Role of Viral and Host factors in Treatment Outcomes of Interferon based therapy in Chronic HCV Pakistani patients

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(2010-NUST-TfrPhD-V&I-31)

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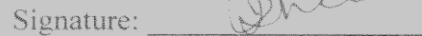
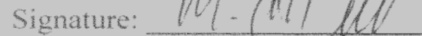
GEC Member 2: Dr. Sheeba Murad Mall Signature: 

GEC Member 3: (External): Dr. Muhammad Idrees Signature: 

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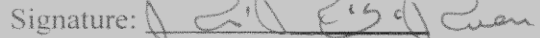
External Evaluator: Dr. Ali Raza Awan Signature: 

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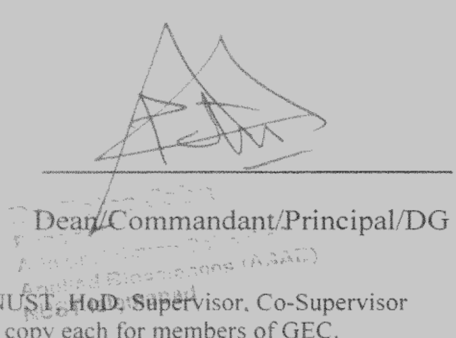

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Dedicated to

My Parents …

For their endless love and prayers

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LIST OF ABBREVIATIONS

ALT	Alanine Transaminase
APC	Antigen Presenting Cells
CD	Cluster of Differentiation
dsRNA	Double Stranded Ribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic acid
F1	Forward primer 1
rs	Reference SNP
HCC	Hepato Cellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
JAK	Jun Activated Kinase
NF-κB	Nuclear Factor kappa B
PRR	Pattern Recognition Receptor

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ABSTRACT

To date the only standard of care for Hepatitis C virus (HCV) infection is combinational therapy of interferon and ribavirin. Interferon based therapy is a treatment of long duration, associated with cost-effects and serious side-effects. The struggle for discovering new direct-acting antiviral agents (DAAs) against HCV infection is still continued. The researchers are making efforts to use combinational therapy against HCV without the involvement of interferon. The majority of patients are treated with interferon plus weight based ribavirin. Therefore, the present study was designed to search for independent markers that may significantly predict the treatment outcomes of interferon based therapy of HCV infection in Pakistani population. This study would assist the clinic doctors and researchers for personalized treatment of HCV infection**.**

Both viral and host factors were considered for the prediction of interferon based therapy of HCV infection in the present study. There were recruited 140 chronic HCV patients and 120 healthy individuals. In the current study; patient characteristics, single nucleotide polymorphisms (SNPs) of important genes, viral genotypes and baseline viral load were considered. SNPs of the host genome were studied by allele specific polymerase chain reaction (AS-PCR), amplification refractory mutation system polymerase chain reaction (ARMS-PCR), and restriction length polymorphism polymerase chain reaction (RFLP-PCR). HCV genotyping was performed by Ohno method. HCV viral quantification was performed by real time PCR.

The results showed that HCV genotype 3 was the most prevalent genotype (81%) with a very high response rate (91%). The distribution of other HCV genotypes was

comparatively less and also showed decreased response rate to interferon based therapy. The level of alanine aminotransferase (ALT) and low baseline viral load were linked with enhanced response rate to combinational therapy of HCV infection. Regarding SNPs in the host genome; SNPs of interleukin 28B (IL28B) clustered RefSNPs (rs) 12979860 and rs8099917, IL18 promoter variants, -607C/A (rs1946518) and -137G/C (rs187238), oligoadenylate synthetase gene 1 (OAS1) at exon 7 splice accepter site (SAS), rs10774671, osteopontin (OPN) -442 C/T (rs11730582), TGF-β1 functional polymorphism at codon 10 T/C (rs1982073) and codon 25 G/C (rs1800471), a single nucleotide variant in intron 6 of GALNT8 rs10849138 were analyzed for their association with natural clearance and interferon based therapy of HCV infection. There was found significant associations of IL28B rs12979860CC genotype, OAS1 rs12979860GG genotype, IL18 -607AA genotype, OPN -442TT genotype with HCV infection and treatment response. In conclusion, HCV genotyping and testing of IL28B polymorphism may be used as predictive markers for the outcomes of interferon based therapy in Pakistani population.

Chapter 1

INTRODUCTION

Hepatitis C Virus (HCV) was discovered as non-A non-B hepatitis virus in 1989 that is mostly transmitted through blood transfusion (Choo *et al.,* 1989). HCV has infected 200 million people around the globe (Davis and Thorpe, 2013). Approximately three to four million people are infected with the HCV each year (Zhang *et al.,* 2013). HCV prevalence is also very high in Pakistan. About 5.5% of the Pakistani population is infected with HCV (Waheed *et al.,* 2009). According to US center of disease control and prevention, in America each year about 25,000 people are getting HCV infection (Llovet *et al.,* 2008). Thus, HCV is not only a serious health problem of developing countries but also a major economical and physical burden of developed countries. HCV infection leads to either acute or chronic hepatitis.

Acute HCV infection is mostly asymptomatic. If the symptoms are present, they are not very specific and usually abate within few weeks. Acute HCV infection in 60-80% leads to the chronic stage. The progress of infection from acute to chronic stage is a slow process and may take 20-30 years. Chronic HCV infection is the leading cause of liver failure and liver transplantation worldwide (Seeff, 2002).

HCV is an enveloped positive stranded RNA virus of approximately 9.6 kb genome belonging to the family, *Flaviviridae*. It comprises of four structural and six nonstructural proteins. Being RNA virus and lack of proofreading in its RNA polymerase enzyme, it possesses eleven genotypes, subgenotypes and quasispecies (Ali *et al.*, 2014).

The natural history of HCV infection shows that 20-40% of infected individuals spontaneously clear the infection within few months of infection. However, the majority of cases are progressed to chronic stage (Kamal, 2008). Thus, HCV infection is a complex interplay between host and virus. There are a commendable number of factors that intervene and make the treatment complicated. Recent evidence has convincingly support this fact that the host and viral factors are key players that determine the response rate to interferon based therapy of HCV infection. Interferon therapy of HCV infection was used even before the discovery of the virus (Hoofnagle *et al.,* 1986). In the beginning, sustained virological response (SVR) was only 6%. SVR is defined as undetectable HCV RNA in patient's serum, six months after the cessation of treatment, while in the vice versa situation patients are referred as non responders (NRs). Rapid virological responders (RVR) are those patients that possess undetectable HCV RNA at week four of treatment. Later on, by increasing the treatment duration to 48 weeks the SVR rate was raised to 16% (Di Bisceglie *et al.,* 1989; Davis, 1989). The addition of ribavirin to interferon therapy elevated the SVR rate to 42% (McHutchison, 1998; Poynard *et al.,* 1998). The discovery of pegylated interferon leads SVR rate to 54-55% (Zeuzem *et al.,* 2000; Lindsay *et al.,* 2001; Reddy *et al.,* 2001). Recently, the addition of protease inhibitors to combinational therapy has increased the SVR rate to approximately 74% (Kwo *et al.,* 2010).

Among the viral factors, HCV genotypes and baseline viral load are important. It is suggested that HCV genotypes 2 or 3 should be treated with pegylated interferon and ribavirin. HCV genotype 1 patients should be treated with pegylated interferon, ribavirin and protease inhibitor. HCV genotype 4 infected patients should be treated with peg-

interferon and ribavirin. The treatment duration is mostly dependent on HCV genotypes. In general, longer the treatment duration, the lesser the chances of relapse (Gary-Gouy *et al.,* 2012). Moreover, the high baseline viral load, mutations in E1-E2, core region, NS5A and interferon sensitivity determining region (ISDR) are associated with treatment failure. However, the results are conflicting and require further investigations (Manns *et al*., 2001; Wohnsland *et al.,* 2007).

Among the host factors, patient characteristics and mutations in the genomic structures are important. Regarding patients characteristics, younger age, low body mass index, low alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP) levels, no other associated diseases, are all positively associated with interferon based therapy of HCV infection. Regarding mutations in the genomic organization single nucleotide polymorphisms (SNPs) have drawn attention. SNPs are considered as the most important host factors in interferon based therapy of HCV infection.

Most of the diseases that badly affected mankind are the result of complex interactions of lifestyle, environmental and genetic factors. SNPs may increase or decrease the chance of getting a disease. Thus, the genetic testing of disease specific SNPs may play a role in estimation of an individual risks of getting a disease and treatment outcomes. Although, the genetic testing of disease associated SNPs may only indicate a somewhat higher or lower risk of developing a disease, but they may also provide valuable information to the individual to make an informed decision about precautionary measure such as changing lifestyles or environment. Moreover, if the disease specific SNP possessed a role in response to the treatment, then the individual

may go for personalized medical therapy. SNPs also give us information about the molecular mechanism of a disease. Thus, from the patient point of view, SNPs can provide a platform for a more personalized tactic of treatment. From the pharmaceutical industrial point of view, SNPs may lead to the new drug targets that would be more effective, targeted and better tolerated. In short, understandings about these small genetic differences in the human genome are growing all the times. Findings of disease specific SNPs are a matter of great interest and a challenge for pharmaceutical industries (Weiner and Hudson, 2002). SNPs have a very critical role in population genetics, drug development, forensic and genetic diseases (Stoneking, 2001).

Recently genome wide association studies (GWAS) have shown that SNPs in the vicinity of IL28 are important in spontaneous clearance and response to interferon based therapy of HCV infection (Ge *et al.,* 2009; Suppiah *et al.,* 2009; Tanaka *et al.,* 2009; Rauch *et al.,* 2010). However, the role of IL28B in HCV genotype 2 and 3 is still controversial. Therefore, IL28B genotyping test is part of the standard of care against HCV genotype 1 only (Bellanti *et al.,* 2012). Genetic variations of different host and viral genes against HCV pathogenesis in various ethnic groups are also reported to possess a significant influence on natural clearance and response to interferon based therapy of HCV infection (Imran *et al.,* 2013a).

In Pakistan, HCV is a major health concern because 75% of patients do not receive standard anti HCV therapy (Interferon + Ribavirin) and the 25% that receive the treatment, the SVR rate is 60-70%. HCV patient's varied treatment response is among one of the major obstacle regarding HCV management. Combination of interferon (IFN) with various formulations (most commonly ribavirin) is the only approved therapy by

food and drugs administration (FDA) for chronic HCV patients. The treatment is currently considered as a standard treatment, but it is both physical and economic burden for the patients. Therefore, the current study was designed to search for some biological markers that can significantly predict the response rate to interferon based therapy of HCV infection. Such potent biological markers would help the clinical doctors to take informative decision regarding patient treatment. Present study scrutinized 140 chronic HCV patients and 120 healthy controls. Both viral and host factors were considered for their role in the combinational therapy (interferon + ribavirin) of HCV infection. From viral point of view, baseline viral load and viral genotypes were considered. While regarding host factors, important SNPs were investigated for their association with treatment response.

To the best of our knowledge, there is no study from Pakistan or neighboring country that has analyzed SNPs of these critical factors for interferon based therapy of HCV infection. The study would assist the search for such viral and host factors that will predict the success or failure of standard IFN therapy response in chronic hepatitis C (CHC) Pakistani patients. Taken together, the findings of current study suggest that HCV genotypes, SNPs of IL28B, IL18, OAS1 and OPN are potential significant predictors of treatment response in chronically infected HCV patients. Furthermore, low baseline viral load and ALT levels were also positively associated with the response to interferon based therapy of HCV infection in local population. This study would benefit the patients to be saved from the adverse side effects of interferon based therapy of HCV infection. Patients would also be saved from cost effects of interferon plus ribavirin treatment. Moreover,

those patients possessing more chances of treatment failure may seek for alternative treatment options of HCV infection**.** The main objectives of the current study were:

- Blood sampling of chronic HCV patients that are undergoing interferon based therapy of HCV infection from provinces, Sindh and KPK of Pakistan
- Follow up of the enrolled chronic HCV patients up to the six months after the end of therapy.
- Determining the genotypes of chronic HCV patients.
- Genetic testing of host genomes of all enrolled subjects for exploring the correlation of important genetic variations (9 SNPs) with interferon based treatment of HCV infection.
- Analysis of viral and host factors with interferon based therapy of HCV infection.

Chapter 2

REVIEW OF LITERATURE

2.1 HISTORY OF HCV

Long time ago, it was believed that jaundice is the result of divine punishment. There occurs imbalance of body humours due to environmental factors leading to skin discoloration, but this opinion was turned down by Hippocrates in 400 BC. For the first time, Jaundice was associated with the liver inflammation and named the hardening feature of the liver as "kirrhos". Since the 17th century, there occurred multiple outbreaks of jaundice. The epidemic outbreaks of the disease suggested that it causes an infectious etiologic nature. In 1908, it was suggested that the virus may be the cause of fulminant hepatitis (White, 1908).

In 1967, there were discovered two types of clinical hepatitis; one was associated with fecal oral transmission while the other was associated with blood transfusion. This leads to the discovery of hepatitis B (Dane and Cameron, 1970) followed by hepatitis A (Feinstone *et al.,* 1973). However, it was revealed by the retrospective study of stored sera that neither of these two viruses was responsible for blood transfusion associated hepatitis. This search for infectious etiology of non-A, non-B hepatitis (NANBH) leads to the discovery of Hepatitis C (Prince *et al.,*1974). HCV was first cloned from a cDNA library of NANBH cases by Choo *et al* in 1989 (Choo *et al.,* 1989). The recombinant peptide consists upon about 10,000 nucleotides. Later on, the recombinant peptide was used to capture antibodies from the sera of NANBH cases confirming the presence of HCV in these patients.

