STUDY OF INHIBITION OF INTERFERON INDUCIBLE GENES BY DEPHOSPHORYLATION OF E2 ENVELOPE GENE OF HCV GENOTYPE 1a

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CENTRE OF EXCELLENCE IN MOLECULAR BIOLOGY UNIVERSITY OF THE PUNJAB LAHORE, PAKISTAN (2012)
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BY

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BE ALL PRAISES TO ALLAH
In the name of ALLAH
the most merciful & compassionate
the most gracious and beneficent
whose help and guidance always solicit
at every step, at every moment
THE HOLY PROPHET MUHAMMAD (S.A.W.W) SAID

“Knowledge is the lost wealth of the Muslim,
Collect it where ever you find it.”
QUAID SAID

Addressing to the students at University, Quaid said that, "It is the responsibility of the students to give up all other interests today and devote all attention toward getting the Education with Honesty, because they have to take up the charge of country matters tomorrow"
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To

Raza ur Rehman
Ibrahim, Fatima
Hepatitis C is a major health problem affecting more than 200 million individuals in the world including Pakistan. Current treatment regimen consisting of interferon alpha and ribavirin does not always succeed to eliminate virus completely from the patient’s body. The mechanism how Hepatitis C Virus (HCV) induces interferon resistance is still indefinable. HCV genotype 1a shows greater hindrance to treatment than genotype 3a. One of the mechanisms by which virus evades the antiviral effect of interferon alpha involves that HCV envelope protein 2 (E2) interacts with Protein Kinase (PKR) which is the interferon-inducible protein kinase and which in turn blocks the activity of its target molecule called eukaryotic translation initiation factor 2 alpha (eIF2α). Sequence analysis of Envelope protein reveals it contains a domain homologous to phosphorylation sites of PKR and the translation initiation factor eIF2alpha. This domain is known as protein kinase (PKR) eukaryotic initiation factor 2 alpha (eIF2α) phosphorylation homology domain (PePHD). This domain in HCV genotype 1 strains is reportedly homologous to PKR and its target eIF2α. Thus envelope protein competes for phosphorylation with PKR. By binding to PKR, PePHD inhibits its activity and therefore cause virus to evade antiviral activity of interferon (IFN).

In the present study the possible role of phosphorylation in envelope 2 protein for interferon resistance was first investigated in silico and then confirmed invivo. Genes coding for envelope 2 protein were isolated from local HCV isolates and their tertiary structure was predicted. Insilico phosphorylation of tertiary structures revealed that two residues S75 and S277 of envelope 2 gene are surface exposed at cytoplasmic domain and may compete with the phosphorylation of PKR protein. Interferon induced antiviral protein PKR has a role in the HCV treatment as dsRNA activated PKR has the capacity to phosphorylate the serine and threonine of E2 protein and dimerization of viral RNA. E2 gene of HCV genotype 1 has an active role in IFN resistance. E2 protein inhibits and terminates the kinase activity of PKR by blocking it in protein synthesis and cell growth. This brings forward a possible relation of E2 and PKR through a mechanism via which HCV evades the antiviral effect of IFN.

A hybrid in-silico and wet laboratory approach of motif prediction, evolutionary and structural analysis has pointed out serine 75 and 277 of the HCV E2 gene as a promising candidate for the serine phosphorylation. It is proposed that Serine phosphorylation of HCV E2 gene has a significant role in interferon resistance. In present study we mutated the two Serine
residues at positions S75 and S277 and their efficacy was checked in respect to interferon resistance. The results of this study suggest that serine residues as predicted have a significant role in interferon resistance, especially S75. It is also suggested that some other factors may be involved along with viral envelope gene 2.

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Although feelings are deep but unfortunately the words are too shallow, that cannot express the depth of my feelings.

All praises and thanks for Almighty Allah, who is the ultimate source of all knowledge to mankind and for His endless blessings for humanity. Who made me reach at present pedestal of knowledge with quality of doing something novel, sensational, and path bearing.

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May Allah Almighty give me the courage to make pace with the expectations of Higher Education Commission, my center of excellence, my family and my country.

Samia Afzal
# ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCV</td>
<td>Hepatitis-C Virus</td>
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<tr>
<td>HDV</td>
<td>Hepatitis D virus</td>
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<tr>
<td>HEV</td>
<td>Hepatitis E virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency Virus</td>
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<tr>
<td>NANBH</td>
<td>non-A, non-B hepatitis</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>5′UTR</td>
<td>5′ untranslated region</td>
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<tr>
<td>SVR</td>
<td>Sustained virological response</td>
</tr>
<tr>
<td>HVR1</td>
<td>Hyper variable region 1</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>Peg IFN</td>
<td>Pegylated Interferon</td>
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<tr>
<td>RBV</td>
<td>Ribavirin</td>
</tr>
<tr>
<td>RVR</td>
<td>Rapid virological response</td>
</tr>
<tr>
<td>EVR</td>
<td>Early virological response</td>
</tr>
<tr>
<td>ETR</td>
<td>End of treatment response</td>
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<tr>
<td>eIF2a</td>
<td>Eukaryotic initiation factor 2alpha</td>
</tr>
<tr>
<td>PePHD</td>
<td>PKR-eIF2a phosphorylation homology domain interferon alpha</td>
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<td>Huh-7</td>
<td>Human hepatoma cells</td>
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INTRODUCTION

Hepatitis C virus (HCV) is a key universal health issue (Khan et al., 2008) affecting approximately 200 million individuals worldwide and over 4 million in the United States alone, where it is the most common blood-borne infection (Alter, 2007). It is the main reason of persistent liver infection and the most familiar sign for liver transplantation (Brown, 2005). In about 60-85% cases HCV develops to cirrhosis and hepatocellular carcinoma (HCC) (Shepard et al., 2005). Presently, in Pakistani population 17 million people are infected with HCV and 8-10% individuals are HCV carriers (Idrees et al., 2008). HCV is a member of genus, hepacivirus, family Flaviviridae and is a positive sense single stranded RNA virus (Bartenschlager, 2000). HCV genome is about 9.6 kb in length (Jhaveri et al., 2005). The large open reading frame of Viral RNA having 5’ and 3’ untranslated regions that is translated into a single polypeptide of 3010 to 3033 amino acids. It is processed by host as well as viral proteases to yield 10 mature individual proteins out of which 3 are structural and 7 are nonstructural (Penin et al., 2004; Brass et al., 2006). Detailed structure of HCV virus is still unclear. However, the infectious viral particles are composed of lipid envelope glycoproteins E1 and E2 (Budkowska et al., 2009).

In spite of much recent advancements, still no vaccine is available against HCV infection. The current therapy for HCV infection is pegylated interferon alpha as a single therapy or in combination with ribavirin (Bisceglie and Hoofnagle, 2002) but it eradicates the virus in only 50-80% of cases and has serious side effects (Keam and Cvetkovic, 2008). IFN system is the first line of protection against viral infection in mammals (Grandvaux et al., 2002). Many viruses have developed mechanisms to dodge the IFN dependent cellular response (Katze et al., 2002). Amongst these HCV is a significant example in which (70-80%), cases runs away the host defenses and develops a chronic infection.
Pathological outcomes of HCV infection changes from individual to individual, unstable from asymptomatic state to liver fibrosis, steatosis, finally to hepatocellular carcinoma (HCC) (Freze et al., 2001; Lauer and Walker, 2001). The factors upon which the success or failure of the antiviral therapy depends are not known completely, and their recognition characterizes a main confront in HCV virology (Bisceglie and Hoofnagle, 2002). IFN-interacts with cells and modifies the expression of a number of genes (Hayashi et al., 20005; Marcello et al., 2006). E2 glycoprotein is the first viral factor that gets in touch with the host cell surface receptors thus it has an important role in vaccine designing and anti-viral drug development thus it has an important role in vaccine designing and drug objective (Cocquerel et al., 2003; Cooper et al., 1999). It elicits the production of neutralizing antibodies against the virus, and is involved in viral morphogenesis. It binds to external loop of CD81, a tetraspanin found on the surface of many cell types including hepatocytes (Pileri et al., 1998). This viral envelope glycoprotein is an obvious candidate for vaccine development as it is chosen target for humoral and cell-mediated immune responses (Kato et al., 1992; Shirai et al., 1999). Primary objective of HCV vaccine is to initiate potent humoral responses against E2 protein (Steinmann et al., 2008).

After the death of infected cells, virus particles are discharged and they can infect the cells present in close proximity. Interferon is released from the infected cells to inform the neighboring cells about the presence of virus. In its rapid response, PKR is produced by these adjoining cells. HCV resistance to IFN-treatment is partially related to inhibition of interferon induced antiviral protein PKR. Interferon induces many protective mechanisms in cells and amongst these the major role in cell protection from many viruses is illustrated by double-stranded RNA (dsRNA)-activated protein kinase PKR (Kaufman, 2000; Pe’ery and Methews, 2000).

Two HCV proteins (NS5A and E2) are involved in IFN resistance through inhibition of the IFN-a induced double stranded-RNA (dsRNA)-activated protein kinase (PKR) (Gale et al., 1997; Taylor et al., 1999). PKR is a kinase enzyme that reveals varied activities. PKR exhibits autophosphorylation of many serine and threonine positions and dimerization of dsRNA. It also phosphorylates the translation initiation factor eIF-2 (a subunit) that directs towards blockage of protein synthesis (Macquillan, 2009). Phosphorylation of many cellular and viral proteins,
including the human immunodeficiency virus transactivator protein, Tat (Mcmillan et al., 1995; Brand et al., 1997) and 90-kDa proteins from rabbit reticulocytes and human cells is mediated by PKR (Thomis et al., 1992; Kumar et al., 1994; Chou et al., 1995; Zamanian-Daryoush et al., 2000). The roles of the other phosphorylation events are as yet unknown. Because of these properties, PKR is taken as an arbitrator of antiviral and anti-inflammatory role of IFN-a (Stark et al., 1998). E2 protein blocks PKR activation to bypass its function (Gale et al., 1998; Stark et al., 1998; Gale et al., 1998; Taylor et al., 1999; Macquillan et al., 2009). Different genotypes of HCV exhibit different rates of response to IFN-alpha and these variants are characterized by mutations that may be accountable for IFN-alpha resistance. Taylor et al. (1999) reported that HCV E2 protein encloses a 12 amino acid sequence domain that is highly homologous to autophosphorylation positions of PKR and initiation factor eIF2. PKR and eIF2 form a phosphorylation homology domain (PePHD). E2 protein inhibits kinase activity of PKR and terminates its blocking role in protein synthesis and cell growth. This suggests that the relationship of E2 and PKR can be considered as a major mechanism by which HCV evades the antiviral effect of IFN.

This study reports the successful cloning of envelope gene and its mutants from local HCV isolates; the clones were then used to generate stable mammalian cell lines harboring mutated envelope glycoproteins of local HCV isolates on their surface. These cell lines were then used to study the effect of interferon alone and in combination with ribavirin. This mammalian cell lines system has proved to be a good model to study the immune response against envelope proteins during HCV infections (Berger et al., 2009) as the availability of animal models is very restricted and expensive. Besides some limitations, this system gives us better understanding of the role of envelope gene in interferon inhibition in mammalian liver cells and the proteins that are released in effect of interferon like PKR. As E2 protein blocks the activation of PKR, so inhibition of binding of E2 protein with PKR may result in efficient interferon response by infected cells. In future this will be very helpful for developing antiviral therapy that can be used alone or in combination with the existing therapy to reduce the prevalence of HCV related liver diseases in local Pakistani population.
LITERATURE REVIEW

2.1 Liver

Human liver is the largest gland in the body located in the right upper abdomen, below the diaphragm in the thoracic region. Adult liver weighs about 1230-1450g for men and for women 1100-1130g. The main cell populations of the liver are, Hepatocytes, Kupffer cells, Liver sinusoidal endothelial cells and Hepatic stellate cells (Kumar et al., 1992).

![Structure and location of human liver](image)

Figure 2.1: Structure and location of human liver. (Adapted from webMD.com)

2.1.1 Functions of liver

Main function of liver is hepatic bile secretion. Liver cells continue to generate bile acid and bile secretion for food digestion. It has a major role in the metabolism of fats. Metabolic functions include synthesis and degradation of carbohydrates and glycogen storage. Liver also plays a key role in maintenance of normal levels of numerous metabolites, including haemoglobin breakdown products (e.g.,bilirubin), coagulation proteins, albumin, cholesterol, hormones, ammonia, water and other electrolytes. Further plasma protein synthesis, hormone production, detoxification, heat generation and blood circulation is also regulated through liver.
Liver can store fat-soluble vitamins and 95% of the human vitamin A is stored in the liver. Liver injury can impair many processes going on in human body. Liver also exhibits self regeneration capacity of its normal size and functions, when excised. (Matton et al., 1993)

2.1.2 Diseases of liver

Common liver diseases include Hepatitis, Fatty Liver, Cirrhosis, steatosis, Hemochromatosis, Obstruction, cholangitis, ascites formation and Liver Cancer. One of the most common clinical presentations in patients with a wide variety of liver diseases is Hepatitis (Kumar et al., 1992).

![Illustration of an infectious liver](adapted from net)

Figure 2.2: Illustration of an infectious liver. (Adapted from net)

2.2 Hepatitis

Hepatitis is an infectious disease characterized by the inflammation of liver. Hepatitis can be (a) due to a group of viruses known as hepatitis viruses or may be (b) due to toxins (notably alcohol), injury, and exposure to drugs, an autoimmune process, or a genetic disorder (C) It can also result from an autonomic condition, in which anomalous immune factors attack the body’s own liver cells.
Hepatitis can take an acute form (short term), or chronic form (chronic persistent hepatitis or chronic active hepatitis). In some cases, acute hepatitis develops into a chronic condition, but chronic hepatitis can also occur on its own.

### 2.2.1 Hepatitis Viruses

Viruses that infect primarily hepatocytes (liver cells) and begin replication are in most cases hepatitis viruses. Viral hepatitis is almost as old as known human history (Szabo et al., 2003). Viral hepatitis has emerged as a major public health problem throughout the world. It is also a cause of significant morbidity and mortality in human beings, both from acute and chronic infection. Hepatitis can be caused by any of the means mentioned above; most commonly it is due to infection by one of several viruses A, B, C, D & E, termed hepatitis viruses. Treatment of hepatitis may range from specific medication to surgery and in severe conditions to liver transplantation.

