Evaluation of the cellular and molecular effects of HCV non-structural proteins on mitochondrial mediated apoptotic pathway

By

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2009-NUST-DirPhD-V&I-43

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National University of Sciences & Technology
Islamabad-Pakistan
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2009-NUST-DirPhD-V&I-43

A dissertation submitted in the partial fulfillment of the requirement for the degree of

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IN

VIROLOGY AND IMMUNOLOGY

Supervisor

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Atta-ur-Rahman School of Applied Biosciences
National University of Sciences & Technology
Islamabad-Pakistan
2016
DEDICATED
TO
MY PARENTS & MY SON
SHAHMIR SHEIKH
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Farakh Javed Shiekh
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<th>Description</th>
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<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy terminal</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetracetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde -3-phosphate dehydrogenase</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HIS</td>
<td>Hyper immune sera</td>
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<tr>
<td>HPI</td>
<td>Hours post infection</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thio-galactopyranoside</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria bertani broth</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>NS3</td>
<td>Non-structural protein 3</td>
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<tr>
<td>NS4A</td>
<td>Non-structural protein 4A</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl sulfonyl fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffer saline with Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>β-ME</td>
<td>Beta mercaptoethanol</td>
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PRESENTLY about 170 million of the world population are suffering with Hepatitis C virus (HCV) that is the major cause of liver diseases, which leads to liver fibrosis, cirrhosis and hepatocellular carcinoma. Approximately 10% of the Pakistani population is infected with HCV, while genotype 3a is the most prominent genotype with the prevalence rate between 75-90%. Genetic heterogeneity is the only main reason to compare the apoptotic pathway in different HCV genotypes. Present study illustrates that HCV non-structural proteins NS3-4A and NS4A of genotype 3a induces apoptosis by mitochondrial-mediated, caspase-3 dependent pathway as confirmed by Hoechst staining. Findings of present study reveal that NS3-4A and NS4A induces cell death in Huh-7 cells. Moreover, our results revealed that NS3-4A and NS4A was not only localized on endoplasmic reticulum but also on the mitochondria. Bax a pro-apoptotic protein translocated to the mitochondria in NS3-4A and NS4A expressing cells, while up-regulated expression of Bax and down-regulated expression of anti-apoptotic Bcl-xL protein was also observed with increased level of cytosolic cytochrome c. High level of mitochondrial superoxide generation, mitochondrial fragmentation and reduction of OXPHOS Complex I activity was also observed. In addition, protein immunoblot assays were done which showed that NS3-4A and NS4A triggers a cascade mechanism of activation starting from caspase-9, then caspase-7 and caspase-3 ends on cleavage of poly (ADP-ribose) polymerase PARP. Collectively findings of the present study suggest that HCV NS3-4A and NS4A alone may possibly induce apoptosis through Bax-triggered, mitochondrial-mediated, caspase-3 dependent pathway.
Introduction
INTRODUCTION

Hepatitis C virus (HCV) isolated in 1989, is a blood borne pathogen causing of acute and chronic liver disease worldwide. HCV infection is the main source of viral hepatitis, steatosis, cirrhosis and liver cancer (Moradpour, et al., 2007). 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 are reported. In Pakistan, approximately 10 % of the population is chronically infected with HCV out of which 85 % is of genotype 3a (Butt, et al., 2011).

Due to significant genetic heterogeneity, the virus can be classified into six genotypes, but the major ones are genotype 1-3. These genotypes are further divided into more than 50 subtypes that differ in their nucleotide sequences by 10-30 % while a nucleotide variation of 30-50 % is found among genotypes (Hoofnagle, 2002). Source of this variation is HCV genome coded its error prone RNA polymerase with high mutation rate (Timm and Roggendorf, 2007).

HCV, a member of flaviviridae family belongs to genus hepacivirus and have a single-stranded RNA genome with 9.6-kb-long size, which encodes a polyprotein of about 3,010 amino acids (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). The polyprotein is post-translationally cleaved by both viral/cellular proteases to produce about 10 polypeptides that include structural (core, E1, E2, p7) and six mature nonstructural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins with diversity in their functions (Ivanov, et al., 2013). C gene encodes the core (capsid) protein, which form the viral particle, include the core which forms the viral nucleocapsid protein and E1, E2 encodes envelope glycoproteins E1 & E2. A short
membrane peptide p7 separates both Structural and non-structural proteins from each other and putative roles of p7 include cation channel activity, productive infection, virion maturation and egress. (Penin, et al., 2004).

HCV non-structural protein NS2 is a small protein that interacts with its adjacent protein forming NS2/NS3 protease catalyzing site. Three residues (His143, Glu163 and Cys184) have been explicitly found to be involved in proteolytic activity (Grakoui et al., 1993).

69 kDa HCV non-structural protein NS3 is a multifunctional protein and is indispensable for HCV replication. NS3 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 integral part of NS2-NS3 proteinase. NS3 protein has a serine protease activity with assistance of cofactor non-structural protein NS4A permits its stabilization and localization at the endoplasmic reticulum (ER) membrane (Bartenschlager, Lohmann et al., 1995). Another important function of NS3-4A serine protease is to cleave its own NS2-NS3 junction with all other (NS3/ NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B) intersections (Brass, et al., 2006; Tang and Grise, 2009). Whereas on the other hand C-terminal domain of NS3 is a superfamily 2 RNA helicase/ NTPase, which unwinds RNA-RNA substrates, resolving secondary structures during RNA replication and also take part in assembly of viral particle (Ma, et al., 2008; Tang and Grise, 2009).

HCV nonstructural protein NS4A a 7 kDa is a small multifunctional protein with 54 amino acids residues. NS4A act as an essential co-factor for the NS3 protease enzyme (Tang and Grise 2009; Joyce and Tyrrell 2010). NS4A has three domain i.e.
membrane anchorage, hydrophobic N terminal domain directs NS3/4A complex to the mitochondrial outer membrane and endoplasmic reticulum, hydrophobic central domain involved in the activation of NS3 that works as a cofactor peptide and its acidic C terminal domain promotes helicase directed ATP hydrolysis at the time of RNA replication (Wolk, et al., 2000; Beran, et al., 2009; Zaidi, et al., 2012). HCV non-structural protein NS4b is a highly hydrophobic protein that strongly interacts with lipid moieties and thereby favors viral replication (Palomares-Jerez et al., 2012).

NS5A is a phosphorylated protein with 56 kDa (basal form) and 58 kDa (hyperphosphorylated form) molecular weight. It is located in the cytoplasm where it is found to be in association with endoplasmic reticulum via its amphipathic α-helix and induces viral replication (Brass et al., 2002; Bartenschlager and Lohmann, 2000). NS5A has a prominent role in HCV induced disease progression primarily because of interferon resistance. HCV non-structural protein NS5b is an RNA dependant RNA polymerase (RdRp) that make use of negative stranded RNA as the template favoring the synthesis of negative stranded RNA (genomic RNA). NS5b of HCV shares a common crystal structure of RdRp showing right hand with the palm, thumb and finger domains (Butcher et al., 2001).

As a result of high genetic mutability among all HCV genotypes they differ in their biology, transmission dynamics, persistence, disease development and sensitivity to therapeutics (Simmonds, 2005; Feld and Hoofnagle, 2005; Gottwein, et al., 2010). High prevalence of genotype 3a is reported worldwide, especially in several countries of South America and Asia including Pakistan. In Pakistan, type 3 is the prominent genotype with the prevalence rate 75–90 % (Qureshi, et al., 2009).
Lack of cheap and efficient \textit{in vitro} cell culturing facilities, no animal model to study HCV pathogenesis, molecular pathways and screening of candidate antiviral drugs are the main limiting factors in the study of HCV (Couto and Kolykhalov, 2006; Butt, \textit{et al}., 2011; Tariq, \textit{et al}., 2012). Although the complete mechanism of HCV associated pathogenesis is not completely understood yet although a significant digits of reports proposed a potential role of apoptosis in HCV infections (Deng, \textit{et al}., 2008; Malhi and Gores, 2008). The apoptotic process is thought to be crucial in establishing the persistent viral infections, viral clearance, antiviral immunity and hepatocellular carcinoma (Jang, \textit{et al}., 2014).

Apoptosis can be better explained as “Scheduled cell death having a sequale of events” coming after one another in a programmed and coordinated manner after any damage or diseased condition of cell. The possible outcomes of cell death as a result of apoptosis include cell deformate like shrinkage of cell size, condensation of chromatin, DNA disintegration, apoptotic body development, vacuolization of cytoplasmic and cell breakdown (Zhao, \textit{et al}., 2012). Mitochondria are very important organelles in cell performing many essential functions and play crucial role during the induction of apoptosis through increasing permeabilization of mitochondrial membrane during oxidative stress (Ivanov, \textit{et al}., 2013). This leading role of mitochondria has been categorize in three phases: Initiation phase; proapototic messengers accumulation, responsible for membrane permeabilization (MP), second decision phase; event of mitochondrial membrane permeabilization (MMP) third and last degradation phase; commencement of caspases and hydrolases activities (Mordon and Blanchemaison, 2008; Sala, \textit{et al}., 2008).

Apoptic cell death occurs through intrinsic and extrinsic pathways. Receptor mediated extrinsic pathway starts with binding of tumor necrosis factor (TNF)-\(\alpha\), \textit{Fas} ligand
and glucocorticoids to their specific receptors and leads to the activation of its subsequent messengers e.g., Caspase-8 activation (Bantel and Schulze-Osthoff, 2003; Chou, et al., 2005; Jang, et al., 2014). This pathway can results in two consequences; cell survival or cell death.

Intrinsic pathway is initiated as a result of ROS production. It is based on the transition in membrane permeabilization of mitochondria as a result of proapoptotic signals which in turn cause the release of inter membrane space proteins (such as cytochrome 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).

There are different reports suggesting apoptotic and anti-apoptotic functions of different HCV proteins, for instance NS3, NS4A, core and E2 are to induce apoptosis (Prikhod'ko, et al., 2004; Benali-Furet, et al., 2005; Nomura-Takigawa, et al., 2006; Lee, et al., 2007). Clones of HCV genotype 2a in human hepatocellular carcinoma cell line have reported to be an efficient replication system for virus and proved HCV induces apoptosis in the cell culture system (Deng, et al., 2008). In Chronic HCV infection apoptosis is related to be involved in liver damage. However, extensive mechanism is still unclear (Valva, et al., 2010). Infection with hepatitis C virus causes hepatocellular death by tempts mitochondrial mediated death signaling pathways (Deng, et al., 2008).
Many findings provide ample evidence of involvement of various structural and non-structural proteins in the induction of extrinsic as well as intrinsic apoptosis pathways. HCV non-structural protein NS4A and NS4B has been studied with reference to the induction of mitochondrial mediated apoptotic pathway. Among HCV non-structural proteins, complex of NS3-4A proteins is the most potent proteins that can damage host cellular machinery for viral and disease propagation, that can favor oxidative stress, production of reactive oxygen species with demolition of mitochondria. However, there is no conclusive study so far that describes potential role of NS3-4A protein in the induction through Bax-triggered, mitochondrial-mediated, caspase-3 dependent pathway. Therefore, the present study was designed to investigate, the cellular and molecular effects of HCV non-structural NS3-4A protein in the induction of mitochondrial death pathway.

1.2 Aims and objectives of the study

Aims and objectives of the current study are:

- Establish a cell culture based system expressing HCV non-structural proteins.
- Evaluate protein expression of different key cellular genes involved in mitochondrial mediated apoptosis in transiently expressing cell culture based system (cell line containing HCV non-structural proteins).
- Investigate the HCV induced mitochondrial mediated apoptotic pathway.
Review of Literature
Chapter 2

Review of literature

REVIEW OF LITERATURE

2.1 Anatomy and Physiology of Liver

A vital lobular organ of the human body located in upper right quadrate of abdominal cavity “Liver” consist of four lobes. Maintenance of homeostasis, energy metabolism, detoxification of toxic substances, processing, storage and distribution of dietary products like lipoproteins, purines, ketones bodies and glucose are the key roles of liver in human body (Malarkey et al., 2005). When different types of functional cells combined to one structural unit in liver then this unit is called as Acinus. Hepatocytes (liver cells) make up 70-80 % of liver volume, cells of bile duct, Kupffer cells, Endothelial cells, Oval cells, Pit cells and Hepatic stellate cells (HSC) form remaining 20-30 % of liver. Liver parenchymal cells cover the whole liver and thus have high rate of metabolic activity. Liver epithelial cells form threads and junctions between them allow the fluids and nutrients movement between neighboring cells. Endothelial cells form Hepatic sinusoids which have large endocytic activity. Mediators like Interferon, Interleukin 1, 6 and nitric oxides secreted by these endothelial sinusoids act as paracrine secretions (Kmiec, 2001; Malik, Selden, and Hodgson, 2002). Macrophages of liver i.e. Kupffer cells have immunomodulatory functions. These cells play important role in the antigen/bacterial destruction, removal of debris and
Figure 2.1: Anatomy of Liver. Liver is composed of various types of functional cells. Liver anatomy showing the location of different cells types. Major portion of liver consist of hepatocytes, rest is made up of endothelial cells. Blood cells migrate through the fenestrations of liver. Adapted from (Saladin, 2012).
dead cells from the blood stream by producing hydrolytic enzymes, hydrogen peroxide, superoxide anions and eicosanoids. Pit cells are intrahepatic leukocytes of liver believed to have Natural killer activity. Pit cells involve in cytotoxic role against viral infections and tumors (Wake, 2004).

2.2 Hepatitis

Hepatitis is a Latin word which is derived from two words Hepa means “liver” and titis means “Inflammation, so hepatitis is the term used for liver inflammation. There are many types of hepatitis like drug induced hepatitis, alcohol, toxins, chemicals, parasitic, bacterial and viral hepatitis. Viral hepatitis is a major health issue around the world, especially of many Asian countries (Lu et al., 2003). Depending on the severity of disease, hepatitis has two clinical conditions acute and chronic. Acute is condition in which disease is progressive and short term which later on convert to chronic condition while, chronic is long term and more aggressive lead to severe symptoms.

2.3 Viral hepatitis

The term viral hepatitis is coined for the viruses targeting hepatocytes (liver cells), therefore, also known as hepatotropic viruses. This group of viruses is not new rather it is as old at the beginning of mankind. It includes a diverse group of viruses named as hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), hepatitis F virus (HFV) and hepatitis G virus (HGV). After infection, they can move from acute to chronic infections. As very little is known about HFV and HGV, so hepatitis A through E will be discussed here. Each of these viruses infects and damages the liver causing the classic symptoms of jaundice with elevated level of liver
enzymes. HAV and HEV use fecal oral route for its transmission and infection mostly resolve after acute stage. HBV, HCV and HDV conversely spread via blood transfusion, sexual, vertical and parenteral, can lead to both acute and chronic infection. All this viral hepatitis can be diagnose by testing patient’s blood by serological and molecular tests (Schiff, 2007).

2.3.1 Hepatitis A virus
HAV belongs from Picornaviridae family and this virus cause acute viral hepatitis which is the most common viral hepatitis worldwide (Feinstone et al., 1975). Route of transmission for the spread of HAV is mainly by the fecal-oral route via contaminated food and water. HAV is endemic in poor and socioeconomically weak countries with lack of good hygienic food, poor sanitation, clean drinking and irrigating water resources. Transmission through injection drug user, sexual contact, blood transfusion and needle stick injury are rarely reported. Mother to fetus HAV transmission is less frequently reported (Mohebbi et al., 2012; Franco et al., 2012).