2.2 HCV EPIDEMIOLOGY

HCV is a major health problem affecting nearly 200 million people worldwide. HCV is the most prevalent in Egypt (22%) (Frank *et al.,* 2000). The highest rate of HCV prevalence in Egypt is mostly attributed to parenteral anti-schistosomal therapy (Choo *et al.,* 1989). In the United States of America approximately, 1.8% of people are positive for HCV antibodies while 2.7 million people have an active HCV infection. In Northern Europe, HCV prevalence is 0.3%, while in Southern Europe and North America its prevalence is 1.5%. HCV prevalence in sub-Saharan Africa is 3% (Madhava *et al.,* 2002). In Pakistan, the overall prevalence of HCV is 6%, but there may be small pockets of high HCV prevalence e.g. HCV prevalence in Gujranwala and Lahore is approximately 23.8% and 15.9% respectively (Hamid *et al.,* 2004).

2.3 ROUTES OF HCV TRANSMISSION

Approximately 50% of HCV transmission cases are attributed to unknown routes (Memon, 2002). The main route of HCV transmission before 1990 was blood transfusion. However, since 1990 the blood screening for HCV antibodies has significantly reduced transfusion related HCV transmission particularly in Asian countries. Currently, the main route of HCV transmission is the use of intravenous drugs. The average prevalence of HCV among the intravenous drug users is 80% (Sulkowski,1998). In Pakistan, there are about 5 million drug users, 15% of them are regular IDU (United Nations Office for Drug Control and Crime Prevention, 2002). In Pakistan, the main drug is heroin. Heroin is a common inhalation drug in Pakistan and Afghanistan. As the availability and quality of heroin is gradually decreasing, so therefore most of the drug users switched from inhalator to injectable drugs. The injectable drugs are comparatively more intensive and

satisfying. In Pakistan, the prevalence of HCV infection among drug users in is 57% ± 17.7% (Achakzai *et al.,* 2007; Altaf *et al.,* 2009; Platt *et al.,* 2009). The distribution of HCV infection among thalassemia and hemophilia population is 8.67% ± 1.75% (Arif *et al.,* 2008).

Health care workers are at the most high risk of HCV transmission. HCV transmission in health care worker mostly occurs by needle stick injuries during recapping of syringes. It is reported that HCV transmission among health care workers ranges from 2% - 10% (Hamid *et al.,* 1999). HCV may also be transmitted by sexual contact. A report from USA showed that 20% of new cases of HCV infection were due to sexual contact (Alter *et al.,* 1999). Saliva can also be the source of virus transmission. Polymerase chain reaction (PCR) also revealed that HCV was detected in the saliva of infected patients. It is still to be determined that the low quantity of virus particles which were detected in the saliva, has the sufficient potential to cause infectivity (Ferreiro, 2005). The other minor routes of HCV transmissions are sharing of tooth brushes, reuse of syringes and needles (Simonsen *et al.,* 1999; Khan *et al.,* 2000). Approximately, 2 million people are annually HCV infected by contaminated health care injections that contribute for almost 40% in distribution of HCV infection worldwide (Simonsen *et al.,* 1999). In developing countries, the re-use of razors by barbers also majorly contribute in distribution of HCV infections (Janjua and Nizamy, 2004).

2.4 HCV GENOME AND STRUCTURE

HCV is a positive stranded RNA-virus which is translated into a single precursor polypeptide chain of about 3300 amino acids. It is very heterogeneous as its nonstructural protein; NS5B, RNA-dependent RNA polymerase (RdRp) is prone to mutation. There are

eleven major genotypes of HCV (Safi *et al.,* 2012). These genotypes are determined on the basis of their nucleotide variations up to 35%. There are also many sub-types of HCV which are based on their sequence variability up to 25%. RdRp has a lack of proof reading, therefore there are also many quasi-species of HCV. HCV genotypes are important predictors of treatment response (Simmonds *et al.,* 2005; Nakano and Sugiyama, 2012).

HCV genome comprises 5'-untranslated region (UTR) containing an internal ribosome entry site (IRES) and an open reading frame (ORF) with four structural proteins (Core, E1, E2 and P7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). At the end of the polypeptide chain there is 3' UTR (Moradpour *et al.,* 2007; Poenisch and Bartenschlager, 2010). The binding site of liver specific MicroRNA-122 (miR-122) is present in 5' UTR as shown in Figure 1 (Jopling *et al.,* 2005).

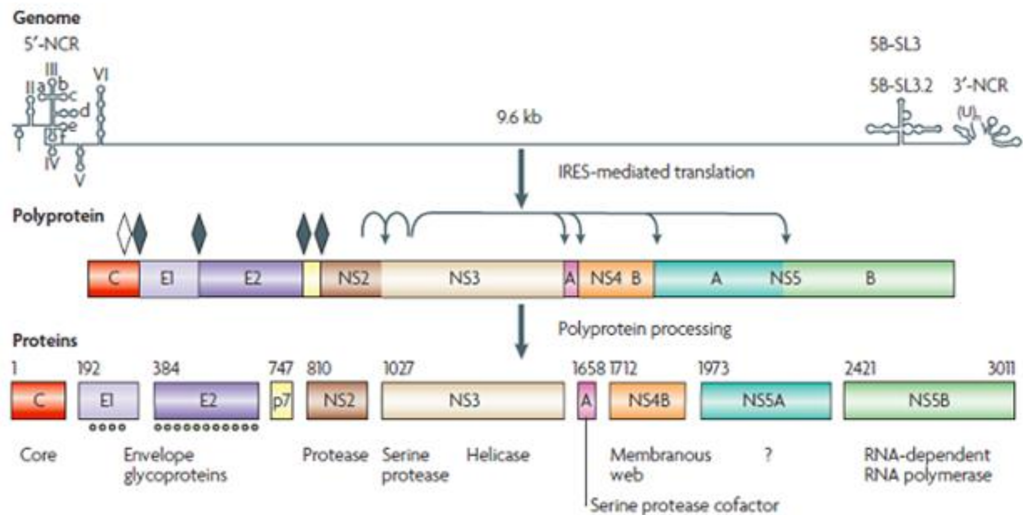


Figure 1.1: HCV genome organization and its proteins. Adapted from Moradpour *et al.,* 2007.

HCV virions have not yet been visualized definitely; however, electron microscopy revealed that its size is 40-70 nm in diameter (Wakita *et al.,* 2005). These virions are tightly associated with lipoproteins. It has been shown that multiple copies of core having genomic RNA and E1, E2 glycoproteins are anchored with the lipid bilayer that is derived from the surrounding cells (Andre *et al.,* 2005).

2.5 HCV LIFE CYCLE

2.5.1 Virus Entry

The first step of the virus life cycle is its entry into the host cell. HCV has a narrow range of host, from chimpanzee to human. The ultimate target of the virus is hepatocytes, but the virus is also reported in B lymphocytes, dendritic cells and endothelial cells (Fletcher *et al.,* 2012). The host receptors that are associated with virus entry into the cells involve CD81 (Pileri *et al.,* 1998), LDL receptor (Agnello *et al.,* 1999), scavenger receptor class B type I (SR-BI) (Scarselli *et al.,* 2002), claudin-1 (Evans *et al.,* 2007), occludin (Ploss *et al.,* 2009), epidermal growth factor receptor (EGFR) (Lupberger *et al.,* 2011) and Niemann-Pick C1–like 1 cholesterol absorption receptor (NPC1L1) (Sainz *et al.,* 2012). The last two receptors EGFR and NPC1L1 are recently recognized receptors; for both of these two receptors pharmacological inhibitors already exist. By coordination of these host receptors, HCV enters into the host cell via clathrin coated endocytosis as shown in Figure 1.2 (Blanchard *et al.,* 2006).

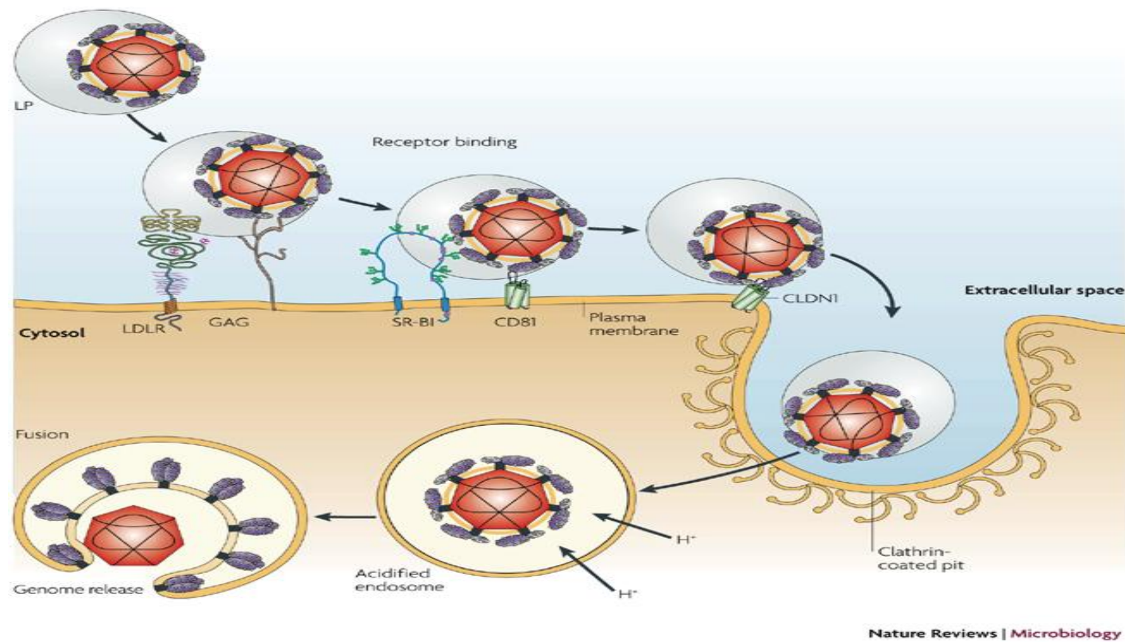


Figure 1.2: HCV entry into the cell. Adapted from Moradpour *et al.,* 2007.

It is noteworthy that structural protein p7 of HCV has a very important role in both HCV virion assembly, release and then again in the entrance into the new cell. The exact mechanism of p7 role in enhancement of virion production is still a mystery. However, there is a supporting evidence that HCV p7 behaves like influenza A viroporin, M2 (Griffin, 2004; Chew *et al.,* 2009; Meshkat *et al.,* 2009). It has a role both in virion secretion and entry. During the process of virion secretion, p7 proteins oligomerise and form ion channels over the lipid membrane of endosome. Hydrogen ions, which enter through the cellular ion channels, exit through the ion channels formed by p7 proteins. By this way, HCV glycoproteins are protected from low pH of endosome. Moreover, p7 oligomers along with HCV-NS2 proteins have a critical role in virion assembly. When the virion attacks another cell through clathrin coated endocytosis, p7 proteins also have a role in the release of virion from endosome. By this time, p7 oligomers remain along the

virion and not transferred towards the lipid membrane. The pH of the endosome rises by the entrance of hydrogen ions through the cellular ion channels of endosome leading to the uncoating of core proteins from endosome. The whole phenomenon is shown in the Figure 1. 3 (Griffin, 2004).

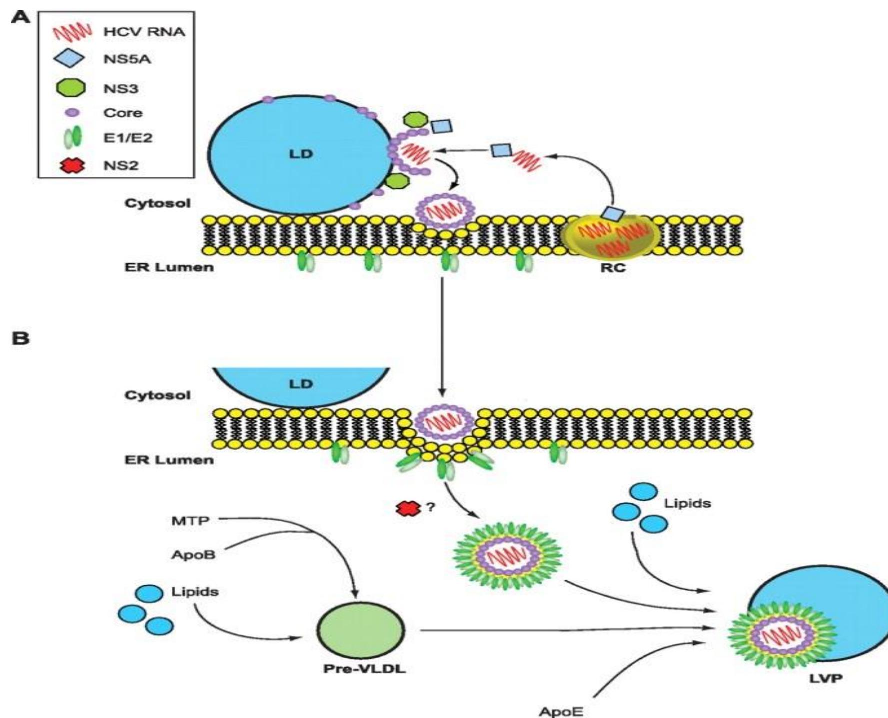


Figure 1.3: HCV assembly/release and entry/uncoating. Adapted from Griffin, 2009.

2.5.2 Translation

The precise mechanism of HCV translation and replication is still unknown. The 5' UTR plays an essential role in translation. Initially, there is produced a large polyprotein precursor which is processed by viral and host proteases into individual peptides. Among the HCV proteins, serine protease NS3 is important. It is an enzyme with a range of catalytic sites involving serine protease, NTPase and helicase activities.

All these actions take place on the surface of rough endoplasmic reticulum (RER) (Morikawa *et al.,* 2011).

