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NS3-4A and NS4A proteins of hepatitis C virus regulate the apoptotic proteins expression in Huh-7 cells

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Abstract

Almost 2 % of the world population is infected with Hepatitis C virus (HCV). Prolonged HCV infection often results in the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) that is severe health problem worldwide. Aim of the current study was to check the role of HCV non-structural NS3-4A and NS4A proteins in the regulation of mitochondrial death pathway. Recombinant vectors containing HCV non-structural NS3-4A and NS4A proteins were transfected in Huh-7 cells and their transfection efficacy were observed. Further confocal microscopy was used to observe the accumulation of HCV the non-structural proteins NS3-4A and NS4A to mitochondria. Western blot analysis shown down regulated expression of Bcl-2protein which is anti-apoptotic in nature in the HCV non-structural NS3-4A and NS4A expressing Huh-7 cells. Bad protein which is basically pro-apoptotic in nature shown up regulated expression in NS3-4A and NS4A expressing Huh-7 cells. Furthermore HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells have shown the activation of executioner procaspase-3 which is the final pro-apoptotic mediator that cleavage the numerous structural and regulatory cellular proteins. In conclusion HCV non-structural NS3-4A and NS4A proteins have shown regulation of apoptotic proteins that are clear indicator of the induction of mitochondrial death pathway.

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Introduction

Hepatitis C virus (HCV) belongs to *Flaviviridae*family and causing acute and chronic infections.In acute stage HCV remain asymptomatic but continual infection establish chronic liver disease typified by inflammation, apoptosis, hepatocellular damage, fibrosis, chronic hepatitis, cirrhosis and ends on hepatocellular carcinoma (Choo*et al.*, 1989;Saito *et al.*, 1990). Worldwide 130-170 million people suffer from HCV infection and in Pakistan HCV is associated withapproximately 366000 lethality's annually (Ali *et al.*, 2009; Lavanchy, 2009).The HCV genome encodes a precursor polyproteinin of approximately 3000 amino acid into about 10 mature, different structural and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). HCV NS4A is 7 kDa non-structural 54 amino acids and playing its role as a cofactor for NS3 to enhance its enzymatic activities, such as RNA and DNA helicases (Kuang*et al.*, 2004;Pang *et al.*, 2002; Reed and Rice 2000) and serine protease (Reed and Rice 2000). NS3/4A protein is a HCV protease consists of two subunits, NS3 and NS4A. Besides playing an essential role in processing viral proteins, the NS3-4A and NS4A have their role in HCV induced cellular apoptosis (Kang *et al.*, 2003).

It is the key factor against viral infection for host cell's defense which inhibits viral particle transmission and persistency (Shen and Shenk, 1995; White, 1996). It has been proposed that HCV injuries to liver-cell are either necrotic changes or apoptotic, are mediated mainly by HCV-specific cytotoxic T lymphocytes (antiviral immune responses) although cytopathic effect induced by HCV should not be neglected. Apoptosis is biochemical cascade mechanism of multicellular organisms for cellular suicide that occurs in response to diverse stimuli (Green, 2000). This self-destruction process occur by using two pathways; mitochondria-mediated and Fas-mediated (Ashkenazi and Dixit., 1998; Gewies*et al.*, 2000). Activation of receptor mediated extrinsic apoptosis pathway carried out through assistance of caspase-8 mediated, in case of HCV infection tumour necrosis factor along with Fas-mediated induces receptor-activated apoptosis (Ruggieri*et al.*, 2007). Intrinsic or mitochondrial death pathway is initiated as a result of ROS production. It is based on the transition in membrane permeabilizationof mitochondria as a result of proapoptotic signals i.e. (activation of BAD and Bax proteins) which in turn cause the release of inter membrane space proteins (such as cyto-chrome c, apoptosis inducing factor (AIF), Endo G and Smac/DIABLO (Second mitochondria-derived activator of caspase /direct IAP binding protein with a low pI) in the cytosol and form apoptosome having ATP and APAF-1(Fischer*et al.*, 2007). Activation of Caspase-9 eventuates following the formation of apoptosome (Galle *et al.*, 1994). Caspase-9 promotes the activation of effector Caspase-3, PARP poly (ADP-ribose) polymerase and downstream apoptotic events (Owen*et al.*, 1994; Kumar, 2007). Present study was designed to investigate, the cellular and molecular effects of HCV non-structural NS3-4A protein in the induction of pro and anti-apoptotic proteins. We examinedthat HCV non-structural proteins NS3-4A in complex and NS4A aloneaccumulates on mitochondria and induce Bax-triggered, Mitochondrion-mediated, Caspase-3 dependent pathway. Our results demonstrated up regulated expression of pro-apoptotic protein BAX and down regulated expression of anti-apoptotic protein Bcl-2 in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells.