2.3.2 Hepatitis B virus
Presently about 350 million peoples around the globe are HBV carriers. It is double stranded DNA virus replicate through reverse transcription and is a member of hepadnavirus family. Mostly transmission through injection drug user, sexual contact, blood transfusion and needle stick injury and mother to fetus transmission of HBV is less primary mode of infection. Identification of Hepatitis B surface Antigens (HBsAg) of HBV can be done by immunochromatographic technique, enzyme linked immune sorbent assay (ELISA) and final confirmation of this viral infection can be by amplification of HBV DNA by polymerase chain reaction
(PCR). But sometime the viral DNA amplification from patient’s serum shows negative results but HBV still there in hepatocytes, this (false negative) dormancy condition is termed as HBV occult infection (Arababadi et al., 2012).

2.3.3 Hepatitis C virus

In 1989 HCV virus was discovered and was known as non-hepatitis A, non-hepatitis B virus (Farci et al., 2002). HCV is positive sense single stranded RNA, enveloped virus belongs to genus Hepacivirus and family Flaviviridae. Based on genotypic analysis HCV genome has been classified into six main genotypes. Based on nucleotide sequences these genotypes 30-33 % on average differs from each other and closely related subtypes which show less heterogeneity of 20-25 % in nucleotide sequences like genotypes 1a and 1b. Error-prone synthesis of RNA in HCV infected patient produces many variants of HCV known as quasispecies. HCV is a fatal blood borne pathogen targeting hepatocytes. At present nearly 160 million peoples are HCV infected and in many cases it remain asymptomatic acute infection but 50 % – 80 % of unclear infected cases progress to a persistent state of viral replication and results in hepatitis (Farci et al., 2002). HCV molecular organization, life cycle, replication and pathogenesis will be explicate below in details.

2.3.4 Hepatitis D virus

In 1977 HDV was discovered (Rizzetto et al., 1977) and termed as “delta agent”. HDV is circular single stranded RNA virus, 36-nm particle size and show gross similarities with main viruses, viral satellites and viroids of plant origin. HBV,
HCV and HDV have common routes of transmission. This is defective RNA virus can only infect persons that have HBV infection which play the role of carrier host. HBV and HDV together cause progressive chronic liver disease which could lead to cirrhosis and cancer of liver in about 80% of patients that have both HBV and HDV infection. This virus is significant cause of severe acute liver injury in many countries of the world (Kumar et al., 1992). Prevalence of HDV infection in HBsAg positive individuals are about 16.6% in Pakistani population. The major source of HDV is the poor hygienic conditions, yet there is some decline in the HDV cases in Pakistan. The study showed that these individuals infected with HBV may have either HDV super infection or co-infection of with HBV (Mumtaz, 2005).

2.3.5 Hepatitis E virus

HEV is RNA based positive polarity single stranded genome virus without envelop is about 32 to 34 nanometers in size. It is the only member of the Hepevirus genus in the family of Hepeviridae. Like HAV the transmission and distribution of this hepatitis virus depend on the geographic location. In 1983 HEV was for the first time isolated from the facial sample of volunteer who was experimentally infected with HEV (Balayan et al., 1983). This virus is generally categorized in four groups i.e. 1, 2, 3 and 4 with single serotype. In about 96% of HEV infected cases, increased level of serum bilirubin and elevated liver enzymes have been observed. But neither HAV nor HEV infection progress to chronic case and the mortality rate in HEV infection is high as compared to HAV in pregnant women especially (Zhao et al., 2009; Li et al. 2006).
2.4 The discovery of Hepatitis C virus

Acute viral hepatitis has history of over 2,000 years described by Hippocrates. Within last thirty years epidemiology, Biology, Immunology, Pathology, Genetics and clinical feature different viral hepatitis have been identified as hepatitis has a very long history in humans. Previously it was speculated that hepatitis might be cause by an infectious agent, after many decades with the development of advance complicated research tools and methodology researcher discovered different viral hepatitis A, B, and C. Detection of hepatitis infection in blood transfusion recipients lead towards the discoveries of these viruses. Patients with increased serum liver enzymes level with symptoms of fever, jaundice, vomiting and nausea were documented before 1940s, after almost 150 years of first ever human blood transfusion (Beeson, 1943). Clinical history and infection pattern in viral hepatitis gave a clue that more than one type of virus is responsible of hepatitis instead of "serum" hepatitis. In 1947 for the first time for the hepatitis A and hepatitis B terms were brought in to differentiate between the two different forms of hypothesized hepatitis (MacCallum, 1947).

With the advancement in diagnosis of viral infection, in 1974 serological diagnostic procedure was discovered for HAV and HBV infection and so their contribution in spread of post transfusion hepatitis was cleared (Prince et al., 1974). Since the serum screening tests for hepatitis A and B in donor blood was not screened it was obvious that there is still another unrevealed infectious agent liable for mass post transfusion hepatitis infections, so for the first time ever “non-A, non-B hepatitis” (NANBH) terminology was used (Alter et al., 1975; Feinstone et al., 1975). With the continuous research progress for about decade, while
digging for this NANBH's causative agent, during that period other viruses causing hepatitis i.e. HDV and HEV were discovered (Balayan et al., 1983; Bonino et al., 1984; Rizzetto et al., 1977). Since 1940 or longer that virus might exist but in 1989 it was identified for the first time. Chiron Corporation in USA unambiguously identified and did genetic mapping of HCV by using very advance tool i.e. molecular biology and recombinant DNA technology (Shepard et al., 2005).

2.5 Epidemiology of HCV

HCV infection is a global health problem and victimizing 180 million people worldwide out of which 130 million are chronic carrier (Shepard et al., 2005). Countries like Canada, Australia, USA, Japan and in most of European countries incidence of HCV infection is about ≥ one percent. While approximately 2 % population of Africa and Southeast Asia infected with HCV (Nakamura et al., 2008). According to epidemiology survey of HCV about 10 million Pakistanis are suffering from HCV infection (Raja and Janjua, 2008) and provincial HCV surveillance data revealed that Punjab province is ranked highest in HCV prevalence. And in male population incidence of this infection is higher as compared to female. More than half of the HCV positive population belong from age group of 40-50 years (Idrees and Riazuddin, 2008). Healthy blood donors show high prevalence of HCV infection and different studies conducted at national level reported about 4 % of blood donor are sub-clinically infected with HCV (Waheed et al., 2009). According to Arif et al. (2008) hemophilic and thalassemic population showed about 49% HCV infection and in health care workers HCV infection percent incidence is almost 5 % (Hamid et al., 2004). Every year Pakistan adds approximately 0.37 million new cases of HCV infections (Ali et al., 2009).
2.6 HCV transmission

In past HCV main mode of transmission was through blood transfusion, now HCV first and foremost spread by means of direct contact to HCV infected blood, body fluid and blood products (Burns et al., 2003). HCV infections in children and HCV positive antibodies in their parents clearly depict that transmission of HCV to them probably by exposure to infected blood or contaminated syringes (Strickland et al., 2005). According to CDC (center for disease control) HCV transmission as compared to Human Immuno deficiency (HIV) via sexual intercourse is infrequently but from the saliva and semen its virions have been isolated (Schultz, 2005). There is high rate of HCV infection in individual with multiple sex partners, sex workers and homosexual however, there are very less cases of sexual transmission have been reported so far. In case of intravenous drug user, sharing of shaving razors, toothbrushes are also potential sources of HCV transmission (Pasquier et al., 2003; Schultz, 2005). Hospital acquired infection of HCV from patient to healthcare worker and from patient to patient is very common in which needle-stick injury contributes about 10 % in the transmission of HCV infections (Foley et al., 2009). Unsterilized razors, scissors, brushes at barber shops, piercing of different body parts and tattooing helps in spreading of HCV infection (Burns et al., 2003).

Transmission via hemodialysis (HD) is very rare in developed countries but this rate is quite high in under developed and in developing countries, in which HCV infected HD machines and biologically unhygienic/infectious practices of healthcare workers are in common practices (Bracho et al., 2005; Nemati et al., 2009). In semen, cervical smear, viginal fluids, saliva, nasal secretion and breast milk HCV
RNA has been also identified (Lock et al., 2006; Aaron et al., 2008). Mother to child (vertical transmission) are slightly high i.e. 5-8 % in pregnant women with increased viral load (HIV/ HCV co-infected). Role of mosquito in the spread of HCV is still unclear. But few studies reported the presence of HCV RNA in mosquito fed on HCV positive blood (Chang et al., 2001).

2.7 Treatment of HCV

HCV treatment regimens are constantly improving with time and have HCV replication inhibiting capabilities and with improvement. First approve drug for the treatment of HCV was recombinant interferon alfa-2b. Interferons are basically cytokines that have antiviral and anti-proliferative activity consequent of multiple gene expression as virus enters the host body. By this way viral replication inhibits and induces anti-proliferative effect on the cell (Neumann et al., 1998). In combination ribavirin increases treatment response rates in HCV infected patients when treatment with interferon and ribavirin in combination given for 24 weeks. But failure to this response is also observed in non respondent and relapse patients (Chevaliez et al., 2007). But treatment of HCV with Peg-IFN has is more effective but that in combination with ribavirin the treatment response is more robust. Those patients that were treated with ribavirin only show poor treatment response. When there is mutation at 415 codon amino acid in NS5B domain (RNA-dependent RNA polymerase) ribavirin shows resistance. Genetic diversity of HCV affect antiviral treatment response in HCV infected patients (Hayashi and Takehara, 2006).

2.8 HCV Genomic organization

HCV viral genome is described as a positive sense single-stranded RNA of almost 9.6 kilo base approximately 10000 nucleotides (Takamizawa et al., 1991). HCV
genome encodes a single long open reading frame (ORF) encoding a polypeptide of 3010 amino acids residues (Fig 2.2) (Grakoui, 1993). ORF of HCV translates through a ~340 nucleotide and 5’ un-translated regions (UTRs) which performs entry site for internal ribosome (IRES). In the absence of translation initiation factors RNA binds with 40S ribosomal subunit in such a way that P site become direct neighborhood for initiation codon (Hellen and Sarnow, 2001). It allows ribosomes to bind ORF’s start codon. Cellular and viral proteases enzymes cleave newly formed HCV polypeptide into three main structural proteins i.e. for core protein/nucleocapsid C, envelop glycoprotein1 E1 and for envelop glycoprotein2 with six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) is E2 (Kato, 1993).

Very less is known about NS1 but it is thought that its functions are linked with E2. NS2 translate in trans-membrane proteins whereas 630 amino acid protein NS3 having three enzymatic activities domains, 180 residues peptide with N terminal and C terminal have serine protease, helicase and nucleoside triphosphate activities respectively. Unwinding of viral genome and cleavage of different non-structural proteins (NS3-4A, NS4A-4B, NS5A-5B) at different junction sites by NS3 protease (Bartenschlager et al., 1993).

NS4 plays the role of co-factor for NS3 protease which is indispensable for polyprotein processing (Failla et al., 1994). NS5a are interferon-resistance protein whereas NS5b play role of RNA-dependent RNA polymerase indispensable for viral replication (Behrens et al., 1996).
Figure 2.2: Hepatitis C virus (HCV): model structure. Adopted from http://www.the-scientist.com/?articles.view/articleNo/24336/title/Culturing-Hepatitis-C/

Figure 2.3: Genome Organization of Hepatitis C virus.
2.9 HCV replication and role of viral proteins

HCV replication mainly take place in hepatocytes but there are many evidences that it can replicate in macrophages, B and T cells in vitro cell lines (Esteban et al., 1996), in gut epithelial cells (Deforges et al., 2004) and nerons (Forton et al., 2004). HCV cell cycle starts with the attachment of this deadly virus with host cell by specific interaction between host cell surface a receptor protein (CD81 a member of tetra-spanin) cell surface protein express on all cells, and protein (E2 glycoprotein evident from in vitro studies) on the HCV surface (Pileri et al., 1998). Furthermore, the virus has ability to enter in host cell via low density lipoprotein receptors binding (Monazahian et al., 1999). While after many structural rearrangements in E1 fusion peptide take part in fusion of membrane of the E1 which facilitates in viral particle entry into the host cytoplasm (Flint et al., 1999).

As virus binds to its receptor, internalization completes with the release of nucleocapsid into the cytoplasm. After decapsidation, translation for polypeptide and replication occur in cytoplasm. Protein translation directly takes place as HCV RNA is positive polarity RNA and this RNA function as mRNA as well. This translation is cap independent unlike other cellular RNAs where cap binding to ribosome machinery is prerequisite for translation. Translation initiation of HCV RNA starts by ribosomal binding of 5’ end of IRES. Rough endoplasmic reticulum (RER) is the main site for HCV RNA translation for synthesis of single polypeptide. For the production of structural and non structural proteins co/post cleavage of this single polyprotein take place via cellular and viral proteases. For the replication of HCV genome, synthesis of negative strand RNA take place by RNA-dependent RNA polymerase (NS5B) which will later on serve as template
for the synthesis of positive sense RNA (El-Hage and Luo, 2003; Gosert et al., 2003). Viral non structural and proteins of host cell form a membranous web known as replication complex nearer to perinuclear membranes is the site for replication and post-translational process. Process like encapsidation of viral genome and enveloping of nucleocapsids take place in ER while final processing and mature at in Golgi apparatus after that newly formed virions release by exocytosis in pericellular spaces (Penin et al., 2004).

2.9.1 Core protein

Core protein contains HCV first 191 amino acids, 3 domain (on the basis of hydrophobicity) basic protein which forms viral nucleocapsid. In the serum of HCV infected patient 21 kDa core proteins has been isolated. This protein is involved in activation of many signaling pathways by interacting with different host cellular proteins. The significant role of core protein is encapsidation of viral genome and assists its replication (Imran et al., 2012). Cytosolic membrane-bound this core protein has been linked with ER, mitochondria, nucleus and lipid droplets, Involved in steatosis and in liver carcinogenesis (Hope et al., 2002; Lerat et al., 2002). Cell functions can be influence as HCV core protein interacts with different host.
Figure 2.4: Replication cycle of HCV. (1) Binding of virus to receptors. (2) Endocytosis. (3) Uncoating. (4) Translation of genomic RNA and proteolytic processing of the polyprotein associated with the endoplasmic reticulum. (5) RNA replication complex formation in the membranous web. (6) Virions morphogenesis (genome replication). (7) Encapsidation of genomic RNA and assembly of virus particles through interactions between the ER and lipid droplets (maturation). (8) Release of virus particles from the cell (Exocytosis). Adapted from New and experimental therapies for HCV (Arema and Jacobson, 2009).
2.9.2 Envelop proteins

HCV consist of highly glycosylated two envelop proteins (E1 and E2) having significant role in HCV entry in host cell. These E1 and E2 proteins are 35 and 72 kDa correspondingly. Envelope proteins are considered to involve in cell-mediate cell entry by identifying receptor proteins of cellular membrane probably for membrane fusion. E1 and E2 proteins leads polyprotein precursor to the ER moves to ER lumen and after cleavage these remain fixed on the inner side of ER lumen. Two hypervariable regions have been recognized in E2 protein which is responsible for mutation, possibly neutralizing antibodies target (Ashfaq et al., 2011).