#### Types of Viral Hepatitis:

1. Hepatitis A.
2. Hepatitis B.
3. Hepatitis C.
4. Hepatitis B with D.
5. Hepatitis E.
6. Hepatitis F virus.

### 2.3 Hepatitis C Virus

HCV is a major causative agent of acute and chronic hepatitis worldwide. The World Health Organization (WHO) estimates that 200 million people, 3% of the world population, are infected with HCV (Lindebach et al., 2007). Including Pakistan where the rate of hepatitis in general population is more than 10% (Idrees et al., 2008). Studies have shown that 75% of individuals infected with HCV can progress to chronic infection, and develop complicated liver diseases including fibrosis, cirrhosis and hepatocellular carcinoma (Liang et al.; 2000). Liver
damage is not directly caused by the virus, but by the interplay between the virus and the immune system that results in the damage of healthy liver tissue (Hoffnagle, 2002).

2.3.1 History of Hepatitis C Virus (HCV)

The term ‘non-A, non- B hepatitis (NANBH) was introduced in the mid 1970s to describe inflammatory liver disease not attributable to infection with hepatitis A virus (HAV) or hepatitis B virus (HBV) (Prince et al., 1974; Alter et al., 1975; Feinstone et al., 1975). Serological analysis confirmed that the etiological agent, in approximately 90% of the remaining hepatitis cases, was neither HAV nor HBV (Knodell, et al., 1975). The NANBH was shown to be transmissible to chimpanzees in 1978 (Alter et al., 1978; Hollinger et al., 1978; Tabor et al., 1978). Filtration studies showed that the agent of NANB is less than 80 nm in size, and thus likely to be a virus. NANBH virus is enveloped, indicated by its sensitivity to chloroform (Bradly et al., 1983; Feinstone et al., 1983). The molecular cloning of NANBH agent was reported in 1989 after a large number of trials were conducted by Chiron’s group. It was found to be a positive stranded RNA virus, designated hepatitis C virus (Choo et al., 1989).

2.3.2 Prevalence of HCV

It is estimated recently that HCV has infected 200 million individuals worldwide including 17 million in Pakistan (with most prevalent genotype 3a) (Narendra et al., 2004; Idrees and Riazuddin, 2008; Akbar et al., 2009). Prevalence of HCV may be different in various regions and various groups of the same community (Idrees et al., 2008). Poverty, high risk sexual behavior, having less than 12 years of education are coupled to an increased risk of infection although the reasons for some of these associations still unclear (Lauer and Walker, 2001). Hepatitis C poses a threat to approximately 3 percent of the world’s population. Every year 3 to 4 million individuals are infected with HCV. HCV infection leads to chronic HCV infection in approximately 70 percent of individuals. Within a mean time of 20 years, 20 percent of chronic HCV patients develop cirrhosis and die of cirrhosis with a rate of 2 to 5 percent per year (Koziel and Peters, 2007).
2.3.3 Route of Transmission

Hepatitis C is a blood borne disease spread mainly by exposure to contaminated blood. Transmission of HCV is possible through the Injection drug use (Karmochkine et al., 2006; Hahn, 2007), blood products, dental exposure, occupational exposure to blood, sexual exposure (Vandelli et al., 2004), Body piercings and tattoos (Thompson et al., 1996), from mother to baby during childbirth and by sharing personal care products. recipients of multiple blood transfusions, intravenous drug abusers have been observed at high risk of infection (Roman et al., 2008; Farhana et al., 2009; Akbar et al, 2009).

2.3.4 Phylogeny of HCV

The gene organization of HCV genome shows similarity to that of the Flaviviridae family. However, HCV genome exhibit low sequence homology with those of flaviviruses and pestiviruses. Therefore, HCV is now classified into a new genus “hepacivirus” which is distinct from the flaviviruses and pestiviruses of the Flaviviridae family (Kato, 2001).

![Figure 2.3: Phylogenetic relationship of HCV with Flaviviridae family.](image)

(Adapted by PG, AKI, Zürich 2002)

2.3.5 Structure of HCV

HCV is 55-65nm in size. The structure of the HCV consists of a core of RNA as a genetic material which is surrounded by protective shell of protein, and further encased in a lipid envelope of cellular origin. It is the only known member of the hepacivirus genus in the family Flaviviridae. There are six major genotypes of the hepatitis C virus, and multiple subtypes.
2.3.6 Genome Organization of HCV

HCV is an enveloped plus strand RNA virus of genus Hepacivirus within the family Flaviviridae (Lindenbach et al., 2007). The HCV genome is a 9.6-kb, uncapped, linear, single-stranded RNA (ssRNA) HCV RNA contains a single long open reading frame of about 9,024 nucleotides, encodes a polyprotein of 3010 amino acids (Kato, 2001). The HCV polyprotein is processed by a combination of cellular (host) and virus encoded proteases into 3 structural proteins (core, and two envelope glycoproteins E1, E2) (Jone et al., 2007; Steinmann et al., 2007) and 7 non structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Dubuisson, 2007; Posta et al., 2008; Akbar et al., 2009).
2.4 HCV Genotypes

Nucleotide sequences of the HCV genome from around the world have heterogeneity among them and on the basis of these genetic differences HCV has been classified into different genotypes. This genetic heterogeneity may be responsible for different antigenic and biological properties of different genotypes (Choo et al., 1991; Zein et al., 1996). There are six major genotypes and almost 80 subtypes identified worldwide (Simmonds et al., 2005). In United States the most common genotype is genotype 1, 75 percent of the total patients harbors genotype 1 in United states followed by genotype 2, which is prevalent in 15 percent of infected people. And least common is genotype 3 (7%). Most common genotype found worldwide is
genotype 1b and it is also common genotype in Asia-Pacific, particularly in Japan, South Korea, China and Taiwan. Apart from genotype 1b, other genotypes like 2a and 2b are also common in these countries (Yu et al., 2001; Simmonds et al., 2005; Suh and Jeong, 2006). Most prevalent genotype in Indian subcontinent, Southeast Asia, Australian and New Zealand is genotype 3 (Nakai et al., 2001; Valliammai et al., 1995). In Pakistan the most common genotype is genotype 3a, which is also the most respondent towards interferon therapy (Idrees and Riazuddin, 2008; Jafri and Subhan, 2008). Genotype 4 is restricted to Middle East and North Africa (Bdour, 2002; Kamal et al., 2006) while genotype 5 is restricted to South Africa (Smuts and Kannemeyer, 1995). In South East Asia genotype 6 is also predominantly found (Fung et al., 2008). Variants of genotype 3 and 6a have been identified in South East Asia and recently were classified as subtypes of genotypes 3 and 6 (Simmonds et al., 2005). For determining the genotypes the most commonly used target is the 5′ untranslated region (5′UTR) of the viral genome (McCaughan et al., 2007). HCV genotypes determine disease outcome and response to interferon treatment and HCV genotypes are important for predicting the sustained virological response (SVR) (Nousbaum et al., 1995; Zein et al., 1996). Genotype 1 is least respondent (Hoofnagle and Seeff, 2006).

### 2.5 Genetic Diversity

A characteristic of RNA viruses is their extreme genetic diversity. The NS5B protein of HCV is an RNA-dependent RNA polymerase that lacks a proof reading mechanism. Thus, mutations within the HCV genome are generated at a rate of approximately 1 mutation per genome per replication cycle. Almost 10 trillion viral particles are produced per day. This results in a population of distinct but closely related viral variants, termed the viral quasispecies that exist within a single individual. By constant mutation, HCV may be able to escape host immunologic detection and elimination and maintain persistent infection. Persistent viremia is associated with a higher hyper variable region 1 (HVR1) (Purcell, 1994; Lemon and Brown, 1995; Walker, 1999; Simmonds, 2004). There is 30-50% variation among viral genotypes and 15-30% among different subtypes while there is 1-5% variation in nucleotide sequence from a single HCV infected patient (Simmonds, 2004; Bartenschlager, 2006). Genotype 1a and 1b is common in Western Europe. Genotype 3 is most frequent in the India Nepal and Pakistan. Genotype 3a has a high prevalence worldwide, infecting up to 50% of patients in several
European countries as well as a high percentage of HCV-infected individuals in many highly populated countries in Asia (e.g. India). In Pakistan the major HCV genotype is 3a followed by 3b and 1a (Idrees and Riazuddin, 2008).

On the basis of nucleotide variation HCV divided into six major HCV genotypes and more than 80 subtypes. More nucleotide variation concentrated in E1 and E2 glycoprotein. The lowest sequence variability between genotypes is found in the 5 UTR which contain specific sequences and RNA secondary structures that are required for replication and translation functions. The sequence variability is due to high replication rate and lack of proofreading activity of RNA dependent RNA polymerase. The rate of nucleotide misincorporation is approximately $10^{-3}$ base substitutions per genome site per year (Major and Feinstone, 1997). All currently recognized HCV genotypes are hepatotropic and pathogenic. However, it has been suggested that different genotypes do vary in their infectivity and pathogenicity, thereby influencing the rate of progression to cirrhosis and the risk of HCC (Simmonds et al., 1999). Furthermore, several distinct but closely related HCV sequences coexist within each infected individual.

### 2.6 HCV Envelope Proteins

Viral genomic RNA is enveloped by a lipid bilayer containing two glycoproteins (E1 and E2). This constitutes the structure of the infectious virion. Cellular peptidases in the lumen of the endoplasmic reticulum (ER) cleave the polyprotein precursor to discharge three structural proteins: two envelope glycoproteins (E1 and E2) and the core protein (Forns and Bukh, 1999). These belong to type I transmembrane proteins that carry a hydrophobic anchor at C-terminal (Wakita et al., 2005). Enveloped viruses fuse their membranes with cellular membranes during entry into cells. HCV encodes two envelope glycoproteins, E1 and E2 which are supposed to be the first viral components that come in contact with the host cell surface (Flint et al., 1999; Forns et al., 2000; Goffard et al., 2005). The HCV envelope glycoproteins can follow two pathways to assemble, a productive pathway leading to the formation of a non covalent heterodimer, and a non productive pathway leading to formation of large disulphide linked aggregates. The non covalent HCV glycoprotein complex is probably involved in entry process into host cells.
These enveloped proteins are targeted to the endoplasmic reticulum (ER) by signal sequence in the preceding polypeptide and cotranslationally separated from each other by host signal peptidase cleavage (Hammond and Helenius, 1994). They remained anchored to endoplasmic reticulum membrane through a hydrophobic sequence located at their COOH-terminus (Dubuisson, 2000). A characterization of HCV envelope glycoproteins associated with HCV pseudotype particles has been shown that the functional unit is a non covalent E1E2 heterodimer. In addition co-expression of both envelope glycoproteins is necessary for the production of infectious pseudotype particles (Bartosch et al., 2003).

2.6.1 Role of HCV envelope glycoprotein in virus entry

Infection begins with the attachment of the virion to the surface of host cell (Forns et al., 2000; Goffard et al., 2005). Attachment is mediated by the binding of a protein present at the surface of native virion to a protein molecule on the cell surface, acting as a virion receptor (OpDe Beeck et al., 2004). The functional data suggest that E1 and E2 can be present in infectious particles. Antibodies specific for E2 block the binding of HCV from infected serum to human cell lines (Zibert et al. 1994; Germi et al. 2001). The chimpanzees immunized with the recombinant envelope glycoprotein (E1E2) were protected against experimental challenge with homologous virus (Choo et al. 1994; Dash et al., 2001). Co-injection of HCV and an antiserum against E2 also protected chimpanzees from infection (Rosa et al., 1996) suggest that antibodies against E1 and E2 can be generated that block the interaction between HCV and host cells. In addition, protection was associated directly with the titer of anti-E1E2 antibodies, suggesting a likely role for antibodies in protection. A role for antibodies in protection has also been
suggested from rare cases of spontaneous resolution of chronic infection in patients (Thompson et al. 1996 & Cocquerel et al., 2003).

2.6.2 CD81 receptor

The E2 glycoprotein interacts with putative cellular receptors, elicits production of neutralising antibodies against the virus, and is involved in viral morphogenesis. It binds to external loop of CD81, a tetraspanin found on the surface of many cell types including hepatocytes (Pileri et al., 1998). Studies with HCVpp and HCVcc have confirmed the involvement of CD81 in HCV entry (Bartosch et al., 2003; Hsu et al. 2003; Zhang et al., 2004; Akazawa et al., 2007). Detailed studies showed that large extracellular domain of CD81 binds with the E2 protein of HCV (Pileri et al., 1998; Higginbottom et al., 2000; Drummer et al., 2002). E2 protein requires intact disulphide bonds in CD81 for binding (Petracca et al., 2000; Kitadokoro et al., 2001; Drummer et al., 2002). The identification of CD81 residues involved in interactions with E2 was done with sE2. Structural differences exist between truncated forms of E2 and the full-length E1E2 and showed different interaction with CD81. This has been further confirmed by checking HCVpp infectivity (Cocquerel et al., 2003; Zhang et al., 2004). CD81 mutation, which has previously been reported to disrupt the E2–CD81 interaction, has been shown to confer susceptibility to HCVpp infection in HepG2 cells (Cocquerel et al., 2003; Brazzoli et al., 2005; Keck et al., 2009). Reduction in CD81 expression by small interfering RNAs was also observed in a dose dependent manner. Altogether, these data demonstrate that CD81 plays a critical role in HCV entry (Patel et al., 2000; Molina et al., 2008).

2.6.3 SR-BI receptor:

The human SR-BI protein is a 509 aa cell-surface glycoprotein (Acton et al., 1994). The human SRBI is post translationally modified by N-glycosylation (Rhainds & Brissette, 2004). It is expressed in a large variety of mammalian tissues and cell types (Bartosch et al., 2003; Rhainds & Brissette, 2004), but its expression is particularly high in the liver, adrenal gland and ovarian tissues (Rhainds & Brissette, 2004). SR-BI was first identified as a binding receptor for low and high density lipoproteins (LDL, HDL) (Acton et al., 1994, 1996; Trigatti et al., 2000). Interaction between E2 protein and SRBI has been shown to be specific (Scarselli et al., 2002; Barth et al., 2005; Grove et al., 2007, 2008). In addition studies with HCVpp are also in
agreement with the involvement of SR-BI in HCV entry (Bartosch et al., 2003, 2005; Lavillette et al., 2005; Voisset et al., 2005). In one study the pre25 incubation of Huh-7 with a polyclonal antibody to SR-BI has been shown to reduce HCVpp infectivity (Bartosch et al., 2003). However, reduction in SR-BI expression by small interfering RNAs was not as effective to inhibit infection as in the case of CD81. In another study it has been reported that silencing of SR-BI expression reduces HCVpp infectivity (Lavillette et al., 2005). Residues that are involved in the E2–SR-BI interaction have not been identified to date. However, several reports suggest that SR-BI may interact with E2 via its hypervariable region 1 (HVR1) segment. Deletion of HVR1 has also been shown to impair the E2–SR-BI interaction (Scarselli et al., 2002) and to reduce HCVpp infectivity (Bartosch et al. 2003; Callens et al., 2005; Bartosch et al., 2005; Voisset et al., 2005). HVR 1 may be involved as a cofactor for inducing infection as HCV clone lacking HVR1 was shown to be infectious in chimpanzee model (Forns et al., 2000). The data shows that HVR region in E2 protein are involved directly or indirectly in the viral attachment and entry but need further confirmation.