2.5.3 Replication

NS4B acting as a cofactor of NS3 protease also has an important role in HCV replication. It is highly hydrophobic in nature playing its role in the assembly of lipid vesicles over the membranous matrix also known as membranous web. HCV NS5B is RdRp which forms double stranded replicative form, from a single positive stranded RNA. There are produced numerous positive strands RNA from a negative strand intermediate. HCV replication takes place on intracellular lipid membranes which structure is also altered by viral proteins. Particularly, the structure of ER is altered to membranous web. Various viral proteins are involved in the membranes alteration, but the most important one is NS4B, as it alone, is capable of triggering these alterations (Egger *et al.,* 2002). There is also the possibility that viral replication occurs on lipid rafts which contain a high amount of cholesterol and sphingolipids. It has been shown that inhibitors of sphingolipid synthesis also inhibited HCV replication (Shi *et al.,* 2003; Aizaki *et al.,* 2004; Matto *et al.,* 2004; Sakamoto *et al.,* 2005). There are also a number of host proteins involved in the process of replication such as vesicle-associated membrane proteins A and B (VAP-A and VAP-B) which bind with NS5A and NS5B (Hamamoto *et al.,* 2005). Similarly, Cyclophilin B possessing peptidyl-prolylcis-trans isomerase activity is also essential for NS5B binding with RNA (Watashi *et al.,* 2005).

2.5.4 Assembly

HCV genome is released from membranous web of ER along with NS5A to allow its contact with core proteins which are present on the lipid droplets (LDs).

There are also transferred other members of replication complex from membranous web to LDs, like NS3 and NS2. NS3 is responsible for the formation of a fast sedimenting core containing particles that are supposed to be non-infectious virions and need further maturation. There occur encapsidation of the viral genome by core proteins. The encapsidated viral genome is translocated inside the ER lumen where E1 and E2 glycoproteins are added. NS2 protein confers infectivity to the virions by interaction of glycoproteins E1 and E2 with immature particles. During the maturation step, nascent viral particle interacts with lumen VLDLs in the form of LDs and other lipid particles such as Apo E to produce LVPs (Jones and McLauchlan, 2010). The whole phenomenon is shown in Figure 1.4.

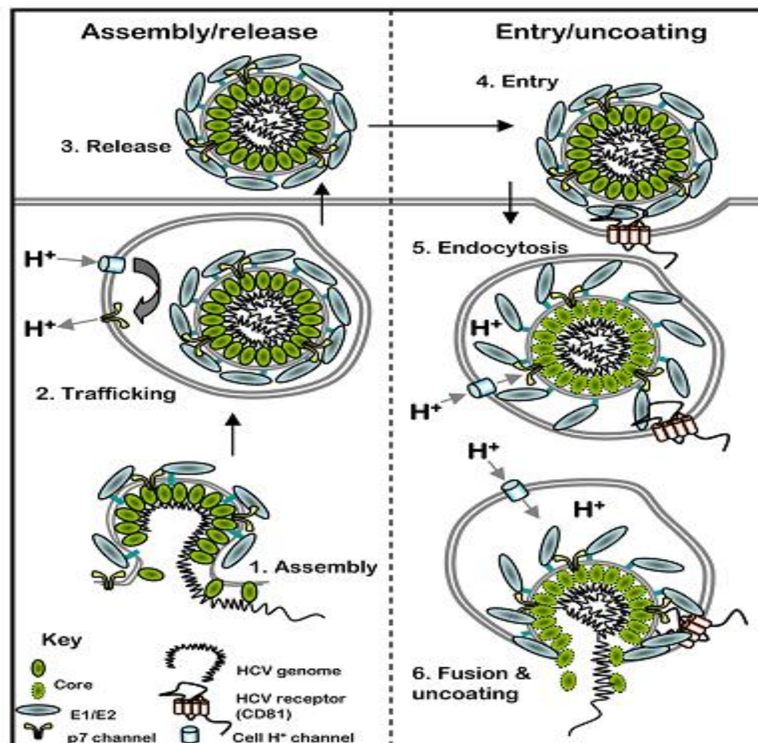


Figure 1.4: Release and assembly of HCV particles. Adapted from; Jones and McLauchlan, 2010).

2.6 VIRUS-LIKE PARTICLES

The serum of HCV infected individual contains different forms of the HCV particles which differ in size and density. There is little known how these different virions are produced. The density of these HCV particles ranges from 1.03 to 1.25 g/ml (Thomssen *et al.,* 1992). Interestingly, the study on Chimpanzees demonstrated that low density HCV particles were more infectious than denser (Choo *et al.,* 1989; Hijikata *et al.,* 1993). Low density HCV particles contained a high amount of triglycerides, core, RNA, and apolipoproteins B and E (ApoB and ApoE). The major components of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were triglycerides, ApoB and ApoE. VLDL and LDL are packed in the ER of hepatocytes and secreted into the blood stream to be transported it to its storage site, adipocytes. It is presumed that HCV virions acquire triglycerides, ApoB and ApoE during assembly. HCV virions exploit the assembly pathway of VLDL and LDL. The transport system of VLDL and LDL to their storage site is interfered by HCV virions. It leads to the accumulation of VLDL and LDL inside the hepatocytes and ultimately to liver steatosis. HCV particles which do not acquire triglycerides, ApoB and ApoE are degraded, as these host components assist HCV from the host immune response and also increases its number of receptors (Andre *et al.,* 2002; Gastaminza *et al.,* 2008; Huang *et al.,* 2007; Nielsen *et al.,* 2006; Perlemuter *et al.,* 2002). The complete HCV life cycle is shown in Figure 1.5.

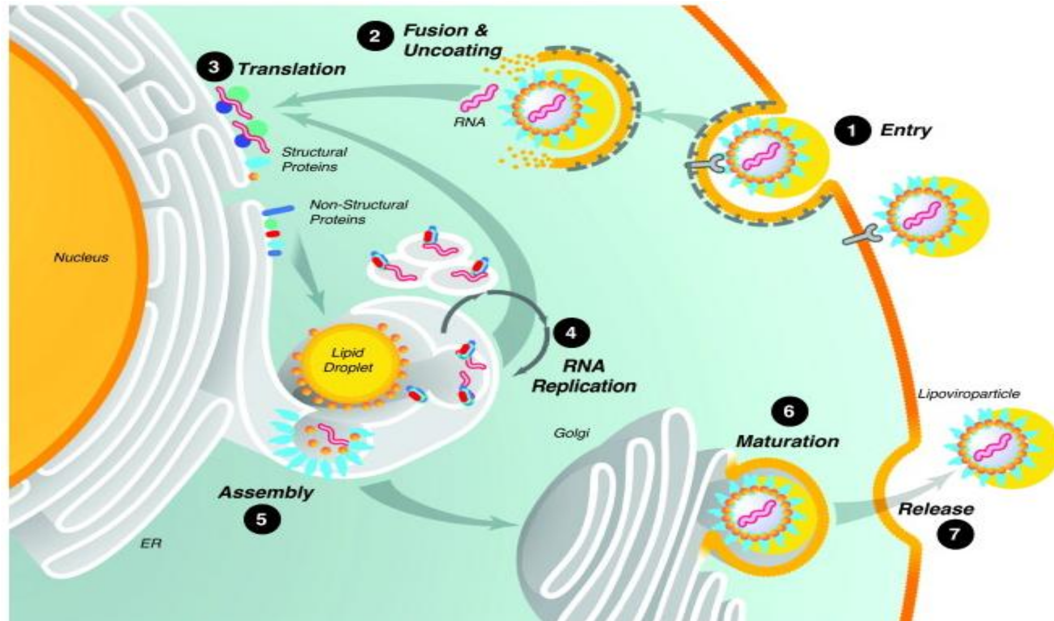


Figure 1.5: Life cycle of HCV. Adapted from Gastaminza *et al.,* 2008.

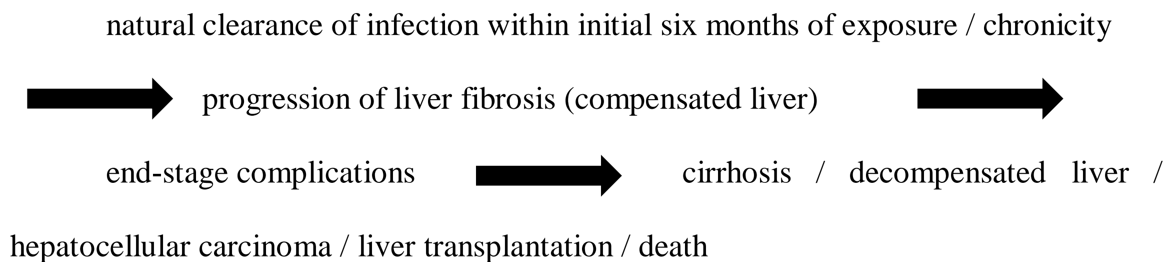
2.7 NATURAL HISTORY OF HCV INFECTION

By definition, acute HCV infection must lead to chronic infection but it is observed that approximately 20% of individuals naturally clear the virus within initial six months of infection (Imran *et al.,* 2013). The actual mechanism of spontaneous clearance of the virus is still unclear. There are no reliable predictors of natural clearance of viral infection. However, several factors are reported to be associated with spontaneous clearance of virus infection. The positively associated factors involved with HCV clearance are low age (Alter *et al.,* 1999; Bellentani and Tiribelli *et al*., 2001; Micallef *et al.,* 2006), female sex (Bakr *et al.,* 2006; Micallef *et al.,* 2006; Page *et al.,* 2009), HCV non-genotype 1 (Micallef *et al.*, 2006), Mexican Americans ethnicity (Alter, 1999) and SNPs of the host genome. However, study from elsewhere has sown that female sex is

poorly associated with the natural clearance of virus infection. Majority cases of acute HCV infection are without symptoms (McCaughan *et al.,*1992; Chung, 2005). Approximately, only 25% - 30% of cases are associated with symptoms within 3-12 weeks after the infection. The main manifestation of infection is fatigue (Alter and Seeff, 2000; Thimme *et al.,* 2001).

2.8 HCV INFECTION AND LIVER FIBROSIS

The following dynamic phases of HCV pathogenesis will help us to understand the natural course of virus infection. Exposure to HCV; mild HCV infection



natural clearance of infection within initial six months of exposure / chronicity

progression of liver fibrosis (compensated liver)

end-stage complications

cirrhosis / decompensated liver / hepatocellular carcinoma / liver transplantation / death

If the individual is not successful in clearing HCV infection; then it ultimately leads to liver fibrosis, hepatocellular carcinoma and finally to decompensated liver. The time taken in the development of liver fibrosis is usually in decades. A study from the Japanese population has shown that it almost took 29 years to develop hepatocellular carcinoma after blood transfusion (Kiyosawa *et al.,*1990). A similar study from America demonstrated that it took 20.6 and 28.3 years for the development of liver cirrhosis and hepatocellular carcinoma respectively (Tong *et al.,* 1995).

2.9 LIVER BIOPSY

Liver biopsy is a very important marker for the determination of the stage of disease and the decision about the treatment options. The HCV patient at the stage of decompensated liver cannot be given interferon therapy as it is associated with serious side-effects. Two types of liver biopsies may be taken for the determination of liver cirrhosis. One is cross-sectional, and the other is longitudinal. On both types of biopsy samples, the METAVIR (Bedossa and Poynard, 1996) or Ishak (Ishak *et al.,* 1995), Histological Activity Index (HAI) staging systems may be applied. Cross-sectional study usually involves the single liver biopsy and the date of first time blood transfusion or intravenous drug use. The longitudinal study of liver biopsies involves two liver biopsies and the rate of fibrosis development between them is assessed. In this method, there is no requirement of estimating the duration of disease or the assumption that the rate of fibrosis development is linear. There are a lot of factors which are involved in acceleration of liver cirrhosis e.g. male sex (Poynard *et al.,*1997) age (Poynard *et al.,* 1997), alcohol intake (Wiley *et al.,* 1998), hepatic injury, hepatic steatosis (Adinolfi *et al.,* 2001), co-infection with other viruses (Benhamou *et al.,* 1999), viral genotype, ethnicity (Poynard *et al.,* 1997), the route of blood transfusion (Roudot-Thoraval *et al.,* 1997) etc. The involvements of these factors suggest that the progression of liver fibrosis is not linear.

2.10 HCV AND ESCAPE FROM THE INNATE IMMUNE SYSTEM

2.10.1 Role of HCV Core Protein in Evasion from the Innate Immune System

In vitro studies of cell lines suggested that core protein in a monomeric form is recognized by TLR2 (Barth *et al.,* 2005). However, the recombinant or serum derived virus particles are not recognized by TLR2. The most probable reason for this difference, in recognition of monomeric core protein and core protein in intact virus might be attributed to changes in structural conformation. The most obvious advantage of this difference in recognition is to the virus. HCV escapes from the innate immune system at early stages at the time of its entrance into the host cell. Once, HCV persists and get hold of hepatocytes; it begins transcriptions and translations. At this stage, the monomeric core proteins that are released from hepatocytes are recognized by TLR2. Thus, this difference in recognition of core protein in intact and monomeric form leads to delayed activation of the innate immune system against the virus (Imran *et al.,* 2012).

2.10.2 Role of HCV NS3/4A Protease in Evasion from the Innate Immune System

TLR3 and retinoic inducible gene 1 (RIG1) are mostly activated as the host innate defense mechanism against RNA viruses such as HCV (Breiman *et al.,* 2005; Katze *et al.,* 2002). The stimulation of these pathogen recognition receptors (PRRs) by double-stranded RNA of HCV leads to signaling cascades. As a result of these two signaling pathways, there is an induction of nuclear factor kappa B (NF-κB), interferon regulatory factor 3 (IRF3) and activation protein 1 (AP1) which are the most important antiviral transcription factors. These transcription factors stimulate the production of type 1 and pro-inflammatory cytokines against HCV infection (Katze *et al.,* 2002). Although, there is no direct interaction of HCV protein with TLR3 or RIG1 but its protein, NS3/4A is

involved in blocking the downstream elements of these signaling pathways (Imran *et al.,* 2012). There are two important adapter proteins, TRIF (Sharma *et al.,* 2003) and Cardif (Meylan *et al.,* 2005) in the downstream of TLR3 and RIG1 signaling pathway respectively. The two adapter proteins are blocked by HCV NS3/4A. By this way, HCV escapes from the host defense mechanisms initiated by the signaling pathways of TLR3 and RIG1.