2.9.3 P7 protein

A polypeptide found between E2 and NS2 genes, a 63-amino acid is known as P7. It is a trans-membrane protein found on ER with two domains one connected through cytoplasmic loop and other is adjusted in the direction of ER luman. It is clear that C-terminal this trans-membrane domin of P7 can perform a signal sequence direct NS2 translocation to cleave by host cell signal peptidases in ER lumen. P7 proteins also form ion channels causing HCV infection’s pathogenic effect. P7 HCV protein has similar features like a group of proteins known as viroporins and this protein is crucial for viral particle assembly and release (Ashfaq et al., 2011).
2.9.4 Nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B)

2.9.4.1 NS2

A trans-membrane protein 21-23 kDa NS2 is indispensable for in vitro and in vivo for viral replication cycle completion (Khromykh and Westaway, 1997). N-terminal amino acids are highly hydrophobic with 3-4 trans-membrane helices anchored in ER membrane. Some part of C-terminal of NS2 and NS2/3 show auto protease activity collectively with of N-terminal NS3. Chelating agents (EDTA) studies showed lose of protease activity of NS2-3 metalloprotease have exogenous zinc medicated protease. This zinc element is important for NS3 structural stabilization present at its active site (Lorenz et al., 2006).

2.9.4.2 NS3

A multifunctional NS3 67 kDa protein’s C-terminal show NTPase/helicase whereas N-terminal performs serine protease activity (Gallinari et al., 1998). NS3 and NS4A are ER membrane bounded proteins (Wolk et al., 2000). N-terminus of NS3 protease of HCV is involved in cleavage between NS3-4A, 4A-4B, 4B-5A and 5A-5B. NTPase/helicase enzymatic activity of the NS3 is crucial for HCV RNA replication. Presumed functions of this protein may be unwinding of double stranded RNA intermediates during replication, RNA secondary structures removal or to split nucleic acid binding proteins from genome. Current advancement in molecular mechanisms understanding NS3 could be use novel antiviral strategy (Serebrov and Pyle, 2004).
2.9.4.3 NS4A

It is 54 amino acids residues containing HCV protein serve as cofactor for NS3. Deletion analysis of this protein’s showed that N-terminal is involved in directing NS3 to the ER membrane (Wolk et al., 2000). NS4A and NS3 interaction is stimulated between NS3 core residues and NS4A C-terminal. This interaction favors active site activation of NS3 which leads to more proficient protease cleavage (Kim et al., 1996). NS5A phosphorylation takes place by NS4A and also directly interacts with NS5A. Amino acids deletion from the central region of NS5A protein revealed NS4A dependent phosphorylation of NS5A (Asabe et al., 1997).

2.9.4.4 NS4B

Small 27 kDa hydrophobic protein NS4B, have significant role in employment many viral proteins (Lundin et al., 2006). This protein directly interacts with NS4A and indirectly with NS3 and NS5A. It is ER localized integral membrane protein and ER membrane also contains non-structural proteins (Lin et al., 1997). Electron microscopy data on NS4B protein of HCV revealed that it from a structure known as membranous web by changing ER membrane morphology (Gretton et al., 2005). It is evident from studies that replication complex formation takes place here as all viral proteins were localized to ER (Egger et al., 2002). Oncogenic and cytopathic effect of NS4B in transgenic mice’s liver is still unclear (Wang et al., 2006).


2.9.4.5 NS5A

A phospho-protein NS5A is hydrophilic in nature without any transmembrane domains, performs different tasks for HCV like activation of cell signaling pathways, interferon responses and viral replication (Macdonald et al., 2004). Some studies reported its linked with other HCV proteins purposed it as part of replication complexes (Neddermann et al., 1999). NS5A mutations are critical for maintaining replicon cell line and HCV replication (Lohmann et al., 1999). NS5A gain its popularity due to its crucial part in interferon response modulating it also contain interferon resistant associated region which impart resistance in HCV virus against treatment with it (Gale et al., 1997). It is suggested that NS5A has interactions with numerous proteins of cell signaling pathways and influencing their function. NS5A can alter the 3 main pathways of MAPK (regulate growth and activation) which are responsible for host cell mitogenic signaling. Both pro-apoptotic and anti-apoptotic pathways are also modulated by NS5A proteins. NS5A is also associated in altering phosphatidylinositol 3-kinase signalling and ROS pathways that are consider to cause transformation in hepatocytes and leading to hepatocellular carcinoma (Macdonald et al., 2004).

2.9.4.6 NS5B

A 65 kDa NS5B protein is a tail anchored protein which substitute for RNA dependent RNA polymerase so have crucial role in HCV new genome synthesis (Behrens et al., 1996). This polymerase activity was discovered after sequence analysis of an amino acid motif GDD (Yamashita et al., 1998). NS5B structural organization is distinctive polymerase (right hand shape) with encircled
active site having sub domains of finger, palm, and thumb (Lesburg et al., 1999). RNA genome serve as template for synthesis of negative strand of a complementary and the succeeding synthesis positive strand RNA of genomic from this negative sense RNA strand intermediate. Playing central part in HCV replicase, now NS5B is potential biomarker for new pharmaceutical (antiviral) discoveries (De Francesco and Migliaccio, 2005).

2.10 Apoptosis and HCV

Apoptosis of hepatocyte is manifested in liver related injury either by metabolic diseases, alcoholic, autoimmune, drug induced or viral hepatitis. For the normal cellular homeostasis apoptosis plays a critical role by removing damaged, abnormal proliferated and aged cells (Shin et al., 1998; Favaloro et al., 2012). Sometime certain stimuli cause collapse apoptosis mechanism leads to form various in tumor which are resistant to cytotoxic therapy (Pitot, 1993). Apoptosis in mammalian cells can be induced via two major pathways 1st death receptor pathway (extrinsic pathway) and 2nd apoptosis pathway (intrinsic pathway) is mitochondrial mediated in response to viral proteins, DNA damage and oxidative stress. In mitochondrial mediated apoptosis expression Bax, Bad, Bak pro-apoptotic genes and Bcl-2 or Bcl-xL anti-apoptotic protein express leading to PM (Fischer, 2007) as shown in figure 2.7.
Figure 2.5: Interference of HCV proteins with the apoptosis cascade. Pro and anti-apoptotic effects of HCV proteins converge at the mitochondria (e.g., NS2, NS3/4A, NS5A, E2, core), partly indirectly via p53 (NS5A, core) and activation of PKB/Akt, c-Jun kinase JNK (core) or NFkB (NS5A). Adapted from (Fischer, Baumert et al. 2007).
HCV proteins perform mitochondrial mediated apoptosis by playing role as antiapoptotic or proapototic proteins, it all depends on the expression system and experimental condition (Marusawa et al., 1999). NS3 and NS5a protein of HCV have anti-apoptotic while core protein has regulatory properties (Macdonald et al., 2004). It is evident from many studies that hepatocytic apoptosis is involved in pathogenic effect of HCV infection. It is thought that core protein of HCV restrains c-myc, TNF-α, cisplatin or Fas mediated apoptosis but mechanism behind this HCV core protein involvement is still not fully known. Due to unavailability of effective tissue culture for HCV or animal model, the study of hepatocyte apoptosis induced by HCV infection (Zekri et al., 2011).

2.11 Apoptosis

Employment of complex signalling mechanism for the removal disease or damaged condition of cells leads by series of steps in a programmed and codinated manner or we can say programmed cell death coordinated with sequale of events. There are many stimuli and causative agents that are consider to take part in the event of apoptosis. This phenomena starts with discrepancy in the normal ongoing functions in the cell. The most important of which is irregularity in redox condition of the cell. Broadly apoptotic stimuli can be categories in two main groups; stress and physiological stimuli Physiological stimuli like (viral, irradiation, UV light and bacterial infections) lead to death by cell surface receptors (TNF or CD95) and initiate mitochondria induced stress apoptosis (Gulbins, 2003).

The leading and vital organelle in performing cellular important functions and even in apoptosis is mitochondria. Whenever cell faces oxidative stress condition the membrane permeability of mitochondria increases leading to induction of
apoptosis. The major function of mitochondria in apoptosis can be categories in 3 parts: Initiation, decision and degradation phases. In initial phase gathering of proapoptotic messengers implicated in MP, in decision phase event of MMP take place and in degradation phase caspases and hydrolases activation happen (Mordon and Blanchemaison, 2008; Sala et al., 2008).

Mitochondria is target organelle for different stress responses type of like reactive oxygen species, ceramide, fatty acids their oxidation products, superoxides, nitric oxide, hydrogen peroxides etc these pro-apoptotic signals leads to mitochondrial MP as a results in release of many mitochondrial proteins in cytosol which ends on mitochondrial induced apoptosis. Metabolites and enzymes concentration for instance NADPH oxidases persuade the strength of MMP (Wang et al., 2008). Mitochondria stop pro-apototic proteins from performing their duties in cytoplasm so we can call it as act as gatekeeper. Protein like cytochrome, caspase 9, mitochondrial activator of caspases, apoptosis-inducing factor, high temperature requiring proteins and endonuclease G release from mitochondria as result of marked increase in leading apoptosis (Gulbins, 2003).

2.12 Apoptosis molecular mechanism

Ubiquitous form of cell death (Apoptosis) takes place in hepatic diseases. Apoptosis is a form of cell death occurring in human liver diseases. Morphologically apoptotic cell show distinctive features cytoplasmic shrinkage, fragmentation of nucleus, condensation of chromatin, plasma membrane blebbing and apoptotic bodies. There are two ways of hepatocytes apoptosis first death receptor (apoptosis via extrinsic pathway) secondly by cellular perturbations
(apoptosis via intrinsic pathway) and both congregate on mitochondria (Goldman, 1994).

i) The extrinsic pathway

Extrinsic mean outside, cell surface transmembrane proteins act as death receptor in extrinsic pathway, these belongs from receptor super-family of tumor necrosis factor and nerve growth factor. These are distinct on basis of their specificity to ligand i.e. Fas ligand, tumor necrosis factor alpha or tumor necrosis factor related apoptosis inducing ligand. These cell surface proteins have N-terminus extracellular that binds with ligands and intracellular C-terminal domain containing a conserve region which is called as death domain. This pathway activate when an external ligand such as tumor necrosis factor, Fas ligand, glucocorticoids, cytokines binds with their specific receptor leading to the down regulation of secondary messengers with the activation of caspase 8. Caspase 8 cleaves proapoptotic BH3 only protein (Bcl-2 family) Bid to tBid proteolytically, leads to activation of Bax and Bak results in mitochondria pore formation. The outcome of this pathway can be either cell survival or death as a result of apoptosis at all depends on signals availability and induced pathways. Activation of transcription factor (NFkB) can also occur due the balance between them and give a cell survival signal (Owen et al., 1994).

ii) The Intrinsic (Mitochondrial mediated apoptosis) pathway

Intrinsic mean starts from inside, this type of apoptosis activates in response to loss of cell-survival factors, DNA damage and different severe intracellular stresses receive and induce by cell’s membrane bound organelle like lysosomes and ER. C-jun N terminus kinase activator of intrinsic pathway of apoptosis can be activated
by DNA damage and steatosis. All these processes transduced by proteins of Bcl-2 family (pro-apoptotic and anti-apoptotic proteins) come together on mitochondrion so called as mitochondrial or Bcl-2-regulated apoptic pathway (Dreschers and Bock, 2003). It starts with the production of ROS modification in MMP as a result of proapoptotic signals induces the release of many intermembrane space proteins e.g Endo G, apoptosis inducing factor, cytochrome c etc induces apoptosome formation which eventuates activates Caspase 9 (Galle et al., 1994). Caspase 9 additional activates caspase 7 and caspase 3 (effectors caspases) ending with the start of membrane degradation, apoptotic bodies formation and fragmentation of DNA (Owen et al., 1994). Figure 2.6 below in brief represents the types and mechanism apoptotic pathways.

2.12.1 Bcl-2 Apoptosis Regulator Family

Bcl-2 is apoptosis regulator family proteins which govern MMP and can perform pro-apoptotic proteins (BAD, Bak, Bok, Bax etc) or can play as anti-apoptotic proteins (Bcl-w, Bcl-2 proper, Bcl-xL). Mitochondrial integrity is control by protein of Bcl-2 family present on the outer membrane of mitochondrial. About 24 years ago B-cell lymphoma-2 (Bcl-2) consequence of up-regulation in follicular B cell lymphoma, it was declared that Bcl-2 doesn’t promotes proliferation in tumors rather it restrains apoptosis (Hockenbery et al., 1993). During research it is discovered that in this family there are about 25 members and on the bases of homology to certain extend this Bcl-2 family is segregate in two sub-families i.e anti-apoptotic and pro-apoptotic subfamilies. Over-expression of this protein is
Figure 2.6: Diagrammatic illustration of the main molecular pathways leading to apoptosis. In the extrinsic pathway upon ligand binding to specific receptors the DISC complex is formed and caspase 8 activated. In the intrinsic pathway release of cyt c from the mitochondria result in the formation of the apoptosome and activation of caspase 9. Caspase 8 and 9 then activate downstream caspases such as caspase 3 resulting in cell death. The two pathways are connected through the cleavage of the BH3 only protein BID. Adapted from (Favaloro et al., 2012).
attributable to translocation of chromosome as it has been observed in many hematological malignancies (Reed et al., 2005). In different model systems for example glucocorticoid-induced apoptosis of lymphoma cells, Bcl-2 had proved its role as inhibitor of apoptosis.

2.12.2 Caspases executors of Apoptosis

Caspases cysteine proteases play important function in inflammation, necrosis and apoptosis and belong to family cysteine-aspartic or we can say cysteine-dependent aspartly-directed proteases. These proteases are found in many membranous organelles and cytoplasm. Based on structural analogy and substrate specificity, 14 different caspases have been found (Kaufmann and Earnshaw, 2000). These are also known as executioner based on their significant role in cells for apoptosis and synchronized at post-translational stage. Produced in inactive form known as pro-caspases with two subunits (smaller and larger) but the effector caspases has very smaller (Chandra et al., 2000). The caspases contains domain that facilitates them to activate after interacting with other molecules which ultimately ends on effector caspase. On C-terminal, there is aspartate amino acid residue a point for cleavage (Fan et al., 2005).

Two terms auto-activation or sometime proteolytic cascade are used for caspase activation which occur when proteolytic cleavage of aspartate residues not less than ultimately separates two subunits transforming inactive form into active form (Ozben, 2007). There are three caspases activation pathways: 1. Cytotoxic T lymphocytes and Natural killer cells release granzyme B which are responsible for caspase-3/-7 activation. 2. TRAIL receptors, Fas, and TNF receptor activate caspase -8/ -10. 3. Cytochrome c and the Bcl-2 protein family (as in case of HCV
infection induced apoptosis) regulated via apoptosome results in activation of -9 caspase. Human twelve caspases -3, to -10 may expose to have a major contribution in programmed cell death. Based on task and caspases activations in signaling pathway they have been categorized in 3 classes: first apoptotic activators -2, 8, 9, 10 (Initiator caspases), second apoptotic executors -3, 6, 7 (Effector caspases) and caspase -1, 4, 5, 11, 12, 13, 14 (Inflammatory caspases).

Activation of effector caspases starts when pro-apoptotic stimuli activate initiator caspases and start their proteolytic cleavage. These caspases then perform their functions by deterioration of cell and expanding the proteins cleavage, obstructing apoptotic activity by releasing apoptotic inhibitors. All catalysis enzymes and regulatory proteins become free progressing in collapse of enzyme activity. Chief effector caspase (8, 9) are caspases and effector caspase (3, 6, 7) destroying proteolytic enzymes leading cells towards apoptosis (Pop and Salvesen, 2009; Li and Yuan, 2008).

There are many contradictory studies that reported many HCV pro-apoptotic or anti-apoptotic proteins are directly involved in apoptosis. In some reports it is declare that core proteins, E1, E2, NS3, NS4A, and NS5A and NS5B HCV proteins activate apoptosis. Some studies reported that these HCV proteins work as anti-apoptotic. Apoptosis induced by HCV by two ways external and internal pathways. Hepatocyte apoptosis occur in patients with HCV chronically infected patients. TNF-R1, TRAIL-R1, Fas and TRAIL-R2 express on liver cells (Tatsuo Kanda et al., 2013).

