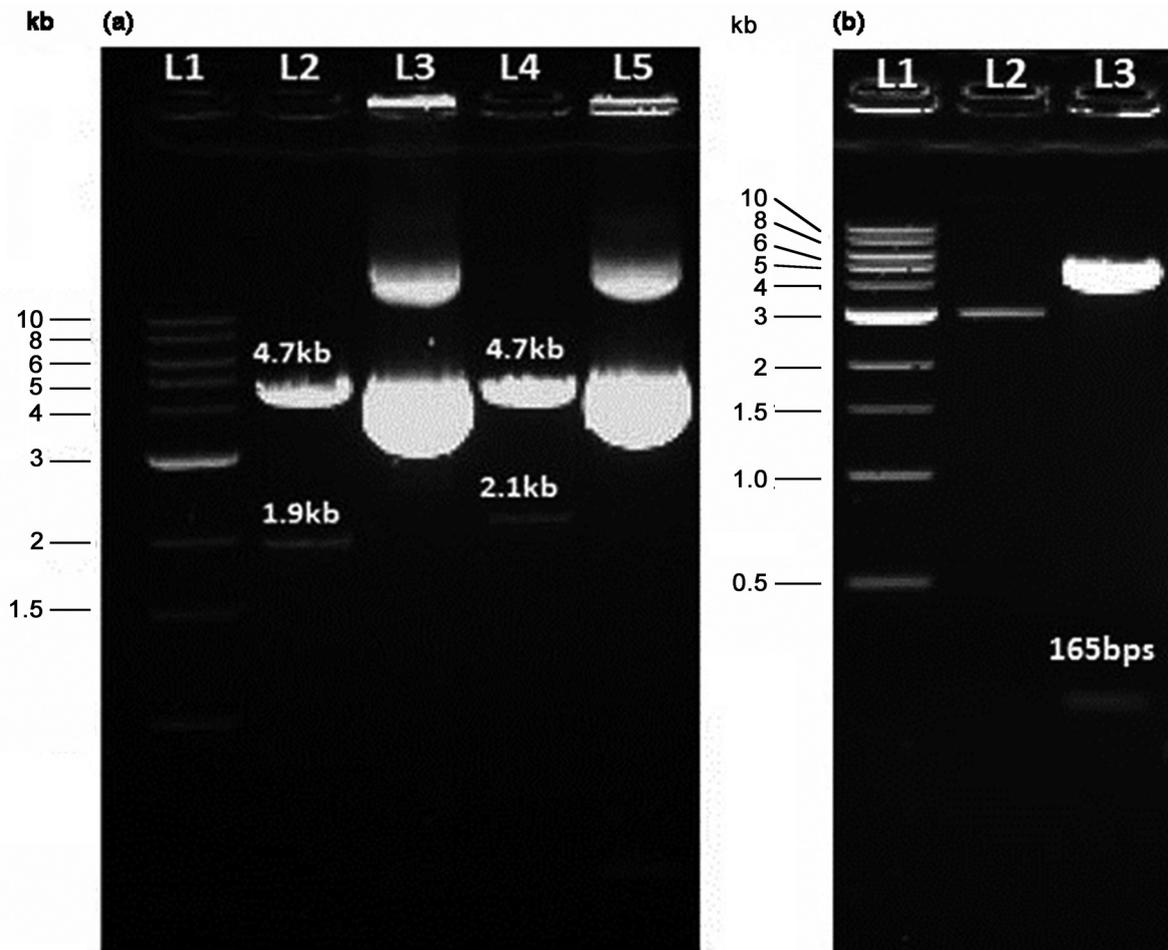
Fig. 2

Restriction digestion confirmations of recombinant pFLAG-CMV2 vectors

Double digestion of recombinant pFLAG-CMV2 vectors. (a) 1 kb ladder (lane 1), digested vector (4.7 kb, lane 2) releasing NS3 (1.9 kb) gene; undigested recombinant vector (pFLAG-CMV2/NS3, lane 3), digested vector (4.7 kb, lane 4) releasing NS3-4A (2.1 kb) gene; undigested recombinant vector (pFLAG-CMV2/NS3-4A, lane 5). (b) 1 kb ladder (lane 1), undigested recombinant vector (pFLAG-CMV2/NS4A, lane 2); digested vector (4.7 kb, lane 3) releasing NS4A (165 bp) gene.