2.7 HCV Induced Pathogenesis

HCV infects hepatocytes and B lymphocytes (Okuda et al., 1999). During replication HCV produces 10 trillion virion particles per day due to its vigorous replication even in the chronic phase of replication (Neumann et al., 1998). RNA dependent RNA polymerase lacking the ability of proofreading is involved in HCV replication. Due to error-prone nature of the RNA-dependent RNA-polymerase HCV exhibits a high degree of genetic variability which results in the production of HCV mutants (Simmonds, 2004; Simmonds et al., 2005). Most of the people infected with HCV develop a persistent viremia and harbors hepatic inflammation and fibrosis. It has been suggested that more than fifty percent of hepatocytes got infected with HCV (Agnello et al., 1998). Components of immune system such as cytotoxic T lymphocytes (Cooper et al., 1999) and helper cells (Lechner et al., 2000) are associated with viral clearance. Loss of these cells especially helper T cells has been associated with the reemergence of viremia in one of the studies (Gerlach et al., 1999). Greater immune mediated control of the virus as well as reduced diversity results in virus clearance (Farci et al., 2000). Superinfection with other genotypes or reinfection with closely related strains supports ineffective immunity against HCV infection (Wyatt et al., 1998). During acute phase of HCV infection, diagnosis is infrequent and
within 7 to 8 weeks (range 2 to 26) clinical features including jaundice, malaise, and nausea may occur, however in most of the cases there are no symptoms or mild manifestation of infection. Very rare form of infection i.e. fulminant infection has been described to occur during this phase although very rarely (Farci et al., 1996).

In most of the cases acute infection leads to a prolonged period of infection with no clinical symptoms called as chronic infection. It may take more than 30 years to develop cirrhosis after infection, due to this long time interval the natural history of infection cannot be assessed easily. Once chronic infection is established viremia is rarely cleared. Chronic infection is usually accompanied by hepatitis, fibrosis and nonspecific symptoms such as fatigue. Fifteen to 20 percent of the people develop cirrhosis which leads to severe complications and death. Factors such as alcohol intake, coinfection with HIV1 and HBV, male sex and older age at infection, enhance the risk of disease progression (Poynard, et al., 1997; Zarski et al., 1998). Whereas factors that decrease the risk of disease progression include female sex and younger age at the time of infection (Laue and Walker, 2001). Chronic HCV infection is also associated with inflammation, hepatic insulin resistance, type 2 diabetes, hepatic steatosis, hepatic fibrosis, cirrhosis, hepatocellular carcinoma, and liver failure (Choi and Ou, 2006; Sheikh et al., 2008; Walters et al., 2009). These may be accompanied by relatively nonspecific symptoms such as fatigue. Severe complications and death usually occur only in persons with cirrhosis. The risk of developing HCC after cirrhosis has established, and is 1 to 4 percent per year (Ikeda et al., 1993). Although the characteristics and complications of HCV are well recognized, but the molecular mechanisms leading to HCV induced pathogenesis remains unclear. Increased oxidative stress is now established to play a major role in the development and the progression of HCV induced pathogenesis including inflammation, fibrosis, altered gene expression and hepatocellular carcinoma (HCC) (Okuda et al., 2002; Lonardo et al., 2004; Choi and Ou, 2006; Ciccaglione et al., 2007; Sheikh et al., 2008). With emerging insight into the pathogenic mechanism involved in liver fibrosis and its progression HCV is now viewed as a true metabolic syndrome rather than a simple viral infection. This syndrome is associated with cirrhosis, portal vein hypertension, hepatic insufficiency, liver failure and HCC (Qadri et al., 2004; Cai et al., 2005).
2.8 HCV treatment

The most important treatment for liver disease is prevention. Currently, there is no antiviral drug that is 100% effective against HCV infection. The standard treatment against HCV is interferon therapy in combination with a nucleoside analogue (Alter and Seef, 2000; Zeuzem et al., 2000; Manns et al., 2001). Unfortunately more than 50% of the patients do not respond to the treatment and become chronic carriers. Therefore, vaccination remains the most effective means of disease prevention. Development of appropriate therapeutic and prophylactic vaccines remains a significant challenge. There is no vaccine against HCV available. Treatment of HCV is designed with an aim of attaining a sustained virological response (SVR) i.e. HCV RNA levels less than 500 IU/ml after 24 weeks post treatment cessation. SVR is considered to be consistent up to 18 years after treatment. Very few individuals (1.7%) have only residual HCV RNA left in the liver tissues, strongly suggesting that SVR represents complete eradication of HCV (Maylin et al., 2008). At present standard therapy for HCV comprise of pegylated IFN (Peg IFN) and ribavirin (RBV), conducted for 12 to 72 week. This treatment regimen is not an ideal treatment for HCV because the success rate is not cent percent as only 40 to 50 percent of patients infected with genotype 1 or 4 develop a SVR, and those infected with genotype 2 or 3 develop a sustained response in 80 percent of patients. Moreover side effects like depression and anemia are also associated with it (Fried and Hadziyannis, 2004).

2.8.1 Interferon monotherapy

Until 1990s the only beneficial therapy for the treatment of HCV was interferon alpha (IFN-α) administered at a dose of 3 million units (MU) three times weekly for 6 months. IFN-α was used alone for treating hepatitis C patients, but at best it Produced sustained viral response rates (SVR) of only around 5%-10%. Many studies supported that longer duration of therapy and higher doses of IFN improves SVR rate. SVR rate was higher (13-19%) for IFN administered for 48 weeks, 3 MU thrice per week than 24 weeks IFN regimen (6%) (Poynard et al., 1998). Further improvement of the SVR was achieved by covalently modifying IFN-α with the polymer polyethylene glycol (PEG). This therapy achieved 50% sustained virological response (SVR) for genotype 1 and 80% for genotype 2 & 3. As pegylated interferon is expensive, standard interferon is still the main therapy for HCV treatment in under developed countries (Munir et al., 2010). This chemical modification produced a molecule (PEG-IFN-α)
that has a longer half-life, better pharmacokinetic properties, and more efficacious antiviral response compared to the unmodified protein.

2.8.2 IFN/Ribavirin combination therapy

As a guanosine analogue, ribavirin was added to IFN-α treatment because it had shown promising results against several RNA and DNA viruses and was found to be particularly useful for treating severe respiratory syncytial virus (RSV) infection in children. Chronic hepatitis C patients if treated with IFN/ribavirin combination therapy instead of IFN monotherapy shows improvement in SVR rate. SVR rate with IFN/Ribavirin combination therapy 3 MU thrice a week and ribavirin 1000–1200 mg/day) is much higher (33% and 41% for 24 and 48 weeks respectively) than IFN α 2b monotherapy (6% for 24 weeks and 16% for 48 weeks) (McHutchison et al., 1998; Poynard et al., 1998).

In order to increase the shelf life of IFN, an inert polyethylene glycol polymer is attached to conventional IFN α, the new form of IFN is called as pegylated IFN-α (PegIFN-α). Such modification causes slower subcutaneous absorption of IFN which reduces its degradation and clearance. PegIFN-α allows less frequent, weekly dosing and still maintains higher sustained IFN blood levels. A significant increase of 54% to 55% in over all SVR rate was observed with PegIFN-α-2b or PegIFN-α-2a plus ribavirin for 48 weeks than conventional IFN/Ribavirin or PegIFN monotherapy (Manns et al., 2001; Fried et al., 2002). PegIFN-α/ribavirin combination treatment is now considered as standard therapy for chronic HCV treatment. A combination of PEG-IFN-α and ribavirin is at present standard treatment regimen used for treating hepatitis C patients. Therapy is expensive, produces unpleasant side effects that include depression, hemolytic anemia, fever and chills, and achieves an SVR that is close to 75%–80% for patients carrying the less common genotypes 2 and 3, but is relatively less effective for the North American genotype-1. Why some HCV genotypes are more responsive than others to PEG-IFN-α and ribavirin therapy remains an open question. More improved versions of ribavirin and several pharmaceutical companies are currently developing PEG-IFN.

Recently, a new formulation of ribavirin called Rebetol (Schering-Plough Kenilworth, NJ) was developed that can be taken orally. Viramidine (Valeant Pharmaceuticals, Costa Mesa,
CA) is an oral prodrug of ribavirin and is currently in phase-III clinical trials. It is converted to ribavirin in the liver and significantly reduces the incidence and severity of anemia as compared to ribavirin. Another modified form of interferon named pegylated interferon-α (IFN-α), (Hoffman-La Roche, Nutley, NJ) has been found to be effective in patients unresponsive to Interferon (Schering-Plough, Kenilworth, NJ). Preliminary results have revealed that a 12-week treatment with Peg-Interferon and ribavirin either eliminated the virus or significantly reduced its load in almost half of the patients who were non-responders to Peg-Interferon and ribavirin treatment.

2.9 Treatment Response

Over the past decade different responses towards standard HCV treatment have been characterized, including on-treatment responses and off-treatment responses. A negative polymerase chain reaction (≤50 IU/mL) after 4 weeks of treatment is defined as Rapid virological response (RVR). Early virological response is defined as an HCV RNA PCR seronegative or HCV RNA decreased at least two logs from baseline after 12 weeks of therapy. EVR is either complete EVR response when HCV RNA is less than 50 IU/ml at 12 weeks or partial EVR response when there is more than 2 log decrease in HCV RNA but still detectable (>50 IU/ml) after 12 weeks. Breakthrough response is another on-treatment response when HCV RNA is seronegative during the treatment but reappears before the treatment ends. End of treatment response is an off-treatment response when PCR is negative at the end of 24 weeks of treatment. If PCR remains negative after 6 months of treatment cessation then it is referred as a sustained virological response (SVR). However if serum HCV RNA reappears after 6 months of completion of therapy then those ETR patients are defined as relapsers. There are patients who consistently don’t respond to the treatment neither during treatment nor after treatment are called as non responders (NR) (Lee and Ferenci, 2008; Yu and Chuang, 2009).

2.9.1 Factors affecting treatment response

The heterogeneity in treatment response is due to number of host related and virus related factors. Major viral factor effecting treatment response is HCV genotype. HCV genotype and subtypes are essential characteristics of a viral strain that remains unchanged during the period of infection. Complexities of the disease and how the virus will respond to the treatment are
genotype dependent. However due to the high HCV replication rate (10 trillion virion particles per day), RNA dependent RNase polymerase lacking the proofreading ability and immune surveillance of the host (Martell et al., 1992; Kato et al., 1993; Zeuzem, 2000), allows a population of heterogenous but closely related copies of HCV called quasi species to circulate in host (Simmonds, 1999). Heterogeneity of quasi species can be estimated by calculation of genetic complexity which is the number of different variants of HCV circulating and genetic diversity which is the mean genetic difference between variants (Zeuzem, 2000). The strongest predictor of a non response to HCV treatment is HCV genotype 1 or 4 and a high baseline viral load (Fried et al., 2002; Hadziyannis et al., 2004). The role of HCV subtypes to the treatment response has not been defined; however quasi species evolution is one of the key predictor of treatment response (Okada et al., 1992). In non responders there is a high degree of quasi species than in those who responds to the treatment (Moribe et al., 1995). In another study it has been shown that quasi species diversity and complexity was found to be same in patients who achieved SVR and those who didn’t as well as those who relapsed after achieving a response (Farci et al., 2002). There are a number of evidences available that prove that rate of SVR is lower in genotype 1 than genotype 2 and 3 (Poynard et al., 2000). Rate of SVR is relatively higher for genotype 2/3 (65%) in contrast to genotype 1 where it was found to be only 30% (McHutchison et al., 1998; Poynard et al., 1998). In Pakistani population higher SVR rate was observed in genotype 2 and 3 (Idrees and Riazuddin, 2009). Therefore for prescription of HCV treatment genotype must be taken into account first.

The genetic heterogeneity of the E2-HVR1 region can also be a factor affecting treatment response. E2-HVR1 used to describe the composition of quasispecies precisely, provided a large number of clones are analyzed. The preliminary studies in this area were based on analysis of single-strand polymorphisms or of a restricted number of clones. A correlation between high levels of quasispecies complexity before Interferon alpha monotherapy and a lack of response to treatment was reported (Moribe et al., 1995; Toyoda et al., 1997). This led to the suggestion that genetic variability ensured a reservoir of potentially resistant strains.
2.10 HCV Resistance to Interferons

Interferons play key roles in mediating antiviral and antigrowth responses and in modulating immune response. The main signaling pathways are rapid and direct. Signal transducers and activators of transcription, and many of the interferon-induced proteins, play important alternative roles in cells, raising interesting questions as to how the responses to the interferons intersect with more general aspects of cellular physiology (George, et al., 1998). A potential explanation for a lack of response to interferon therapy of HCV infection is an underlying deficient cellular response to interferon with a blunted response to interferon signaling (Taylor et al., 2007). The mechanisms utilized by IFN-α to block HCV replication are neither well understood (Jiang et al., 2008) nor are the reasons for the limited effectiveness of IFN therapy known (Bisceglie, et al., 2002; Pawlotsky, 2003). Although many trials of the efficacy of IFN in eradicating HCV have been conducted, the results have been far from complete elimination of HCV in all treated patients (Chayama, et al., 2003). Among the many interferon-inducible gene products, the double-stranded RNA (dsRNA)-activated protein kinase PKR has been shown to play an important role in cell defense against infections by many viruses through suppressing protein synthesis (Kaufman, 2000). PKR is a serine/threonine kinase that exhibits distinct activities: dimerization upon binding to dsRNA and autophosphorylation at many serine and threonine sites and phosphorylation of the α subunit of translation initiation factor eIF-2, a modification that leads to the inhibition of protein synthesis (MacQuillan et al., 2009). Through this capacity, PKR is thought to be a mediator of the antiviral and antiproliferative actions of IFN-α (Stark et al., 1998). To bypass PKR activation and the inhibition of host protein synthesis, E2 proteins were shown to block PKR activation (Gale et al., 1997; Gale et al., 1998).