Material and methods

Reagents and antibodies

Chemical reagents and kits used in this study wereProLong® Gold Antifade Reagent with DAPI (Invitrogen), Immuno western blot chemiluminesent HRP substrate (Millipore). Primary antibodies used in this study include the following: rabbit polyclonal anti-Bad (Cell Signaling); rabbit monoclonal anti-β-actin (Cell Signaling); rabbit polyclonal anti-Bcl-2 (Cell Signaling); rabbit polyclonal anti-Procaspase 3 (Cell Signaling).

Cell culture and expression vectors

Human hepatoma cell lines Huh-7 used in this study was grown in high-glucose DMEM (Gibco)

supplemented with 10 % fetal bovine serum (Hyclone), 1 % MEM non-essential amino acids (Gibco), 100 units/ mL penicillin (Gibco) and 100 mg/ mL streptomycin (Gibco). pEGFP-C1, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A recombinant vectors were constructed by amplifying HCV non-structural NS3-4A and NS4A genes separately from Infectious cDNA clone of HCV genotypes 3a (pS52 strain), accession no GU814263 (Gottwein*et al.*, 2010). For transient expression of each HCV protein, Huh7 cells were transfected with each recombinant vector by using trans-LT1 transfection reagent (Mirus, USA).

Immunofluorescence analysis

The cells expressing HCV non-structural NS3-4A and NS4A proteins were grown on glass cover slips. After removal of medium from plates cells were fixed with 4 % paraformaldehyde for 10 mints at room temperature. After fixation cells were washed five time using PBS, mounted with ProLong® Gold Antifade Reagent with DAPI to stain nuclei and were observed under a fluorescence microscope (Olympus).

Western blot analysis

Proteins bands run by SDS-page transferred to Hybond-C extra nitrocellulose membrane electrophoretically using tank blotting apparatus (BioRAD). After being blocked with 5 % skimmed milk for 1h at room temperature followed by washing with TBS containing 0.05 % Tween 20 (TBS-T), the blots were incubated with appropriate primary antibodies (1:1000) overnight at 4 ^{o}C. After being washing three times with TBS-T blot was incubated with particular horseradish peroxidase (HRP) labeled secondary antibodies (1:10000) at room temperature for 1 hr. After being washed three times with TBS-T, the membrane were incubated with chemiluminescent HRP substrate for 1 minute at room temperature to visualized the positive bands using Kodak image station (Digital science, 440) according to the manufacturer's instructions.

Results

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

Cells containing recombinant vectors GFP-tagged NS3-4A and NS4A proteins were grown on glass coverslips and fixed 48 hours post transfection, mounted withProLong® Gold Antifade reagent with DAPI to stain the nuclei and observed under fluorescence microscope. Fig. 1 (A) depicts the transfected GFP-tagged NS3-4A and NS4A expressing cells in green, while nuclei counterstained with DAPI in blue.

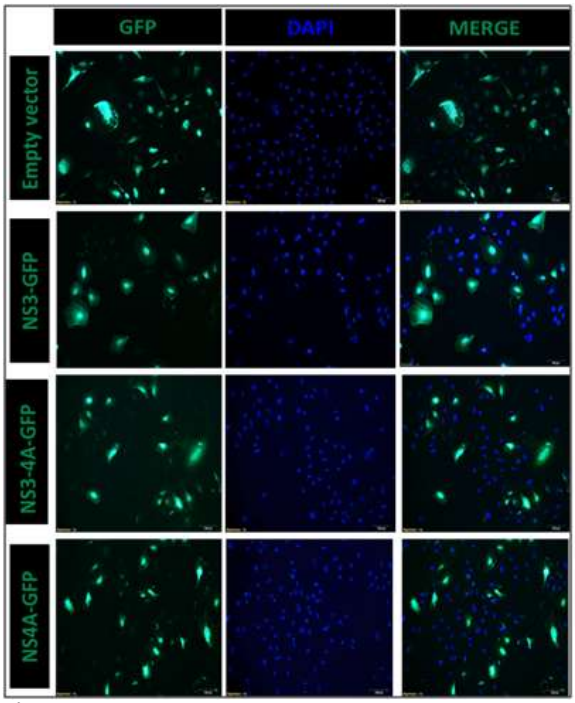

Fig. 1. Immunofluorescence analyses of GFP-tagged NS3, NS3-4A and NS4A proteins.Figure depicts transfected GFP-tagged NS3, NS3-4A and NS4A expressing cells in green, while nuclei counterstained with DAPI in blue.