Results

Amplification and construction of recombinant vectors of NS3, NS3-4A and NS4A genes

HCV non-structural NS3, NS3-4A and NS4A genes were successfully amplified from cDNA of HCV genotypes 3a (pS52 strain) using two different sets of primers for each gene i.e. one set of primers for pEGFP-C1 clones and other set of primers for pFLAG-CMV2 clones. Positive clones were confirmed by restriction digestion analysis. All the plasmids yielded after double digestion bands of appropriate size, pEGFP-C1/NS3 of 1.9 kb, pEGFP-C1/NS3-4A of 2.1 kb and pEGFP-C1/NS4A of 165 bp, and 4.7 kb (Fig. 1a,b),

and for pFLAG positive clones, pFLAG-CMV2/NS3 of 1.9 kb, pFLAG-CMV2/NS3-4A of 2.1 kb and pFLAG-CMV2/NS4A of 165 bp, and 4.7 kb (Fig. 2a,b). Positive clones were also confirmed by sequencing. Maps of all clones are shown in (Fig. 3a,b).

Western blot analysis of FLAG-tagged NS3, NS3-4A and NS4A proteins

Transient expression of FLAG-tagged NS3, NS3-4A and NS4A proteins was analyzed by Western blotting. Cell lysates were harvested 72 hr post transfection and were detected with anti-FLAG antibody. Specific band representing fusion complex of FLAG with NS4A protein of 7 K (NS4/FLAG,
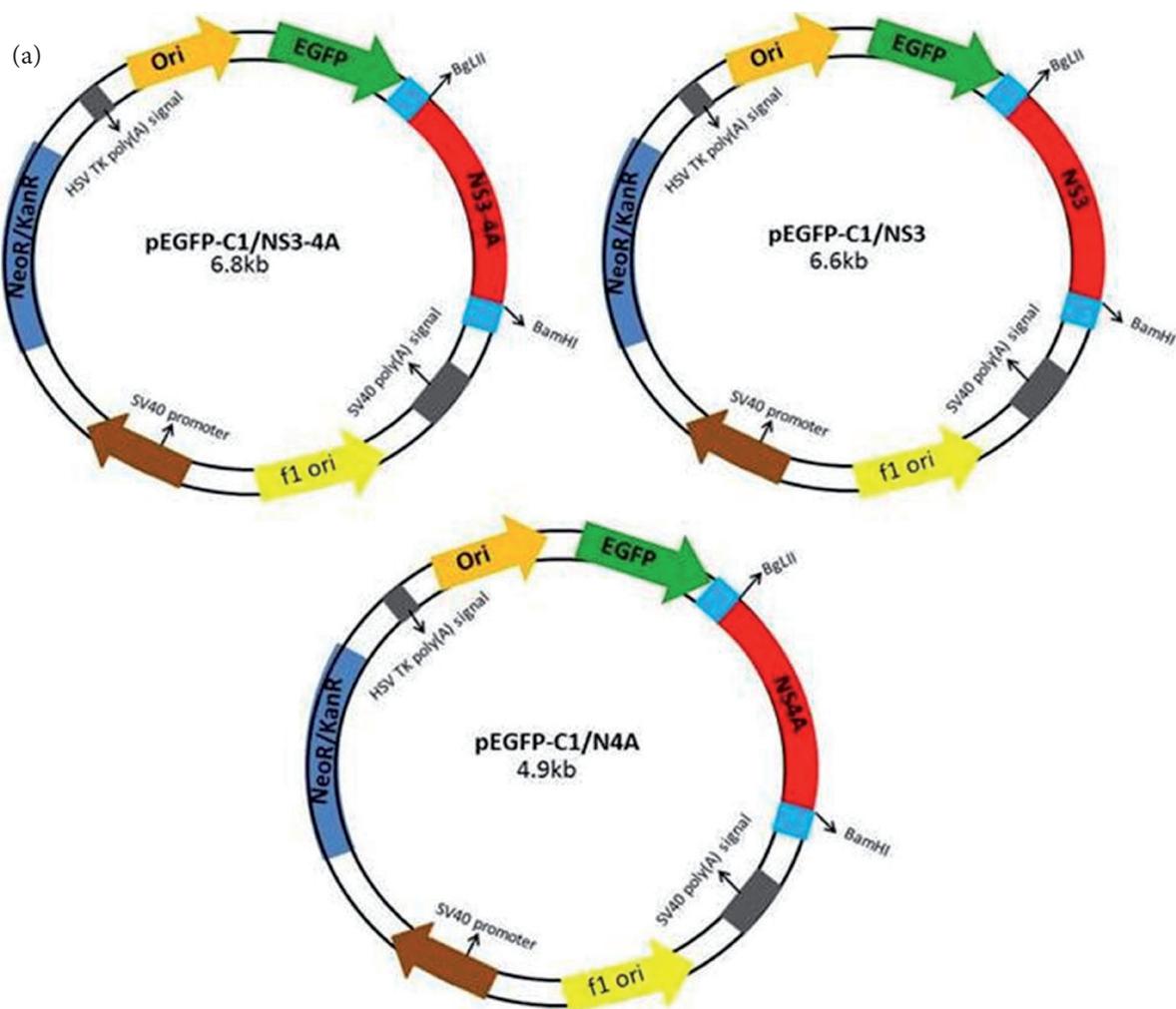
Fig. 3