2.10.3 Role of HCV NS5A Protease in Evasion from the Innate Immune System

TLR4 recognizes HCV NS5A protein (Sene *et al.,* 2010). However, the signaling cascade is also blocked by NS5A. Studies on Huh-7 cell line suggested that NS5A decreased the expression of TLR4. NS5A is not directly involved in reduction of TLR4 expression; it decreases the expression of CD14 and MD-2 molecules that are involved in formation of TLR4 receptor complex. Moreover, cell lines studies demonstrated that the activity of MyD88 is also inhibited by NS5A attachment. MyD88 is an important adapter protein of TLR4 signaling pathway (Tamura *et al.,* 2011). NS5A also stimulates the induction of anti-inflammatory cytokines such as IL10, TGFβ, IL6 which in turn leads to down-regulation of proinflammatory cytokine such as IL12 (Ma *et al.,* 2001).

2.11 HCV AND ESCAPE FROM THE ADAPTIVE IMMUNE SYSTEM

2.11.1 Role of HCV Core Protein in Evasion from the Adaptive Immune System

Co-culturing of monocytes and T-cells from HCV infected patients along with HCV core protein results in the production of APCs with a limited ability to drive Th-17 differentiation as compared to healthy controls. Further research showed that the defective Th-17 differentiation is due to impaired production of IL17 by these cells

(Triantaflou *et al.,* 2006; Watanabe *et al.,* 2006; McGeachy and Cua, 2008). The impaired IL17 induction is attributed to impaired IL6 production by antigen presenting cells (APCs). Thus, core protein stimulates TLR2 that results in the production of a series of cytokines; among these cytokines, IL6 is important as its production is impaired. Decreased IL6 induction by APCs leads to impaired stimulation of IL17 which in turn results in defective Th-17 differentiation. By this way, HCV exploits the host adaptive immune system of Th-17 cells (Dolganiuc *et al.,* 2004).

During the initial step of virus entry into the host cell, there is no activation of TLR2 signaling pathway as the intact core protein cannot activates TLR2. However, core protein is able to induce TLR9 signaling pathway. There is a production of IFN-α via TLR9 signaling pathway. IFN- α is an antiviral cytokine. It inhibits HCV replication but, once the virus is persisted somehow, then there is also production of monomeric core proteins of HCV. The HCV monomeric core protein leads to the production of IL10 and TNF-α by stimulating TLR2 signaling pathway. IL10 and TNF-α exert an inhibitory effects on TLR9-induced IFN-α production. Moreover, rIL10 and TNF-α suppress apoptosis of plasmacytoid cell (PDC) in a dose dependent manner. Apoptosis of the infected cell is a host defense mechanism against the spread of infection. In conclusion, HCV help itself against IFN-α by the production of IL10 and TNF-α and also maintains its persistence in the PDC by inhibiting PDC apoptosis (Payvandi *et al.,* 1998; Rissoan *et al.,* 1999; Gary *et al.,* 2002; Duramad *et al.,* 2003).

2.11.2 Role of HCV NS3/4A protease in Evasion from the Adaptive Immune System

NS3/4A also plays an important role in evasion from the adaptive immune system. It abrogates TLR3 and RIG1 signaling pathways by inhibiting the two important

adapter proteins of these signaling pathways, TRIF and Cardif. These two pathways are essential for the activation of NF-κB and IRF3, which in turns leads to the failure in induction of IFN-α and IFN-β. Moreover, NS3/4A suppresses or delay the CD8 T cell responses which are essential for the elimination of virus (Iwasaki and Medzhitov, 2004; Hoebe and Beutler, 2004).

2.11.3 Role of HCV NS5A in Evasion from the Adaptive Immune System

NS5A is a ligand of TLR4 (Sene *et al.,* 2010). It stimulates the production of IL10 and TGFβ via TLR4 signaling pathway. Blocking of IL10 or its receptors result in decreased production of TGFβ by NS5A in a dose dependent manner. Thus, TGFβ production is mainly dependent on autocrine activity of IL10. IL10 is produced by P38 and P13K pathway (Ma *et al.,* 2001). P13K is an endogenous suppressor of IL12, which is a pro-inflammatory cytokine and support HCV persistence (Fukao *et al.,* 2002).

NKG2D is pathogen recognition receptors that are mainly expressed on natural killer (NK) cells and CD8 T cells (Bauer *et al.,* 1999). These receptors are important component of the host immune system as viruses, bacteria and other pathogens have developed different strategies to evade this host defense mechanism. On the normal cells, NKG2D are not expressed (Groh *et al.,* 2002). These are expressed as a result of some stress such as DNA damage or an invasion by some pathogen. TGFβ, which is produced by the activation of TLR4 signaling pathway via HCV NS5A, causes the down-regulation of these receptors. The down-regulation of NKG2D receptors on NK cells and CD8 T cells has functional consequences. It causes impaired production of IFNγ and CD107a degranulation by NK cells (Sene *et al.,* 2010). The exact role of NKG2D in HCV infection is still to be cleared (Oliviero *et al.,* 2009; De Maria *et al.,* 2007).

2.12 STANDARD TREATMENT OF HCV INFECTION

Interferon monotherapy was used for HCV infection even before the discovery of the virus. The success rate was only 6% - 10% (Davis *et al.,* 1989; Di Bisceglie *et al.,* 1989). The successful treatment against HCV is defined as sustained virological response (SVR), avirimia for six months after the completion of treatment. The continuous struggle for improving the response rate led to the discovery of broad-spectrum antiviral agent, ribavirin. Ribavirin is a guanosine nucleotide analogue. The addition of ribavirin leads to the improvement in success rate up to more than 30% (McHutchison *et al.,* 1998; Poynard *et al.,* 1998). The success rate was further improved by the addition of pegylated interferon instead of simple interferon. Pegylated interferon increases the half-life of interferon as polyethylene is covalently attached with interferon. The success rate was attained up to 50% (Manns *et al.,* 2001; Fried *et al,* 2002; Hadziyannis *et al.,* 2004). Currently, there is another addition to combinational therapy of HCV infection, Ribvirin or Telaprevir. The triple therapy (pegylated interferon, ribavirin and protease inhibitor) is considered as a standard of care against HCV genotype 1 (Imran *et al.,* 2013b).

More than two decades have passed since the discovery of interferon and ribavirin as a treatment of HCV infection; their exact mechanisms of action are still unknown. Interferon is considered not to directly act on the virus; it binds to the host cell receptors and stimulates the induction of interferon stimulated genes (ISGs). There are more than 300 ISGs which confers antiviral activity (Feld and Hoofnagle, 2005). ISGs most probably block HCV replication. HCV escapes the action of ISGs by interfering the transcription of these genes by its core protein (De Lucas and Bartolome, 2005; Choi and

Ou, 2006a). Moreover, HCV NS3/4A protease also inhibits interferon amplification loop (De Lucas and Bartolome, 2005; Karayiannis, 2005; Choi and Ou, 2006b).

Ribavirin alone has no appreciable effect on HCV infection (Bodenheimer *et al.,* 1997), but its combination with interferon accelerates the response rate. The most probable reason for this augmented immune response is attributed to down-regulated effect on inhibitory pathways of interferon. It also inhibits host inosine monophosphate dehydrogenase (IMDH) enzyme that result in depletion of GTP pool, essentially required for the viral RNA synthesis (Lau *et al.,* 2002). Additionally, ribavirin has a direct effect on HCV RdRp by binding its nucleoside binding region and inducing mutations in viral RNA synthesis. All these functions of ribavirin were concluded from in-vitro studies. It is still unclear that any or all of these functions of ribavirin will exist in in-vivo also (Feld and Hoofnagle, 2005).

2.12.1 Side-effects of Interferon Based Therapy

Although interferon based therapy of HCV is a gold standard treatment of HCV infection as it is successful in more than half number of patients but 10% - 20% of patients have to stop the treatment due to a lot of serious side-effects (Manns *et al.,* 2006). Major side effects of interferon based therapy are fever, fatigue and headache (Hadziyannis *et al.,* 2004). The most severe side effect of interferon based therapy is depression which is most likely due to effect of interferon on serotonin activity. Moreover, dose dependent haemolyticanaemia is also caused by ribavirin that leads to the discontinuation of treatment in 32% - 42 % of cases (Fried *et al,* 2002).

2.13 FUTURE THERAPIES OF HCV INFECTION

HCV comprises four structural proteins and six non-structural proteins. There is the development of anti-viral drugs against each viral protein as shown in table 1.1 (Imran *et al.*, 2013b). NS3/4A protease inhibitors and nucleoside / nucleotide inhibitors against are currently in the most advanced stages of clinical developments.

Table 1.1: Future drugs against hepatitis C virus infection.

Company	**Drug**	**Mechanism of action**	**Stage of clinical development**
Biotron Limited	BIT225	Inhibit HCV P7 protein	Completed phase IIa
Roche	Danoprevir	Inhibit NS3 protein	Phase II
Merck & Co.,	Vaniprevir	Inhibit NS3 protein	Phase II
Merck	Boceprevir	Inhibit NS3 protein	**APPROVED**
Vertex	Telaprevir	Inhibit NS3 protein	**APPROVED**
Tibotec/Janssen	TMC-435	Inhibit NS3 protein	Phase III
BoehringerIngelheim	BI 201335	Inhibit NS3 protein	Phase III
Gilead and Achillion Pharmaceuticals	ACH-806	NS4A antagonist	Phase 1b/2
Eiger BioPharmaceuticals	Clemizole hydrochloride	Inhibitor of NS4B:RNA	Phase 1b
Med Chem express	Anguizole	inhibitor of HCV RNA replication	Phase 1b
Bristol Myer Squibb	BMS-790052	NS5A inhibitor	Phase III

Bristol Myer Squibb	BMS 824	NS5A inhibitor	Phase II
Gilead Sciences Inc	GS-5885	NS5A inhibitor	Phase II
GlaxoSmithKline	GSK2336805	NS5A inhibitor	Phase II
Presidio Pharmaceuticals	PPI-668	NS5A inhibitor	Phase II
Pharmasset	PSI-7851	NS5B inhibitor	Phase II
Abbott	ABT-072	Non-Nucleoside Polymerase Inhibitor	Phase II
Abbott	ABT-333	Non-Nucleoside Polymerase Inhibitor	Phase III
SantarisPharma	SPC3649	Lock Nucleic Acid mRNA122 inhibitor	Phase 1
Human Genome Sciences	Zalbin	Immunomodulator	Phase III
Novartis / Debiopharm	Debio 025	Cyclophilin Inhibitor	Phase III
SciClonePharma / Sigmatau	Zadaxin - thymalfasin	Immunomodulator	Phase III

Recently two protease inhibitors, Boceprevir or Telaprevir are approved as a part of combinational therapy against HCV genotype 1.

2.13.1 Boceprevir

Boceprevir is a ketoamide inhibitor of HCV NS3 protease. It forms a reversible covalent bond with NS3 and blocks its protease activity (Mederacke *et al.,* 2009; Sarrazin and Zeuzem, 2010). Boceprevir monotherapy against HCV infection is not effective due to the development of viral resistance within a week of therapy. Hence, it is highly recommended that four tablets of Boceprevir, each 200 mg should be given to the patients after 7-9 hours with the optimal dose of peg-interferon plus ribavirin (Malcolm *et al.,* 2006). The published data have shown that the response rate to interferon based therapy is different in black and non-black African-American patients; therefore the response rate of triple therapy was studied in two different cohorts. The improved SVR rate to triple therapy of black African-American cohort was 19% - 30%, while for non-black African-American cohort, it was 27% - 28%. The triple therapy against HCV genotype 1 is equally efficient; whether it is used with IFNα 2a or IFNα 2b as a part of standard treatment (Poordad *et al.,* 2011).

2.13.2 Telaprevir

Telaprevir also known as VX-950 is a linear peptidomimetic inhibitor possessing a ketoamide serine trap warhead for HCV NS3/4A. Its monotherapy against HCV infection is also not recommended due to the immediate emergence of viral resistance. Two tablets of Telaprevir are recommended, each 375 mg after every 7-9 hour along with the optimal dose of peg-interferon plus ribavirin. Telaprevir was given for 12 weeks in phase III stage of clinical development due to the development of severe side-effects (McHutchison *et al.,* 2010). Two large studies of treatment naive patients showed that triple therapy was more effective than double therapy. However, triple therapy is mostly

dependent on HCV genotype. The best results are shown by HCV genotype 1 as compared to HCV genotype 2 and 3 (Jacobson *et al.,* 2011; Chatel-Chaix *et al.,* 2010). Retreatment of the relapse patients by double therapy showed SVR rate of 24% - 34%, while by triple therapy it was 69% - 80%. Hence, relapse patients are more suitable for treatment with triple therapy.

In conclusion, different therapies are developing against HCV infection but not a single therapy can completely eradicate HCV infection. Interferon is still used as a part of combinational therapy. The addition of direct acting antiviral agent to interferon therapy also causes the addition of new side-effects that were not previously reported with interferon plus ribavirin treatment. The future therapy of HCV infection is expected to be the combination of only direct acting antiviral agents without the involvement of interferon and ribavirin to get rid-off its side-effects during the course of therapy (Imran *et al.,* 2013b).

2.14 ROLE OF VIRAL FACTORS IN INTERFERON BASED THERAPY

There are multiple viral factors which also have an important role in the interferon based therapy of HCV infection. The low viral titer before the treatment is the predictor of SVR. HCV genotype is also an important marker of treatment response. The treatment is easy for HCV genotype 3 as compared to other genotypes. However, no patient should be left without treatment purely on the basis of HCV genotype as its predictive value is 55%. (Yuen and Lai, 2006). Genetic variations in domain III portion of 5′ UTR were observed more commonly in SVR patients as compared to breakthrough patients. These mutations were insertion mutations and induced altered Watson-Crick base pairing that

led to decreased RNA stability and binding affinity with ribosomes. Thus, genetic variations of 5′ UTR domain III are positively associated with interferon based therapy of HCV infection (El Awady*et al.,* 2009). Genetic variations of HCV core, p7, NS2, NS3, NS5 are also noteworthy. These genetic variations were not specific for their positions in viral proteins. The random genetic mutations in viral sequences were more common in SVR patients as compared to NR patients. A mutation in HCV core protein at residue 71 was found to be a significant predictor of treatment response in triple therapy of HCV infection (Akuta *et al.,* 2010). How these genetic variations of virus play roles in treatment response is still to be discovered.