In case of hepatocytes hepatic apoptosis the role of Fas and TNF-R1 are well documented on the bases of both in vitro and in vivo agonist antibodies anti-Fas
and TNFα induced hepatotoxicity. TRAIL induced hepatotoxicity is still under discussion but it was thought that minor role in apoptosis progression however its expression is caused by DNA damage. HCV non-structural NS3 protein induces host cell transformation and tumor suppressor p53 and the N-terminus of NS3 forms a complex which in turn inactivates actinomycin D-induced apoptosis. Many research studies have revealed that HCV proteins interact with host cells proteins and thought that induce hepatocarcinogenesis (Tatsuo Kanda et al., 2013).
Material & Methods
3.1 Materials and Methods

3.1.1 Expression vectors and host cells

Human hepatoma cell line (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).

HCV non-structural proteins NS3, NS3-4A and NS4A were amplified from cDNA clone of HCV genotypes 3a (pS52 strain), accession no GU814263 and cloned in the pEGFP-C1 and pFLAG-CMV2 vectors. Recombinant vectors containing NS3, NS3-4A and NS4A genes pEGFP-C1/NS3, pEGFP-C1 /NS3-4A, pEGFP-C1 /NS4A, pFLAG-CMV2/NS3, pFLAG-CMV2 /NS3-4A and pFLAG-CMV2 /NS4A were transfected using trans-LT1 transfection reagent in Huh7 cells (Mirus 2300, USA). HCV infection in this study was carried out using cell culture-derived HCV Jc1 genotype 2a HCVcc (HCV infectious in cell culture) that were maintained in the presence of 0.4 mg/mL G418 (Invitrogen). HCVcc infection was carried out at multiplicity of infection (MOI) of 1.

3.1.2 Chemicals and consumables

All the chemicals were purchased from Sigma St. Louis, USA and Fisher scientific, USA.

3.1.3 Enzymes

DNA amplification enzyme Platinum® Taq DNA Polymerase High Fidelity and Proteinase K were purchased from (Life Technologies Invitrogen, USA).
Restriction endonucleases include (BglII & BamHI), T4 DNA ligase, antarctic phosphatase was purchased from (New England Biolabs Inc. MA, USA). Rnase cocktail enzyme mix was obtained from (Life Technologies Ambion, USA).

3.1.4 Antibodies

Antibodies used in present study were purchased from (Cell Signaling Technology, USA, Sigma, USA, Abcam, USA and Thermo Scientific, USA, Molecular Probe, USA). Primary antibodies were included the following: rabbit polyclonal anti-BAX; rabbit monoclonal (Cell Signaling); anti-β-actin (Cell Signaling); rabbit polyclonal anti-Bcl-xL (Cell Signaling); rabbit polyclonal anti-Caspase 7 (Cell Signaling); rabbit polyclonal anti-Caspase 9 (Cell Signaling); rabbit polyclonal anti-Caspase 3 (Cell Signaling); rabbit polyclonal anti-Cytochrome c (Cell Signaling); rabbit polyclonal anti-PARP (Cell Signaling); rabbit polyclonal anti-GFP (Cell Signaling); mouse monoclonal anti-FLAG (Sigma); rat monoclonal anti-KDEL (Abcam); mouse monoclonal anti-HCV core (Thermo Scientific). The secondary antibodies used for immunofluorescence were Alexa Fluor 350, 488, 568, 594, or 647 anti-donkeys, mouse, rabbit, or goat IgG (Molecular Probe). The secondary antibodies used for immunoblot analysis were RP-conjugated anti-mouse IgG (Cell Signaling), HRP-conjugated anti-rabbit IgG (Cell Signaling).
3.1.5 Antibiotics

Table 3.1: List of antibiotics used in the present study

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock solution</th>
<th>Final concentration in use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg/mL</td>
<td>100 µg/mL</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 mg/mL</td>
<td>50 µg/mL</td>
</tr>
</tbody>
</table>

3.1.6 Molecular weight markers

1 Kb ladder, Mass DNA ladder and 100 bp DNA ladders were obtained from (Biolabs, USA). Protein molecular weight marker was purchased from MBI (BioRad, USA).

3.1.7 Reagents

Reagents used in the study were included the following: dNTPS mix (Invitrogen Life Technologies, USA), Trans-LT1 transfection reagent (Mirus, USA), Super signal (West Femto Maximum Sensitivity Substrate) (Thermo Scientific, USA), Immuno Western blot (Chemiluminescent HRP Substrate (Millipore, USA), MitoTracker CMXRos Red stain (Invitrogen), MitoSOX™ Red mitochondrial superoxide stain (Invitrogen), Hoechst 33342 stain (Invitrogen), Mounting agent ProLong® Gold Antifade Reagent with DAPI (Invitrogen).

3.1.8 Media

(A) Bacteria

(a) Luria Bertani (LB) Broth (per liter)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LB (Sigma)</td>
<td>25 g</td>
</tr>
</tbody>
</table>
Dissolve 25 g LB broth to the 900 mL dH₂O. After mixing adjust the volume up to 1 L.

**(b) Luria Bertani (LB) Agar (per liter)**

| LB (Sigma) | 37 g |

Dissolve 37 g LB agar to the 900 mL dH₂O. After mixing adjust the volume up to 1 L.

**(B) Media used for cell lines**

(a) DMEM (Gibco)

(b) RPMI (Gibco)

(c) Fetal bovine serum (Hyclone)

(d) 1% MEM non-essential amino acids (Gibco)

(e) Penicillin / streptomycin (Gibco)

(f) Geneticin® Selective Antibiotic (G418 Sulfate) (Invitrogen)

### 3.1.9 Buffers

**(a) 1X Tris EDTA (TE) Buffer (500 mL)**

<table>
<thead>
<tr>
<th>Tris HCl</th>
<th>1 M</th>
<th>5 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.5 M</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

**Buffers for Agarose Gel Electrophoresis**

**(a) Tris Acetate EDTA (TAE) Buffer, pH 8.3 (per Liter)**

<table>
<thead>
<tr>
<th>Tris base</th>
<th>242 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (0.5M)</td>
<td>100 mL</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 mL</td>
</tr>
</tbody>
</table>
Mix all chemicals together in 800 mL dH$_2$O. After mixing adjust to volume up to 1 L.

(b) Ethidium-bromide dye (10 mg/ mL)

<table>
<thead>
<tr>
<th>Ethidium- bromide</th>
<th>10 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli Q water</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

(c) 6X loading dye for agarose gel electrophoresis (Biolabs, USA)

**Reagents and Buffers for SDS-Page**

(a) RIPA buffer  

<table>
<thead>
<tr>
<th>Quantity (1mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M TRIS pH 7.4</td>
<td>50 μL</td>
</tr>
<tr>
<td>2.5 M NaCl</td>
<td>60 μL</td>
</tr>
<tr>
<td>10 % NP40</td>
<td>100 μL</td>
</tr>
<tr>
<td>10 % DOC</td>
<td>25 μL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>10 μL</td>
</tr>
<tr>
<td>10 mM PMSF</td>
<td>10 μL / 255 μL</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>74 5μL</td>
</tr>
</tbody>
</table>

(b) Acrylamide (30 %)

<table>
<thead>
<tr>
<th>Quantity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>29.2 g</td>
</tr>
<tr>
<td>N, N' methylene bisacrylamide</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>60 mL</td>
</tr>
</tbody>
</table>
The solution was stirred to dissolve the acrylamide. The volume was made up to 100 mL and the solution was filtered through Whatman filter paper no. 1 before use.

(c) Ammonium per sulfate 12.5% (w/v)

(d) TEMED 8.4% (v/v)

(e) Resolving buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>9.08 g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

Dissolve in 60 mL of distilled water and adjust pH to 8.8 with conc. HCl and make up to 100 mL.

(f) Stacking buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>3.03 g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

Dissolve in 60 mL of distilled water and adjust pH to 6.8 with conc. HCl and make up to 100 mL.

(g) 10 X Tris-Glycine buffer (per/liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>30.3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144.1 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
</tbody>
</table>

(h) Coomassie brilliant blue staining solution (per Liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brilliant blue (R250)</td>
<td>1 g</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>100 mL</td>
</tr>
</tbody>
</table>
Methanol  
Milli Q water  

(i) Lysis Buffer/ Loading dye

- Tris base 1.51 g
- SDS 4 g
- Glycine 20 g
- Bromophenol blue 0.002 g

Dissolve in 60 mL of water and make up to 90 mL. Add 10 mL β-mercaptoethanol just before use.

Reagents for Western blot Analysis

(a) Transfer buffer (per liter)

- Tris Base 3 g
- Glycine 14.4 g
- Methanol 200 mL

(b) Blocking buffer 5% non-fat milk in TBS

(c) Wash buffer 0.1% Tween 20 in TBS (TBS-T)

Reagents for cell viability assay

(a) Plaque Staining Solution

- Crystal violet 0.2%
3.1.10 Instrumentation

Sorvall RT7 plus (Ultra Centrifuge), Eppendorff (5415R) Centrifuge, Premium Biotech Visible Spectrophotometer Ultra space 1000, Kodak (Digital Science) image station (440), i cycler (Bio-Rad), Weighing Balance (OHAUS), PH Meter (Accumet Basic Fisher Scientific), Incubator (Boeuel Scientific), Kodak (M35A-OMAT Processor), Transilluminator (Fisher Biotech), Olympus X cite (Series 120Q) EXFO, ELx 800 Universal Microplate Reader (Bio-TEK Instruments), New Brunswick Scientific Excella E25 Incubator Shaker Series, Castel Autoclave System, Mini-PROTEAN Tetra Handcast System (Bio-Rad), Mini-Sub Cell GT System (Bio-Rad), Wide Mini-Sub Cell GT System (Bio-Rad).

3.2 Methodology

3.2.1 Primer designing

Primers for amplification of HCV non-structural NS3, NS3-4A and NS4A genes and primers for the sequencing of positive clones were designed according to mammalian expression vectors (pFLAG-CMV2 & pEGFP-C1) using online available oligo calculator and synthesized from (IDT-Integrated DNA Technologies, USA). Primers sequences are given in the Table 3.2.

3.2.2 Amplification of HCV non-structural NS3, NS3-4A and NS4A genes

Infectious cDNA clone of HCV genotypes 3a (pS52 strain), accession no GU814263 was used for the amplification of HCV non-structural NS3, NS3-4A & NS4A genes. These genes were amplified using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). The reaction mixture and cycling conditions for PCR amplification of each gene are given in Table 3.2.
**Table 3.2:** Nucleotide sequences of the synthetic primers for sequencing and PCR:

All primers were successfully annealed to their respective targets. The sequences were designed from reported sequences of HCV genomes (NZL & pS52 of genotype 3a) available at NCBI. Sequences of the restriction enzymes used for each primer is italicized while stop codons in reverse primers are underlined.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Primer ID</th>
<th>Primer Sequence 5’ to 3’</th>
<th>Restriction site</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR PRIMERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>NS3-GFP F</td>
<td>ATCTAAAAAGATCTGGCCGGTGAGGTGTTG</td>
<td>BglII</td>
<td>NS3 (1.9 kb)</td>
</tr>
<tr>
<td>2.</td>
<td>NS3-FLAG F</td>
<td>ATCTAAAAAGATCTGGCCGGTGAGGTGTTG</td>
<td>BglII</td>
<td>NS3-4A (2.1 kb)</td>
</tr>
<tr>
<td>3.</td>
<td>NS3 R</td>
<td>TTAATTGGAATCCTCAAGTGGTTAATTCCAG</td>
<td>BamHI</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>NS4A-GFP F</td>
<td>ATCTAAAAGATCTGGCCGGTGAGGTGTTG</td>
<td>BglII</td>
<td>NS4A (168 bp)</td>
</tr>
<tr>
<td>5.</td>
<td>NS4A-FLAG F</td>
<td>ATCTAAAAGATCTGGCCGGTGAGGTGTTG</td>
<td>BglII</td>
<td>NS4A (168 bp)</td>
</tr>
<tr>
<td>6.</td>
<td>NS4A R</td>
<td>TTAATTGGAATCCTCAAGCACCCTCCATC</td>
<td>BamHI</td>
<td></td>
</tr>
<tr>
<td><strong>SEQUENCING PRIMERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>pFLAG-CMV2 F</td>
<td>ATAAACCCCCCGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>pFLAG-CMV2 R</td>
<td>TTAGGACAGGGCTGTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>pEGFP-C1 F</td>
<td>AGCACCCAGCTCGCCCTGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>pEGFP-C1 R</td>
<td>GAAATTTGTGATGCTATTGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.3 Amplification of NS3 gene using pFLAG-CMV2 primers

Reaction mixture

\[ \begin{align*}
10 \text{ mM dNTPs} & \quad \text{(1 µL)} \\
2 \text{ mM MgSO}_4 (25 \text{ mM}) & \quad \text{(2 µL)} \\
\text{HF Buffer (10X)} & \quad \text{(5 µL)} \\
\text{NS3-FLAG F (10 pmole/µL)} & \quad \text{(1 µL)} \\
\text{NS3-FLAG R (10 pmole/µL)} & \quad \text{(1 µL)} \\
\text{Template (DNA 640 ng/µL)} & \quad \text{(1.5 µL)} \\
\text{HF Taq polymerase (1U)} & \quad \text{(0.2 µL)} \\
\text{Nuclease free water} & \quad \text{(38.3 µL)} \\
\text{Total volume} & \quad \text{(50 µL)}
\end{align*} \]

The following conditions were followed for amplification of NS3 gene.

**PCR cycling conditions**

<table>
<thead>
<tr>
<th>Initial denaturation (Stage 1)</th>
<th>94°C 2 minutes</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Stage 2)</td>
<td>94°C 40 seconds</td>
<td>X 35</td>
</tr>
<tr>
<td></td>
<td>55°C 40 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68°C 2:30 minutes</td>
<td></td>
</tr>
<tr>
<td>(Stage 3)</td>
<td>68°C 7 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 4)</td>
<td>04°C Hold</td>
<td></td>
</tr>
</tbody>
</table>
3.2.4 Amplification of NS3 gene using pEGFP-C1 primers

**Reaction mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM dNTPs</td>
<td>1 µL</td>
</tr>
<tr>
<td>2 mM MgSO₄ (25 mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>HF Buffer (10 X)</td>
<td>5 µL</td>
</tr>
<tr>
<td>NS3-FLAG F (10 pmole/µL)</td>
<td>1 µL</td>
</tr>
<tr>
<td>NS3-FLAG R (10 pmole/µL)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Template (DNA 640 ng/µL)</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>HF Taq polymerase (1U)</td>
<td>0.2 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>38.3 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

The following conditions were followed for amplification of NS3 gene.