Maps of recombinant vectors containing NS3, NS3-4A and NS4A genes

(b)
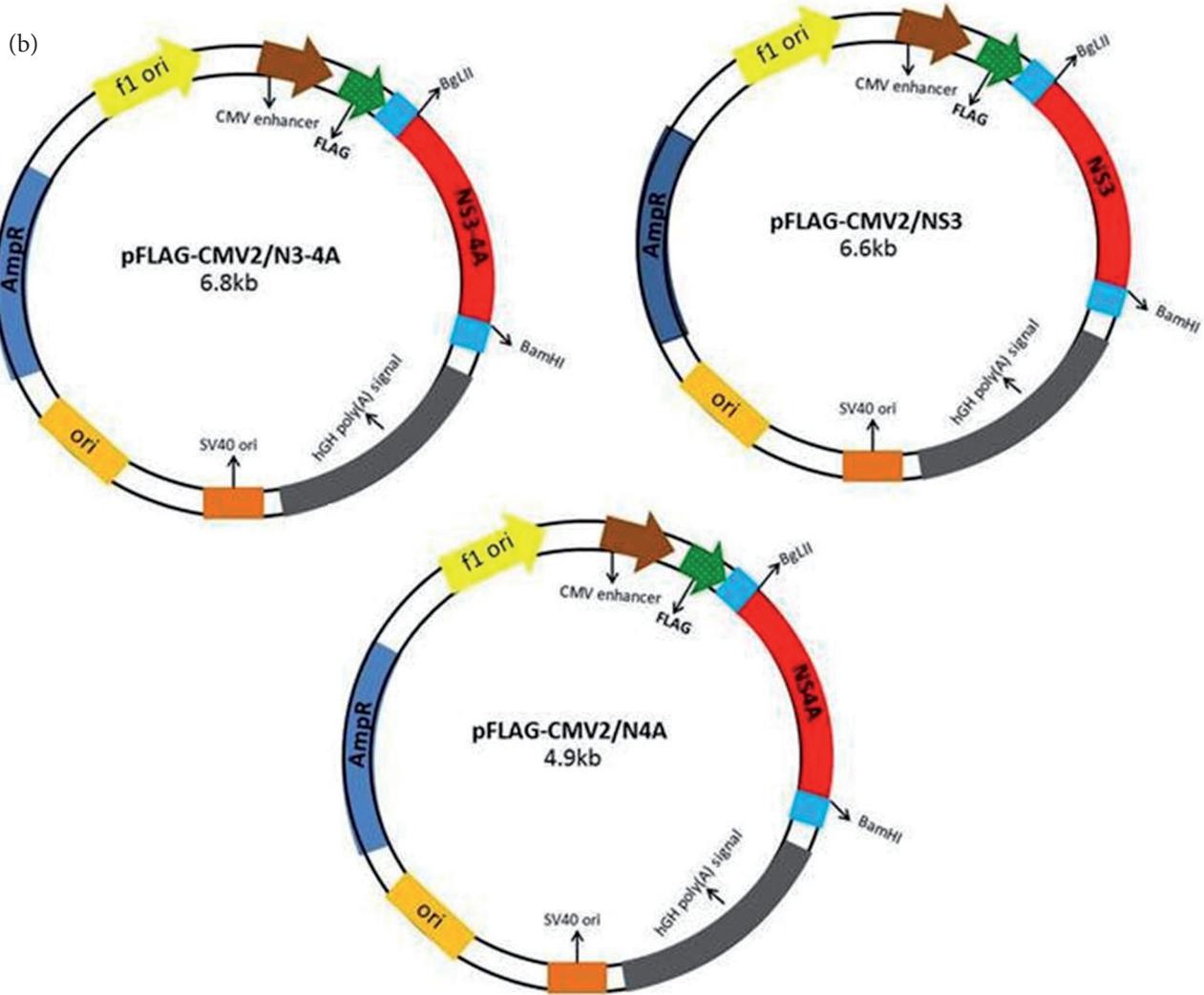
Fig. 3

Maps of recombinant vectors containing NS3, NS3-4A and NS4A genes

(a) pEGFP-C1/NS3-4A of 6.8 kb size, pEGFP-C1/NS3 of 6.6 kb and pEGFP-C1/NS4A of 4.9 kb size. (b) pFLAG-CMV2/NS3-4A of 6.8 kb size, pFLAG-CMV2/NS3 of 6.6 kb, and pFLAG-CMV2/NS4A of 4.9 kb size.

Fig. 4a lane 2), with NS3A protein of 72 K (NS3A/FLAG, Fig. 4b lane 1), and with NS3-4A protein of 72 K (NS3-4A/FLAG, Fig. 4b lane 2) were present. β-actin was used as an internal loading control.