2.11 Inhibition of Interferon and E2 Gene

The E2 glycoprotein interacts with putative cellular receptors, elicits production of neutralising antibodies against the virus, and is involved in viral morphogenesis. It binds to external loop of CD81, a tetraspanin found on the surface of many cell types including hepatocytes (Pileri et al., 1998). This viral envelope glycoprotein is a preferred target for humoral (Kato et al., 1992) and cell-mediated immune responses (Shirai et al., 1999). Taylor et
al., (1999) reported that HCV E2 protein contains a 12 amino-acid sequence domain that is highly homologous to the autophosphorylation site of PKR and initiation factor eIF2a, a target of PKR (the PKR-eIF2 a phosphorylation homology domain [PePHD]). They showed that the E2 protein inhibited the kinase activity of PKR and blocked its inhibitory effect on protein synthesis and cell growth, suggesting that the interaction of E2 and PKR may be one of the mechanisms by which HCV circumvents the antiviral effect of IFN. They also reported that E2 proteins with a PePHD sequence identical to genotypes 2 and 3 HCV, which are known IFN-sensitive genotypes, showed only a weak inhibitory effect against PKR activity.

2.11.1 PePHD Domain of HCV E2 Gene

One of the major ways by which cellular Interferon alpha inhibits viral replication involves the Interferon-alpha-inducible double-stranded RNA-activated Protein Kinase R (PKR). Indeed the Interferon alpha binds to the PKR and leads to its autophosphorylation which in turn initiates the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2a) by the PKR. This phosphorylation inhibits the RNA transcription. Indeed, eIF2a is necessary to initiate the translation by forming a complex with GTP and met-tRNA and then allowing binding to the 40S ribosomal subunit. The E2 glycoprotein, or more precisely, a 12-amino acid domain of this protein located between residues 659 and 670 and known as PePHD (PKR-eIF2a phosphorylation homology domain), is involved in PKR inhibition. Taylor et al (1999) showed that PePHD was the main domain of E2 able to bind PKR in vitro. The PePHD motif sequence is very similar to that of the auto-phosphorylation sites of PKR and its target, eIF2a, and this similarity is more marked for genotype 1 viruses than for genotype 2 and 3 viruses. E2 glycoproteins of genotype 1 viruses (HCV-1 E2) behave in vitro as pseudosubstrates, inhibiting the kinase activity of PKR. In mammalian cells, the stimulation of translation by HCV-1 E2 is consistent with the hypothesis of PKR inhibition. This inhibitory activity has also been observed in a yeast model (Saccharomyces cerevisiae expressing HCV1 E2). The replacement of HCV-1 E2 by proteins identical to those of genotype 2 and 3 viruses abolishes this inhibitory effect. An interaction between genotype 1 strains and PKR, via PePHD, has therefore been proposed to account for the intrinsic resistance of the strains of this genotype to Interferon alpha (Taylor et al., 1999).
The clinical relationship between aminoacid sequence of PePHD and the outcome of Interferon therapy has been a matter of controversy. A few studies have addressed polymorphism of the PePHD region in patients carrying strains of genotypes 1, 2 and 3. A small number of patients infected with HCV genotype 1b were studied by Abid and colleagues. They initially showed that in some patients responding to treatment, the virus had a PePHD sequence identical to that of the HCV-J strain calling Taylors hypothesis into question (Abid et al., 2000). For HCV 2a/b isolates, conflicting results about the association of PePHD mutations and treatment response have been published (Saito et al., 2003; Watanabe et al., 2003). A number of studies focusing exclusively on the diversity of PePHD sequences in genotype 1b HCV before treatment have since shown strong conservation of this motif, regardless of the response subsequently obtained (Polyak et al., 2000; Guady et al., 2005).

2.12 Role of PKR in HCV Resistance to Interferons

PKR belongs to a small family of enzymes that uses as their substrate the smallest (α) subunit of polypeptide chain initiation factor (eIF-2α) (Samuel, 1993). PKR phosphorylates serine 51 of eIF-2α limiting the functions of eIF-2α resulting in a concomitant inhibition of mRNA translation (Clemens and Elia, 1997). Type 1 interferons (IFNs), through transcriptional induction of interferon stimulated genes, upregulate a wide range of biological effector molecules, some of which have direct antiviral activity against HCV (Staeheli, 1990). This RNA regulated protein kinase (PKR) was found to be responsible for some of the antiviral activity of the IFNs (Masters and Samuel, 1984). It is known that HCV envelope protein E2 (Taylor et al., 1999) bind PKR and inhibit its antiviral function in vitro. In addition it has been shown that HCV protein expression is directly dependent on PKR expression in vitro (Tokumoto et al., 2007) and PKR has antiviral activity against HCV in cell culture (Jiang et al., 2008). Inhibition of PKR activity diminishes phosphorylated eIF-2α protein levels, resulting in maintenance of normal protein synthesis and cell growth, which may be beneficial for HCV persistence (Gale et al., 1997). However, the clinical significance of HCV proteins interacting with PKR protein remains to be determined (Paterson et al., 1999; Taylor, 2001). These hepatocytes could have upregulated PKR protein levels in response to endogenous type 1 IFN release in the local microenvironment,
although it appears that PKR in an uninduced state has antiviral activity against HCV in vitro (Kang et al., 2009).
METHODOLOGY

3.1 Collection of samples and their storage

For this study blood samples of patients were kindly donated by Molecular Diagnostics Lab of National Centre of Excellence in Molecular Biology, University of the Punjab Lahore. Blood samples were collected in BD Vacutainer tubes (Becton Dickenson) and were centrifuged at 2000g for 10 minutes. For serum separation, serum was aliquoted and stored at -70 °C. Repeatedly freezing and thawing of samples considerably reduces the yield of viral RNA, so we stored samples for long time at -70 °C, as it did not cause any major affect on viral RNA recovery. Viral genotype was checked before RNA isolation. Genotype was determined by the Molecular Diagnostics lab, National Centre of Excellence in Molecular Biology, University of the Punjab by applying the genotyping assay for the detection of hepatitis C virus genotypes and subtypes in Pakistan (Ohno et al., 1997). The samples with 1a genotype were used for RNA isolation that was used for next analysis.

3.2 Inclusion Criteria

Only those samples were included in the current study which was anti-HCV ELISA positive (anti-HCV ELISA, DRG Germany), and negative for hepatitis B virus surface antigen (HBsAg; DRG Germany). All patients were adults (≥18) with serum RNA positive for HCV, and had not taken any therapy at the time of sample collection. Patients showed their full willingness and consent for the participation in this study.

3.3 Exclusion Criteria

Patients having any infection other than Hepatitis C (co-infection with Hepatitis B or delta virus), genotype other than 1a, having low viral load, insufficient sera samples and or lacking required information such as age, sex, mode of transmission etc were excluded from the study.
3.4 Study Design

This study was designed to establish a correlation of Dephosphorylation of E2 Envelope gene of HCV genotype 1a and its effect on inhibition of the genes that are induced by the interferon, when it is introduced in the body.

3.5 Primer Designing and Synthesis

Primers for the amplification of Envelope protein E2 were designed by using existing data for our gene. Widely recognized strain; Genbank accession number \textbf{AF\textunderscore 009606} was used as reference strain. Two sets of primers were designed for PCR amplification of E2 gene (structural protein) of HCV using Primer 3 software: (http://bionformatics.weizmann.ac.il/cgi-bin/primer/primer3.cgi).

The primers were synthesized by the DNA Core Facility of National Centre of Excellence in Molecular Biology, University of the Punjab. The names, sequences and expected product size of primers are shown in table-3.1.

<table>
<thead>
<tr>
<th>primer name</th>
<th>Sequences (5’- 3’)</th>
<th>product size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 OS</td>
<td>TCCTGGTAGTGCTGCTGCTA</td>
<td></td>
</tr>
<tr>
<td>E2OAS</td>
<td>GTATTACGAGGTTCTCCAAAAGC</td>
<td>1149</td>
</tr>
<tr>
<td>E2 IS</td>
<td>GAAACCCACGTCACCCGGGGAA</td>
<td></td>
</tr>
<tr>
<td>E2 IAS</td>
<td>CGCCTCCGCTTGGGATATGAGTAACA</td>
<td>1089</td>
</tr>
</tbody>
</table>

3.6 RNA Isolation

Commercially available GF-1 Viral Nucleic Acid Extraction kit (Vivantis, Cat#GF-RD-300) was used in this study. HCV RNA was extracted from 200 µl serum according to the procedure given in the kit protocol with little modification. Briefly,
1. Added 50 µl Proteinase K, 215 µl Buffer VL (lysis buffer) containing carrier RNA into 200 µl serum. Incubated it at 65 °C for 10 min.
2. Added 280 µl absolute ethanol and mixed thoroughly.
3. Transferred the sample into a column assembled in collection tube. Centrifuged it at 5,000 x g for 1 min. Discarded the flow through.
4. Washed the column with 500 µl wash buffer 1 and centrifuged it at 5,000 x g for 1 min. Discarded the flow through.
5. Washed the column twice with 500 µl of wash buffer 2 and centrifuged at 5,000 x g for 1 min.
6. Placed the column in a microcentrifuge tube and added 30 µl nuclease free water in it. Allowed to stand for 2 min. Centrifuged at 5,000 x g for 2 min. to obtain RNA. Stored the RNA at -20 °C.

3.7 Complimentary DNA Synthesis

The extracted RNA was reverse transcribed into cDNA to create a suitable template for amplification of the E2 gene. Anti sense primer designed specifically for HCV virus genotype 1a were used to create negative sense cDNA. This cDNA was then used as template for amplification of E2 gene. Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RTase) (Invitrogen) was used to generate the reverse transcribed cDNA. The reaction mixture for the preparation of cDNA, for a single reaction, contained the following reagents:

- 5X FSB* 4.0 µl
- dNTPs (10 mM) 2.0 µl
- DTT (dithiothreitol), (0.1 M) 0.5 µl
- Antisense Primer (E2OAS) (10 pmol/µl) 1.0 µl
- M-MLV RTase (200U/µl) 100.0 U
- Ribolock RNase Inhibitor (40U/ µl) 0.2 µl
- Extracted RNA (20-50ng) 10.0 µl
- dH2O (DEPC treated) Adjusted to final volume of 20 µl

*5X First Strand Buffer contained 50 mM Tris-HCl (pH 8.3), 7.5 mM KCl, and 3mM MgCl2
Cycling Profile

The reaction mixture was incubated as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 °C</td>
<td>50 minutes</td>
</tr>
<tr>
<td>42 °C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>95 °C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>20 °C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

This cDNA generated is stable at 4 °C for short time storage and for long time storage -20 °C will be used. This cDNA will serve as template for PCR amplification of E2 gene.

3.8 PCR Amplification of E2 Gene

The amplification was performed with the help of Taq polymerase (Fermentas), by using outer sense (E2OS) and outer antisense (E2OAS) primers. Reaction mixture for a single reaction is:

10X PCR Buffer*  
MgCl₂ (25 mM)  
dNTPs (2.5mM)  
E2OAS (10 pmol/µl)  
E2OS(10 pmol/µl)  
Taq DNA polymerase (2U/µl)  
RT-PCR product  
dH₂O (nuclease free)  

2.0 µl  
2.4 µl  
1.0 µl  
1.0 µl  
1.0 µl  
1.0 µl  
4.0 µl  
up to 20.0 µl
* 10X PCR Buffer contained 50 mM Tris-HCl (pH 8.8 at 25°C), 200 mM (NH₄)₂SO₄, 0.1% (v/v) Tween 20

**Thermal Cycling Profile**

There are three major steps in the polymerase chain reaction, which were repeated for 35 cycles. This was done on an automated thermal cycler (ABI PCR system 2700), which could heat and cool the tubes with the reaction mixture in a very short time.

![Thermal Cycling Profile Diagram](image)

With the first round PCR product, a nested PCR reaction was performed for each sample. The reaction mix was same for this PCR except the outer primers were substituted with inner primers and the first round PCR fragment was used as template. Cycling profile was the same as described for 1st round PCR.

**3.9 Agarose Gel Electrophoresis**

PCR product was checked on 1.2 % agarose gel that was prepared by adding 1.2 g of agarose in 100 ml of 1X Tris Acetate EDTA (TAE) buffer. A clear solution was obtained by boiling this mixture. Cool it for 5 minutes to about 60 °C. Ethedium bromide (20μl) was added to make final concentration of 0.5 μg/ml. Mixed it carefully by mild was positioned 0.5-1.0 cm above the plate so that a complete well was formed. Slightly cool agarose solution was poured into the caster. Keep the gel caster at room temperature. When gel was completely set, carefully
removed the comb. The gel caster was kept in a horizontal gel tank (electrophoretic chamber) containing 1X TAE. A sufficient quantity of buffer was added in the gel tank up to depth of approx. 1 mm. Samples were prepared by adding 3 µl of 6X loading dye to 20 µl sample DNA. DNA sample was loaded in the wells of prepared gel. An appropriate DNA marker was also run with the samples. The gel was run for 35-40 min at 75 volts. After appropriate running, amplified PCR products were visualized under UV transilluminator in the Gel Documentation System (GDS). Amplified DNA products were visible in the form of light bands under UV light due to the intercalation of fluorescent dye ethidium bromide with DNA. Gel image was saved in gel documentation System.

**3.10 Gel Elution**

For purification of DNA from agarose gel GF-1 Gel DNA Recovery Kit (Vivantis Cat# GF-GP-100) was used and the manufacturer’s protocol was followed, as:

1. Net weight of gel slice was determined and 1 volume of buffer GB was added to the 1 volume of excised gel in a microcentrifuge tube.
2. Incubated the tube at 50 °C until gel has completely melted.
3. Transferred the sample in a column placed in a collection tube. Centrifuged it at 10,000 x g for 1 minute. Discarded the flow through.
4. Added 750 µl Wash Buffer in the column. Centrifuged at 10,000 x g for 1 minute and discarded the flow through.
5. Centrifuged the column again at 10,000 x g for 1 minute to remove the residual ethanol.
6. Placed the column in a new microcentrifuge tube and added 25-30 µl elution buffer in it. Allowed it to stand for 5 min.
7. Centrifuged it at 10,000 x g for 1 minute to obtain pure DNA. Stored this DNA at -20 °C.
8. A small amount of eluted DNA along with the control was run on agarose gel to determine the quantity of pure DNA obtained.
9. A nanodrop spectrophotometer (Nanodrop Technologies) was used to know the accurate quantity of this pure DNA.
3.11 Sequence Analysis of PCR Product

Sequence analysis of the eluted DNA was performed in both directions using both gene specific reverse or forward primers in separate reactions. Sequence analysis was carried out according to the manufacturer’s instructions (Big Dye Deoxy Terminators; Applied Biosystems, Weiterstadt, Germany). Sequencing was performed on automated Genetic Analyzer (Applied Biosystems; 3100 DNA Analyzer). Reaction mixture for single reaction was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big Dye</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>5X sequencing buffer</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Forward or reverse gene specific primer (10pM)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Sterile dH2O</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Total Reaction Volume</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Thermal Cycling Profile:

1. Added 2 µl of 3 M Sodium Acetate and 2 µl of 125 mM EDTA to the 10 µl of sequencing PCR reaction.
2. Added 26 µl of 100% ethanol and incubated at RT for 15 minutes.
3. Spun down for 30 minutes at 2800 rpm at 4 ºC.
4. Removed ethanol and added 36 µl of 70% ethanol.
5. Spun again for 15 minutes at 2800 rpm at 4 ºC.
6. Ethanol was removed and air dried the pellet at 50 °C for 2 min. or by keeping it at room temp. overnight.
7. 12 µl formamide was added and transferred the product into microtiter plate.
8. Heat shocked at 95 °C for 5 minutes.
9. Chilled on ice for 5 minutes.
10. Then handed over to core sequencing lab facility of CEMB for sequence analysis.