**PCR cycling conditions**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Condition</th>
<th>Duration</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation (Stage 1)</td>
<td>94°C 2 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>2</td>
<td>(Stage 2)</td>
<td>94°C 40 seconds</td>
<td>X 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C 40 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C 2:30 minutes</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(Stage 3)</td>
<td>68°C 7 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>4</td>
<td>(Stage 4)</td>
<td>04°C Hold</td>
<td></td>
</tr>
</tbody>
</table>
3.2.5 Amplification of NS3-4A gene using pFLAG-CMV2 primers

Reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM dNTPs</td>
<td>1 μL</td>
<td></td>
</tr>
<tr>
<td>2 mM MgSO₄ (25 mM)</td>
<td>2 μL</td>
<td></td>
</tr>
<tr>
<td>HF Buffer (10X)</td>
<td>5 μL</td>
<td></td>
</tr>
<tr>
<td>NS3-FLAG F (10 pmole/μL)</td>
<td>1 μL</td>
<td></td>
</tr>
<tr>
<td>NS3-FLAG R (10 pmole/μL)</td>
<td>1 μL</td>
<td></td>
</tr>
<tr>
<td>Template (DNA 640 ng/μL)</td>
<td>1.5 μL</td>
<td></td>
</tr>
<tr>
<td>HF <em>Taq</em> polymerase (1U)</td>
<td>0.2 μL</td>
<td></td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>38.3μL</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>50 μL</td>
<td></td>
</tr>
</tbody>
</table>

The following conditions were followed for amplification of NS3-4A gene

**PCR cycling conditions**

<table>
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<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
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<td>94°C</td>
<td>2 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 2)</td>
<td>94°C</td>
<td>40 seconds</td>
<td>X 35</td>
</tr>
<tr>
<td>(Stage 2)</td>
<td>55°C</td>
<td>40 seconds</td>
<td></td>
</tr>
<tr>
<td>(Stage 2)</td>
<td>68°C</td>
<td>2:30 minutes</td>
<td></td>
</tr>
<tr>
<td>(Stage 3)</td>
<td>68°C</td>
<td>7 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 4)</td>
<td>04°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>
3.2.6 Amplification of NS3-4A gene using pEGFP-C1 primers

Reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM dNTPs</td>
<td>1 μL</td>
</tr>
<tr>
<td>2 mM MgSO₄ (25 mM)</td>
<td>2 μL</td>
</tr>
<tr>
<td>HF Buffer (10 X)</td>
<td>5 μL</td>
</tr>
<tr>
<td>NS3-FLAG F (10 pmole/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>NS3-FLAG R (10 pmole/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Template (DNA 640 ng/μL)</td>
<td>1.5 μL</td>
</tr>
<tr>
<td>HF Taq polymerase (1U)</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>38.3 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

The following conditions were followed for amplification of NS3-4A gene.

**PCR cycling conditions**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Initialdenaturation</th>
<th>94°C 2 minutes</th>
<th>94°C 40 seconds</th>
<th>55°C 40 seconds</th>
<th>68°C 2:30 minutes</th>
<th>Final anneal</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Stage 1)</td>
<td>94°C 2 minutes</td>
<td>X 1</td>
<td></td>
<td></td>
<td></td>
<td>X 1</td>
<td></td>
</tr>
<tr>
<td>(Stage 2)</td>
<td>94°C 40 seconds</td>
<td></td>
<td>X 35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Stage 3)</td>
<td>68°C 2:30 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X 1</td>
<td></td>
</tr>
<tr>
<td>(Stage 4)</td>
<td>04°C Hold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.7 Amplification of NS4A gene using pFLAG-CMV2 primers

**Reaction mixture**

- 10 mM dNTPs
- 2 mM MgSO$_4$ (25 mM)
- HF Buffer (10X)
- NS3-FLAG F (10 pmole/μL)
- NS3-FLAG R (10 pmole/μL)
- Template (DNA 640 ng/μL)
- HF Taq polymerase (1U)
- Nuclease free water

Total volume: 50 μL

The following conditions were followed for amplification of NS4A gene.

**PCR cycling conditions**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Initial denaturation (Temp/Time)</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Stage 1)</td>
<td>94°C 2 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 2)</td>
<td>94°C 40 seconds 55°C 40 seconds 68°C 40 seconds</td>
<td>X 35</td>
</tr>
<tr>
<td>(Stage 3)</td>
<td>68°C 7 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 4)</td>
<td>04°C Hold</td>
<td></td>
</tr>
</tbody>
</table>
3.2.8 Amplification of NS4A gene using pEGFP-C1 primers

Reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM dNTPs</td>
<td>1 μL</td>
</tr>
<tr>
<td>2 mM MgSO$_4$ (25 mM)</td>
<td>2 μL</td>
</tr>
<tr>
<td>HF Buffer (10 X)</td>
<td>5 μL</td>
</tr>
<tr>
<td>NS3-FLAG F (10 pmole/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>NS3-FLAG R (10 pmole/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Template (DNA 640 ng/μL)</td>
<td>1.5 μL</td>
</tr>
<tr>
<td>HF Taq polymerase (1U)</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>38.3 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

The following conditions were followed for amplification of NS4A gene.

PCR cycling conditions

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Stage 1)</td>
<td>94°C</td>
<td>2 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 2)</td>
<td>94°C</td>
<td>40 seconds</td>
<td>X 35</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>40 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>40 seconds</td>
<td></td>
</tr>
<tr>
<td>(Stage 3)</td>
<td>68°C</td>
<td>7 minutes</td>
<td>X 1</td>
</tr>
<tr>
<td>(Stage 4)</td>
<td>0°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>
3.2.9 Agarose gel electrophoresis

Agarose gel 1-1.5 % premixed with 10 mg/mL Ethidium- bromide were used for the resolving of PCR products when run containing TAE buffer in the gel electrophoresis chamber. Size of the desired band was determined either by 1 kb or 100 bp ladder, while gel was run at 70 V for 60 minutes and observed in UV light (Tran illuminator).

3.2.10 Purification of DNA products from agarose gel

Amplified DNA products of the particular genes were eluted from agarose gel using DNA clean and concentrator kit according to the manufacturer’s instructions. Briefly, particular DNA sample was run on 1 % agarose gel prepared in TAE buffer. The required fragment was sliced out under UV light and transferred to 1.5 mL microcentrifuge tube. 2-5 volumes of DNA binding buffer to each volume of DNA sample was added in the tube, mixed and incubated at 55 °C in water bath for approximately 10-15 minutes, to dissolve the agarose completely. Then mixture were transferred to Zymo-spin column, centrifuged for 30 sec at room temperature and discarded the flow-through. Next, 200 μL wash buffer was added to the column and centrifuged for 30 sec at room temperature. Washing step was repeated and then column was air dried, poured with 20-30 μL pre-warmed nuclease free water, incubated at room temperature for 1 min and spun down for 1 min at 13,000 rpm for the elution of DNA. Concentration of eluted DNA was estimated by running on 1 % agarose gel, with Mass DNA ladder.
3.2.11 Construction of pEGFP-C1 recombinant vectors

Respective amplified DNA products of NS3, NS3-4A and NS4A separately and expression vector pEGFP-C1 were digested with selected restriction enzymes, gel purified and further ligated using T4 DNA ligase overnight at 4 °C. 2-5 µl of ligation mixture was mixed with 100 µl to Z-competent™ E. coli strain competent cells and incubated on ice for 30 minutes. The tubes were placed at 42 °C in a water bath for 90 seconds and on ice for 1 minute. After the addition of 400 µl LB medium, tubes were then incubated at 37 °C for 30 to 60 minutes. After that 150 µl of transformation culture was plated onto LB agar plate containing ampicillin (100 ug / mL).

3.2.12 Construction of pFLAG-CMV2 recombinant vectors

Respective amplified DNA products of NS3, NS3-4A and NS4A separately and expression vector pFLAG-CMV2 were digested with selected restriction enzymes, gel purified and further ligated using T4 DNA ligase overnight at 4 °C. 2-5 µl of ligation mixture was mixed with 100 µl to Z-competent™ E. coli strain competent cells and incubated on ice for 30 minutes. The tubes were placed at 42 °C in a water bath for 90 seconds and on ice for 1 minute. After the addition of 400 µl LB medium, tubes were then incubated at 37 °C for 30 to 60 minutes. After that 150 µl of transformation culture was plated onto LB agar plate containing Kanamycin (100 ug / mL).
3.2.13 Selection of positive clones

Positive clones of particular genes were separately screened out first through restriction digestion (BamHI and BglII) and then by sequencing (Eton Biosciences, USA). First, plasmids from 32 selected colonies were isolated by “QIAprep® Miniprep Kit” according to manufacturer’s instruction. Further, were digested using selected restriction enzymes and was incubated in an eppendorf tube at 37 °C for 1-2 hrs. The digested DNA was checked by agarose gel electrophoresis. Selected clones were further confirmed by automated sequencing, which was performed commercially form Eton Biosciences, USA.

3.2.14 Large scale preparation of plasmid DNA

High concentration of plasmid DNA was prepared by using “Qiagen® plasmid midi kit” according to manufacturer’s concentration. Shortly, 150 mL LB broth containing ampicillin (100 μg/ mL) was inoculated with a transformed E. coli colony and incubated overnight in a shaking incubator at 37 °C. Further cells were harvested by centrifuging at 6000 g for 10 min at 4 °C and bacterial pellet was resuspended in 4 mL ice chilled buffer P1. 4 mL P2 buffer was poured in the tube for 5 min to lysed the cells. After 5 min 4 mL ice chilled P3 buffer was poured in the and immediately inverted 4 - 6 times and incubated on ice for 15 min. Further tube was centrifuged at 13,000 g for 30 min at 4 °C and supernatant was placed in a new centrifugation tube. A QIAGEN-tip column was equilibrated by the addition of 4 mL buffer QBT and allowed it to empty. Then supernatant was applied to the column and allowed to drain through by gravity than washed the column by the
addition of 10 mL QC buffer. The plasmid DNA was eluted by the addition of 5 mL QF buffer and collected in new tube. For cleaning purpose 3.5 mL isopropanol was added to the eluted DNA and further mixed and centrifuged at 17,000 g for 30 min at 4 °C. Further, supernatant was discarded and the pellet was washed with 2.5 mL 70 % ethanol and was centrifuged at 17,000 g for 10 min at 4 °C. Discard the supernatant and was allowed the pellet to completely dry for 5-10 min and finally resuspended in 100 μL TE buffer or nuclease free water. DNA concentration was determined using spectrophotometer at 595nm.

3.2.15 Cell culture and transfection

Huh-7 cells were maintained in Dulbecco’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) at 37 °C with 5 % CO2. Cells were seeded in 60mm plates and cultured until they became 50-60 % confluent. Constructed plasmids about 1-2 μg of pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A, pFLAG-CMV2 (non-expressing control), pFLAG-CMV2/NS3, pFLAG-CMV2/NS3-4A & pFLAG-CMV2/NS4A were separately transfected in 40 % confluent cells with trans-LT1 transfection reagent. 6hrs post transfection media (with transfection reagent and plasmid) was changed.

3.2.16 Preparation of whole cell lysate for SDS page

Whole cell lysate for SDS PAGE was prepared by washing twice with PBS 72hrs post transfection. Cells were scraped out using scraper and poured in 1.5 mL
microcentrifuge tube. Further these cells were dissolved in freshly prepared 1 mL RIPA buffer (1 M TRIS pH 7.4, 2.5M NaCl, 10 %NP40, 10 % DOC, 10 %SDS, 10 mM PMSF and proteinase inhibitor), incubated on ice for 30 mints and sonicated 6-8 times. After being sonication extracts were centrifuged at 12000 rpm for 20 mints at 4 °C, supernatants were poured in new ice chilled 1.5 mL microcentrifuge tube and stored at -80 °C. Protein Assay kit (Bio-Rad) was used according to manufacturer’s instructions to measure protein concentration.

3.2.17 SDS polyacrylamide gel electrophoresis

Protein was separated by SDS polyacrylamide gel electrophoresis. 8 and 10 % gels were used for the separation of protein bands. Following recipe were followed for the preparation of above mentioned gels (Table 3.3). After addition and mixing of all ingredients in the tube, TEMED was added in it to allow the gel to polymerize. Gel was poured between the assembled gel plates up to 2/3 portion.

Small amount of butan-2-ol was added on the top of the resolving gel to allow a smooth interface on the gel. After polymerization of the gel butan-2-ol was removed from the top of the gel and washed twice with distilled H₂O. Further, stacking gel was poured on the top of resolving gel and comb was inserted to the gel for the preparation of wells for loading of protein samples. After polymerization comb was carefully removed and placed the plates in the electrophoresis tank having 1X gel running buffer. Protein samples were mixed with 5X SDS loading buffer and boiled for 5 mints at 100 °C. 50ug of each sample was loaded in each lane of the gel and precision plus protein standard dual color
was also loaded in one lane to estimate the size of loaded samples. Finally gel was electrophoresed at 25 V for 1 hour.

**Table 3.3:** Composition of different SDS-PAGE gels

<table>
<thead>
<tr>
<th>S. NO</th>
<th>Chemical</th>
<th>Resolving 8%</th>
<th>Resolving 10%</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
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<td>1.</td>
<td>40 % Acrylamide</td>
<td>2.4 mL</td>
<td>3 mL</td>
<td>0.75 mL</td>
</tr>
<tr>
<td>2.</td>
<td>1.5 M Tris-HCl</td>
<td>4 mL</td>
<td>4 mL</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>1 M Tris-HCl</td>
<td>-</td>
<td>-</td>
<td>2.25 mL</td>
</tr>
<tr>
<td>4.</td>
<td>Water</td>
<td>5.3 mL</td>
<td>4.7 mL</td>
<td>3 mL</td>
</tr>
<tr>
<td>5.</td>
<td>10 % SDS</td>
<td>150 μL</td>
<td>150 μL</td>
<td>75 μL</td>
</tr>
<tr>
<td>6.</td>
<td>10 % APS</td>
<td>150 μL</td>
<td>150 μL</td>
<td>75 μL</td>
</tr>
<tr>
<td>7.</td>
<td>TEMED</td>
<td>5 μL</td>
<td>5 μL</td>
<td>5 μL</td>
</tr>
</tbody>
</table>

**3.2.18 Western Blotting**

For western blotting proteins bands were transferred to Hybond-C extra nitrocellulose membrane electrophoretically using tank blotting apparatus. The gel was removed from the tank and stacking gel discarded from the resolving gel. A blotting sandwich was made where the gel was placed with a piece of nitrocellulose membrane, two pieces of 3 MM Whatman paper and two fiber pads.
The sandwich was fixed in a cassette and was placed into the transblot apparatus and filled the tank with transfer buffer (Tris glycine buffer). Electrotransfer was carried out at 100 mA for 1:30 hrs or 25 mA at 4 °C overnight.

After successfully transferred, blot was blocked with 5 % skimmed milk for 1 hr at room temperature followed by one washed with TBS-T buffer (0.05 % Tween 20). Further, blots were incubated with appropriate primary antibodies (1:1000) overnight at 4 °C. After being washing three times with TBS-T buffer 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 buffer, 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.

3.2.19 Immunofluorescence Microscopy

The cells expressing NS3, 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 1 X PBS. MitoTracker CMXRos Red was used to stain mitochondria in live cells for 10 mints before fixation. Next, cells were blocked with blocking buffer (3 % BSA, 5 % Goat serum, 0.05 % Triton X-100) for 1 hr at room temperature. After blocking cells were incubated with particular primary antibodies (1:1000) overnight at 4 °C. Further, cells were washed thrice with PBS
and then incubated with particular Alexa flour secondary antibodies (1:10000) in the dark. After secondary antibody incubation cells were washed thrice with PBS. Further, nuclei were stained with DAPI. Images were visualized under a 100X oil objective using an Olympus FluoView 1000 confocal microscope. Quantification of images was conducted with ImageJ or MBF ImageJ softwares.

3.2.20 Crystal violet cell viability assay

The effect of cell viability was determined by measuring crystal violet dye absorbance of living cells. Briefly, cells were transfected with pEGFP-C1 (empty vector/ non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A, pFLAG-CMV2 (empty vector/ non-expressing control), pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A and pFLAG-CMV2 /NS4A recombinant vectors in three different sets of experiments. 72 hrs post transfections cells were treated with crystal violet dye, stabilized with 33 % acetic acid and fixed with 4 % paraformaldehyde. Optical density was measured at wave length 595 nm using premium biotech visible spectrophotometer ultra-spec 1000.