Western blot analysis of GFP-tagged NS3, NS3-4A and NS4A proteins

Transient expression of GFP-tagged NS3, NS3-4A and NS4A proteins were analyzed by Western blotting. Cell lysates were harvested 72 hr post transfection and were detected with anti-GFP antibody. Fig. 5 shows specific band representing GFP of 27 K (Fig. 5 lane 1), fusion complex of GFP and NS4A protein of 33 K (NS4A/GFP, Fig. 5 lane 2)

with NS3 protein of 98 K (NS3/GFP, Fig. 5 lane 3), and with NS3-4A protein of 98 K (NS3-4A/GFP, Fig. 5 lane 4). β-actin was used as an internal loading control.

Immunofluorescence analysis of FLAG-tagged NS3, NS3-4A and NS4A proteins

Cells containing recombinant vectors FLAG-tagged NS3, NS3-4A and NS4A proteins were grown on glass coverslips and fixed 48 hr post transfection. Cells were incubated first with anti-FLAG mouse antibody followed with Alexa Fluor 488 anti-mouse (green) antibody and then mounted with ProLong® Gold Antifade reagent with DAPI and observed under fluorescence microscope. Green fluorescence was vis-
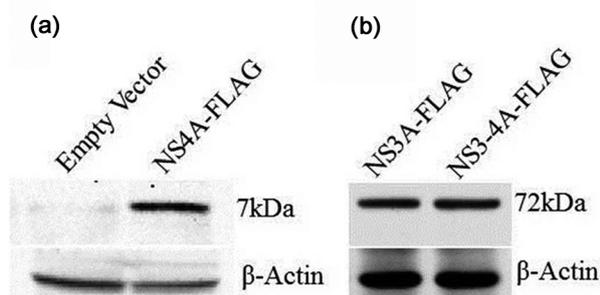
Fig. 4

Western blot analyses of FLAG-tagged NS3, NS3-4A and NS4A proteins of HCV

Western blot of (a) fusion complex of FLAG with NS4A protein of 7 K (NS4A/FLAG, lane 2). (b) fusion complex of FLAG with NS3 protein of 72 K (NS3/FLAG, lane 1) and fusion complex of FLAG with NS3-4A protein of 72 K (NS3-4A/FLAG, lane 2). β-actin was used as an internal loading control.
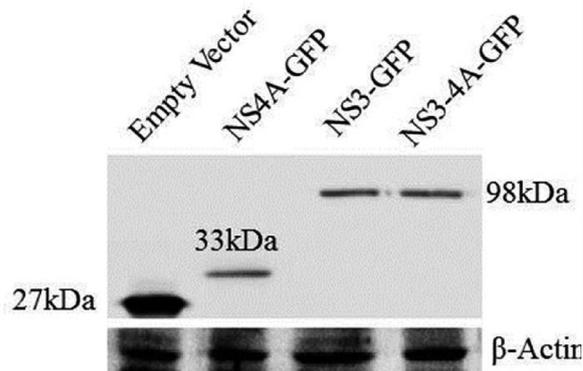
Fig. 5

Western blot analysis of GFP tagged NS3, NS3-4A and NS4A proteins of HCV

Western blot of GFP protein of 27 K (lane 1), fusion complex of GFP with NS4A protein of 33 K (NS4A/GFP, lane 2), fusion complex of GFP with NS3 of 98 K (NS3/GFP, lane 3), and fusion complex of GFP with NS3-4A protein of 98 K (NS3-4A/GFP, lane 4). β-actin was used as internal loading control.

ible in all transfected cells indicating successful transfection (Fig. 6a). Transfection efficiency was 58% for cells transfected with non-expressing control/empty vector pFLAG-CMV2, 47% for pFLAG-CMV2/NS3, 50 % for pFLAG-CMV2/NS3-4A, and 49 % for pFLAG-CMV2/NS4A (Fig. 6b).

Immunofluorescence analysis of GFP-tagged NS3, NS3-4A and NS4A proteins

Cells containing recombinant vectors GFP-tagged NS3, NS3-4A and NS4A proteins were grown on glass coverslips and fixed 48 hr post transfection, mounted with ProLong® Gold Antifade reagent with DAPI to stain the nuclei and observed under fluorescence microscope. Green fluorescence was visible in all transfected cells indicating successful transfection (Fig. 7a). Transfection efficiency was 60% for cells transfected with non-expressing control/empty vector pEGFP-C1, 43% for pEGFP-C1/NS3, 42% for pEGFP-C1/NS3-4A, and 49% for pEGFP-C1/NS4A (Fig. 7b).