### 3.13 Phylogenetic Analysis

Protein sequences of envelope gene for genotype 1a from different countries of the world (Japan, France, USA, and UK) were obtained from NCBI. All sequences (GQ898898, EU482831, EU234064, EU362889, DQ061315, AY958052, AY958057, AY956468, AB520610, and AF529293) were then aligned with local envelope gene by using CLUSTALW (Thompson et al., 1994). A neighbor joining tree was generated using PHYLIP (Felsenstein, 1981).

### 3.14 Identification of Phosphorylation sites in the Envelope protein:

To predict in silico phosphorylation sites in the local sequences of the envelope genes a free on line software Expasy Proteomics Server of the Swiss Institute of Bioinformatics (SIB) was used. The possible outcome of predication was studied with the help of NetPhos 2.0 software as described by Blom et al. (1999). This software checks the possible post translation modification in the local 1a local strain primary sequence in the amino acid. As a result of analysis, the target motif for different kinases is visualized (Blom et al., 1999; Obenauer, 2003). Scansite is kept at low stringency in order to determine the maximum number of sites that may participate in phosphorylation, upon which further predication is done. Protein Structure Analysis An ab-initio model was designed by using I-TASSER as no template model was available in protein Data Bank (Pettersen et al., 2004). Data was uploaded and models were generated. A 3D structure of predicted phosphorylation sites was generated by using two servers, Chimera (Pettersen et al., 2004) and SWISS PDB viewer (Guex and Peitsch, 1997) were used to obtain 3D structure. It was checked that potential phosphorylation sites are surface exposed or hidden inside. Five sites were observed at the exposed surface of model (aa: S75, S95, S118,
Two sites S75 and S277 were chosen as potential phosphorylation sites and primers were designed for these sites. The sequences of mutagenic primers used in this study are shown in Table 2.

### Table 2: Primer sequences used for the site directed mutagenesis of E2 gene 1a genotype.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 75</td>
<td>CCTGAGAGGTGGCCGGCTGTAACC</td>
</tr>
<tr>
<td>AS 75</td>
<td>GGCCAACCTCTCAGGCGAGCCTGAA</td>
</tr>
<tr>
<td>S 277</td>
<td>GACGATAGGGACAGGGCGAGCTCAGC</td>
</tr>
<tr>
<td>AS 277</td>
<td>CCTGTCCCTATCGTCAGATCACAA</td>
</tr>
</tbody>
</table>

### 3.15 Site Directed Mutagenesis

Principle of the PCR-based Site Directed Mutagenesis Method (SDM) allows mutation of one or more bases in newly synthesized DNA. This protocol used a new primer design that promoted primer-template annealing by eliminating primer dimerization and also permitted the newly synthesized DNA to be used as the template in subsequent amplification cycles. Our gene of interest was amplified into two separate PCR fragments using four designed primers. Each fragment was produced by pairing one anchor primer with one mutagenic primer. The two mutagenic primers contain a desired mutation. After first PCR Cohesive ends of each fragment remain complementary and undergo specific ligation between each other, resulting in mutation. Each reaction was performed with either a primer pair that included a forward anchor primer and a reverse mutagenic primer or a pair that included a reverse anchor primer and a forward mutagenic primer as shown in figure 3.1A & B.
3.16 **Sequence Analysis:**

To confirm the mutation, sequence analysis of mutated E2 gene \((mE2)^*\) was performed by using both forward and reverse E2 primers as mentioned earlier. Two different strands with mutations at serine 75\((m_1E2)^*\) and Serine 277 \((m_2E2)^*\) were checked.

\((mE2)^*=\text{mutated E2 gene}\)

\((m_1E2)^*=\text{Mutation at S75}\)

\((m_2E2)^*=\text{Mutation at S277}\)

3.17 **Cloning of \(m1E2\) gene in mammalian expression vector pcDNA3.1 (+)**
For cloning of \( m1E2 \) gene into pcDNA restriction sites were required. Gene Sequence was put in the neb cutter (http://tools.neb.com/NEBcutter2/) and 0 cutter enzymes were selected to identify the restriction enzyme that did not cut the core gene. Restriction sites for HindIII and EcoR1 restriction enzyme were added in the forward and reverse primers at 5 prime site respectively. The names and sequences of these primers are given in table 3.3.

**Table 3.3: Primer sequences having restriction sites used for the amplification of \( mE2 \) gene of local prevalent 1a genotype.**

<table>
<thead>
<tr>
<th>primer name</th>
<th>Sequences (5’- 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE2F</td>
<td>GCAAGCTTGCATGGCCGAAACCCACGTCACCGGGGAA</td>
</tr>
<tr>
<td>RE2R</td>
<td>GCGAATTCCGCTCCGCTTGGGATATGAGTAACA</td>
</tr>
</tbody>
</table>

**3.17.1 PCR Amplification Using Primers having restriction sites:**

Using eluted product of amplified \( m1E2 \) gene as a template, PCR reaction was made in which primers having restriction sites were used. The mixture was prepared as follows:

- 10X PCR Buffer 2.0 µl
- MgCl\( _2 \) (25 mM) 2.4 µl
- dNTPs (2.5mM) 1.0 µl
- RE2F (10 pmol/µl) 1.0 µl
- RE2R (10 pmol/µl) 1.0 µl
- dH\( _2 \)O (nuclease free) up to 20.0 µl
- Taq DNA polymerase (2U/µl) 1.0 µl
- Template 4.0 µl

**Thermal Cycling Profile**

- **Hold**
  - 94°C 2:00 min
  - 94°C 0:45 S

- **35 cycles**
  - 54°C 1:30 min
  - 72°C 0:45 S

- **Hold**
  - 72°C 7:00 min
  - 4°C ∞
PCR product was run on 1.2% agarose gel stained with ethidium bromide, and required bands were gel eluted as described previously.

**3.17.2 Restriction Digestion of pcDNA3.1 and m1E2 Gene**

1. In order to generate sticky ends at both sites of m1E2 gene before ligation, pcDNA3.1/ZeoR(+)(Invitrogen, Life Technologies) and core gene were digested with enzymes EcoR1 (50U/µl) and HindIII (10U/µl).

<table>
<thead>
<tr>
<th>Tube 1:</th>
<th>Tube 2:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (m1E2 gene)</td>
<td>20µl</td>
</tr>
<tr>
<td>EcoR1 (50U/µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>HindIII(10U/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>10X Tango Buffer*</td>
<td>3 µl</td>
</tr>
<tr>
<td>d.H₂O</td>
<td>4.5 µl</td>
</tr>
</tbody>
</table>

*10X Tango Buffer contained 33 mM Tris-acetate (pH 7.9 at 37 °C), 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA

1. Both the eppendorfs were incubated for 12-16 hours at 37 °C.
2. Digested products were run on 1.2% agarose gel stained with ethidium bromide, and required bands were gel eluted as done previously.

**3.17.3 Ligation Reaction:**

The digested m1E2 gene was ligated in the mammalian expression vector pcDNA3.1 vector (Invitrogen). For ligation, reaction mixture was as follow:
The ligation reaction was incubated at 14 °C for overnight for about 12 to 16 hours.

3.17.4 Transformation

1. The ligation mixture was used to transform chemically-competent cells. Equal amount of sterile water was added in the ligation reaction, and mixed gently. From this mixture, 25 µl was used to transform chemically-competent 100 µl aliquot of TOP10F’ cells.
2. The mixture was incubated on ice for 30 min. The cells were given heat shock at 42 °C for 50 s and kept on ice for 2 minute.
3. 500 µl of LB broth was then added to the cells and incubated at 37 °C in a 225 rpm shaker for 1 hour. Competent cells that have taken up plasmid are then selected by spreading the culture onto a Luria-Bertani (LB) agar plate containing 100 μg/ml ampicillin and 12.5 μg/ml of tetracyclin.
4. Colonies were selected by incubating the plate overnight at 37 °C.

3.17.5 Selection of Clones

1. To identify bacteria harboring cloned m/E2 gene, individual colonies were then used to inoculate a 5 ml LB culture containing 100 µg/ml ampicillin and 12.5 µg/ml tetracyclin, shaking at 225 rpm overnight at 37 °C.
2. After 16 hours of incubation, 5 µl of culture was taken in an eppendorf and centrifuged at 13000 rpm for 2 min.
3. Supernatant was discarded and pellet was resuspended in 50 µl of 1X T.E buffer.
4. Heat shocked at boiling point (100 °C) in water bath for 10 min.
5. Centrifuged at 13000 rpm for 2 min.
6. Supernatent containing the plasmid was used as template for the PCR reactions. These PCR reactions were prepared both with gene specific primers having restriction sites (RE2F & RE2R) and vector-specific primers, T7 (TAATACGACTCACTATAGGG) and BGH (TAGAAGGCACAGTCGAGG).
7. Following amplification, PCR products were checked on a 1.2%, ethidium bromide-stained agarose gel, a successful cloning reaction being visualized under UV lamp as a product at approximately 1089bp with gene specific and 1212-bp with vector specific primers.

3.17.6 Mix Composition for Colony PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer*</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>25mM MgCl2</td>
<td>2.4 µl</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>T7 / E2IS Primer (10 pmol/µl)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>BGH / E2IAS Primer (10 pmol/µl)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>DNA Taq polymares (2U/µl)</td>
<td>2.0 U</td>
</tr>
<tr>
<td>Above supernatant</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>dH2O (DEPC treated)</td>
<td>Adjusted to final volume of 20 µl</td>
</tr>
<tr>
<td>Total Reaction Volume</td>
<td>20.0 µl</td>
</tr>
</tbody>
</table>

* 10X PCR Buffer contained 50 mM Tris-HCl (pH 8.8 at 25°C), 200 mM (NH₄)₂SO₄, 0.1% (v/v) Tween 20

Cycling Profile for vector specific PCR

![Cycling Profile](image-url)
Cycling Profile for gene specific PCR

\[
\begin{array}{c|c|c|c|c}
\text{Hold} & 94^\circ C & 94^\circ C & 35 \text{ cycles} & 72^\circ C \\
2:00 \text{ min} & 45 \text{ sec} & 54^\circ C & 1:30 \text{ min} & 10:00 \text{ min} \\
& & 45 \text{ sec} & & 4^\circ C \\
\end{array}
\]

3.17.7 Plasmid isolation

Plasmid was isolated by using commercially available GF-1 Plasmid DNA Extraction kit (vivantis Cat # GF- PL-100).

1. 5 ml of bacterial culture containing plasmid was pelleted by centrifugation at 6,000 x g for 2 min. and decant the supernatant completely.
2. 250 µl S1 (lysis buffer) containing RNase A was added to the pellet and completely resuspended the cells by pipetting.
3. Added 250 µl S2 and gently mixed by inverting tube several times to obtain a clear lysate. Incubated at room temp. for 5 min.
4. 400 µl of Buffer NB (neutralization Buffer) was added and gently mixed the tube by inverting it several times until a white precipitate formed. Centrifuged at 13,000 x g for 10 min.
5. Transferred the supernatant in a column assembled in a collection tube and centrifuged at 10,000 x g for 1 min. Discarded the flow through.
6. Washed the column with 700 µl Wash buffer and centrifuged at 10,000 x g for 1 min. Discarded the flow through.
7. Centrifuged the column at 10,000 x g for 1 min. to remove the residual ethanol.
8. Placed the column in a clean tube. Added 50 µl of Elution Buffer directly on the column membrane. Centrifuged it at 10,000 x g for 1 min. to elute DNA. Stored the DNA at -20 °C.

### 3.17.8 Cloning Conformation

Successful cloning was confirmed through:

#### 3.17.8.1 PCR

To confirm the insert in pcDNA 3.1 vector, PCR was run with gene specific primers having restriction sites and vector specific primers using isolated plasmid DNA as template. The PCR product was run on 1.2% agarose gel. All other conditions were same as that of the regular PCR as described above.

#### 3.17.8.2 Digestion

Digestion of pcDNA 3.1 vector having \( m_1E2 \) gene was done with Fermentas restriction enzymes EcoRI and HindIII. The reaction mixture was made as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI (50U/µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>10X Tango buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>HindIII(10U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>10 µl</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>6.5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37 °C for overnight.
Resolved the digested product on 1.2% agarose gel stained with ethidium bromide as done previously.

#### 3.17.8.4 Sequencing

Clones were sequenced and protocol followed as described before. To obtain sequence for an entire \( m_1E2 \) clone, four primer sequencing runs are generally required. The primers used for this were T7, BGH, RE2F, RE2R primer.
3.18 Cloning of $m_2E2$ genes in mammalian expression vector pcDNA3.1 (+)

Same procedure was adopted as for cloning of $m_1E2$ gene in pcDNA3.1 vector mentioned in section 2.14.

3.19 Expression Studies of mutated Envelope ($m_1E2$ & $m_2E2$) Genes

3.19.1. Cell Culturing

Human liver hepatoma (Huh-7) cell line was obtained from American Type Cell culture (kindly donated by Dr. Zafar Nawaz), and were grown in Dulbeccos modified Eagles medium (DMEM) (ICN technologies USA) supplemented with streptomycin (100 ug/ml), penicillin (100 U/ml) and 10%Fetal bovine serum (FBS) (Gibco life science technologies USA).