BAD and Bcl-2 regulation in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells

The protein expression levels of BAD and Bcl-2 were analyzed by immunoblotting with anti-BAD and Bcl-2 antibodies. Huh-7 cells were transfected with pEGFP-C1 (non-expressing control/ empty vector), pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3-4A and NS4A proteins, Huh-7 cells treated with staurosporine served as positive control, while only Huh-7 cells serve as mock (negative control). BAD protein expression were observed up regulated in positive control, HCV non-

structural NS3-4A and NS4A proteins expressing Huh-7 cells, normal expression were observed in mock, pEGFP-C1 (non-expressing control/ empty vector) expressing Huh-7 cells (Fig.3A). In the case of Bcl-2protein expression, down regulated expression were observed in positive control, HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells, while normal expression in mock, pEGFP-C1 (non-expressing control/ empty vector) expressing Huh-7 cells (Fig.3B).

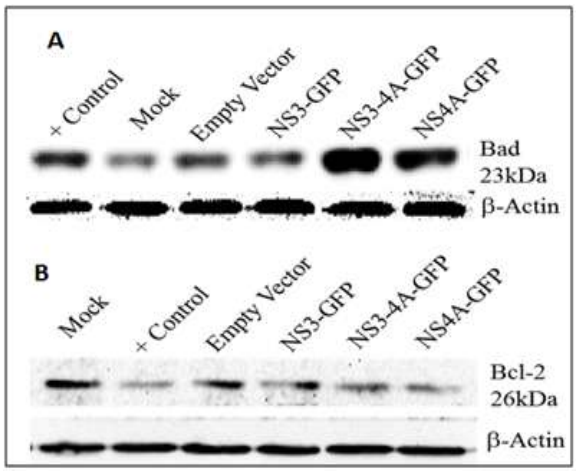

Fig. 2. (A)Western blot analysis of pro-apoptotic Bad protein. Blot was probed with anti-Bad antibody and up regulated expression was observed in positive control (Huh-7 cells treated with Staurosporine), pEGFP-C1/NS3-4A vector expressing HCV non-structural NS3-4A protein & in pEGFP-C1/NS4A vector expressing HCV non-structural NS4A protein. β-actin was used as an internal loading control. (B) Western blot analysis of anti-apoptotic Bcl-2 protein. Blot was probed with anti-Bcl-2 antibody and down regulated expression observed in positive control (huh-7 cells treated with Staurosporine), pEGFP-C1/NS3-4A vector expressing HCV non-structural NS3-4A protein & in pEGFP-C1/NS4A vector expressing HCV non-structural NS4A protein. β-actin was used as an internal loading control.

Immunoblot analysis of procaspase-3 in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells

To analyze the protein expression of procaspase-3 cells were transfected with pEGFP-C1 (non-expressing control/ empty vector), pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3-4A and NS4A proteins. Huh-7 cells treated with staurosporine served as positive control, while only Huh-7 cells serve as mock (negative control). Blot were treated with anti procaspase-3 antibody, down regulated expression of procaspase-3 were observed in positive control, HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells, while normal expression were observed in mock, pEGFP-C1 (non-expressing control/ empty vector) expressing Huh-7 cells (Fig. 4).

Discussion

HCV infection is one of the leading causes of acute and chronic hepatitis and accounts for more than 50% of adult liver transplantations in the Western world. HCV uses complex mode of action to evade and disrupt the total host immune responses. Concerning, HCV current treatment, only about half of the HCV infected patients showed good response to the pegylated interferon plus ribavirin combination therapy, despite the fact that remaining half of the HCV infected patients did not. HCV induces several complex pathways leading to generation of reactive oxygen species, hepatic inflammation, hepatic fibrosis and HCC (Pekow*et al.*, 2007; Bieche *et al.*, 2005). With the advancement of research in molecular virology study related to HCV proteomics revealed many underlying pathways involved in HCV associated pathogenesis. Fluorescence and confocal microscopy is a very powerful tool for labeling of different organelles and proteins within a living cell to investigate there interaction with other organelles, protein 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 experimentations. Hepatitis C infection in human liver as well as different experimental models is associated with increased hepatic oxidative stress (Liu *et al.*, 2003;Qadri*et al.*, 2006; Ghazani*et al.*, 2006). The previous study indicated that HCV and its certain proteins NS4A and NS4B modulate the cellular signaling pathways in such a way that these proteins accumulates on the mitochondria and ultimately disturb the mitochondrial dynamics. In the