Discussion

HCV uses complex mode of action to evade and disrupt the total host immune responses, which ultimately establish acute and chronic infection. These infections ultimately result in liver cirrhosis, steatosis and hepatic failure, contributing to millions of infected people per year (Racanelli and Rehermann, 2003). The asymptomatic nature of this dangerous viral infection is serious threat to Pakistani population. With the advancement of research in molecular virology study related to HCV proteomics revealed many underlying pathways which are very informative for the field of drug design and diagnostics. Due to the lack of robust HCV infected cell lines, efficient cell culture system and host-viral genes interaction are areas which need more focus and attention for new research (Sanchez-Quijano and Lissen-Otero, 2006). Concerning HCV current treatment, only about half of the HCV infected patients showed good response to the pegylated interferon and ribavirin combination therapy. Keeping in view these studies, data are clearly indicating that some other means to deal with this deadly disease are needed (Trujillo-Murillo Kdel *et al.*, 2004).

Constructions of intergenotypic and intragenotypic chimeras of strain (JFH-1) genotype 2a and HCV genotype 1b cloned genome (bicistronic replicons) are working efficiently to get the advantage of boosted replication (Blight *et al.*, 2000; Lindenbach *et al.*, 2005). In this study novel clones of HCV genotype 3a strain pS52 and of genotype 4a known as strain ED43 were constructed. However robust cell culture model for expression of individual hepatitis C virus proteins of different genotypes, especially genotype 3a are still missing. (Gottwein *et al.*, 2010) From Pakistan Butt *et al.* (2011) have only reported the construction of few mammalian expression plasmids encoding some genes of HCV genotype 3a.

In the present study we successfully amplified and cloned the full length NS3, NS3-4A and NS4A genes of HCV genotype 3a separately in pFLAG-CMV2 and pEGFP-C1 expression vectors. Positive clones were confirmed by restriction digestion analysis and then by sequencing from both directions. Each particular clone was transfected separately to Huh-7 cells and expression was analyzed by Western blot. Expression of transfected cells was also analyzed by florescence microscopy. pFLAG-CMV2 plasmid used in the present study has the ability of intracellular transient expression of N-terminal Met-FLAG which is fused with
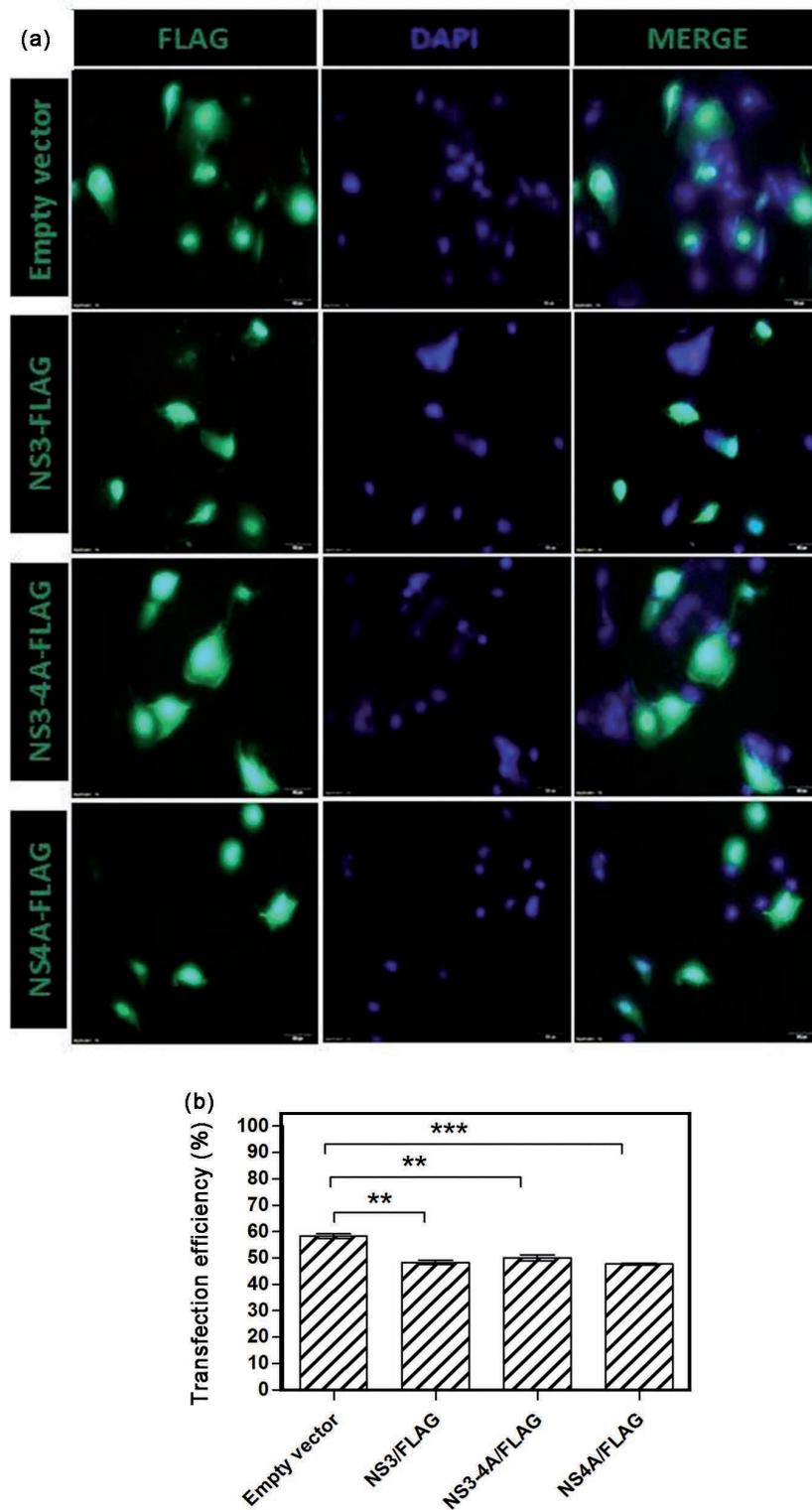
Fig. 6