3.20 Establishment of Stable Cell Lines

Three different cell lines of Huh-7 and CHO for expressing E2 protein, $m_1E2$ protein and $m_2E2$ protein of HCV of genotype 1a were established to study the role of Envelope protein in interferon inhibition. The steps involved are given below:

3.20.1. Linearization of Plasmids

Plasmids having all the above mentioned genes and pcDNA3.1 (+) were linearized before transfection to facilitate their stable integration in Huh 7 cells genome. Restriction enzyme $Bgl$ II (Fermentas) was used to linearized pcDNA3.1+/core and pcDNA3.1 (+). Before using $Bgl$ II, it had been confirmed using software NEBcutter V2.0 that this enzyme was quite safe for important sequences required for protein synthesis of inserted gene. $Bgl$ II cut the pcDNA3.1 (+) only at single site (nucleotide number 13, far away from CMV promoter). $Bgl$ II did not cut at any nucleotide site of inserted genes. Following materials were added in two separate reaction tubes.

<table>
<thead>
<tr>
<th>Tube 1:</th>
<th>Tube 2:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector (Required gene)</td>
<td>pcDNA 3.1 vector</td>
</tr>
<tr>
<td>10µl</td>
<td>10µl</td>
</tr>
<tr>
<td>$Bgl$ II (10U/µl)</td>
<td>$Bgl$ II (10U/µl)</td>
</tr>
<tr>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-------</td>
</tr>
<tr>
<td>10X Buffer O</td>
<td>2 µl</td>
</tr>
<tr>
<td>d.H2O</td>
<td>11 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Reaction tubes were incubated at 37 °C for 4 hours and digested products were run on 1.2% agarose gel stained with ethidium bromide, and required bands were gel eluted as done previously.

### 3.20.2. DNA Transfection and Single Cell Selection

1. When cells become 70% confluent in 60 mm petri plate, then they were used for transfection
2. Old media was removed since it contained antibiotic and FBS. Cells and washed with 100 ul of 1X PBS.
3. 4 ml of DMEM media without antibiotic and FBS was added in the petri plate and plate was incubated at 37°C for 15-20 mins.
4. 8 µl of linearized plasmid having core gene was added in 500 ul of serum and antibody free medium in one tube and 6 µl of lipofctamine was added in 500 ul of serum and antibody free medium in another tube. Contents of both the tubes were mixed, and incubated at room temperature for 20 min. This mixture was added drop by drop to the cells. Same procedure was performed for pcDNA3.1 vector alone (control).
5. After 96 hours posttransfection, cells were subcultured into 60 mm petri-dishes and were grown in DMEM supplemented with selective agent G418 (1mg/ml).
6. Transfected cell lines were grown in G418 (1mg/ml) continuously for more than 1 month (40days).
7. Single colonies were isolated using sterile filtered tips employed on sterile pippett, under sterile conditions. Single clones were transferred to 24 well culturing plates, grown in the presence of G418 (500µg/ml).
8. After 7 days single clones were trypsinized and shifted into 6 well plates. Upon 70% confluence, single clones with stable expression were splitted into 60 mm petri-dishes. Expression of core gene was verified by reverse transcriptase PCR (mRNA), real time PCR and western blotting (protein)
3.20.3 Verification of Stable Cell Lines

3.20.3.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):

To characterize the stable cell lines, we have screened the expression profiles of cell line by Reverse Transcriptase PCR and followed the same protocol as described above.

1. A 10 ul of RNA was reverse transcribed using reverse transcriptase (Invitrogen Technologies USA) according to the manufacturer's instructions as mentioned above.
2. PCR was performed using sense and antisense primers to produce gene specific fragments, as mentioned above.
3. PCR products were analyzed on 1.2% agarose gels stained with ethidium bromide and photographed under UV Trans illuminator.

3.20.3.2. Protein Extraction from Transfected Cells

1. Media was removed from the transfected plates and 500 μl of trypsin was added to the cells.
2. Incubated at room temperature for 2-3 mins to detach the cells from the surface.
3. 1000 μl of DMEM media was added in the plate and mixed well to resuspend the cells in the media.
4. Media was shifted to a new sterile eppendorf and spinned for 3 minutes at 8000 rpm.
5. Pellet of cells was washed by adding 500 μl of 1X PBS, resuspending the cells and centrifuged for 3 minutes at 8000 rpm.
6. Supernatant was discarded and 60 μl of lysis solution was added to the pellet.
7. Vortexed for 5 seconds and kept on ice for 10 minutes.
8. Centrifuged at 13000 rpm at 4 °C for 10 minutes and supernatant was shifted to a new sterile eppendorf.
9. Added 45 μl of 2X sample loading dye and heat shocked in boiling water for 5 minutes and immediately transferred on ice for 5 to 10 minutes.
3.20.3.3 Western Blot

1. A 30 μl volume of transfected cell lysates was loaded onto a polyacrylamide gel possessing a 5% stacking gel and 12% resolving gel. A 10 μl of prestained protein marker (Fermantas LOT # SM1811) was loaded alongside to assess the apparent MW of proteins expressed. Running at a constant voltage of 60V for 90 minutes.

2. Proteins were then transferred to nitrocellulose membranes using a semi-dry blotting apparatus (Bio-Rad) at 15V for 1.5 hrs.

3. The membrane was blocked for 1 hour at 37 °C with a 5% skim milk solution, and washed three times with 10 ml of 1XPBS-T.

4. A primary antibody for E2 protein (sc-65457) was added, each at a concentration of 10 μl in 500 μl of 1XPBS.

5. After incubating at 4 °C for overnight, the membrane was washed 3 times with 1X PBST.

6. A secondary antibody, rabbit anti-mouse IgG, conjugated to alkaline phosphatase(Sigma), was added at a dilution of 1/1000 μl in 1XPBS, incubated at room temperature for one hour. The membrane was washed for three times with 1XPBS-T.

7. Proteins were visualized by adding Substrate solution of BCIP/NBT (sigma) to the membrane and incubated at room temperature for 30 minutes.

3.21 Study of in vitro interferon response

Invitro interferon response was checked in Huh-7 cells and CHO cells. Huh-7 and CHO cells were seeded in DMEM with 10% fetal bovine serum and 1 % penicillin and streptomycin at 37 °C in an atm. of 5% CO₂. Cells were grown up to the density of 1 million cells per 60mm plate. 10IU, 100IU and 1000IU of Interferon alfa-2a (F. Hoffmann-La Roche Ltd, Basel) were added to each plate. Viability of cells was checked after 24, 48 and 72 hours. The cells were trypsinized and cell number was counted by haemocytometer.

We hypothesized that Huh-7 cells have lost the rapid inducibility of IFNs because of presence of E2 gene of HCV which block the expression of interferon while high expression of interferon will be obtained in case of infection with mutants.
RESULTS

4.1 Complimentary DNA (cDNA) Synthesis and PCR Amplification of E2 Gene

Hepatitis C Virus RNA of local 1a genotype, isolated from the serum sample of a chronic HCV carrier was taken for further studies. The serum was negative for hepatitis B virus surface antigen (HBsAg) but positive for antihepatitis C virus antibody (HCV IgG). The primers used for the amplification of E2 gene were designed based on knowledge of existing sequence data for these genes. The cDNA was synthesized using gene specific outer anti sense primer E2OAS (10pm). cDNA was stored at -20 for long term storage, and it was used as a template for first round PCR amplification. Outer set of primers (E2OAS and E2OS) used in first round of PCR amplification. This first round PCR product was 1149-bp and was used as template in second round of PCR which was nested PCR using E2IAS and E2IS internal primers. The PCR products obtained after amplification were run on 1.2% agarose gel stained with ethidium bromide (0.5 mg/ml). The amplified products were visualized by UV transillumination. Figure 4.1 shows a 1089-bp fragment produced by nested PCR that were identified by comparison with a 1Kb molecular size marker.
These bands were excised from the gel and DNA was eluted using gel elution kit according to the manufacturer’s protocol. This eluted product was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies), and used for further studies.

### 4.2 Sequencing PCR

Identification of amplified product, that might carried sequence of HCV E2 gene of 1a genotype was confirmed by sequencing PCR. Sequencing was done by using either forward or reverse primers of the inner set, which is E2IS and E2IAS in separate reactions. Figure 4.2 shows representative chromatogram of sequence of E2 gene of HCV1a.

![Representative chromatogram of sequence of E2 gene of HCV genotype 1a Pakistani isolate.](image)

Sequences of amplified E2 gene of HCV 1a showed 89% homology with reported sequences. The sequence was submitted to NCBI Genbank data base. The assigned Accession number for the local E2 gene sequence is GU736411.

### 4.3 Comparison of E2 gene of genotype 1a published sequences

Documented sequences for 1a genotype from diversified areas of the world were compared with local envelope gene sequence to get the percentage nucleotide identity (PNI) (Table 4.1). Sequence dissimilarities in the envelope genes explained the different levels of
disease outcomes in patients infected with same genotype. The local envelope gene sequence was compared with other reported sequences for 1a genotypes from different regions of the world to find out the percentage nucleotide identity (PNI). The sequence variations in the envelope genes accounts for different degrees of disease progression in patients infected with same genotype. The study of these nucleotide variations may help to design genotype specific therapy to prevent and resolve HCV infections.

Table 4.1: Comparison of local and published envelope gene sequences

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Genotype</th>
<th>Country</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GQ898898</td>
<td>1a</td>
<td>PAKISTAN</td>
<td>96%</td>
</tr>
<tr>
<td>EU482831</td>
<td>1a</td>
<td>USA</td>
<td>92%</td>
</tr>
<tr>
<td>EU234064</td>
<td>1a</td>
<td>USA</td>
<td>90%</td>
</tr>
<tr>
<td>EU362889</td>
<td>1a</td>
<td>USA</td>
<td>90%</td>
</tr>
<tr>
<td>AY956468</td>
<td>1a</td>
<td>USA</td>
<td>91%</td>
</tr>
<tr>
<td>DQ061315</td>
<td>1a</td>
<td>USA</td>
<td>90%</td>
</tr>
<tr>
<td>AY958052</td>
<td>1a</td>
<td>UK</td>
<td>91%</td>
</tr>
<tr>
<td>AY958057</td>
<td>1a</td>
<td>UK</td>
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</tr>
<tr>
<td>AF529293</td>
<td>1a</td>
<td>FRANCE</td>
<td>89%</td>
</tr>
<tr>
<td>AB520610</td>
<td>1a</td>
<td>JAPAN</td>
<td>88%</td>
</tr>
</tbody>
</table>

4.4 Phylogenetic Analysis

All the above mentioned sequences from all over the world were then aligned with local envelope gene by using CLUSTALW. A neighbor joining tree was generated using PHYLIP.
Figure 4.3: Phylogeny of local envelope gene sequence with published sequences for 1a genotype from different localities of the world.

### 4.5 Identification of net phosphorylation sites

Possible phosphorylation sites were calculated in the cytoplasmic domain of the E2 protein that can be implicated in the interferon resistance. Twelve putative sites (Figure 4.4) were chosen as potential phosphorylation sites (S-6, Thr-4, Tyr-2). Stringency of Phosphorylation site predictors was increased as they have tendency to over-predict. Only those motifs were selected that showed a NetPhos score of 0.8 or greater. After it they were also analyzed by Scansite. Finally six sites were predicted by both servers (Table 4.2).
Figure 4.4: Phosphorylation sites software calculated in the local HCV envelope gene sequence.

An online server NetSurfP was used to find the surface accessibility of local envelope sequence, as the phosphorylation sites should be exposed on the surface of proteins (Table 4.2). Discovery Studio and SWISS PDB Viewer were applied to visualize 3D protein structure of the putative sites. PDB structure was built up by using the server I-TASEER (Ab-initio protein structure predictor) (Zhang, 2008). After this analysis two phosphorylation sites (S75 and S277) were found to be most reliable sites (Figure 4.5). Scansite was used for finding the phosphorylation interaction motifs and found that S75 and S277 interact with the CLK2 Kinase and YWHAZ which were then investigated in GeneCards and was confirmed from UniGene and UniProt (Table 4.3).

Table 4.2: Summary of predicted tyrosine phosphorylation sites.

<table>
<thead>
<tr>
<th>Site*a</th>
<th>aa*b</th>
<th>Context*c</th>
<th>NetPhos*d</th>
<th>Scansite*e</th>
<th>NetSurfP*f</th>
<th>I-TASSER*g</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>S</td>
<td>ERLASCKPL</td>
<td>0.977</td>
<td>Y</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>95</td>
<td>S</td>
<td>YANGSGPEH</td>
<td>0.969</td>
<td>_</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>118</td>
<td>S</td>
<td>VPAQSVCGP</td>
<td>0.885</td>
<td>_</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>277</td>
<td>S</td>
<td>DRDRSELSP</td>
<td>0.978</td>
<td>Y</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>221</td>
<td>T</td>
<td>GPWITPRCL</td>
<td>0.951</td>
<td>Y</td>
<td>E</td>
<td>No</td>
</tr>
<tr>
<td>211</td>
<td>Y</td>
<td>PEATYSRCG</td>
<td>0.863</td>
<td>Y</td>
<td>B</td>
<td>Yes</td>
</tr>
</tbody>
</table>
a) Phosphorylation sites in the local 1a sequence of the envelope gene.
b) S indicates Serine; T is for Threonine and Y for Tyrosine predictions.
c) Region where the phosphorylation sites are available.
d) Predicted sites by NetPhos with a score of $\geq 0.8$. Dashes indicate lack of phosphorylation sites in that position.
e) Y indicates predicted sites in sequence on “Low Stringency”. Dashes indicate lack of phosphorylation sites in that position.
f) E indicates Exposed sites and B indicates Buried sites. Surface accessibility calculated by NetSurfP.
g) Ab-initio 3D model was constructed by using I-TASSER server.

Table 4.3: Interacting enzymes predicted by Scansite.

<table>
<thead>
<tr>
<th>Site*</th>
<th>Enzyme*b</th>
<th>Gene Card</th>
<th>UniGene *c</th>
<th>UniProt*c</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>Clk2</td>
<td>CLK2</td>
<td>Yes</td>
<td>P49760</td>
<td>CDC2-like kinase (CLK)</td>
</tr>
<tr>
<td>277</td>
<td>pST_bind</td>
<td>YWHAZ</td>
<td>Yes</td>
<td>P63104</td>
<td>Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide</td>
</tr>
<tr>
<td>---</td>
<td>Clk2</td>
<td>CLK2</td>
<td>Yes</td>
<td>P49760</td>
<td>CDC2-like kinase (CLK)</td>
</tr>
</tbody>
</table>

- Phosphorylation sites in the envelope gene.
- Observed enzymes by the Scansite.
- Gene’s availability on UniGene and UniProt.
4.6 Site Directed mutagenesis

Various mutagenesis protocols based on the PCR method have been developed. In this study; to introduce two separate mutations at positions S75 and S277 in envelope gene E2, two PCR fragments were produced in separate reactions with the primer pairs listed in Table 3.2 as mentioned in section 3.17 (Figure 4.6). After PCR, the PCR product was purified and two ligation steps were performed separately. Fragments F1, F2 and F3, F4 were ligated separately to produce ligation products. The end ligation product, represented by the 1089bp band was purified from the agarose gel (Figure 4.7).