2.15 ROLE OF HOST FACTORS IN INTERFERON BASED THERAPY

2.15.1 Age

Age is defined as a decline in body functions and the immune system. Elderly people are comparatively more at risk for invasion by pathogens (Aspinall *et al.,* 2007). The immune system of aged people for spontaneous clearance of pathogens or response to treatment is also weak due to decreased number of CD4 and CD8 T cells, naive T cells, B cells. There is also a shift from Th1 to Th2 cytokines production and expression of PRRs. All these changes are associated with failure in clearance of pathogen and treatment outcomes (Ginaldi *et al.,* 2001). Phase III studies (NV15801/NV15942) of 569 HCV genotype 1 patients treated with peg-interferon and ribavirin demonstrated that the treatment response rate in young patients was higher as compared to aged patients. Moreover, the relapse rate was also higher in elderly patients than younger patients (Dey and Chaudhury, 1997).

2.15.2 Sex

Formally, it was considered that female sex favors response to interferon based therapy (Poynard *et al.,* 1998) but later on large respective studies concluded that there is no effect of gender on interferon based therapy of HCV infection (Manns and McHutchison, 2001).

2.15.3 Insulin Resistance

Insulin resistance (IR) is a condition where high concentration of insulin is required to maintain normal glycemia and appropriate consumption of glucose. Both genetic factors and environmental factors may contribute in the development of IR. The genetic factors may generate abnormalities either in insulin gene or its receptors and members of the signaling pathway. The environmental factors may involve various pathogens. IR is often associated with HCV infection (Zein *et al.,* 2005). The risk of developing type 2 diabetes (T2D) increases 11 times when the patient is infected by HCV (Harrison, 2006). Moreover, it was found that HCV infection and IR led to the development of extra-hepatic manifestations and a decreased response rate to interferon therapy. IR may also be induced by HCV core protein. HCV core protein plays its role by proteosomal degradation of insulin receptor substrate 1 and 2 and blocks intracellular insulin signaling pathway. On the other side, interferon therapy of HCV infection is also influenced by IR. IR leads to upregulation of SOCS3 cytokines, which interfere with interferon signaling pathways (Walsh *et al.,* 2006). Thus, HCV may not only induces IR but may further exploits this weakness for its defense against interferon action.

2.15.4 Race

Racial differences also influence the rate of SVR against HCV infection (Reddy *et al.,* 1999). Peg-interferon plus ribavirin treatment of African-American and Caucasian-American demonstrated that the success rate of therapy was 28% and 52% respectively (Conjeevaram *et al.,* 2006). Moreover, the viral breakthroughs were also more frequent among the African-American as compared to Caucasian-American (Conjeevaram *et al.,* 2006). The mechanisms behind these observed differences are poorly understood and may be attributed to multiple factors. Some factors among them are high obesity rate and prevalence of HCV genotype 1 among African-American (McHutchison *et al.,* 2000). Furthermore, the distribution of treatment responsive SNP, IL28B rs12979860 was less prevalent among African-American than Caucasian-Americans. The distribution of this particular SNP among the African-American was 16% with a treatment success rate of 47 %, while among the Caucasian-Americans, it was 39% with a treatment success rate of 81% (Clark *et al.,* 2010).

2.15.5 Obesity

Individuals with body mass index (BMI) ≥ 30 kg/m2 are defined as obese. It is estimated that 30% HCV infected patients are obese (Bressler *et al.,* 2003; Hickman *et al.,* 2003). Obesity is linked with a lower treatment response to interferon based therapy of HCV infection (Camma *et al.,* 2004). There are proposed different mechanisms to explain how obesity decreases the response rate to interferon based therapy of HCV infection. The first mechanism suggested that obesity is an inflammatory condition resulting in abnormal response to interferon based therapy of HCV infection. The second mechanism highlighted that obesity has a role in the development of liver steatosis,

fibrosis, cirrhosis and hepatocellular carcinoma that leads to poor response to interferon therapy. According to third mechanism the bioavailability of peg-interferon is decreased by obesity (Charlton *et al.,* 2006). A recent large study concluded that there is no significant effect of BMI on the treatment outcomes of HCV infection. However, it is established that weight loss plays an important in the treatment outcomes of HCV infection as it causes down regulation of liver enzymes and fibrosis development (Hickman *et al.,* 2002).

2.15.6 Hepatic steatosis

Disease progression in HCV infection is also accelerated by hepatic steatosis (Commentary *et al.,* 2001). In case of HCV genotype 3, the virus itself is the major cause for inducing hepatic steatosis while in case of other HCV genotypes obesity and central adiposity are the key factors that are involved in the induction of hepatic steatosis (Khattab *et al.,* 2010).

2.15.7 Alcohol

Alcohol intake is also responsible for the poor treatment response to interferon based therapy of HCV infection. Alcohol causes histological activity and liver fibrosis. In chronic HCV infection, even the moderate intake of alcohol leads to the progression of disease and a poor treatment response (Poynard *et al.,* 1997; Singal and Anand, 2007).

2.16 SINGLE NUCLEOTIDE POLYMORPHISMS

There are many genetic variations in the human genome. Most of these genetic variations are not important to us, but some genetic variations are medically relevant. The presence of some genetic variations may predispose us to infectious disease or may weaken the immune system for disease. In some more extreme situation mere the

presence or absence of a genetic variation in the genome may be the cause of a life threatening disease e.g. sickle cell anemia. Most of these genetic variations come in the form of SNPs. SNPs are haphazardly distributed in our genome and make each individual unique (Brookes, 1999). Approximately, 99.9% of an individual genome is matched with the genome of other individuals in a community. In each individual genome, one base pair (bp) after every 500 – 1000 bp differs from the one which is the most prevalent in the population. It is supposed that three to six million small genetic variations are present in our genome. When such variations are present only in 1% of the population, they are referred as SNPs (Stoneking, 2001).

2.17 SNPS AND THE TREATMENT RESPONSE

SNPs may modify the body response to drug actions by the following three ways. Firstly, SNPs may play their role by abnormal absorption of drugs. Secondly, some drugs require to be further processed in the body. Genetic variations of the body may interfere with the internal processing of the drug. Thirdly, after taking the drug, it uses the body transport system to deliver to its site of action. The body mostly uses proteins as a transport system. A genetic variation of the transport protein or some other protein that interferes the transport system may leads to the inappropriate concentration of the drug to its site of action (Chakravarti, 2001).

In-conclusion, disease relevant SNPs may provide us a molecular basis of the disease and its treatment outcomes. SNPs may play a significant role in a personalized treatment. For pharmaceutical industries also, SNPs are of prime importance in drugs research and development (Weiner and Hudson, 2002).

2.18 HOST SNPs AND HCV INFECTION

HCV infection is one of the most important examples where genetic information is utilized for the treatment of infection. Recently, genetic testing of IL28B is recommended by FDA before interferon based therapy of HCV genotype 1 infection. Currently candidate gene approaches are in use for the treatment of HCV infection (Mosbruger *et al.,*2010). Table 2.2 shows those SNPs in the host genome that play critical roles in progression of HCV infection or treatment outcomes. SNPs, which are highlighted blue boldtyped, are discussed in detail as these polymorphisms were also selected in the current study.

Table 2.2: Association of host SNPs with treatment response to HCV infection

Gene symbol	Function	SNP	Effect on interferon therapy
		IFN –λ	
IL29 (IFN-λ1)		**rs8099917**	TT genotype is favorable
IL28A (IFN-λ2)	Inhibit viral replication	rs12980275	AA genotype is favorable
IL28B (IFN-λ3)		**rs12979860**	CC genotype is favorable
IFN - γ	Inhibit viral replication	-768G	Enhances promoter activity 2-3 folds
MBL	Pathogen recognition receptor	O/A at exon 1 At promoter region: MBL2*H,Land X,Y.	X or O mutations linked with non-responsiveness
CTLA4	Down regulates T cell functions	-318 C/T 49 G/A	-318C, 49G are favorably linked with therapy response.
IL10	Anti-inflammatory, Down regulates MHC1and MHC II molecules	−1082, −819, −592	−819T and −592A are positively associated
IL18	Pro-inflammatory cytokine Induces IFN-γ	**-607 C/A,** **-137 G/C**	-607A and -137C are positively associated
TRAIL	Induces apoptosis in virally infected cells	rs4242392	Poorly associated

TGFβ1	Multifunctional cytokine	codon 10T/C , codon 25G/C	Positively associated
Mx1	Antiviral activities	G/T at nt -88	Positively associated
Osteopontin	Induces Th1 response	nt 442, nt 1748	T/T at nt -442 G/G or G/A at 1748 Positively associated with SVR
LMP7	HLA-1 antigen presentation	LMP7-K	Positively associated with SVR
OAS1	converts ATP into 2'-5' linked oligomers of adenosine	at exon 7 SAS	AA genotype is poorly associated

2.18.1 IL28B and HCV Infection

Interferon-λ was first reported in 2003. The three members of the family of interferon λ are IFN λ1, 2, and 3 are also recognized as IL29, IL28A and IL28B respectively. The three members show approximately 80% homology with each other. Although, IFN-λ homology with IL10 and IFN-α is minimal but like IL10, IFN-λ also encompasses 5 - 6 exons (Gad *et al.,* 2009; Hwang *et al.,* 2006; Sheppard *et al.,* 2003). Most of the immune cells, alveolar epithelial cells, hepatocytes, cell lines and neuronal cells produce IFN-λ, but the major source of its production are dendritic cells DCs (Ank *et al.,* 2008; Coccia *et al*., 2004; Iversen *et al.,* 2010; Sommereyns *et al.,* 2008; Wang *et*

al., 2009). Like all other interferons, IFN-λ is also mainly produced against viruses. IFN-λ is mainly produced by stimulation of TLR signaling pathways (Marcello *et al.,* 2006). The production of IFN-λ is reported against a series of viruses such as influenza virus A, HBV, HIV, dengue virus, cytomegalovirus, herpes simplex virus 1, Encephalomyocarditis virus (EMCV), and HCV (Hou *et al.,* 2009; Hwang and Chen, 2006; Marcello *et al.,* 2006; Sheppard *et al.,* 2003). It was shown that IFN-λ not only inhibits viral replication but also possessed immune-modulatory functions. It plays an important role in the differentiation and maturation of the immune cells (Megjugorac *et al.,* 2009; Mennech and Uze, 2006; Wolk *et al.,* 2008). Thus, IFN-λ also plays its part in the modulation of the adaptive immune system against viruses.

In 2002, there was the development of the human HapMap project which greatly facilitates genome wide association studies (GWAS) of various infectious diseases. This technique differs from the candidate gene approach in which the distribution of pre-identified relevant gene in the population is studied. The first study of GWAS for HCV infection involved 1651 HCV patients from mixed ethnicity (American European, Hispanic and African) (Ge *et al.,* 2009). These patients received the peg-interferon α plus ribavirin treatment for HCV infection. An astonishing association was found between the treatment outcomes and a cluster of seven SNPs in the vicinity of IL28B gene. The most important of these SNPs was rs12979860. It demonstrated a significant value; P = 1. 063×10^{25}. The GWAS study showed that not a single SNP of some other gene was associated with the treatment outcomes of HCV infection. The SVR rate among the patients was 78%, 38% and 28% for IL28B rs12979860 genotypes, C/C, T/C and T/T

respectively. Thus, the protective allele against HCV infection at IL28B rs12979860 was C irrespective of ethnicity.

The results of many other studies from Australian, Hispanic, European, African and Japanese population also confirmed the conclusions of Ge *et al*., (2009) studies (Labie and Gilgenkrantz, 2010; Mangia *et al.,* 2010; McCarthy *et al.,* 2010; Montes-Cano *et al.,* 2010; Mosbruger *et al.,* 2010, Rallon *et al.,* 2010; Thomas *et al.,* 2009). Soon after the landmark discovery of Ge *et al.,* (2009) candidate gene approach for IL28B genetic testing demonstrated that IL28B rs12979860 also possessed a role in the spontaneous clearance of HCV infection in African and Caucasian population. The results showed that individuals possessing the risk allele are three times less likely to spontaneously clear HCV infection than individual possessing favorable allele. These results were further strengthened by another large GWAS study (Rauch *et al.,* 2010).

Regarding IL28B rs12979860, it is clear that the response rate to interferon based therapy of HCV infection is not dependent on ethnicity, but its dependence or independence on HCV genotype is still unclear. The reported data suggest that there is an association between IL28B rs12979860 polymorphism with interferon based therapy in HCV genotype 1 but not in HCV genotype 2 and 3 (Novick *et al.,* 1994; Stark *et al.,* 1998). However, two other studies noted the association of IL28B rs12979860 polymorphism with HCV genotype 2 and 3 (De Veer *et al.,* 2001; Darnell, 1997). Interestingly, one study indicated that the treatment predictive capability of IL28B rs12979860 polymorphism for genotype 3 was even greater than genotype 1 (0.0007) (Darnell, 1997).

The other two important SNPs of IL28B, rs12980275, and rs8099917 showed an association of treatment outcomes of HCV infection with ethnicity (Doyle *et al.,* 2006; Lange and Zeuzem, 2011). Interferon-λ based HCV drugs are possible candidates for treating HCV infection. These drugs have relatively fewer side-effects than IFN-α and β base drugs as there are a comparatively less number of IFN-λ receptors, permitting more targeted therapy (Imran *et al.,* 2013a).