Immunofluorescence analyses of FLAG-tagged NS3, NS3-4A and NS4A proteins

(a) Cells transfected with pFLAG-CMV2/NS3, NS3-4A and NS4A labeled with anti-FLAG antibody and AF488 secondary antibody (green). Nuclei were stained with DAPI (blue). (b) Transfection efficacy of pFLAG-CMV2 transfected cells in percentage. Transfection efficacy of pFLAG-CMV2 (non-expressing control/empty vector) was 58%, of pFLAG-CMV2/NS3 48%, of pFLAG-CMV2/NS3-4A 50%, and of pFLAG-CMV2/NS4A 49%.
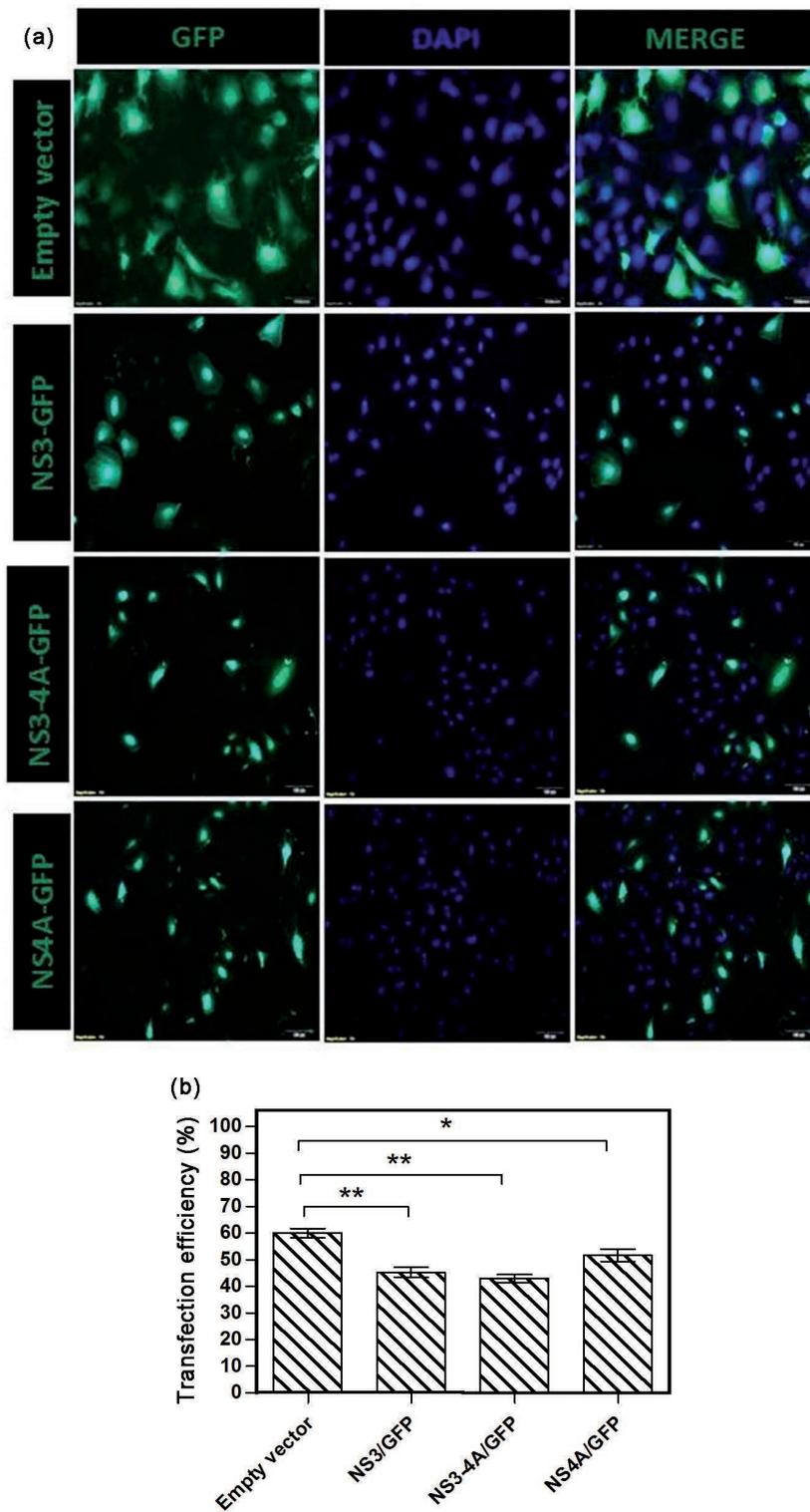
Fig. 7