Figure 4.5: Visualsating phosphorylation sites S277 and S75 in Tertiary structure of the envelope gene.

Figure 4.6: Figure showing two separate PCR fragments for both mutations at position S75 and S277. Lane 1-2: PCR product for the sense primer of E2 gene and antisense mutated primer for S75 (F1). Lane 3-4: PCR product for the antisense primer of E2 gene and sense mutated primer for S75 (F2). Lane 5: 50 base pair DNA ladder. Lane 6-7: PCR product for the sense primer of E2 gene and antisense mutated primer for S277 (F3). Lane 8-9: PCR product for the antisense primer of E2 gene and sense mutated primer for S277 (F4). Lane 10: 50 base pair DNA ladder.
Figure 4.7: Ligation results of fragments $m_1E2$ & $m_2E2$.

Lane 1-2: 1089bp bands obtained after ligation of F1 and F2 fragments ($m_1E2$). Lane 3-4: bands obtained after ligation of F3 and F4 fragments ($m_2E2$). Lane 5: 1kb plus ladder.

This mutagenesis strategy produced near-perfect end products of the desired mutations. The end product was checked by DNA sequence analysis. The mutagenesis efficiency at the desired two sites was estimated at 100%, without any additional mutations. The desired mutated sequences are shown in figure 4.8 a and b.

(A)

(B)
4.7 Cloning of HCV mutated E2 genes in mammalian expression vector pcDNA3.1

In order to characterize the role of mutated E2 genes in disease progression we cloned the amplified PCR products obtained after site directed mutagenesis, in mammalian expression vector pcDNA 3.1 myc/his. The vector has a CMV promoter which represents an effective mean to transduce eukaryotic cells for transient and stable expression studies. The amplified sequences encoding mutations in E2 gene were digested, purified and cloned in expression vector between HindIII and EcoRI sites.

4.7.1 PCR Amplification Using Primers Having Restriction Sites

In order to clone any gene into pcDNA it is necessary to amplify that gene by using primers having restriction sites RE2F and RE2R (table. 3.3). The primers artificially introduced a start codon at the 5’ end of the proposed signal peptide of E2 which permits expression of the gene in mammalian cell culture. PCR products obtained after amplification were run on the etidium bromide (0.5 mg/ml) stained 1.2% agarose gel and were visualized by UV transillumination. The 1089-bp fragments produced by PCR were identified by comparison with
a molecular mass marker (Fig. 4.9). These bands were excised from the gel and further used in digestion reaction.

![Image of gel with bands](image)

**Figure 4.9: PCR amplification using primers having restriction sites:**
Lane 1-2: amplified product of m1E2, 4-5: amplified product of m2E2. Lane 3, 6: negative control. Lane 7: 1kb marker.

### 4.7.2 Digestion of Vector and Amplified Genes

For construction of the mammalian expression vector, pcDNA3.1 (+) vector was used, which has a CMV promoter (Figure 4.10).
Figure 4.10: The figure above summarizes the features of the pcDNA3.1(+) and pcDNA3.1(-) vectors.
Adapted from www.invitrogen.com

Both the \textit{m}_1\text{E}2 and \textit{m}_2\text{E}2 genes and vector pcDNA3.1 (+) were double digested with enzymes \textit{HindIII} (10U/\mu l) and \textit{EcoRI} (50U/\mu l), and were gel purified (Figures 4.11 & 4.12).
Figure 4.11: **Double digestion of vector pcDNA 3.1.** (invitrogen)
Lane-1: 1kb marker, lane-2 cut, lane-3 uncut vector. Vector size is 5.5kb.

Figure 4.12: **Double digestion of amplified PCR products**
Lane1-2: digested PCR products of m1E2. Lane3-4: digested PCR products of m2E2. Lane-5: 1kb marker

### 4.7.3 Ligation in Expression Vector pcDNA 3.1(+)

The digested PCR products were ligated in the mammalian expression vector pcDNA 3.1(+) (Fig 4.13). The ligation reaction was used to transform bacterial cell strain TOP 10F by heat shock method. Competent cells that have taken up plasmid were selected by spreading the culture onto a Luria-Bertani (LB) agar plate containing 100 μg/ml ampicillin and 12.4 μg/ml of tetracyclin. Colonies were selected by incubating the plate overnight at 37 °C. To identify bacteria harboring mutated E2 genes, individual colonies were used to directly inoculate PCR
reactions. The resulting colonies were analyzed by restriction digestion and the positive clones were analyzed by PCR and DNA sequencing.

![Diagram of HCV E2 envelope protein coding vector](image)

**Figure 4.13: HCV E2 envelope protein coding vector**

### 4.7.4 Cloning Confirmation

#### 4.7.4.1 Colony PCR

Screening of transformed bacterial colonies for the presence of required genes was done by colony PCR (Figure 4.14). In each sample, a single transformed bacterial colony was used directly as template in a PCR reaction using appropriate restriction gene specific and vector-specific primers T7 (TAATACGACTCACTATAGGG) and BGH (TAGAAGGACACAGTCGAGG). The successful cloning of E2 gene was confirmed by the presence of a PCR product with vector specific primer that was approximately 1212-bp. The amplified products were run on 1.2% agarose gel stained with ethidium bromide (0.5 mg/ml) and were visualized by UV transillumination.
Figure 4.14: Colony PCR for screening of positive clones:

Lane 1: 1kb ladder. Lane 2-3: Positive clones for m1E2. Lane 4-5: Positive clones for m2E2. Lane 6: lambda hind marker

These results provided a proof for the presence of gene of interest in the expression vector as these primers originates from the outer regions of the cloned genes. Results of Figure 4.14 show that all of 4 clones gave positive results when amplified with vector specific primers. Further these clones were confirmed by doing restriction digestion analysis and DNA sequencing.

4.7.4.2 Restriction Digestion

The cloning efficiency was estimated by Percentage of positive colonies (PPC). PPC was checked through restriction digestion of plasmid miniprep DNA from randomly picked colonies. Clones when checked by double digestion with HindIII (10U/µl) and EcoRI (50U/µl) gave the exact product of 1089-bp. Digested DNA were checked on 1.2% agarose stained with ethidium bromide under U.V illuminator (Figure 4.15).
4.7.4.3 Sequence Analysis

To identify the vector constructs having the desired insertion sequence in the required orientation we have sequenced the clones with the applied biosystems prism dye termination method with restriction gene specific as well as vector specific primers. The data was analysed for different clones. This data also confirmed the presence of artificially introduced start codon at the start of the gene sequences required for its translation in the expression studies. The sequence was submitted to NCBI Genbank data base. The assigned Accession numbers for the local E2 gene sequence is HM853672.

4.8 Characterization of Mutated HCV E2 Proteins

The functional effect of the expression vectors was studied systematically by transduction of human hepatoma cells with mutated E2 proteins coding vector (pcDNA).
4.8.1 Linearization of Plasmids

pcDNA vector having mutated envelope genes was linearized with BglII enzyme. These linearized vectors were cut and purified for further study.

![Image of gel electrophoresis](image)

Figure 4.16: Linearized vector pcDNA with envelope genes.

Lane 1: showing 1kb DNA size marker, Lane 2: showing the linearized empty vector pcDNA 3.1 (+) (5.4 kb), Lanes 3,4: linearized vector pcDNA/m1E2 and m2E2 (6.0-kb), Lane 5: showing λHindi marker.

4.8.2 Confirmation of Stable Cell Lines

4.8.2.1 Reverse Transcriptase-Polymerase Chain Reaction(RT-PCR):

The main criteria for functional clones are the formation of viral RNAs of correct size. To detect in vitro expression of mutated HCV E2 genes RNA, Huh-7 cell lines were transfected with the cloned full length mutated E2 genes encoding vector. RT PCR expression was checked (Figure 4.17). RNA isolated from Huh-7 cell line was used as negative control. The product size of approximately 1089-bp confirmed the presence of required mRNA in the transfected cells.
Figure 4.17: RT-PCR was done to characterize the Envelope Protein producing cell lines. Lane 1, 2&3 RT-PCR amplified genes of E2, m1E2, m2E2 from cells transfected with pcDNA & lane 4: 1 Kb marker.

4.8.2.2 Western blot analysis

Expression of HCV envelope proteins coded by expression vector was checked with protein extracts of transfected Huh-7 cells using mouse monoclonal sera against E2, (Santa Cruz Biotechnologies Inc. USA). The Western blot analysis identified specific bands of the expected molecular weight of 40 kDa (Fig 4.18). The migration pattern of the mutated envelope proteins were not influenced by mutation. Taken together, these data indicate that the viral envelope proteins were efficiently expressed after mutation.
Figure 4.18: Western blot of envelope proteins extracted from selected cell line.

Lane: 1 Protein prestain ladder, Lane:2 protein from cell lines transfected with pcDNA/m\(_1\)E2 vector, Lane:3 protein from cell lines transfected with pcDNA/m\(_2\)E2 vector, Lane:4 protein from cell lines transfected with pcDNA/E2 vector.

**Cell viability assay in response to Interferon Treatment:**

**Cell viability assay with Huh 7 Cells infected with E2 gene:**

Huh-7 cells conceded with E2 gene were seeded in 6-well plate 1 day prior transfection. Next day media was removed and cells were treated with different concentrations of interferon i.e. 10 IU, 100 IU, 1000 IU and 15000 IU. Cells were kept for different time periods at 37°C and 5% CO\(_2\). Cell viability assay was done at day 1, 2 and 3. No. of cells were gradually decreased in concentration and time dependent manner. Maximum decrease was observed when 1000 IU of interferon was given at day 3. Further increase in interferon concentration up to 1500 IU represents no contrasting effect on viability of cells (Table 4.4).
Table 4.4: The influence of different doses of interferon practiced at different time periods in Huh-7 cells infected with HCV E2 gene.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Interferon different Doses</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10IU</td>
<td>100IU</td>
</tr>
<tr>
<td>1st day</td>
<td>215000</td>
<td>21000</td>
<td>190000</td>
</tr>
<tr>
<td>2nd day</td>
<td>205000</td>
<td>190000</td>
<td>175000</td>
</tr>
<tr>
<td>3rd day</td>
<td>195000</td>
<td>195000</td>
<td>185000</td>
</tr>
</tbody>
</table>

Fig. 4.19: Shows the relationship of different dosages of interferon with different duration

Avg. No. of Viable cells

Different Doses of Interferon (IU/ml)
Table 4.5: The linked effect of various dosages of interferon with three consecutive days in Huh 7 cell lines carried E2 gene mutated at S75.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Interferon different Doses</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10IU</td>
<td>100IU</td>
</tr>
<tr>
<td>1st day</td>
<td></td>
<td>215000</td>
<td>200000</td>
</tr>
<tr>
<td>2nd day</td>
<td></td>
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<td>175000</td>
</tr>
<tr>
<td>3rd day</td>
<td></td>
<td>195000</td>
<td>165000</td>
</tr>
</tbody>
</table>

Figure 4.20: Represent the association of different doses of interferon with different time periods
Table 4.6: The outcome of four suggestive amount of interferon in three different time intervals in Hu-7 cells conceded with E2 Mutated gene S277.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Interferon different doses</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10IU</td>
<td>100IU</td>
<td>1000IU</td>
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<tr>
<td>1st day</td>
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<tr>
<td>3rd day</td>
<td>195000</td>
<td>160000</td>
<td>130000</td>
</tr>
</tbody>
</table>

Figure 4.21: Represent the association of different doses of interferon with different time periods
Cell viability assay with CHO Cells:

CHO cells were seeded in 6-well plate at 37°C and 5% CO₂. Transfection was done after 1 day. After removing old media the cells were treated with 10 IU, 100 IU, 1000IU and 15000IU of interferon. Cell viability assay was done after 1, 2 and 3 days. No. of cells gradually decreased in concentration and time dependent manner. At a dose of 1000 IU maximum decrease in no. of cells was observed. Again Further increase in interferon conc. brings no effect on viability of cells.

Table 4.7: The outcome of four suggestive amount of interferon in three different time intervals in CHO cells conceded with E2 Mutated gene at S75.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Interferon different doses</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10IU</td>
<td>100IU</td>
</tr>
<tr>
<td>1st day</td>
<td>215000</td>
<td>195000</td>
<td>170000</td>
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<tr>
<td>2nd day</td>
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<tr>
<td>3rd day</td>
<td>195000</td>
<td>155000</td>
<td>115000</td>
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</table>

Figure 4.22: Represent the effect of four different doses of interferon in three consecutive days
Table 4.8: The influence of different doses of interferon practiced at different time periods in CHO cells infected with HCV E2 gene.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Interferon different doses</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10IU</td>
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<tr>
<td>1st day</td>
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<td>215000</td>
<td>210000</td>
</tr>
<tr>
<td>2nd day</td>
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<td>205000</td>
<td>195000</td>
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<tr>
<td>3rd day</td>
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<td>195000</td>
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</tbody>
</table>

Fig. 4.23: Shows the relationship of different dosages of interferon with different duration
Table 4.9: The linked effect of various dosages of interferon with three consecutive days in CHO cell lines carried E2 gene mutated at S277.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Interferon different doses</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>10IU</td>
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<td>1st day</td>
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<td></td>
<td>195000</td>
<td>175000</td>
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Figure 4.24: Represent the effect of four different doses of interferon in three consecutive days
DISCUSSION

The most common types of hepatitis virus are A, B and C. Hepatitis C virus (HCV) is a major cause of viral hepatitis in human population at the globe (Cox et al., 2005; Lindebach et al., 2001, 2005 & 2007) including Pakistan as well where the rate of hepatitis in general population is above 10% (Idrees, 2008; Ali, 2009). Chronic HCV develops a sustained liver viremia in number of infected patients (Sheikh et al., 2008). HCV induced complex diseases encompassing fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC) (Joyce et al., 2009). Currently, there is no antiviral drug fully effective against HCV infection (Hahn, 2007). Current treatment strategy in practice for the chronic Hepatitis C patients is a combined therapy of IFN-α and ribavirin for 6 to 12 months (Munir et al., 2010). But unluckily more than 50% of treated subjects do not respond to the treatment (Alter and Seeff, 2000; Guo et al., 2001; Manns et al. 2001; Hoffnagle et al., 2003). The response rate of antiviral therapy is highly effective in genotype 3a and is less effective in genotype 1a (Idrees and Riazuddin, 2008). Safe and effective vaccines are available for hepatitis A and B which result to develop immunity in 90-95% of treated people. In many cases adverse side effects in response to the therapy have been observed thus vaccines remains the only choice for disease prevention and further spreading. The development of standard genetic vaccine against prevalent HCV genotypes can help to minimize HCV infections (Legrand et al., 2007).