present study we examined the regulation of proteins involved in the mitochondrial mediated apoptosis in the presence of HCV non-structural NS4A protein alone and in complex with NS3 (NS3-4A) in the Huh-7 cell lines. Huh-7 cells were transfected withparticular clone was transfected separately to Huh-7 cells and expression was analyzed using Western blot assays. Expression of transfected cells were also analyzed using florescence microscopy (Fig. 1). A pro-apoptotic protein Bax,translocates and anchors on the outer mitochondrial membrane (OMM), while Bcl-2 or Bcl-xL anti-apoptotic proteins inhibit the Bax and BAD protein to integrate in the mitochondria. Current study we have observed the up regulated expression of BAD protein and down-regulated expression of Bcl-2 protein in HCV non-structural NS3-4A and NS4A expressing Huh-7 cells (Fig. 3).

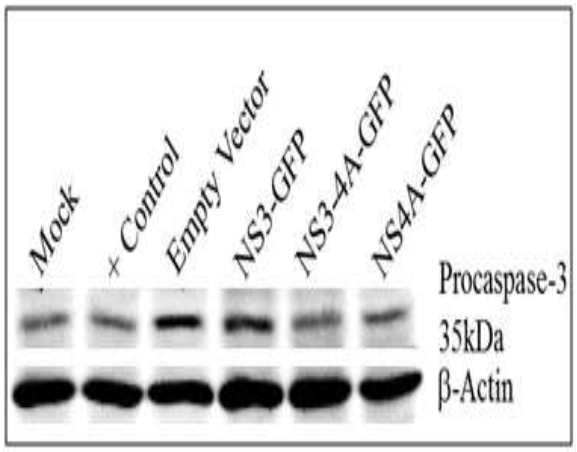

Fig. 3. Western blot analysis of Caspase-3 protein in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells.Huh-7cells were transfected with pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A containing HCV non-structural NS3, NS3-4A and NS4A proteins. Cells treated with staurosporine served as positive control, while only Huh-7 cells serve as mock. 72 hrs post transfection cells were harvested and used for western blot analysis.Blot was probed with anti procaspase-3 antibody and down regulated expression was observed only in positive control (treated with Staurosporine), pEGFP-C1/NS3-4A & pEGFP-C1/NS4A containing HCV non-structural NS3-4A and NS4A proteins expressing cells. β-actin was used as an internal loading control.

This result overall demonstrate that HCV non-structural protein NS4A alone and in complex with NS3 (NS3-4A) accumulate on mitochondria that disturb the mitochondrial dynamics and ultimately up and down regulate the expression of anti and pro-apoptotic proteins. Western blot analysis of procaspase-3 in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig. 4) demonstrate that active caspase-3 cleaved its substrate PARP into two fragments i.e. p89 and p24 leading the cell towards apoptosis by DNA fragmentation, Nuclei condensation, membrane blebbing, nuclear breakdown, apoptotic bodies etc. In conclusion current study suggests the directly involvement of HCV non-structural NS3-4A and NS4A proteins in the regulation of apoptotic proteins.

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Reference

Ali SA, Donahue RM, Qureshi H, Vermund SH. 2009. Hepatitis B and hepatitis C in Pakistan: prevalence and risk factors. International Journal of Infectious Diseases **13,** 9-19. http://dx.doi.org/10.1016/j.ijid.2008.06.019

Ashkenazi A, Dixit VM. 1998. Death receptors: signaling and modulation. Science 281, 1305-1308. http://dx.doi.org/10.1126/science.281.5381.1305

Bieche I, Asselah T, Laurendeau I, Vidaud D, Degot C. 2005. Molecular profiling of early stage

liver fibrosis in patients with chronic hepatitis C virus infection. Virology **332,** 130-144. http://dx.doi.org/10.1016/j.virol.2004.11.009

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989.Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244,** 359-362. http://dx.doi.org/10.1126/science.2523562

Fischer R, Baumert T, Blum HE. 2007. Hepatitis C virus infection and apoptosis. World Journal Gastroenterology **13,** 4865-4872.

Galle J, Herzog C, Schollmeyer P, Wanner C. 1994. Oxygen-derived radicals stimulate renin release of isolated juxtaglomerular cells. FEBS Letter **351,** 314-316. http://dx.doi.org/10.1016/0014-5793(94)00853-1

Gewies A, Rokhlin OW, Cohen MB. 2000. Cytochrome *c* is involved in Fas-mediated apoptosis of prostatic carcinoma cell lines. Cancer Res. **60,** 2163-2168.