Immunofluorescence analyses of GFP-tagged NS3, NS3-4A and NS4A proteins

(a) Cells transfected with pEGFP-C1/NS3, NS3-4A and NS4A (green). Nuclei were stained with DAPI (blue). (b) Transfection efficacy of pEGFP-C1 transfected cells in percentage. Transfection efficacy of pEGFP-C1 (non-expressing control/empty vector) was 59%, of pEGFP-C1/NS3 46%, of pEGFP-C1/NS3-4A 45%, and of pEGFP-C1/NS4A 55%.

desired (NS3/NS4A/NS3-4A) proteins and detected by using anti-FLAG antibody. While pEGFP-C1 expression vector has the red-shifted variant of wild-type GFP protein which is fused with desired (NS3/NS4A/NS3-4A) proteins and can easily be detect by using anti-GFP antibody.

Fluorescence and confocal microscopy is a very powerful tool for labeling and observing of different organelles and proteins within a living cell to investigate their interaction with other organelles, and to investigate the transfected proteins expression in the eukaryotic cell. Plasmids selected for the current study were selected on their ability to be used for fluorescence and confocal microscopy experiment. These efficient cell culture based systems have the ability to express individual HCV proteins NS3, NS3-4A and NS4A separately in Huh-7 cells. Beside the investigation of role of NS3, NS4A and NS3-4A proteins in the induction of mitochondrial mediated apoptosis pathway, these transiently expressing cell lines can also be used in the development of novel antiviral strategies to inhibit the replication of this noxious pathogen. These cell culture based systems can also play a dynamic role to test the novel drugs for its inhibitory action, the evaluation of vaccine candidates, characterization of humoral immune responses and also in the evaluation of different signaling pathways.

References

Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE (1992): The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. N. Engl. J. Med. 327, 1899–1905. http://dx.doi.org/10.1056/NEJM199212313272702

Bartenschlager R, Lohmann V, Wilkinson T, Koch JO (1995): Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J. Virol. 69, 7519–7528.

Beran RK, Lindenbach BD, Pyle AM (2009): The NS4A protein of hepatitis C virus promotes RNA-coupled ATP hydrolysis

by the NS3 helicase. J. Virol. 83, 3268–3275. http://dx.doi.org/10.1128/JVI.01849-08

Blight KJ, Kolykhalov AA, Rice CM (2000): Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974. http://dx.doi.org/10.1126/science.290.5498.1972

Brass V, Moradpour D, Blum HE (2006): Molecular virology of hepatitis C virus (HCV): 2006 update. Int. J. Med. Sci. 3, 29–34. http://dx.doi.org/10.7150/ijms.3.29

Butt S, Idrees M, Rehman IU, Ali L, Hussain A, Ali M, Ahmed N, Saleem S, Fayyaz M (2011): Establishment of stable Huh-7 cell lines expressing various hepatitis C virus genotype 3a protein: an in-vitro testing system for novel anti-HCV drugs. Genet. Vaccines Ther. 9, 12. http://dx.doi.org/10.1186/1479-0556-9-12

Chevaliez S, Pawlotsky JM (2006): HCV Genome and Life Cycle.

Couto LB, Kolykhalov AA (2006): Animal Models for HCV Study.

el-Khattib AA, Abdelhakam SM, Ghoraba DM, Ibrahim WA, Sayed MM (2012): Outcome of antiviral therapy in Egyptian Hepatitis C Virus (HCV) genotype 4 patients with advanced liver fibrosis. Eur. J. Intern. Med. 23, e34–35. http://dx.doi.org/10.1016/j.ejim.2011.10.003

Feld JJ, Hoofnagle JH (2005): Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 436, 967–972. http://dx.doi.org/10.1038/nature04082

Gottwein JM, Scheel TK, Callendret B, Li YP, Eccleston HB, Engle RE, Govindarajan S, Satterfield W, Purcell RH, Walker CM, Bukh J (2010): Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and in vivo pathogenesis studies. J. Virol. 84, 5277–5293. http://dx.doi.org/10.1128/JVI.02667-09

Idrees, M, Riazuddin S (2008): Frequency distribution of hepatitis C virus genotypes in different geographical regions of Pakistan and their possible routes of transmission. BMC Infect. Dis. 8, 69. http://dx.doi.org/10.1186/1471-2334-8-69