The most important query about IL28 SNPs is that whether these SNPs have any effect on IFN-λ production. Two studies on peripheral blood mononuclear cells (PBMCs) proposed that the protective allele is related with up-regulation of IFN-λ3 in PBMCs (Soh *et al.,*1994; Kotenko *et al.,* 2003). The results were not replicated by other group (Pestka *et al.,* 2004). However, one consistent finding of all the studies was the high viral titer in patients with protective allele than those patients possessing risk allele (Darnell, 1997). It seems to be irrational as pre-treatment high viral load is associated with poor response to interferon therapy. The most possible reason may be no effect of protective or risk allele on viral load (Marcello *et al.,* 2006).

In-conclusion, the future therapies of HCV infection would be the competition between peg-interferon α and peg-interferon λ1. Moreover, the presence of the risk allele of IFNλ3 may lead us towards alternative treatment options. HCV provides us the opportunity to step forward for personalized treatment options. Thus, only those patients may take the treatments that are likely to benefit from it. The most important point is to solve the puzzle, how IFNλ3 polymorphism affects acute HCV infection and the treatment response. Furthermore, it is still a challenge to search how these polymorphisms influence IFNλ3 structure. Unlocking the immune-modulatory functions

of IFNλ3 will help us to understand its antiviral mechanisms and toxicities. Overall, the link between treatment outcomes of HCV infection and IFNλ SNPs is a major step and raises new questions to be solved (Kelly *et al.,* 2011).

2.18.2 TGFβ and HCV Infection

TGFβ is secreted by most of the cells. The major sources of TGFβ secretion are macrophages. It is secreted in the latent form, complexed with two polypeptides. The active TGFβ is produced by the action of serum proteinases such as plasmin (Kitamura *et al.,* 1997). TGFβ is secreted in three different isoforms TGFβ1, TGFβ2, TGFβ3. TGFβ is a chemo-attractant cytokine with multiple functions. The main function is its role in cellular differentiation, apoptosis, degradation and production of extra-cellular matrix (Massague *et al.,* 1998; Derynck *et al.,* 2001).

TGFβ receptor comprises of two transmembrane units, TβR1 and TβR II. The ligand binding leads to TβR II activation which phosphorylates TβR1. TβR1 phosphorylates the receptor specific R-Smad and common receptor Co-Smad. The cmplexes of R-Smad and Co-Smad are translocated to the nucleus and induces transcription and apoptosis (Schuster and Krieglstein, 2002). HCV NS5A protein was shown to block TGFβ signaling pathway via binding TβR1. There is a direct interaction between NS5A protein and TβR1 leading to inhibition of R-Smad complexes and their translocation to the nucleus. Blocking of TGFβ signaling pathway also invites infection by other viruses such as HBV, adenovirus and human papiloma virus as the proteins of these viruses also inhibit the members of the TGFβ signaling cascade (Choi, 2006).

Dysregulation of TGFβ cytokines were associated with the progression of liver fibrosis and persistence of HCV infection (Okumoto *et al.,* 2004). TGFβ is a well known

suppressor of NK cells that are the major sources of IFN-γ and IL12 production. It also inhibits the cytotoxicity and proliferation of NK cells. Over-suppression of NK cells may leads to HCV persistence (Rook *et al.,* 1986). TGFβ1 SNP, -509T was associated with higher plasma level of TGFβ (Grainger *et al.,* 1999). Moreover, -509CC genotype and "C" allele was found to be associated with the natural clearance of HCV infection. TGFβ -509CC genotype is responsible for a low secretion of TGFβ, which in turn leads to a weak suppression of NK cells and better immune response against HCV infection. Furthermore, it is reported that TGFβ down-regulates the expression of NKG2D on NK cells at the transcriptional level. Blocking the activity of TGFβ by RNA interference leads to increased activity of NK cells (Castriconi *et al.,* 2003; Taniguchi *et al.,* 2004). It is still unknown that whether TGFβ polymorphisms at promoter level that regulates its secretion causes different activity of NK cells or IFN-γ and IL12 secretion (Kimura *et al.,* 2006).

HCV core protein is reported to up-regulate TGFβ secretion (Taniguchi *et al.,* 2004), leading to enhanced liver fibrosis and the development of hepatocellular carcinoma (Kimura *et al.,* 2006). TGFβ polymorphism at codon 10 position is considered to influence its export efficacy (Grainger *et al.,* 1999). Moreover, the functional polymorphism of TGFβ at codon 25 is associated with graft rejection after liver transplantation in chronic HCV patients (Eurich *et al.,* 2011).

2.18.3 Oligoadenylatesynthetase Gene and HCV Infection

Oligoadenylatesynthetase (OAS) is an enzyme that is induced by interferon stimulation and double stranded RNA. It uses adenosine triphosphate (ATP) to produce 2', 5'-oligoadenylates (2-5As). 2-5As binds and stimulates RNaseL system which in turns

leads to the degradation of double stranded RNA and blocks the synthesis of viral protein. Hence, this protein possesses a significant antiviral activity (Hamano *et al.,* 2005; Xiang *et al.,* 2003). Recent study has proved that genetic variations of OAS1 gene were associated with asymptomatic west nile virus (WNV) infection and disease progression (Bigham *et al.,* 2011). Additionally, associations between OAS1 SNPs and diabetes mellitus 1 (Fedetz *et al.,* 2006), multiple sclerosis and (Cagliani *et al.,* 2012) prostate cancer (Mandal *et al.,* 2011) are also reported.

The gene family of 2′–5′ OAS involves three members, OAS1, OAS2 and OAS3. Regarding HCV infection, OAS1 is of special importance. It is positioned on chromosome 12q24.2, and released in four different isoforms; p42, p44, p46 and p48 (Nguyen and Keeffe, 2005). The published data has suggested a significant role of OAS-RNaseL system in natural clearance of viral infections (Mashimo *et al.,* 2002; Perelygin *et al.,* 2002; Lucas and Mashimo, 2003). Cell line studies of HCV infection demonstrated that HCV core protein activates OAS1 gene promoter. Further research in the same direction revealed that interferon based therapy of cell lines enhanced HCV core activity to induce OAS1 gene (Naganuma *et al.,* 2000). It was concluded from these studies that any genetic variation of OAS gene may be important determinant in the treatment outcomes of virus infection. Search for SNPs in OAS1 gene exposed 36 SNPs. There was found one SNP at 5' UTR, three in promoter, eight in exon and twenty four in introns. Twelve of these genetic markers were associated with the enzymatic activity of OAS1 gene. Although, it is an important component of the host immune system against virus infections but its sequence is not strictly conserved. The strongest genetic association

between OAS1 gene and virus infection was found at exon 7 splice accepter site (El Awady *et al.,* 2009).

2.18.4 IL18 and HCV Infection

Interleukin-18 (IL18) is an important member of IL1 family. Unlike IL1, IL18 is a pivotal mediator of Th1 cytokines (Dinarello,1996; Dinarello *et al.,* 1998; Okamura *et al.,* 1995). However, the common function between the two cytokines is the induction of pro-inflammatory cytokines (Puren *et al.,* 1998). IL18 plays an important role in both the innate and adaptive immune system. IL18 was first discovered in 1989 as IFN-γ inducing factor (Nakamura *et al.,* 1989). IL18 induces the production of IFN-γ mainly from NK cells (Tsutsui *et al.,* 1996) and T cells (Okamura *et al.,* 1995). IL18 is produced by a number of cells such as dendritic cells, osteoblasts, T-cells, B-cells, corneal epithelial cells, and glucocorticoid-secreting adrenal cortex cells, but the major sources of its production are macrophages (Dinarello, 2000). IL18, like IL1β is produced in the precursor form which is cleaved by caspase 1 to produce active mature IL18. IL18 is mainly produced in response to LPS and other microbe antigens stimulation along with IL12 to promote inflammatory response. In vitro studies also demonstrated that IL18 may promote Th2 response in the absence of IL12 (Nakanishi *et al.,* 2001). The bioactivities of IL18 are regulated by IL18 binding protein a (IL18BPa). The bioactivity of IL18 is mainly associated with inflammatory diseases such as septic shock, colitis, crohn's disease, rheumatoid arthritis, atherosclerosis, myasthenia gravis and multiple sclerosis. Antitumor activities are also suggested for IL18 (Micallef *et al.,* 1997).

In HCV infection, IL18 is mainly regulated by TNFα. TNFα regulates the production of IL18BP that in turn leads to the modulation of IL18 and fibrosis

progression (Zecchina *et al.,* 2001). In chronic HCV infection, the level of IL18 and its receptors was raised and linked with the poor treatment outcomes (Asakawa *et al.,* 2006). Interferon therapy of HCV infection increased the level of IL18BP, 3-24 folds (Kaser *et al.,* 2002). Two important genetic variations are reported in the promoter region of IL18; IL18 -607 and IL18 -137. The minor alleles (-607A and -137C) were linked with lower promoter activity while the major alleles (-607C and -137G) were linked with higher promoter activity. The association of these two polymorphisms of IL18 with some other diseases such as cardiovascular diseases (Tiret *et al.,* 2005) crohns disease (Glas *et al.,* 2005) human immunodeficiency virus (HIV) infection (Segat *et al.,* 2006), HBV (Zhou *et al.,* 2013; Kimura, 2002) and HCV (Manohar *et al.,* 2009) are also reported.

2.18.5 Osteopontin and HCV Infection

Osteopontin (OPN) is a phosphoprotein secreted by the most tissues and cells. It has a high degree of posttranslational modification that can also differ from cell to cell. As OPN is involved in most of the body immune system, it was discovered in many laboratories with different names. The protein was discovered for the first time in 1979 as a transformation associated protein (Senger *et al.,* 1979) and later on it was named as secreted phosphoprotein 1 (SPP1). OPN was also defined as an early T cell activation gene 1 (Patarca *et al.,* 1989). Lastly, the name "osteopontin" was proposed for this protein which means "bone bridge" (Oldberg *et al,* 1986). OPN in the human genome is positioned at chromosome 4q13 with seven exon (Young *et al.,* 1990). One of these exons is non-coding (Hijiya *et al.,*1994). The most abundant modification of OPN is serine phosphorylation as there are 12 serine phosphorylations and one threonine modification (Neame and Butler,1996). OPN is now considered as a chemo-attractant and

a cytokine molecule with significant roles in apoptosis, inflammation, wound healing and tumor metastasis (Giachelli and Steitz, 2000; Chabas, 005). Moreover, it is a key cytokine for the induction of T helper type 1 (Th1) cytokines.

OPN expression is regulated by other cytokines, hormones and various growth factors (Sodek *et al.,* 2000). Over expression of OPN is observed in various diseases which highlight its importance in these diseases. In rheumatoid arthritis (RA), OPN expression is highly increased. One group of researchers concluded that OPN knockout mice are protected against RA (Yumoto *et al.,* 2002). However, another group failed to demonstrate such association between OPN and RA (Jacobs *et al.,* 2004). Thus, the exact role of OPN in RA is still a question mark. OPN over expression is also experienced in a verity of cancers including breast, colorectal, lung and stomach. Thus, managing a plasma level of OPN will assist in the treatment outcomes of these diseases (Wang *et al.,* 2000; Wang and Denhardt, 2008). It is established that there exist numerous polymorphisms in the promoter region of OPN which influence the immunological response to infectious diseases (Patarca *et al.,* 1993; Cantor and Shinohara 2009).

Over expression of OPN was also observed in inflammatory liver diseases (Morimoto *et al.,* 2004). Treatment with OPN neutralizing antibodies reduced inflammation related liver injuries (Lund *et al.,* 2009). OPN expression level was also accelerated in fulminant hepatitis most probably due to enhanced production from macrophages and kupffer cells (Tsutsui *et al.,* 1996). Alcoholic hepatitis also leads to enhanced expression of OPN. The high level of OPN was linked with disease severity in alcoholic patients (Naveau *et al.,* 2012; Patouraux *et al.,* 2012). Plasma level of OPN may be used as a prognostic marker of HBV related hepatocellular carcinoma (Zhao *et al.,*

2008). Furthermore, it was also suggested to be a prognostic marker of HCV infection as high level of plasma OPN was associated with increased liver cirrhosis and damage (Huang *et al.,* 2002). Interferon based therapies of HCV infection were also reported to have an association with promoter polymorphisms of OPN. These SNPs were positioned at promoter region, -155, -616, -1748 and -442. The most significant association of OPN and HCV infection was shown at -442 (Wang *et al.,* 2000; Naito *et al.,* 2005). A recent study also demonstrated that OPN polymorphism at -442 may be used as a therapeutic marker of combinational therapy of HCV infection (Sakr *et al.,* 2013).

2.18.6 GALNT8 and HCV Infection

Probable polypeptide N-acetylgalactosaminyltransferase 8 (GALNT8) is placed in the family of O-linked UDP-GalNAcglycosyltransferase (ppGalNTase) (Ten Hagen *et al.,* 2003). It participates in the biosynthesis of mucin-like-o-glycan by transferring N-acetyl-D-galactosamine residue to a serine or threonine residue on a receptor protein. Mucin type-o-glycans are associated with a number of glycoproteins. Glycans or sugar residues are the important component of most proteins and lipids and serves as a PRRs (Varki, 1993). Glycans are essential for the correct folding of proteins in the endoplasmic reticulum (ER) (Jentoft,1990), and possessed a role in trafficking of proteins (Ellgaard and Helenius, 2001; Helenius and Aebi, 2001). Glycans are also reported for receptor function of endogenous structures, cell to matrix or cell to cell interactions (Paulson, 1989).

The family of polypeptide GaLNc-transferase is highly conserved as there are only 20 isoforms of this enzyme out of 20 - 25000 genes (Bennett *et al.,* 1999). During the process of embryo development, each isoform has different spatiotemporal pattern

suggesting a unique role of each isoform (Kingsley *et al.,* 2000). The differential expression pattern of different isoforms was also observed in adult tissues (Hagen *et al.,* 2003). The structural analysis of GaLNc-transferase showed that it comprises a short cytoplasmic domain, transmembrane domain, a stem region of variable length, a catalytic site containing "DXD" motif (Hazes, 1996; Imberty *et al.,* 1997).