Ghaziani T, Shan Y, Lambrecht RW, Donohue SE, Pietschmann T, Bartenschlager R, Bonkovsky HL. 2006. HCV proteins increase expression of heme oxygenase-1 (HO-1) and decrease expression of Bach1 in human hepatoma cells. Journal of Hepatology **45,** 5-12. http://dx.doi.org/10.1016/j.jhep.2005.12.020

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. Journal of Virology **84,** 5277-5293. http://dx.doi.org/10.1128/jvi.02667-09

Green DR. 2000. Apoptotic pathways. Cell, **102,** 1-4. http://dx.doi.org/10.1016/s0092-8674(00)00003-9

Kang X, ChenX, He Y, GuoD, GuoL, ZhongJ, ShuHB. 2013. DDB1 is a cellular substrate of NS3/4A protease and required for hepatitis C virus replication.Virology **435,** 385-94. http://dx.doi.org/10.1016/j.virol.2012.10.025

Kuang WF, Lin YC, Jean F, Huang YW, Tai CL, Chen DS, Chen PJ, Hwang LH. 2004. Hepatitis C virus NS3 RNA helicase activity is modulated by the two domains of NS3 and NS4A. Biochemical and Biophysical Research Communications **317,** 211-217. http://dx.doi.org/10.1016/j.bbrc.2004.03.032

Kumar S. 2007. Caspase function in programmed cell death. Cell Death and Differentiation **14,** 32-43. http://dx.doi.org/10.1038/sj.cdd.4402060

Lavanchy D. 2009. The global burden of hepatitis C. Liver International **29,** 74-81. http://dx.doi.org/10.1111/j.1478-3231.2008.01934.x

Liu C, Gaca M, Swenson ES, Vellucci VF, Reiss M, Wells RG. 2003. Smad2 and Sn-mad3 are differentially activated by transforming growth factor beta in quiscent and avctivated hepatic stellate cells in TGF- beta independent. Journal of Biological Chemistry **278,** 1172-1178. http://dx.doi.org/10.1074/jbc.m207728200

Owen RA, Molon-Noblot S, Hubert MF, Kindt MV, Keenan KP, Eydelloth RS, 1994. The morphology of juxtaglomerular cell hyperplasia and hypertrophy in normotensive rats and monkeys given an angiotensin II receptor antagonist. Toxicologic Pathology **22,** 606-19. http://dx.doi.org/10.1177/019262339402200605

Pang PS, Jankowsky E, Planet PJ, Pyle AM.2002. The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding. The EMBO Journal **21,** 1168-1176. http://dx.doi.org/10.1093/emboj/21.5.1168

Pekow JR, Bhan AK, Zheng H, Chung RT. 2007 Hepatic steatosis is associated with increased

frequency of hepatocellular carcinoma in patients with hepatitis C-related cirrhosis. Cancer **109,** 2490-2496.
http://dx.doi.org/10.1002/cncr.22701

Qadri I, IwahashiM, Kullak-Ublick GA, Simon FR. 2006. Hepatocyte Nuclear Factor (HNF) 1 and HNF4 Mediate Hepatic Multidrug Resistance Protein 2 Up-Regulation during Hepatitis C Virus Gene Expression. Molecular Pharmacology **70,** 627-636.
http://dx.doi.org/10.1124/mol.106.023499

Reed KE, Rice CM. 2000. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Current Topics in Microbiology and Immunology **242,** 55-84.
http://dx.doi.org/10.1007/978-3-642-59605-6_4

Ruggieri A, Harada T, Matsuura Y, Miyamura T. 1997. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology **229,** 68-76.
http://dx.doi.org/10.1006/viro.1996.8420

Saito T, Miyamura A, Ohbayashi H, Harada T, Katayama S, Kikuchi Y, Watanabe S, Koi M, Onji Y, Ohta. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proceedings of the National Academy of Sciences **87,** 6547-6549.
http://dx.doi.org/10.1073/pnas.87.17.6547

Shen Y, Shenk TE. 1995. Viruses and apoptosis. Current Opinion in Genetics & Development **5,** 105-111.
http://dx.doi.org/10.1016/s0959-437x(95)90061-6

White E. 1996. Life, death, and the pursuit of apoptosis.Genes & Development **10,** 1-15.
http://dx.doi.org/10.1101/gad.10.1.1