3.2.21 Subcellular Fractionation

For the isolation and separation of mitochondrial and cytosolic fractions from experimental cells, Huh-7 cells were transfected with pEGFP-C1 (non-expressing control/ empty vector), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A. 3 days post-transfection, cells were scraped and pure cytosolic and mitochondrial fractions were isolated using mitochondrial isolation kit for cultured cells according to the manufacturer’s instructions. Briefly, 800 μL Reagent A were added in the homogenized cells and incubated on ice for 2 mints. Next, cells were
dounce homogenized then mixed with 800 μL Reagent C and centrifuged at 700 g for 10 mints at 4 °C. Supernatant were collected in fresh 1.5 mL microcentrifuge tube and again centrifuged at 12,000 g for 15 mints at 4 °C. Supernatant were removed and pellet (mitochondria) were washed with 500 μL Reagent C and centrifuged at 12,000 g for 5 mints at 4 °C. Supernatant (Cytosolic fraction) were collected in new tube while pellet (mitochondrial fraction) were resuspended in TE buffer. Equivalent amounts of protein from each fraction were analyzed by Western blotting with the indicated primary and secondary antibodies.

3.2.22 Mitochondrial Superoxide Estimation

Huh-7 cells were seeded on glass cover slips and transfected with pEGFP-C1 (non-expressing control/ empty vector), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A for the estimation of mitochondrial superoxide (byproduct of oxidative phosphorylation of complexes I and III of the electron transport chain). 72 hrs post transfection cell were washed with PBS and stained with MitoSOX™ Red mitochondrial superoxide indicator 10 μM for 10 min at 37 °C. After being fixed with 4 % paraformaldehyde, superoxide production was estimated at 100X oil under florescence microscope (Olympus).

3.2.23 Measurement of Mitochondrial Complex I Enzyme Activity

The activity of mitochondrial oxidative phosphorylation respiratory chain complex I (NADH dehydrogenase) in NS3, NS3-4A and NS4A expressing cells were measured by using mitochondrial complex I activity assay kit according to the
manufacturer’s instructions. Briefly, Huh-7 cells were transfected with pFLAG-CMV2 (non-expressing control/ empty vector), pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A and pFLAG-CMV2 /NS4A. Huh-7 cells were used as negative control (mock) while Huh-7 cells infected with HCVcc used as positive control. Cells were harvested 72 hrs post transfection and processed for further experimentation.

3.2.24 Hoechst Staining

Cells were seeded on glass cover slips and transfected with pEGFP-C1 (non-expressing control/ empty vector), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A. At various time points (24hrs, 48hrs and 72hrs) cells were fixed using 4 % paraformaldehyde, washed five times with PBS and stained with 10mM Hoechst 33342 at room temperature for 10 min. After incubation cells were gently washed and mounted. The morphology of the nuclei of the cells was examined at 100 X oil under florescence microscope (Olympus).

3.2.25 Statistical analysis

To analyze the significance of numerical data, Student’s t-test was performed by using Graph Pad Prism version 5.0 (GraphPad Software, Inc., CA, USA). Data are presented as mean ± standard error of the mean. p-values < 0.05 were considered statistically significant (p < 0.05, p < 0.01 and p < 0.001).
Results
RESULTS

4.1 Amplification and construction of recombinant vectors

Genes for HCV non-structural proteins NS3, NS3-4A and NS4A were successfully amplified from cDNA clone 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. Figure 4.1(A & B) shows the double digestion of positive clones of pEGFP-C1 /NS3 containing NS3 (1.9 kb), pEGFP-C1 /NS3-4A containing NS3-4A (2.1kb) and pEGFP-C1/NS4A containing NS4A (165 bp), while figure 4.2(A & B) shows the double digestion of positive clones of pFLAG-CMV2 /NS3 containing NS3 (1.9 kb), pFLAG-CMV2 /NS3-4A containing NS3-4A (2.1 kb) and pFLAG-CMV2 /NS4A containing NS4A (165 bp).

Maps of above mentioned recombinant vectors are shown in Figure 4.3 (A & B).
Figure 4.1: 1% agarose gel electrophoresis shows the results of double digestion of recombinant pEGFP-C1 vectors. (A) Lane 1 refer to 1 kb ladder, lane 2 refers to undigested recombinant vector (pEGFP-C1/NS3), lane 3 refer to digested vector (4.7 kb) releasing NS3 (1.9 kb) gene, lane 4 refer to undigested recombinant vector (pEGFP-C1/NS3-4A) while, lane 5 refer to digested vector (4.7 kb) releasing NS3-4A (2.1 kb) gene. (B) Lane 1 refer to undigested recombinant vector (pEGFP-C1/NS4A), lane 2 refer to digested vector (4.7 kb) releasing NS4A (165 bp) gene while, lane 3 refer to 1 kb ladder.
Figure 4.2: 1% agarose gel electrophoresis shows the results of double digestion of recombinant pFLAG-CMV2 vectors. (A) Lane 1 refer to 1 kb ladder, lane 2 refer to digested vector (4.7 kb) releasing NS3 (1.9 kb) gene, lane 3 refers to undigested recombinant vector (pFLAG-CMV2/NS3), lane 4 refer to digested vector (4.7 kb) releasing NS3-4A (2.1 kb) gene while, lane 5 refer to undigested recombinant vector (pFLAG-CMV2/NS3-4A). (B) Lane 1 refer to 1 kb ladder, lane 2 refer to undigested recombinant vector (pFLAG-CMV2/NS4A), while, lane 3 refer to digested vector (4.7 kb) releasing NS4A (165 bp) gene.
Figure 4.3 (A): Maps of recombinant pEGFP-C1 constructs. pEGFP-C1/NS4A vector containing HCV NS3-4A gene, pEGFP-C1/NS3 vector containing HCV NS3 gene while pEGFP-C1/NS4A vector containing HCV NS4A gene.

Figure 4.3 (B): Maps of recombinant pFLAG-CMV2 constructs. pFLAF-CMV2/NS3-4A vector containing HCV NS3-4A gene, PFLAG-CMV2/NS3 vector containing HCV NS3 gene while pFLAG-CMV2/NS4A vector containing HCV NS4A gene.
4.2 Sequencing of GFP-tagged recombinant vectors

Positive clones of pEGFP-C1/NS3, pEGFP-C1/NS3-4A & pEGFP-C1/NS4A were confirmed by commercial sequencing (Eton Biosciences, USA) from both directions using forward and reverse primers. Figure 4.4 depicts the representative chromatogram and sequence homology of HCV non-structural NS3 gene with reported sequences. Figure 4.5 shows the representative chromatogram and sequence homology of HCV non-structural NS3-4A gene with reported sequences while; Figure 4.6 is showing the representative chromatogram and sequence homology of HCV non-structural NS4A gene with reported sequences.
Figure 4.4: Representative chromatograms and sequence homology of HCV non-structural NS3 gene with reported sequences.
Figure 4.5: Representative chromatograms and sequence homology of HCV non-structural NS3-4A gene with reported sequences.
Figure 4.6: Representative chromatograms and sequence homology of HCV non-structural NS4A gene with reported sequences.
4.3 Sequencing of FLAG-tagged recombinant vectors

Positive clones of pFLAG-CMV2/NS3, pFLAG-CMV2/NS3-4A, pFLAG-CMV2/NS4A were confirmed by commercial sequencing from both directions using forward and reverse primers. Figure 4.7 depicts the representative chromatogram and sequence homology of HCV non-structural NS3 gene with reported sequences. Figure 4.8 shows the representative chromatogram and sequence homology of HCV non-structural NS3-4A gene with reported sequences while; Figure 4.9 is showing the representative chromatogram and sequence homology of HCV non-structural NS4A gene with reported sequences.
Figure 4.7: Representative chromatograms and sequence homology of HCV non-structural NS3 gene with reported sequences.
Figure 4.8: Representative chromatograms and sequence homology of HCV non-structural NS3-4A gene with reported sequences.
Figure 4.9: Representative chromatograms and sequence homology of HCV non-structural NS4A gene with reported sequences.
4.4 Western blot analysis of FLAG-tagged HCV non-structural NS3, NS3-4A and NS4A proteins

Transient expression of FLAG-tagged HCV non-structural NS3, NS3-4A and NS4A proteins were analyzed by western blotting. Cell lysates were harvested 72 hrs post transfection using RIPA buffer and were analyzed with anti-FLAG antibody. Figure 4.10 (A) shows no band (non-expressing control) in lane 1 while specific band of 7 kDa representing the fusion complex of FLAG with NS4A protein in lane 2. On the other hand figure 4.10 (B) shows specific band of 72 kDa representing the fusion complex of FLAG with NS3 protein in lane 1 while specific band of 72 kDa representing fusion complex of FLAG with NS3-4A protein in lane 2. Internal loading control β-actin was used.
Figure 4.10: Western blot analyses of pFLAG-CMV2/NS3, pFLAG-CMV2/NS3-4A, pFLAG-CMV2/NS4A vectors expressing HCV non-structural NS3, NS3-4A and NS4A proteins. (A) Lane 2 shows specific band of 7 kDa representing the fusion complex of FLAG with NS4A protein. (B) Lane 1 shows specific band of 72 kDa representing the fusion complex of FLAG with NS3 protein, while lane 2 shows specific band of 72 kDa representing fusion complex of FLAG with NS3-4A protein. β-actin was used as an internal loading control.
4.5 Western blot analysis of GFP-tagged HCV non-structural NS3, NS3-4A and NS4A proteins

Transient expression of GFP-tagged HCV non-structural NS3, NS3-4A and NS4A proteins were analyzed by western blotting. Cell lysates were harvested 72 hrs post transfection using RIPA buffer and were analyzed with anti-GFP antibody. Figure 4.11 shows specific band of 27 kDa representing GFP protein in lane 1, 33 kDa representing the fusion complex of GFP with NS4A protein in lane 2, 98 kDa representing fusion complex of GFP with NS3 in lane 3 while specific band of 98k Da representing the fusion complex of GFP with NS3-4A protein in lane 4. β-actin was used as internal loading control.
Figure 4.11: Western blot analysis of pEGFP-C1/NS3, pEGFP-C1/NS3-4A & pEGFP-C1/NS4A vectors expressing HCV non-structural NS3, NS3-4A and NS4A proteins. Specific band of 27 kDa representing GFP protein in lane 1, 33 kDa representing the fusion complex of GFP with HCV non-structural NS4A protein in lane 2, 98 kDa representing fusion complex of GFP with HCV non-structural NS3 protein in lane 3 while, specific band of 98 kDa representing the fusion complex of GFP with HCV non-structural NS3-4A protein in lane 4. β-actin was used as internal loading control.
4.6 Immunofluorescence analysis of GFP tagged recombinant vectors

To determine the GFP tagged HCV non-structural NS3, NS3-4A and NS4A protein expression transiently, cells were fixed 48 hours post transfection on glass coverslips, mounted with DAPI containing ProLong® Gold Antifade Reagent to stain the nuclei and observed under fluorescence microscope. Figure 4.12 (A), depicts the transfected GFP tagged pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing NS3, NS3-4A and NS4A proteins expressing Huh-7 cells in green, while nuclei counterstained with DAPI in blue. Figure 4.12 (B) depicts the graphical representation of transfection efficacy of GFP tagged expressing cells in percentage, pEGFP-C1 (non-expressing control) shows 59 % transfection, pEGFP-C1/NS3 containing NS3 protein shows 46%, pEGFP-C1/NS3-4A containing NS3-4A protein shows 45 % while pEGFP-C1/NS4A containing NS4A protein shows 55 % transfection.
Figure 4.12 (A): Immunofluorescence analysis of pEGFP-C1/NS3, pEGFP-C1/NS3-4A & pEGFP-C1/NS4A vectors expressing HCV non-structural NS3, NS3-4A and NS4A proteins. Huh-7 cells transiently expressing pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (left panel) were stained with DAPI (middle panel) to stain nuclei. Overlaid pictures (right panel) showing transfected and untransfected cells.
Figure 4.12 (B): Graphical representation of pEGFP-C1/NS3, pEGFP-C1/NS3-4A & pEGFP-C1/NS4A vectors expressing HCV non-structural NS3, NS3-4A and NS4A proteins. pEGFP-C1 (non-expressing control) shows 59% transfection efficiency, NS3/GFP shows 46%, NS3-4A/GFP shows 45% while NS4A/GFP shows 55% transfection efficiency.
4.7 Immunofluorescence analysis of FLAG tagged recombinant vectors

FLAG tagged HCV non-structural NS3, NS3-4A and NS4A expression transiently, cells were fixed 48 hours post transfection on glass coverslips, incubated first with anti-FLAG antibody mouse and second with Alexa Fluor 488 anti-mouse (green) antibody and then mounted with ProLong® Gold Antifade Reagent with DAPI to stain the nuclei and observed under fluorescence microscope. Figure 4.13 (A) depicts the transfected FLAG tagged pFLAG-CMV2 (non-expressing control), pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A, pFLAG-CMV2 /NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins expressing Huh-7 cells in green and nuclei counterstained with DAPI in blue. Figure 4.13 (B) depict the graphical representation of transfection efficacy of FLAG tagged expressing Huh-7 cells in percentage, pFLAG-CMV2 (non-expressing control) shows 58 % transfection, pFLAG-CMV2 /NS3 containing NS3 protein shows 48 %, pFLAG-CMV2 /NS3-4A containing NS3-4A protein 50 % while pFLAG-CMV2 /NS4A containing NS4A protein shows 49 % transfection.
Figure 4.13 (A): Immunofluorescence analysis of pFLAG-CMV2/NS3, pFLAG-CMV2/NS3-4A, pFLAG-CMV2/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins. Huh-7 cells transiently expressing pFLAG-CMV2 (non-expressing control), pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A and pFLAG-CMV2 /NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (left panel) were incubated first with anti-flag mouse antibody and second with Alexa Fluor 488 anti-mouse antibody (left panel) and counter stained with DAPI (middle panel) to stain nuclei. Overlaid pictures (right panel) showing transfected and untransfected cells.
Figure 4.13 (B): Graphical representation of pFLAG-CMV2/NS3, pFLAG-CMV2/NS3-4A, pFLAG-CMV2/NS4A vectors expressing HCV non-structural NS3, NS3-4A and NS4A proteins. pFLAG-CMV2 (non-expressing control) shows 58 % transfection efficiency, NS3/FLAG containing NS3 protein shows 48 %, NS3-4A/FLAG containing NS3-4A protein shows 50 % while NS4A/FLAG containing NS4A protein shows 49 % transfection efficiency.
4.8 HCV non-structural NS3-4A and NS4A proteins induces cell death

One of the most common applications of crystal violet dye use is to determine the maximum cell viability by checking the absorbance of the dye taken up by the cells (Gillies, Didier et al., 1986; Shaik, Chatterjee et al., 2004; Thomas, Finnegan et al., 2004). To check the cell viability in pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins. Huh-7 cells transfected in three sets of experiments were used for each vector and cell viability was determined by measuring crystal violet dye absorbance of living cells. pEGFP-C1 (non-expressing control) shows 100% cell viability, pEGFP-C1/NS3 containing NS3 protein shows 65%, pEGFP-C1/NS3-4A containing NS3-4A protein shows 45% and pEGFP-C1/NS4A containing NS4A protein shows 46% cell viability (Fig. 4.14 B). These results depict maximum cell death in NS3-4A and NS4A expressing Huh-7 cells (Fig. 4.14 A).
Figure 4.14: Cell death induced by HCV non-structural NS3-4A and NS4A proteins. Huh-7 cells transiently expressing pEGFP-C1 (empty vector/ non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins were examined for cell viability by crystal violet cell viability assay. (A) Huh-7 cells transfected with above mentioned vectors stained with crystal violet dye which clearly showing the cell death in HCV non-structural NS3-4A and NS4A proteins expressing
cells. (B) Graphical representation of cell viability of vectors-harbouring cells. \( P \) values were calculated by using an unpaired Student’s t-test (mean ± SEM; \( n=03; ***p<0.001, **p<0.01 \)).