Recently, SNPs of GALNT8 were reported to have an association with interferon based therapy of HCV infection in 1088 Japanese population. These patients were given interferon monotherapy and a significant association between treatment outcomes and GALNT8 SNP in intron 6 (rs2286580) was found. The study was further replicated by additional study of 328 patient's cohort. However, there was found no association of rs2286580 polymorphism with the peg-interferon plus ribavirin treatment in chronic HCV patients from the same population. The peak association of combinational therapy and GALNT8, intron 6 was observed at rs10849138. Thus, SNPs of GALNT8 also have an influence on treatment outcomes of HCV infection (Nakano *et al.,* 2013).

Chapter 3

MATERIALS AND METHODS

3.1 COLLECTION OF BLOOD SAMPLES

Informed consent from all patients and healthy controls was taken before their enrollment in the current genetic and clinical study. The study was reviewed and approved by the ethical committee of Atta-Ur-Rahman School of applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad. The patients were recruited in the study from November 2010 to July 2013. The patients were selected from two different provinces of Pakistan; Khyber Pakhtunkhwa (KPK) and Sindh. Ninety six patients from KPK and ninety patients from Sindh were selected for the current study. However, finally we were left with only 75 patients from KPK and 65 patients from Sindh. Forty five patients were excluded from the current study due to incomplete follow up of the patients.

3.2 PATIENT'S INCLUSION CRITERIA

The inclusion criteria of the current study included patients infected with HCV as initially confirmed by the detection of HCV antibody in patient's serum. The levels of two markers of liver damage; serum Aspartate aminotransferase and Alanine aminotransferse (ALT and AST) were higher than normal level. Moreover, all the patients had HCV RNA level higher than 1000 IU/ml as quantified by real time reverse transcriptase Polymerase chain reaction qRT-PCR. All enrolled chronic HCV patients were treatment naïve. Only those patients were included in the study that had any final

fate of therapy and were followed up to six months after the cessation of combinational therapy.

3.3 PATIENT'S EXCLUSION CRITERIA

Patients with liver damage caused by infection other than HCV, patients co-infected with other diseases such as HBV, HIV, patients with decompensated liver, patients with hepatocellular carcinoma and patients who discontinued the planned course of treatment, were all excluded from the current study.

3.4 STANDARD TREATMENT OF HCV PATIENTS

The patients were given the treatment of interferon α 2a plus weight based ribavirin for at least six months and maximum for one year. 3 million units/0.5ml of interferon α 2a was given to the patients three times a week and weight based ribavirin 800 mg – 1200 mg tablets were given each day. After three months of treatment, patients were monitored for viral RNA quantification and the levels of liver enzymes to make decision about continuation or discontinuation of combinational therapy. Approximately six months after the end of proposed treatment duration, patients were classified into two groups, sustained virological responders (SVRs) and non-responders (NRs). SVRs were those patients, who were avirimia six months after the cessation of treatment while NRs were those patients, who were possessing viral RNA six months after the end of therapy.

3.5 HEALTHY CONTROLS

One hundred and twenty healthy controlss were also included in the current study. These healthy controls were mostly from Islamabad and cities in vicinity.

3.6 STORAGE OF COLLECTED BLOOD SAMPLES

The blood samples from all chronic HCV patients and healthy controls were taken in 5ml K3 ethylene diamine tetra acetic acid (EDTA) tubes (BD vacutainer TM, Franklin Lakes, New Jersey, USA) through 5ml sterilized syringes (BD 0.6mm X 25mm, 23 G X 1 TW, Lahore, Pakistan). The blood samples collected in the field were directly dispatched to Viral Hepatitis Laboratories ASAB, NUST, Islamabad (Pakistan). Collected samples were stored at 4 degree Celsius (°C) before processing for extraction of genomic DNA.

3.7 DNA EXTRACTION FROM WHOLE BLOOD

DNA was extracted from the whole blood using commercially available kit (Gentra Puregene, Hilden, Germany) by following manufacturer's instructions. Initially, 300 µl blood was taken in 1.5 ml centrifuge tube (Axygen®, California, USA), then there was added 900 µl RBC lysis buffer. The mixture was left for five minutes incubation at room temperature. During the incubation period, the mixture was inverted gently for ten times. The mixture was centrifuged for one minute at 13,000 × g by the use of microcentrifuge (spectrafuge 24D Labnet, Edison, New Jersey, USA). The supernatant was discarded, leaving a white pellet with about 10-15 µl of residual liquid. The pellet was vigorously vortexed by means of Reax top (Heidolph, Schwabach, Germany). The vortexing was continued until the white pellet was completely disappeared and the cells were completely re-suspended in the residual liquid. Then 300 µl cell lysis buffer was added and pipetted up and down to lyse the cells. After that the solution was kept for at least five minutes to get it cooled. Then 100 µl protein precipitation solution was added to the cell lysates and vortexed for half a minute. The mixture was centrifuged at 13,000

× g for one minute. Proteins were precipitated in the form of dark brown pellet. Supernatant possessing genomic DNA was transferred into another 1.5 ml centrifuge tube that already contained 300 µl isopropanol solution. The centrifuge tube was inverted multiple times till a clear suspended white thread of DNA was observed. Centrifugation was performed at 13,000 × g for two minutes to sediment DNA. The supernatant was discarded and the tubes were dried on absorbent paper. After drying, 300 µl of 70% ethanol was added to the centrifuge tube and followed by centrifugation at 13,000 × g for one minute. Ethanol was poured off, tube was drained on a clean absorbent paper and air dried for one hour. DNA hydration solution ranged from 50-100 µl was added, depending on quality of DNA and mixed thoroughly by vortexing. DNA samples were stored at -4 °C for short period and at -20°C for long term storage.

3.8 QUANTIFICATION OF EXTRACTED DNA

To find the quality of extracted DNA, 5 µl of extracted DNA sample was run on 1% agarose gel. 1% (w/v) agarose gel was prepared by melting 0.4g of agarose in 40ml of 1X TBE buffer (0.89 M Tris-Borate, 0.032 M EDTA, pH 8.3) in microwave oven for one and a half minutes. Ethidium bromide solution (0.5µg/ml final concentration) was added to stain DNA. Extracted DNA sample was mixed with a loading dye (0.25% bromophenol blue prepared in 40% sucrose solution) and loaded into the wells. Electrophoresis was performed at 100 volts for forty minutes in 1X TBE running buffer. After electrophoresis, extracted DNA was visualized by placing the gel under UV Transilluminator (Biometra, Goettingen, Germany). The extracted DNA was also quantified by using Nandrop (Eppendorf Biophotometer, Germany).

3.9 PCR FOR THE DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS

The list of primers used for the PCR reactions and the size of PCR products are shown in table 3.1. Primers were designed manually by first searching the gene sequence and then designed primers for the desired SNPs. Three different techniques were used for the detection of SNPs in the studied genes.

3.9.1 Allele Specific Polymerase Chain Reaction (AS-PCR)

It is a type of selective PCR to detect one of the two alleles of a gene to find out SNP. The selective amplification is achieved by designing two forward primers and one reverse primer. Let forward primer 1 is for allele "A" and forward primer 2 is for allele "B" at a particular position. DNA sample from an individual is taken in two separate tubes. In one PCR tube forward primer one, reverse primer and other components of PCR mixtures are added. In the second PCR tube forward primer two, reverse primer and other components of PCR mixtures are added. If the desired PCR product is amplified only in tube 1 then the individual possesses allele "A" at that particular position, if the desired PCR product is amplified in tube 2 then the individual possesses allele "B" at that particular position. Alternatively, if the desired PCR product is present in both tubes then the individual possesses both alleles at that particular position. The difference between forward primer 1 and forward primer 2 lies at single nucleotide at 3'-end only. This single nucleotide at 3'-end also represents SNP position of that particular gene. Thus, selective amplification is achieved by the primer matching or mismatching at one of the two alleles at 3'-end of the primer.

3.9.2 Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR)

ARMS-PCR is almost similar to AS-PCR. The only difference is the use of two additional primers that serve as an internal control. The reverse primer of an internal control serve as reverse primer for allele specific primer 1, allele specific primer 2 and internal forward primer. Similar to AS-PCR, an individual DNA sample is taken in two different PCR tubes. In PCR tube 1, allele specific primer 1, internal reverse and internal forward primer along with other PCR components are used while in PCR tube 2 allele specific primer 1, internal reverse and internal forward primer along with other PCR components are used. Like AS-PCR, the results are interpreted as; if the desired PCR product is present in tube 1 then it contains allele "A". Alternatively, if the desired amplification occurs in tube 2 then it contains allele "B". However, if the desired PCR product is present in both tubes then the individual contains both alleles and heterozygous for that particular genetic mutation.

3.9.3 Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP-PCR)

RFLP-PCR is a common technique which is used for SNP analysis. This technique is based on the fact that SNP usually leads to the creation or abolishment of the restriction site of a restriction enzyme (Narayanan, 1991). The first step of RFLP-PCR is the amplification of the genomic region spanning the desired SNP. The PCR product is digested with the appropriate restriction enzyme that can digest at SNP. The presence or absence of the restriction site of the enzyme leads to the production of different size products, allele identification can be performed by gel electrophoresis of the digested

PCR product. Primers used in the current study for AS-PCR, ARMS-PCR and RFLP-PCR are shown in the table 3.1.

Table 3.1: List of primers used for the detection of SNPs

Gene	Primer sequence	Product size (bp)
RFLP-PCR primers of IL28B rs8099917		
Forward primer	5′- GTGCATATGTTTTCTGAC -3′	430
Reverse primer	5′- GAGGCCCCTCACCCATGC -3′	
RFLP-PCR primers of IL28B rs12979860		
Forward primer	5′- CCAGGGCCCCTAACCTCTGCA -3′	139
Reverse primer	5′- GGGAGCGCGGAGTGCAATTCA -3′	
AS-PCR primers of IL28B rs12979860		
Forward primer	5′- AGGGAGCTCCCCGAAGGCGC -3′	190
Forward primer	5′- AGGGAGCTCCCCGAAGGCGT -3′	
Reverse primer	5′- CCTATGTCAGCGCCCACAATTCCCA -3′	
AS-PCR primers of TGFβ polymorphism at codon 10		
Forward primer 1	5′-TCCGGGCTGCGGCTGCTGCC -3′	297
Forward primer 2	5′-TCCGGGCTGCGGCTGCTGCT-3′	
Common reverse primer	5′-GTCGGCCTCAGGCTCGGGC-3′	
AS-PCR primers of TGFβ polymorphism at codon 25		
Forward primer 1	5′-TACTGGTGCTGACGCCTGGCCG-3′	254

Forward primer 2	5′-TACTGGTGCTGACGCCTGGCCC-3′	
Common reverse primer	5′-GTCGGCCTCAGGCTCGGGC-3′	
AS-PCR primers of OAS1 polymorphism at rs10774671		
Forward primer	5′-TGCAATGCAGGAAGACTCC-3′	203
Reverse primer	5′-TGCAGGTCCAGTCCTCTTCT-3′	
AS-PCR primers of IL18 -607 polymorphism		
Common forward primer	5′-TAACCTCATTCAGGACTTCC-3′	
Sequence specific primer 1	5′-GTTGCAGAAAGTGTAAAAATTATTAC-3′	196
Sequence specific primer 2	5′-GTTGCAGAAAGTGTAAAAATTATTAA-3′	196
Common reverse primer	5′-CTTTGCTATCATTCCAGGAA-3′	301
AS-PCR primers of IL18 -137 polymorphism		
Common forward primer	5′-AGGAGGGCAAAATG CACTGG-3′	
Sequence specific primer 1	5′-CCCCAACTTTTACGGAAGAAAAG-3′	261
Sequence specific primer 2	5′-CCCCAACTTTTACGGAAGAAAAC-3′	261

Common reverse primer	5′-CCAATAGGACTGATTAT TCCGCA-3′	446
AS-PCR primers of Osteopontin -442 polymorphism		
Reverse primer 1	5′-TTGTTCAAGCCTGCAAGGAGTTCAGAA-3′	442
Reverse primer 2	5′-TTG TTCAAGCCTGCAAGGAGTTCAGAG-3′	
Forward primer	5′-GTCCTTAAGATACGCAGAGCATTTGC-3′	
AS-PCR primers of GALNT8 polymorphism in intron 6 at rs10849138		
Forward primer 1	5′-CTGGGTCAGACCTGAAGG-3′	340
Forward primer 2	5′-CTGGGTCAGACCTGAAGC-3′	
Common reverse primer	5′-GCACTGGCCTCTAACTTTGGA-3′	

The major recipe of PCR mixture used for the analysis of all SNPs is shown following.

Nuclease free water (RO582, Fermentas, USA)	12 µl
Taq buffer	2.5 µl
2mM dNTPs	2.5 µl
2.5mM $MgCl_2$	2.5 µl
Forward Primer 1 (10 pmole)	1 µl
Forward Primer 2 (10 pmole)	1 µl
Reverse Primer (10 pmole)	1 µl
DNA template	2 µl
Taq Polymerase (EP0402, Fermentas, USA)	0.5 µl
Total volume	25 µl

PCR condition of all reactions is shown in the Figure 3.1.