Ivanov AV, Bartosch B, Smirnova OA, Isaguliants MG, Kochetkov SN (2013): HCV and oxidative stress in the liver. Viruses 5, 439–469. http://dx.doi.org/10.3390/v5020439

Joyce MA, Tyrrell DL (2010): The cell biology of hepatitis C virus. Microbes Infect. 12, 263–271. http://dx.doi.org/10.1016/j.micinf.2009.12.012

Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T (2003): Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 1808–12517. http://dx.doi.org/10.1053/j.gastro.2003.09.023

Kolykhalov AA, Mihalik K, Feinstone SM, Rice CM (2000): Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo. J. Virol. 74, 2046–2051. http://dx.doi.org/10.1128/JVI.74.4.2046-2051.2000

Lam AM, Frick DN (2006): Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. J. Virol. 80, 404–411. http://dx.doi.org/10.1128/JVI.80.1.404-411.2006

Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating

JA, Rice CM (2005): Complete replication of hepatitis C virus in cell culture. Science 309, 623–626. http://dx.doi.org/10.1126/science.1114016

Ma Y, Yates J, Liang Y, Lemon SM, Yi M (2008): NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly. J. Virol. 82, 7624–7639. http://dx.doi.org/10.1128/JVI.00724-08

Moradpour D, Penin F, Rice CM (2007): Replication of hepatitis C virus. Nat. Rev. Microbiol. 5, 453–463. http://dx.doi.org/10.1038/nrmicro1645

Moriishi K, Matsuura Y (2007): Evaluation systems for anti-HCV drugs. Adv. Drug Deliv. Rev. 59, 1213–1221. http://dx.doi.org/10.1016/j.addr.2007.04.015

Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R (2006): Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad. Sci. USA 103, 7408–7413. http://dx.doi.org/10.1073/pnas.0504877103

Pietschmann T, Zayas M, Meuleman P, Long G, Appel N, Koutsoudakis G, Kallis S, Leroux-Roels G, Lohmann V, Bartenschlager R (2009): Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. PLoS Pathog. 5, e1000475. http://dx.doi.org/10.1371/journal.ppat.1000475

Qureshi S, Batool U, Iqbal M, Qureshi O, Kaleem R, Aziz H, Azhar M (2009): Response rates to standard interferon treatment in HCV genotype 3a. J. Ayub. Med. Coll. Abbottabad. 21, 10–14.

Racanelli V, Rehermann B (2003): Hepatitis C virus infection: when silence is deception. Trends Immunol. 24, 456–464. http://dx.doi.org/10.1016/S1471-4906(03)00178-9

Sanchez-Quijano A, Lissen-Otero E (2006): [Treatment of viral hepatitis (II). Treatment of chronic hepatitis C]. Enferm. Infect. Microbiol. Clin. 24, 518–525; quiz 526.

Scheel TK, Rice CM (2013): Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nat. Med. 19, 837–849. http://dx.doi.org/10.1038/nm.3248

Simmonds P (2004): Genetic diversity and evolution of hepatitis C virus - 15 years on. J. Gen. Virol. 85, 3173–3188. http://dx.doi.org/10.1099/vir.0.80401-0

Tang H, Grise H (2009): Cellular and molecular biology of HCV infection and hepatitis. Clin. Sci. (London) 117, 49–65. http://dx.doi.org/10.1042/CS20080631

Tariq H, Manzoor S, Parvaiz F, Javed F, Fatima K, Qadri I (2012): An overview: in vitro models of HCV replication in different cell cultures. Infect. Genet. Evol. 12, 13–20. http://dx.doi.org/10.1016/j.meegid.2011.10.009

Timm J, Roggendorf M (2007): Sequence diversity of hepatitis C virus: implications for immune control and therapy. World J. Gastroenterol. 13, 4808–4817.

Trujillo-Murillo Kdel C, Garza-Rodriguez Mdel L, Martinez-Rodriguez HG, Barrera-Saldana HA, Bosques-Padilla F, Ramos-Jimenez J, Rivas-Estilla AM (2004): Experimental models for hepatitis C virus (HCV): new opportunities for combating hepatitis C. Ann. Hepatol. 3, 54–62. http://dx.doi.org/10.1186/1476-5926-2-S1-S54

Williams R (2006): Global challenges in liver disease. Hepatology 44, 521–526. http://dx.doi.org/10.1002/hep.21347

Wolk B, Sansonno D, Krausslich HG, Dammacco F, Rice CM, Blum HE, Moradpour D (2000): Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. J. Virol. 74, 2293–2304. http://dx.doi.org/10.1128/JVI.74.5.2293-2304.2000

Zaidi N, Kanwal N, Ghazal A, Fatima K, Javed F, Qadri I (2012): Phylogenetic and structural analysis of HCV nonstructural protein 4A from Pakistani patients. Virus Genes 44, 1–7. http://dx.doi.org/10.1007/s11262-011-0672-y