While pFLAG-CMV2 (non-expressing control), pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A and pFLAG-CMV2 /NS4A vectors containing NS3, NS3-4A and NS4A proteins were also used to check the cell viability. Huh-7 cells transfected in three sets of experiments were used for each vectors. pFLAG-CMV2 (non-expressing control) shows 100 % cell viability, pFLAG-CMV2 /NS3 containing NS3 protein shows 62 %, pFLAG-CMV2 /NS3-4A containing NS3-4A protein shows 45 % and pFLAG-CMV2 /NS4A containing NS4A protein shows only 48 % viability (Fig. 4.15 B). These results depict maximum cell death in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig. 4.15 A).
Figure 4.15: Cell death induced by HCV non-structural NS3-4A and NS4A proteins. Huh-7 cells expressing pFLAG-CMV2 (non-expressing control), pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A, pFLAG-CMV2/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins were examined for cell viability by crystal violet cell viability assay. (A) Huh-7 cells transfected with above mentioned vectors stained with crystal violet dye which clearly showing the cell death in HCV non-structural NS3-4A and NS4A proteins expressing cells. (B)
Graphical representation of cell viability of vectors-harbouring cells. $P$ values were calculated by using an unpaired Student’s t-test (mean ± SEM; n=03; ***p<0.001, **p<0.01).

4.9 **Intracellular localizations of HCV non-structural NS3, NS3-4A and NS4A proteins**

We examined the intracellular localization of HCV non-structural NS3, NS3-4A and NS4A proteins to endoplasmic reticulum and mitochondria. GFP tagged pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing NS3, NS3-4A and NS4A proteins were used to observe localization. KDEL antibody was used to stain Endoplasmic reticulum while MitoTracker CMXRos Red was used to stain mitochondria in live cells. Confocal immunofluorescence microscopic analysis showed that all HCV non-structural NS3, NS3-4A and NS4A proteins were localized on endoplasmic reticulum (Fig.4.16). While in the case of Mitochondria, only HCV non-structural NS3-4A complex and NS4A proteins shown significance localization to the perinuclear region (Fig.4.17). These analyses suggest that HCV non-structural NS4A protein alone or in complex with NS3 induces mitochondrial perinuclear clustering.
Figure 4.16: Intracellular localizations of HCV non-structural NS3, NS3-4A and NS4A proteins on endoplasmic reticulum. Huh-7 cells transiently expressing pEGFP-C1 (empty vector/ non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (left panel) were stained with KDEL-ER marker primary antibody (middle panel). Overlaid pictures show in the (right panel). Yellow color in the overlaid pictures showing that HCV non-structural NS3, NS3-4A & NS4A localized on endoplasmic reticulum.
Figure 4.17: Intracellular localizations of HCV non-structural NS3, NS3-4A and NS4A proteins on mitochondria. Huh-7 cells transiently expressing pEGFP-C1 (empty vector/non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (left panel) were stained with mitotracker (CMXROS) to stain mitochondria (middle panel). Overlaid pictures are shows in the (right panel). Yellow color in the overlaid pictures showing that HCV non-structural NS3-4A & NS4A localized on mitochondria.
4.10 HCV non-structural NS3-4A and NS4A proteins induces mitochondrial fragmentation/fission

To examine the induced mitochondrial dynamics changes in HCV non-structural NS3-4A, NS4A proteins expressing Huh-7 cells, transfected cells were stained with membrane potential dependent mitotracker-Red. The mitochondria network was severely damaged in pEGFP/NS3-4A and pEGFP/NS4A expressing cells, while in pEGFP-C1 (non-expressing control) and pEGFP/NS3 expressing cells shows filamented mitochondria with no damage (Fig. 4.18A). Graphical representation of percentage of fragmented mitochondria shows almost 82% fragmented mitochondria in pEGFP/NS4A containing NSA protein expressing cells, 78% in pEGFP/NS3-4A containing NS3-4A protein expressing cells, while 4% in pEGFP/NS3 containing NS3 protein expressing cells and 1% in pEGFP-C1 (non-expressing control) (Fig. 4.18B). Hence these results collectively suggest that HCV non-structural NS4A protein alone and in complex (NS3-4A) localized into mitochondria and disrupt its morphology.
Figure 4.18 (A): HCV non-structural NS3-4A and NS4A proteins induce mitochondrial fragmentation. Huh-7 cells transiently expressing pEGFP- C1 (empty vector/ non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (middle panel) were stained with mitotracker (CMXROS) to stain mitochondria (right panel). Overlaid pictures are showing in the (left panel). HCV non-structural NS3-4A & NS4A proteins expressing cells (right panel) clearly shows the mitochondrial fragmentation. Areas marked with green boxes were enlarged (zoom-in).
Figure 4.18 (B): Graphical representation of HCV non-structural NS3-4A and NS4A proteins induces mitochondrial fragmentation. Cells with fragmented mitochondria were counted randomly. *P values were calculated by using an unpaired Student’s t-test (mean ± SEM; n=03; ***p<0.001, *p<0.05).
4.11 BAX translocation and Bcl-X<sub>L</sub> regulation in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells

BAX is multidomain protein of Bcl-2 family a pro-apoptotic protein and essential for all events of apoptosis via the intrinsic pathway (Oltvai, Milliman <em>et al.</em>, 1993; Wei, Zong <em>et al.</em>, 2001). Normally BAX is expressed in an inactive state in the cytosol and in response to apoptotic stimuli, conformational alterations regulates its activation and ultimately translocate the BAX towards mitochondria (Crow, Mani <em>et al.</em>, 2004). While Bcl-X<sub>L</sub> is an anti-apoptotic protein, mostly localize to the outer membrane of mitochondria and inhibit the cytochrome c release to the cytosol (Krajewski, Tanaka <em>et al.</em>, 1993; Kluck, Bossy-Wetzel <em>et al.</em>, 1997).

GFP tagged pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins were transfected to analyze the BAX translocation. Confocal immunofluorescence analysis shows the maximum BAX protein translocation towards mitochondria in HCV non-structural NS3-4A and NS4A expressing cells, while NS3 and pEGFP-C1 (non-expressing control) shows negligible translocation (Fig. 4.19A). Immunoblotting technique was used to analyze the expression levels of BAX and Bcl-X<sub>L</sub> proteins were analyzed using anti-BAX and anti-Bcl-X<sub>L</sub> antibodies. Transfection of Huh-7 cells were done with pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins, cells treated with staurosporine served as positive control, while Huh-7 cells serve as mock. BAX protein expression were observed up regulated in positive control huh-7 cells, HCV non-structural NS3-4A and NS4A proteins expressing cells, while down regulated in mock cells, pEGFP-C1 (non-expressing control) and in pEGFP-C1/NS3 containing HCV non-structural NS3 protein expressing cells (Fig. 4.19 B). In the case of Bcl-X<sub>L</sub>
protein expression, down regulated expression were observed in positive control Huh-7 cells, pEGFP-C1/NS3-4A containing HCV non-structural NS3-4A and in pEGFP-C1/NS4A containing HCV non-structural NS4A protein expressing cells, while up regulated expression was observed in mock cells, pEGFP-C1 (non-expressing control) and in pEGFP-C1/NS3 containing HCV non-structural NS3 protein expressing cells (Fig. 4.19 C). These results overall demonstrate that HCV non-structural protein NS3-4A, NS4A containing Huh-7 expressing cells and positive control shown down regulated expression of Bcl-X₁ protein which is anti-apoptotic in nature while shown up regulated expression of BAX protein which is pro-apoptotic in nature hence, 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.
Figure 4.19 (A): HCV non-structural NS3-4A and NS4A induces BAX translocation to mitochondria. Huh-7 cells transiently expressing pEGFP-C1 (empty vector/ non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (left panel) were incubate with BAX primary antibody rabbit (second left panel) and were stained with mitotracker (CMXROS) to stain mitochondria (second right panel). Yellow color in the overlaid pictures (right panel) showing that in HCV non-structural NS3-4A & NS4A proteins expressing cells, BAX protein induces translocation to mitochondria.
Figure 4.19 (B-C): Western blot analysis of pro-apoptotic BAX protein and Bcl-XL protein.

Blot was probed with anti-BAX 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. (C) Western blot analysis of anti-apoptotic Bcl-XL protein. Blot was probed with anti-Bcl-XL 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.
4.12 HCV non-structural NS3-4A and NS4A proteins increased mitochondrial superoxide generation in Huh-7 cells

Oxidative phosphorylation of complexes I and III of electron transport chain produce mitochondrial superoxide as byproduct (Liu, Fiskum *et al*., 2002; Kudin, Bimpong-Buta *et al*., 2004). In the presence of viral proteins or other environmental stress ROS level become increases dramatically. For detection of superoxide in the mitochondria of live cells a MitoSOX™ Red mitochondrial superoxide indicator a fluorogenic dye with highly selective detection was used. To estimate the mitochondrial superoxide generation in GFP tagged pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing NS3, NS3-4A and NS4A proteins expressing cells, 72 hrs post transfection cells were stained with MitoSOX™ Red dye. Immunofluorescence images show highly increased superoxide generation in pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors expressing HCV non-structural NS3-4A and NS4A proteins expressing cells and almost none in pEGFP-C1 (non-expressing control) and pEGFP-C1/NS3 containing HCV non-structural NS3 protein expressing cells (Fig. 4.20 A). Figure 4.20 B depicts the graphical representation of percentage of superoxidation in mitochondria, in which pEGFP-C1/NS4A containing HCV non-structural NS4A proteins expressing cells shows 84 % superoxide generation, pEGFP-C1/NS3-4A containing HCV non-structural NS3-4A protein shows 79 %, pEGFP-C1/NS3 containing HCV non-structural NS3 protein shows 4 % and pEGFP-C1 (non-expressing control) shows 3 % generation.
Figure 4.20 (A): HCV non-structural NS3-4A and NS4A proteins induced the mitochondrial superoxide generation. Huh-7 cells transiently expressing pEGFP- C1 (empty vector/ non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins (left panel) were stained with MitoSOX™ Red mitochondrial superoxide (right panel). Overlaid pictures show in the (middle) panel. Right panel pictures showing that HCV non-structural NS3-4A & NS4A proteins expressing cells generate high amount of superoxide species.
Figure 4.20 (B): Graphical representation of HCV non-structural NS3-4A and NS4A proteins increased the mitochondrial superoxide generation. Cells with increased level of superoxide generation were counted randomly. $P$ values were calculated by using an unpaired Student’s t-test (mean ± SEM; n=03; ***p<0.001).
4.13 Cytochrome c translocation in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells

During mitochondrial mediated apoptosis outer membrane permeabilization allowed cytochrome c release to cytosol (Goldstein, Waterhouse et al., 2000). To assess the cytochrome c translocation, Huh-7 cells were transfected with pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins, cells treated with staurosporine served as positive control, while Huh-7 cells serve as mock. Immunoblot analysis of mitochondrial fractions shows down regulated expression in positive control, NS3-4A and NS4A expressing cells and up regulated in mock, pEGFP-C1 (non-expressing control) and in HCV non-structural NS3 protein expressing cells (Fig. 4.21 A), while cytosolic fraction shows up regulated expression in positive control, NS3-4A and NS4A expressing cells and down regulated in mock, pEGFP-C1 (non-expressing control) and in NS3 expressing cells (Fig.4.21 B). These results collectively represented the mitochondrial membrane damage and increase in membrane permeability.
Figure 4.21: Cytochrome c translocation in HCV non-structural NS4A and NS3-4A proteins expressing cells. Huh-7 cells were transfected with pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins. Huh-7 cells treated with staurosporine served as positive control, while only Huh-7 cells serve as mock. (A) Down regulated expression was observed in mitochondrial fractions in positive control (treated with Staurosporine), pEGFP-C1/NS3-4A & pEGFP-C1/NS4A vectors containing HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells. β-actin was used as an internal loading control. (B) Up regulated expression was observed in cytosolic fractions in positive control (Huh-7 cells treated with Staurosporine), pEGFP-C1/NS3-4A & pEGFP-C1/NS4A vectors containing HCV non-structural NS3-4A and NS4A proteins expressing huh-7 cells. β-actin was used as an internal loading control.
4.14 Effect of HCV non-structural NS3-4A and NS4A proteins on the mitochondrial oxidative phosphorylation system

Previous studies show that HCV infection affects the oxidative phosphorylation (Piccoli, Scrima et al., 2007; Kim, Syed et al., 2013). Hence, we designed an experiment to compare the effects of OXPHOS Complex I activity between whole HCV genome and individual HCV non-structural proteins i.e. NS3, NS4A and NS3-4A. Huh-7 cells infected with HCVcc for 3 days while only Huh-7 cells serve as mock. Graphical representation shows the percentage activity of OXPHOS Complex I in Transfection of Huh-7 cells was done with pFLAG-CMV2 /NS3, pFLAG-CMV2 /NS3-4A, pFLAG-CMV2 /NS4A vectors containing HCV non-structural proteins and pFLAG-CMV2 vector serve as (non-expressing control). 72 hrs post transfection cells were harvested and mitochondrial I enzyme activity was analyzed as per manufacturer’s instruction. NS3-4A protein expressing cells shows total 16 % reduction, NS4A protein expressing cells shows 19 % reduction while NS3 protein expressing cells shows only 15 % reduction (Fig.4.22 B). Expression levels of HCV non-structural NS3, NS3-4A and NS4A proteins used in assay were confirmed by immunoblotting (Fig.4.22 C). From these results we inferred that HCV non-structural NS3, NS3-4A, NS4A proteins individually and HCVcc leads to overall 15-20 % reduction of OXPHOS Complex I activity as compared to mock.
Figure 4.22 (A): Graphical representation of OXPHOS Complex I activity between whole HCV genome (HCVcc) and Huh-7 cells (mock). Huh-7 cells infected with HCVcc and harvested 3 days post infection while only Huh-7 cells used as mock. Activity of mitochondrial I enzyme was measured according to manufacturer’s instruction. $P$ values were calculated by using an unpaired Student’s t-test (mean ± SEM; n=03; **p<0.01).
Figure 4.22 (B): Graphical representation of the effect of HCV individual HCV non-structural NS3, NS3-4A, NS4A proteins on the reduction of OXPHOS Complex I activity. Huh-7 cells expressing HCV non-structural NS3, NS3-4A, NS4A proteins and pFLAG-CMV2 vector (non-expressing control) were transfected for 72 hrs and used to analyze the activity of mitochondrial I enzyme. \( P \) values were calculated by using an unpaired Student’s t-test (mean ± SEM; \( n=03; **p<0.01, *p<0.05 \).
Figure 4.22 (C): Western blot analysis of HCV non-structural NS3, NS3-4A and NS4A proteins used for the assay reduction of OXPHOS Complex I activity. β-actin was used as an internal loading control.
4.15 Caspase -3, 7, 9 activation and poly (ADP-ribose) polymerase (PARP) cleavage in HCV non-structural NS3-4A and NS4A expressing Huh-7 cells