In Pakistan the most prevalent genotype of HCV is 3a (Butt et al., 2010; Rafique et al., 2011) like in other south Asian countries such as in our neighboring country India (Chowdhury et al., 2003; Singh et al., 2004). Idrees and Riazuddin in (2008) reported that in Pakistan genotype 3a is (49%) whereas genotype 1a is (8%) and when compared the observed genotype data to the previous genotype data reported from Pakistan (Shah et al., 1997; Idrees, 2001), it represents that the frequency of genotype 1 is increasing in this country without any cease in increase in the frequency of genotype 3. It seems that in coming 15–20 years the current most frequent genotype 3a will be substituted by less common genotype 1 (a or b). If this scenario actually happens in Pakistan, it will change and complicate more the present consequences of HCV that will be alarming health issue in this country as in aspect of treatment response rate because genotype 1 is highly resistant to antiviral therapy (Munir et al., 2010).
Viral RNA was extracted from the serum sample of the patients infected by local genotype 1a Pakistani isolate which had never treated before. RNA was reverse transcribed into cDNA using antisense primer of the outer set, from which E2 gene was amplified by using nested PCR. The entire amplified product of HCV E2 sequence was confirmed by sequencing, and the obtained sequence was submitted to GenBank (Accession# GU736411) data base. The local E2 sequence was compared with other reported sequences of HCV-1a genotype on HCV database to find the percentage nucleotide identities (PNI). Our Pakistani isolates HCV-1a shared maximum identity with the HCV isolates from USA where the prevalent genotype is genotype 1a (Flor et al., 2007).

IFN usually causes the transcription of some antiviral genes. However, detailed principal mechanisms exploring high IFN-a resistance in HCV genotype 1a infected patients are still ambiguous. A double stranded RNA activated PKR phosphorylates the translation initiation factor (eIF2) and thus inhibits its protein synthesis ability. PKR protein has been demonstrated to have antiviral activity against HCV and interacts with HCV proteins in vitro (Kang et al. 2009). It has also a putative site of virus-mediated resistance to IFN-a (Gale et al. 1998; Taylor et al. 1999). PKR protein has many constitutive biological functions other than antiviral activity (Clemens and Elia 1997), which may explain the moderate to strong PKR expression in non-HCV infected liver. The most important function of PKR protein other than aforementioned functions is that it plays a key role in the resistance of HCV to IFN-a (Korth and Katze 2000). Direct inhibition of PKR activity by the HCV E2 protein has been investigated in vitro (Gale et al. 1998; Taylor et al. 1999).

A clear relationship was seen in a study of E2 protein of HCV genotype 1a and IFN inhibition pathway. It was shown that it blocks PKR functions such as inhibition of viral protein synthesis and kinase activity, through a 12 amino acid sequence E2-PePHD domain, and for PKR this domain can perform the function of pseudosubstrate. PKR autophosphorylation sites are highly identical to the PePHD domain in HCV genotype 1a than other genotypes (Gaudy et al., 2005). It has been demonstrated that E2 protein of HCV genotype 1 shares homology with PKR and eIF2 phosphorylation sites and therefore inhibits PKR by binding to it in vitro in mammalian and yeast cells. HCV therefore has evolved a mechanism in the form of E2-PKR
interaction to block IFN activity. The possible consequences of PKR inhibition is not only IFN resistance and infection persistence but also promote irregular cell growth promotion which ultimately leads to hepatocellular carcinoma (HCC). Since E2 PePHD domain of HCV genotype 3a share less homology to PKR and eIF2 phosphorylation sites than to genotypes 1a and 1b. Therefore, it can be the most satisfactory reason that patients with 3a genotype react to interferon treatment more proficiently as compared to genotype 1a that shows maximum resistance to IFN therapy (Glue et al., 2000; Zeuzem et al., 2000). The association of E2-PePHD domain and its behavior during interferon therapy is quite indistinct. Some studies also explained the modifications happening in the PePHD domain during treatment. In one study it was suggested that HCV defeats host defense system as well as the recommended therapy retort in patients who are mostly non responders (Chayama et al., 2000). HCV has developed different strategies to undo the antiviral effects of PKR (Mathews et al., 1996; Guo et al., 2001) and it is assumed that IFN resistance may be due to phosphorylation of envelope proteins particularly of E2 protein. Our study focused on detecting such sites in our local isolate of HCV genotype 1a. To check the local 1a sequences of the envelope genes in silico, N-linked phosphorylation analysis was done by free online software Expasy proteomics server of the Swiss Institute of Bioinformatics (SIB). Twelve potential phosphorylation sites were predicted in the local 1a sequence GU736411. After further analysis two phosphorylation sites (S75 and S277) were confirmed at the surface of folded E2 protein that interacted with CLK2 Kinase and YWHAZ. IFN normally acts by inducing transcription of many antiviral genes, including the double stranded RNA activated protein kinase (PKR), which inhibits protein synthesis by phosphorylation of the translation initiation factor eLF2. HCV have evolved the strategies to overcome the antiviral effects of PKR (Mathew et al., 1996; Guo et al., 2001) and phosphorylation of envelope glycoproteins especially of E2 protein may accounts for IFN resistance.

The aim of this research was to study the possible phosphorylation sites in the cytoplasmic domain of the E2 protein which may be possibly involved in interferon resistance. After this analysis PCR amplification of E2 and mutated E2 gene was performed with restriction primers that were capable to artificially introduce start codon at the 5’ end of these genes and restriction sites for HindIII and EcoRI enzymes at 5’ and 3’ ends respectively. Starting codon is very essential for expression of the any gene in mammalian cell culture (Fournillier Jacob et al.,
In molecular cloning technique the recombinant plasmid of HCV E2 wild and mutated genes was constructed that can express envelope genes into mammalian cell culture and successful cloning into pcDNA 3.1(+) was confirmed by restriction digestion and sequencing procedures (Lagging et al., 1995; Liu et al., 2002). In most circumstances, amplification of clinical samples yields multiple PCR products which attribute to the presence of quasispecies as reported by Farci et al., (2000). Sequence variation in envelope genes of HCV also account for difference in disease pathogenesis in different individuals with same genotype and can be pivotal candidate for the development of effective vaccines (Brass et al., 2006; Gastaminza et al., 2006).

The expression of envelope protein may have important function in vaccine discovery and drug targeting as it is considered to be the first viral element that comes in contact with the receptors of host cell surface (Bartenschlager, 2006). To check the expression of HCV envelope protein 2 and its mutants we have generated different stable cell lines that collectively produce both normal and mutated envelope proteins. G418 was used for the selection of positive clones. No consistent difference in morphology and growth rate of cells was observed. RNA expression was checked through RT-PCR and protein expression was examined with mouse anti-E2 antibody. The expressed proteins have a molecular weight of approximately 40KDa that is reported size of E2 protein. No change in protein size and conformation after mutation was observed.

The effect of interferon therapy on these mutated E2 proteins was also checked. The clinical efficacy of combined chemotherapy with IFN-α has already been established (Leung et al., 2002). Interferon response was checked in Huh-7 and Chinese Hamster Ovary (CHO) cells. Different doses of interferon were given to Huh-7 and CHO cells for different periods of time. Interferon α-2a response was checked at a dose rate of 10 IU/ml, 100 IU/ml, 1000 IU/ml and 1500IU for time period of 24 hours, 48 hours and 72 hours. The mechanism of IFN-mediated HCV inhibition in Huh7 cells is poorly defined but appears to be linked to a reduction in viral protein synthesis and the eventual inhibition of viral RNA amplification (Guo et al., 2004). Interferon dose of 1500IU represents no contrasting effect on the cell viability thus further doses of interferon stopped.

We hypothesized that envelope protein 2 of HCV fights with the phosphorylation of PKR which in turn effects the transcription of eIF2 and increases viral protein synthesis. In order to
study the role of phosphorylation in causing interferon resistance, we stably transfected the mutated envelope protein 2 in Huh-7 cells and CHO cells. Stable pools were screened with G418. Interferon induction was done and it was observed that mutant 1 ($m_1E2$) with dephosphorylation at S75 showed good response to interferon treatment while mutant 2 ($m_2E2$) with dephosphorylation at S277 responded to interferon therapy but response was low. The response to interferon was in dose and time dependent manner in both cell lines transfected with $m1E2$ and $m2E2$. With increase in interferon concentration number of viable cells was decreased in a dose dependent manner. Further increase brings no increase in cell number. Cells viability was checked at day 1, 2 and 3. It was observed that cell number is gradually decreased with increasing time period. Maximum inhibition was observed at 72 hours post induction in both $m1E2$ and $m2E2$ stable cell lines. Results showed that phosphorylation site S75 are involved in causing interferon resistance and by mutating this site interferon resistance in host may be inhibited. No such previous study was conducted on the related topic for the comparison of our findings.
Conclusion

To date no therapy is effective against HCV infection. Interferon is the only effective antiviral approach but unfortunately effective against small no of patients due to resistance against treatment. Envelope protein 2 is important in causing interferon resistance but mechanism by which virus cause mutation in PKR is not clear. The present research is based on study the role of envelop protein 2 in causing interferon resistance. S75 and S277 of E2 protein competes with PKR for phosphorylation. By mutating these sites interferon resistance can be checked. This study may help in developing effective treatment against HCV infection.
REFERENCES


APPENDIX

0.5 M EDTA (1000 ml)
- EDTA 186.1 g/L
- NaOH Few pellets to dissolve EDTA
- Distilled water To make volume up to one liter.

10X TBE Buffer (1000 ml)
- Trisma base 108g
- Boric acid 55g
- EDTA (0.5 M) 40ml
- dH2O To make volume up to one liter

TE Buffer (1000 ml)
- 0.5 M EDTA 0.2ml
- 1M HCl 1.0ml
- dH2O 98.8ml

0.5 Tris-HCl (1000ml)
- Trisma 3.025g
- dH2O To make volume up to one liter
- HCl To adjust pH up to 7.5

DNA Loading Dye (10ml)
- EDTA (0.5M) 200μl
- Glycerol (100%) 3ml
- Bromophenol Blue 0.025g
- Xylene Cyanol 0.025g
- dH2O To make volume 10ml.

2% Agarose Solution (100ml)
- Agarose 2g
- 1X TAE Buffer 100ml
- Ethidium Bromide (5mg/ml) 10μl

Luria Bertani Broth Medium (1000ml)
Tryptone 10g
Yeast Extract 5g
NaCl 10g
dH2O Up to 1000ml
pH 7.5 adjusted by 1N NaOH and autoclaved

**Luria Bertani Agar Medium (1000ml)**
Tryptone 10g
Yeast Extract 5g
NaCl 10g
Agar 10g
dH2O Up to 1000ml
pH 7.5 adjusted by 1N NaOH and autoclaved.

**Sol-I (10% Glycerol)**
50% glycerol 100ml
dH2O 400ml autoclaved

**50% Glycerol Stock**
100%glycerol 200ml
dH2O 200ml and autoclaved

**1M CaCl2 Stock (300ml)**
M. Wt of CaCl2 110.99g/mol
CaCl2 33.3g
dH2O 300ml and autoclaved

**1M MgCl2 (100ml)**
M. Wt of MgCl2 230.30g/mol
MgCl2 23.30g
dH2O 100ml and autoclaved

**Sol-III (5ml)**
0.1M CaCl2 2.5ml
50% glycerol 2.5ml
Stored at 4°C

**Antibiotics:**
Tetracyclin (Stock 12.5g/ml)
  Tetracyclin  0.125g
  70% ethanol  10ml

Ampicillin (Stock 100g/ml)
  Ampicillin  1g
  dH2O  10ml

Kanamycin (Stock 50g/ml)
  Kanamycin  0.5g
  dH2O  10ml

Reagents of Western Blotting:

Phosphate Buffered Saline (PBS)
  NaCl  8 g
  KCl  0.2 g
  KH2PO4  0.24 g
  Na2HPO4  1.44 g
  Dissolve in 1 liter of distilled water adjust pH to 7.4 and autoclaved.

Phosphate Buffered Saline-Tween 20 (PBST)
  Tween-20  500 μl (0.05 %)
  1x PBS  1 liter

10% SDS
  SDS  100g
  dH2O  900ml

10% APS (ammonium per sulphate)
  APS  0.1g
  Water  1ml

4X TRIS-SDS Buffer pH 8.8.
  Trizma base  18.2g
  SDS  0.4g
  Adjust pH to 8.8 with concentrated HCl, make up volume to 100ml, filter sterilize and store
  at 4ºC.

4X TRIS-SDS Buffer pH 6.8
  Trizma base  6.05g
SDS 0.4g
Adjust pH to 6.8 with concentrated HCl, make up volume to 100ml, filter sterilize and store at 4ºC.

10X Running Buffer
- Trizma base 30.05g
- Glycine 142.5g
- SDS 10g
Dissolve in 1 liter of distilled water.

Transfer Buffer
- Trizma base 3.032g
- Glycine 14.416g
- Methanol 200ml
Adjust volume upto 1 liter.

SDS-PAGE Solutions
12% separating gel
- Water 2.8ml
- Acrylamide (30%) 3.2ml
- 4xTris-SDS (pH8.8) 2.5ml
- APS (10 %) 26.7μl
- SDS (10%) 10μl
- TEMED 5.3μl

4% stacking gel
- Water 2.5ml
- Acrylamide (30%) 0.533ml
- 4xTris-SDS (pH6.8) 1ml
- APS (10 %) 31.5μl
- SDS (10%) 10μl
- TEMED 6.3μl

Coomassie Stain
- Coomassie blue 2.5g
- Methanol 455ml
- Glacial acetic acid 91ml
Adjust volume to 1 liter with distilled water.
**Coomassie Destain**

- Methanol: 250ml
- Glacial acetic acid: 70ml
- Adjust volume to 1 liter with distilled water

**2X Sample Buffer**

- 4x TRIS-SDS Buffer (pH 6.8): 2.5ml
- Glycerol: 2ml
- SDS: 0.4g
- β-mercaptoethanol: 200ul
- Bromophenol blue: 200ul
- Adjust volume upto 10 ml with distilled water

**Blocking Solution**

- Skim milk: 1.25g
- 1x PBS: 25ml