After destruction of mitochondrial functions, next step of intrinsic apoptosis is to activate procaspases (Crow, Mani et al., 2004). Once cytochrome c released to cytosol it works together with Apaf-1 (apoptotic protease activating factor 1) to activate procaspase-9 an initiator caspase (Lawen, 2003). Activation of caspase-9 then activates procaspase-7 and 3 (effector caspases). Further caspase-3 plays an important role in the cleavage of PARP (poly (ADP-ribose) polymerase) (Boulares, Yakovlev et al., 1999). Active caspase-3 become capable to cleave cellular substrate PARP which ultimately induces apoptosis is the cell i.e. blebbing of membrane, DNA fragmentation and deterioration of cell structure (Coleman, Sahai et al., 2001; Leverrier and Ridley, 2001; Sebbagh, Renvoize et al., 2001).To confirm the activation of caspase-9, 7, 3 and cleavage of PARP cells were transfected with pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins. Cells treated with staurosporine served as positive control, while Huh-7 cells serve as mock. Blot treated with anti procaspase 9 antibody shown 47 kDa band representing procaspase-9 in all experimental cell while beside 47 kDa, a polypeptide of 35 kDa representing the cleavage form of procaspase-9 have also observed in positive control, HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig.4.23 A). Blot treated with anti procaspase-7 antibody shown 35 kDa band of procaspase-7 in all experimental cells while beside 35 kDa, a polypeptide of 19 kDa band has also observed in positive control, HCV non-structural NS3-4A and NS4A protein expressing Huh-7 cells representing the active form of caspase-7 (Fig.4.23 B). For capase-3 activation blot were treated with anti procaspase-3 antibody, all experimental cells shown procaspase-3 expression with 35 kDa band while positive control,
HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells have shown a polypeptide of 17 kDa band representing the caspase-3 active form (Fig. 4.23C). Consistent with the caspase-3 activation, western blot analysis of all experimental cells shown the full length inactive form of PARP protein with 116 kDa band while positive control, HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells shown the cleavage of PARP protein into a smaller unit of 85 kDa band (Fig. 4.23 D).
Figure 4.23: (A) Western blot analysis of Caspase-9 protein in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells. Huh-7 cells 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-9 antibody and activation of procaspase-9 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.
Figure 4.23: (B) Western blot analysis of Caspase-7 protein in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells. Huh-7 cells 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-7 antibody and activation of procaspase-7 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 Huh-7 cells. β-actin was used as an internal loading control.
Figure 4.23: (C) Western blot analysis of Caspase-3 protein in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells. Huh-7 cells 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 activation of procaspase-3 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 Huh-7 cells. β-actin was used as an internal loading control.
Figure 4.23: (D) Western blot analysis of PARP protein in HCV non-structural NS3-4A and NS4A proteins expressing cells. Huh-7 cells 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 PARP antibody and cleavage of PARP 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 Huh-7 cells. β-actin was used as an internal loading control.
4.16 Chromatin condensation in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells

Apoptosis ends on chromatin condensation which is one of the most important occasion. Caspase-3, -7 (execution caspases) activates cytoplasmic endonucleases and proteases which degrades nuclear material and cytoskeletal proteins in the cell (Elmore, 2007). Endonuclease CAD (Caspase-activated DNAses) degrades chromosomal DNA and cause the chromatin condensation (Sakahira, Enari et al., 1998). To analyze the condensed nuclei in HCV non-structural NS4A and NS3-4A proteins expressing Huh 7 cells, cells were transfected with pEGFP-C1 (non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins. Cells were harvested at different time points (24 hrs, 48 hrs & 72 hrs) and stained with Hoechst 33342 stain and were observed under the florescence microscope. More condensed nuclei were observed at 72 hrs post transfection in pEGFP-C1/NS3-4A and pEGFP-C1/NS4A vectors containing HCV non-structural NS3-4A and NS4A proteins while pEGFP-C1 (non-expressing control), pEGFP-C1/NS3 vector containing HCV non-structural NS3 protein expressing cells shown negligible amount of condensed nuclei (Fig. 4.24 A). Fig. 4.24 B depicts the graphical representation of percentage cells with condensed nuclei in which pEGFP-C1/NS3-4A vector containing HCV non-structural NS3-4A shown 48 % condensed nuclei, pEGFP-C1/NS4A vectors containing HCV non-structural NS4A shown 50 % while pEGFP-C1 (non-expressing control) and pEGFP-C1/NS3 vector containing HCV non-structural NS3 shown only 10-15 % condensed nuclei at 72 hrs post transfection.
Figure 4.24 (A): Chromatin condensation in HCV non-structural NS3-4A and NS4A proteins expressing cells. Huh-7 cells transiently expressing pEGFP-C1 (empty vector/non-expressing control), pEGFP-C1/NS3, pEGFP-C1/NS3-4A, pEGFP-C1/NS4A vectors containing HCV non-structural NS3, NS3-4A and NS4A proteins Huh-7 cells were harvested 24 hrs (left panel), 48hrs (middle panel) and 72 hrs (right panel) post transfection. Nuclei of the cells were stained with Hoechst 33342 stain. 72hrs post transfected stained nuclei showing more condensed
nuclei (bright) in pEGFP-C1/NS3-4A & pEGFP-C1/NS4A vectors containing HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells.

Figure 4.24 (B): Graphical representation of chromatin condensation in NS3-4A and NS4A expressing cells. Cells with condensed nuclei were counted randomly. $P$ values were calculated by using an unpaired Student’s t-test (mean ± SEM; n=03; ***p<0.001).
Discussion
Hepatitis C virus (HCV) is a major cause of viral hepatitis in humans. It is a blood borne pathogen that targets hepatocytes and moves from acute to chronic phase, favoring complicated hepatic and extra-hepatic pathologies like apoptosis, fibrosis and cirrhosis, eventually leads to hepatocellular carcinoma (HCC) (Qadri, et al., 2004; Howe et al., 2004; Joyce et al., 2009). A strong association is being documented between chronic HCV infection with genotype 3a and HCC (63.44 % of tested HCC patients) in Pakistani population (Idrees et al., 2009). HCV shows hepatocyte specific tropism, where it propagates, infects the neighboring hepatocytes and takes control over the host machinery by perturbing normal signaling pathways to favor its replication (Clement, et al., 2009; Joyce et al., 2009).

HCV induces several complex pathways leading to generation of reactive oxygen species, hepatic inflammation, hepatic fibrosis and HCC (Fartoux et al., 2005; 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 but the factors involved in HCV induced pathogenesis are still unclear (Sheikh et al., 2008; Joyce et al., 2009).

Many viruses including HCV have been shown to induce oxidative stress during replication and/or protein expression. 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; Ghaziani 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 which lead the cells towards the mitochondrial mediated apoptotic pathway.

In the present study we examined the possible effects of HCV non-structural NS4A protein alone and in complex with NS3 (NS3-4A) in the induction of apoptosis via mitochondrion mediated pathway. 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, Kolykhalov et al. 2000; Lindenbach, Evans et al. 2005). In previous study novel clones of HCV genotype 3a infectious cDNA named as strain S52 and of genotype 4a known as strain ED43, however robust cell culture model for expression of individual hepatitis C virus proteins of different genotypes, especially genotype 3a are still missing. (Gottwein, Scheel et al. 2010) From Pakistan Butt et al. (Butt, Idrees 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 & NS4A genes of HCV genotype 3a separately in pFLAG-CMV2 and pEGFP-C1 expression vectors. Positive clones were confirmed by restriction digestion analysis and sequencing from both directions. Each particular clone was transfected separately to Huh-7 cells and expression was analyzed using Western blot assays. Expression of transfected cells were also analyzed using fluorescence microscopy after having probed with particular fluorescent antibodies. pFLAG-CMV2 expression vector used in the present study has the ability for intracellular transient expression of N-terminal Met-FLAG which is fused with desired HCV non-structural NS3, NS3-4A and NS4A 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 HCV non-structural NS3, NS3-4A and NS4A proteins and can easily detected by using anti GFP antibody.

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. Efficient cell culture based systems have the ability to express individual HCV non-structural NS3, NS3-4A and NS4A protein separately in Huh-7 cells.

Apoptosis, programmed/ planned cell death or cell suicide, is a normal physiological process and occurs normally during development, aging, hemostasis mechanism and defense mechanism in pathological conditions (Kerr, Winterford et al. 1994; Elmore 2007). Different studies suggest that apoptosis occurs in the human hepatocytes mostly by mitochondria-mediated apoptosis pathway but still it is under investigations that whether apoptosis is mediated by T lymphocytes or by HCV replication itself. (Bantel and Schulze-Osthoff 2003; Deng, Adachi et al. 2008). HCV infection prompts Bax-induced, mitochondrial mediated, caspase-3 dependent apoptosis when infected Huh-7.5 cells with chimeric J6/JFH1 strain of HCV genotype 2a was used in their experiments (Deng, Adachi et al. 2008). Zhu et al. was successful in culturing a human hepatoma novel cell line (LH86) permitted JFH-1 HCV genotype 2a infection which induced cell apoptosis which was related to the viral replication in the cell.

Different structural and non-structural proteins of HCV are reported to be involved in the induction of the mitochondrial-mediated caspase-3 dependent apoptosis pathway when expressed in different cell culture systems. (Chou, Tsai et al. 2005; Chiou, Hsieh et al. 2006; Nomura-Takigawa, Nagano-Fujii et al. 2006). HCV Core protein expressing in Huh7 cells activated TRAIL-mediated apoptosis during the chronological induction of caspase-8 activation by
triggering of mitochondrial mediated apoptosis signaling pathway (Chou, Tsai et al. 2005). HCV E2 protein of HCV have the capability to induce apoptosis because they have the genetic similarity with the envelope proteins of other viruses of the flaviviridae family i.e. dengue and Langat virus (Chiou, Hsieh et al. 2006). While Nomura-Takigawa et al. reported that HCV non-structural NS4A (genotype 1b) is probably involved in the induction of apoptosis through mitochondrial mediated apoptosis while HCV non-structural NS3-4A complex is more prone to translocate towards mitochondria as compared to HCV non-structural NS3 protein alone. Our study clearly shown that HCV non-structural NS4A protein (genotype 3a) alone and in complex with NS3 (NS3-4A) induces apoptosis via Bax-induced, mitochondrial mediated, caspase -3 dependent pathway.

Our results indicated the significant number of cell death in Huh-7 cells expressing HCV non-structural NS3-4A and NS4A proteins in contrast to NS3 expressing and vector transfected non-expressing control cells (Fig. 4.1). Intracellular compartmentalization of HCV protein is considered as an important aspect and it is clearly understood that HCV structural and non-structural proteins accumulates on ER for maturation (Moradpour, Penin et al. 2007), while some of HCV protein were reported to be localized on mitochondria as well. Our results clearly shows the localization of HCV non-structural NS3, NS3-4A and NS4A proteins to ER (Fig. 4.2A) while only NS3-4A complex and NS4A shown localization on the mitochondria (Fig. 4.2B) and this result confirms that both HCV non-structural NS3-4A and NS4A proteins localized on mitochondria and disturb the mitochondrial dynamics. Furthermore our results clearly showed the significant mitochondrial fragmentation in HCV NS3-4A and NS4A expressing Huh-7 cells (Fig. 4.3). Bax proteins and Bcl-2 proteins belong to Bcl-2 family that takes part in prominent role in mitochondrial mediated apoptotic pathway and in addition to the regulation of
cytochrome c release from mitochondria to cytosol (Borner 2003; Qi, Li et al. 2010). 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 protein to integrate in the mitochondria. In the current study we have observed increased accumulation of Bax and conformational changes on mitochondria in the HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig 4.4A). The current study also demonstrated the up regulated expression of Bax protein and down-regulated expression of Bcl-2 protein in HCV non-structural NS3-4A and NS4A expressing Huh-7 cells (Fig. 4.4B & C). These results overall demonstrate that HCV non-structural protein NS3-4A, NS4A containing Huh-7 expressing cells and positive control shown down regulated expression of Bcl-X\textsubscript{L} protein which is anti-apoptotic in nature while shown up regulated expression of BAX protein which is pro-apoptotic in nature hence, 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.

I observed increased production of mitochondrial superoxide in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig. 4.5) because mitochondrial superoxide is an important supply of reactive oxygen species (ROS) in the cell and may contribute to prompt the conformational changes of Bax protein, its dimerization and mitochondrial translocation (D'Alessio, De Nicola et al. 2005; Nie, Tian et al. 2008). Bax protein ultimately integrates into the outer mitochondrial membrane (OMM), and from mitochondria cytochrome c releases into the cytosol. The present research found HCV non-structural NS3-4A and NS4A proteins expression prompted the release of cytochrome c into the cytosol (Fig. 4.6). Activation of caspases takes part as dominant character in the completion of apoptosis and in the same manner
released cytochrome c from mitochondria activates effector caspases. Normally cytochrome c is located on the mitochondrial inner membrane although its cofactors Apaf-1 and procaspase-9 are expressing in the cytosol. Once Cytochrome c is released to the cytosol forms a multimeri Apaf-1/cytochrome c complex and ultimately activates the procaspase-9. After the activation of caspase-9, it cleaves and activate the downstream caspase i.e. caspase-7 and -3 (Budihardjo, Oliver et al. 1999; Denault and Salvesen 2002; Lawen 2003; Gogvadze, Orrenius et al. 2006). Further 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. (Boulares, Yakovlev et al. 1999; Loeffler and Kroemer 2000; Lockshin and Zakeri 2001). Western blot analysis in our study demonstrates the activation of caspase-9, caspase-7, caspase -3, and cleavage of PARP in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig. 4.8 A, B, C & D).

Various research studies clearly indicate that affects of HCV infection on the respiratory chain complex I enzyme (NADH dehydrogenase) of mitochondria via oxidative phosphorylation which ultimately promote the destruction of mitochondrial outer membrane (Korenaga, Okuda et al. 2005; Korenaga, Wang et al. 2005; Piccoli, Scrima et al. 2007; Kim, Syed et al. 2013). In the present study we compare the consequences of OXPHOS Complex I activity of HCV infected cells (HCVcc) with HCV non-structural NS3, NS3-4A and NS4A proteins expressing Huh-7 cells in which our results shown that there is a 15 % reduction in HCVcc and NS3 expressing cells (Fig. 4.7A) and 18-20 % reduction in HCV non-structural NS3-4A and NS4A proteins expressing Huh-7 cells (Fig. 4.7B) as compared mock. These results clearly suggest that HCV non-structural NS3-4A and NS4A proteins accumulate on mitochondria hence promote the destruction of mitochondrial outer membrane and induces the mitochondrial-mediated apoptosis.
Chromatin condensation which is the terminal stage of apoptosis is the clear evidence of apoptosis induced cell death. Chromatin condensation has been observed in NS3-4A and NS4A expressing cells at 72 hrs post transfection (Fig. 4.9). In conclusion, current study clearly demonstrates the directly involvement of HCV genotype 3a non-structural NS3-4A and NS4A proteins in the triggering of Bax translocated, mitochondrial-mediated, Caspase-3 dependent apoptotic pathway.

**Future prospects**

The role of NS3-4A and NS4A proteins in mitochondrial-mediated apoptosis pathway can also be confirm by synthesis of the mutants of these particular proteins by producing mutation at different conserved regions in their genes. Beside the investigation of HCV non-structural NS3, NS4A and NS3-4A proteins role in the induction of mitochondrial mediated apoptosis pathway, these transiently expressing cell lines can also be used and facilitate 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.
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Chapter 6

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