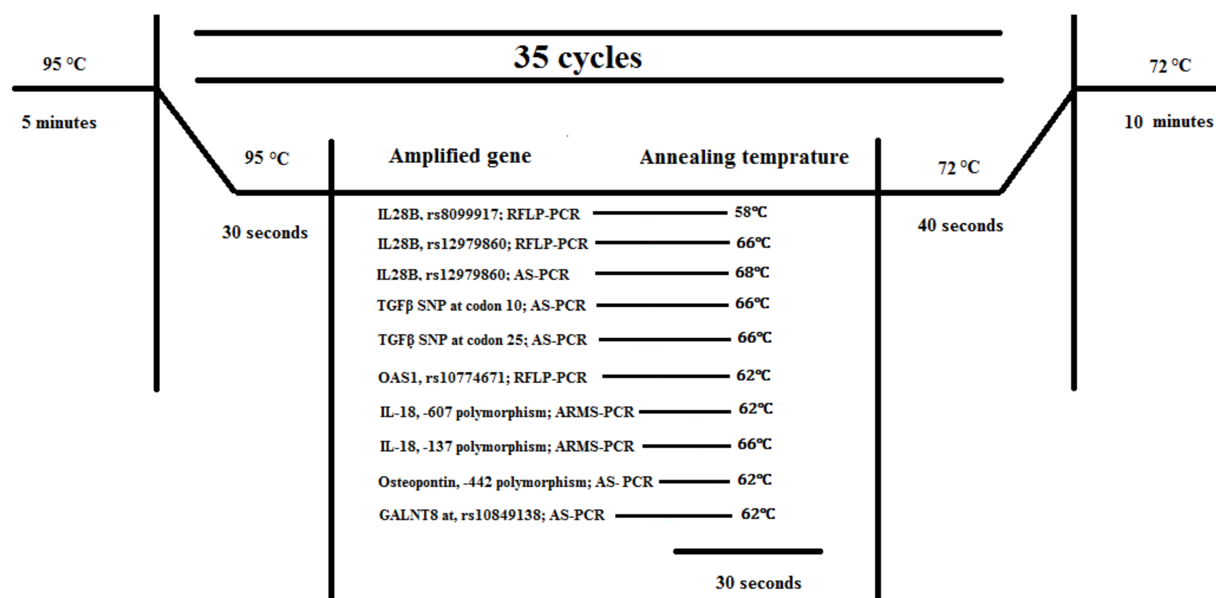


Figure 3.1: PCR conditions of all reactions

3.10 VIRAL RNA EXTRACTION

HCV RNA was extracted by the use of RTA® Viral RNA Isolation Kit (Catalog No. 09006100). The frozen serum samples were thawed at room temperature. 6 µl of carrier RNA was taken in a 1.5 ml eppendrof tube followed by the addition of 600 µl RL (RBC lysis buffer) and 150 µl serums. The mixture was mixed properly by pulse-vortexing for 20 times. The mixture was kept at room temperature for ten minutes. After a brief centrifugation, there was added 600 µl pure ethanol and pulse shaked for twenty times following a brief centrifugation. 700 µl of the mixture was transferred into the column placed in a collection tube. The solution was centrifuged at 10,000 × g for one minute, collection tube was discarded. The column was placed in a new collection tube and the remaining 700 µl mixture was transferred into it followed by a centrifugation. Again, the collection tube was discarded. The column was placed in a new collection tube for the step of washing. 700 µl of washing buffer 1 was added to the column followed by centrifugation at 10, 0000 × g for one minute. The collection tube was discarded. The column was placed in other collection tube; there was added 700 µl washing buffer 2 followed by the centrifugation at 14,000 × g for one minute. The spin column was placed in a new eppendrof tube of 1.5 ml and there was added 50-100 µl of solution E followed by incubation at room temperature for 3 min. Finally, the spin column was centrifuged at 14,000 rpm for one minute. The spin column was discarded. The eppendrof tube containing viral RNA was kept at -20˚C for short term storage and at -80 ˚C for long term storage.

3.11 HCV GENOTYPING

The extracted RNA was used for the cDNA synthesis. The final PCR reaction mixture of 20 µl contained 10 µl extracted RNA possessing approximately 50 ng RNA, 1.87 µl RNase free water, 1x MMLV buffer, 400 µM dNTPs, 10 units of RNase inhibitor (fermentas), 20 units of Moloney murine leukemia virus reverse transcriptase enzyme (M-MLV RTaseFermentas), and 20 pM of antisense primer. The sequence of antisense primer was was 5′-GAGACGGGTATAGTACCCCATGAGAGTCGGC-3′. The PCR was performed by AB Veriti 96 well thermocycler and cycling condition was; incubation at 37°C for 55 minutes and then followed at 95°C for 5 minutes. Synthesized cDNA was stored at 4°C.

3.11.1 First Round PCR (Genotyping)

4 µl of the synthesized cDNA was used for first round PCR of HCV genotyping. Forward primer was; 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and reverse primer was; 5′-GAGACGGGTATAGTACCCCATGAGAGTCGGC-3′. The reaction mixture contained; 2x M-MLV buffer, 200 µM dNTPs, 3 mM MgCl2, 8.2 µl RNase free water, 20 pM of each primers (Forward and Reverse) and 2 units of Taq polymerase enzyme (Fermentas). Cycling condition was; incubation at 94°C for 05 minutes followed by 25 cycles, each cycle was; incubation at 94°C for 45 s, at 52°C for 45 s, at 72°C for 55 s. The final extension was performed at 72°C for 07 min.

3.11.2 Second Round PCR (Genotyping)

The first round product was used in the second round. There were used two separate PCR tubes for Mix1 and Mix2 primers. Mix1 and Mix2 were mixtures of primers. The primers contained in each mixture are shown in table 3.1. The primers of

Mix1 were specific for genotype 1b, 3b, 2a, 2b, while the primers of Mix2 were specific for genotype 1a, 3a, 5a, 6a and 4. Each mixture contained 20 pm of each respective primer in both Mix1 and Mix2.

Table 3.2: List of primers used for HCV genotyping.

Name	Mix 1 primers	Name	Mix 2 primers
S7	AGACCGTGCACCATGAGCAC	**S7**	AGACCGTGCACCATGAGCAC
S2a	AACACTAACCGTCGCCCACAA	**G1a**	GGATAGGCTGACGTCTACCT
G1b	CCTGCCCTCGGGTTGGCTAAG	**G3a**	GCCCAGGACCGGCCTTCGCT
G2a	CACGTGGCTGGGATCGCTCC	**G4**	CCCGGGAACTTAACGTCCAT
G2b	GGCCCCAATTAGGACGAGAC	**G5a**	GAACCTCGGGGGGAGAGCAA
G3b	CGCTCGGAAGTCTTACGTAC	**G6a**	GGTCATTGGGGCCCCAATGT

For both mixtures, the PCR receipe was; 2 units of Taq polymerase enzyme (Fermentas), 20 pm of each primers, 2x M-MLV buffer, 200 µM dNTPs, 3 mM MgCl2, 3.2 µl RNase free water, and 5 µl of template (first round product). The reaction condition was; denaturation at 95˚C for 5 minutes followed by 25 cycles of denaturation at 94˚C for 45 sec, annealing at 52˚C for 45 sec, extension at 72˚C for 45 sec. The final extension was performed at 72˚C for 10 min. Finally, the amplified product was run on 3% agarose gel with commercially available DNA marker (Fermentas). Products of HCV genotypes were visualized under UV light of the gel documentation system (Wealtec) and compared with 100-bp DNA marker.

3.12 VIRAL LOAD DETERMINATION

40 µl of Master Mix was added to 10 µl of RNA template or positive control into 100 µl PCR tube. The PCR tubes were properly closed and centrifuged briefly. Then the tubes were placed in PCR machine. The PCR program was run according to the following conditions.

Table 3.3: Real Time PCR Thermal Cycling Conditions

STEPS	TEMPERATURE	TIME
Reverse Transcription	42 ˚C	5.00 min
Initial Denaturation	95 ˚C	10 sec
Denaturation	95 ˚C	5 sec
Annealing Extension	58 ˚C	40 sec
Extension	72 ˚C	10 sec
Fluorescence Detection	FAM, JOE	
Number of Cycles	50	

3.13 STATISTICAL ANALYSES

Statistical analysis of the data was performed by SPSS software version 13. Age of the individuals in healthy control group, SVR group and NR group was taken as mean±SD. Independent T-test was applied on age to find any correlation with treatment response to interferon based therapy of HCV infection. Viral load of HCV was taken as range and median. Independent T-test was applied on viral load to find any association of viral load with interferon based therapy of HCV infection. Phi coefficient test was applied to explore any correlation of treatment response of HCV infection with age.

Hardy-Weinberg equilibrium was applied on the distribution of SNPs to find the expected frequencies of various genotypes in the studied population. Pearson chi square analysis was applied on the observed distribution frequencies of different genotypes in the enrolled subjects. A P-value ≤ 0.05 was considered to be statistically significant.

3.14 BIOINFORMATIC ANALYSES FOR GENETIC POLYMORPHISM

The single nucleotide genetic variations were identified by putting the number of RefSNP (rs) of each SNP in National Centre for Biotechnology Information (NCBI) database. After approaching the gene sequence, there were designed primers manually. The primers were checked for their specific binding by Primer-blast software. Moreover, a theoretical restriction map program of online software Webcutter V2.0 was used to find the restriction sites of the restriction endonucleases and the size of the digested product.

Chapter 4

RESULTS

4.1 DEMOGRAPHIC PROFILES OF PATIENTS

Initially, 186 chronic HCV patients were enrolled for the present study but finally 140 patients completed the proposed duration of combinational therapy of interferon and ribavirin and possessed any final fate of therapy. There were 115 chronic HCV patients (72 male and 43 female) that were categorized as sustained virological responders (SVRs) with the mean age of 36.28±12.11. Twenty five chronic HCV patients (11 male and 14 female) were classified as non responders (NRs) with the mean age of 37.32±8.24. There was no significant difference in the age of responders and non responder groups (P = 0.608). One hundred and twenty healthy controls (60 male and 60 female) with the mean age of 25.11±8.21 were also considered in this study. There was a significant variability in the baseline viral load of SVRs and NRs group (P = 0.241). Statistical analysis of other clinical parameters of chronic HCV patients showed that there was a significant variation in baseline Alanine transaminase (ALT) level of the responder and non-responder group of patients (P = 0.001). Regarding other clinical parameters; serum glutamic oxaloacetic transaminase or aspartate aminotransferase (SGOT or AST) level and bilirubin level, we found no significant differences in responder and non responder group of patients. The P value in the two groups of patients for SGOT level, ALT level and bilirubin levels were 0.267, 0.347 and 0.420 respectively as shown in table 4.1.

Table 4.1 Demographic profiles of patients and healthy control controls.

Variables	SVRs	NRs	Healthy Controls	P Values
Numbers	115	25	120	-
***Age**	36.28±12.11	37.32±8.24	25.11±8.21	0.608
Gender	72 male; 43 female	11 male; 14 female	60 male; 60 female	0.086
****Baseline viral load**	$1.11X10^5$ ($0.045x10^5$ - $54.7x10^5$	$5.64X10^5$ ($0.4x10^5$ - $98.7x10^5$)	-	0.241
Baseline alanine aminotransferase (IU/L	66.3±33	90.3±33.6	-	**0.001**
Base line SGOTM (IU/L)	72.6±42	82.5±30	-	0.267
Alkaline phosphatase (U/L)	311.5.6±143	340 ±105.1	-	0.347
Bilirubin level (mg/dl)	1.3±0.87	1.4 ±0.86	-	0.420

4.2 HCV GENOTYPING

There are 11 major HCV genotypes with more than 100 sub-genotypes (Safi *et al.,* 2012). HCV genotyping is very imperative from the patients' point of view as different HCV genotypes respond in a different way to combinational therapy of interferon and ribavirin. Thus, HCV genotyping is significant for clinical management of infection. The distribution of HCV genotypes varies from region to region (Idrees *et al.,* 2008). Type 1a and 1b are the most prevalent HCV genotypes worldwide, particularly in United States of America and Japan (Ashfaq *et al.*, 2011). HCV genotype 2 is mostly distributed in North America and Europe. HCV Genotype 3 is mostly distributed in East-South Asia while in middle East, Egypt and central Africa, HCV genotype 4 is most commonly present (Kamal and Nasser, 2008). HCV genotype 5 is common in South Africa. HCV genotype 6 is prevalent in Hong Kong (Chao *et al.*, 2011), while 7, 8, 9 are common in Vietnamese population. HCV Genotype 10 and 11 are acknowledged in Indonesian population (Lavanchy, 2011).

HCV genotyping was performed by Ohno as described in chapter 2. Firstly, HCV RNA was extracted according to the manufacturer's instruction. Extracted RNA was used to synthesize cDNA. The first round and second PCR were performed using different sets of primers as shown in table 3.2. The second round PCR product is shown in Figure 4.1.

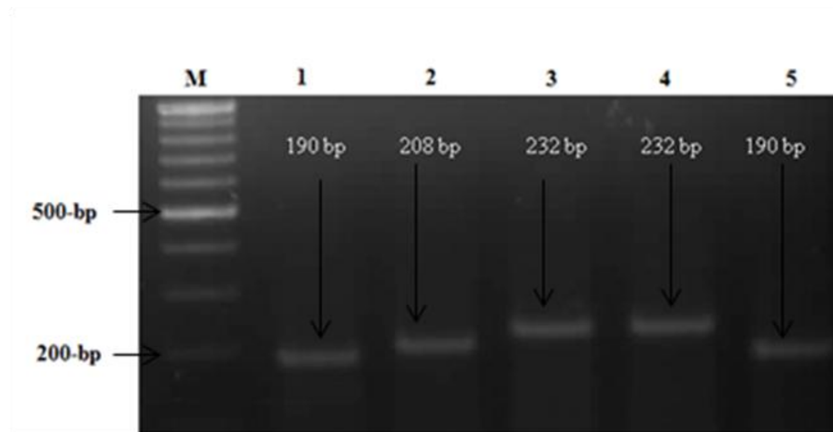


Figure 4.1: Electrophoretic analysis of HCV genotypes of five patients. Patient's numbers are represented by "Numerals". Products in lane number 1 and 5 represent HCV genotype 2a (product size190bp, in Mix1). Lane 2 represents genotype 1a (product size 208-bp in Mix 2). Lane number 3 and 4 indicate HCV genotype 3a (Product size 232bp, in Mix 2).

4.2.1 HCV Genotypes in the Present Study

The current study demonstrated that most of the patients were infected with HCV genotype 3 and then genotype 1. Further classification into sub genotypes showed that the distribution of sub-genotype 3a and sub-genotype 3b was 56.4% and 15% respectively. The prevalence of sub-genotypes 1a and 1b were 10% and 3.57% respectively while the prevalence of untypeable and co-infection patients were 6.42% and 7.14% respectively as shown in Figure 4.2. There were no significant variations in the distributions of HCV genotypes in the two provinces (KPK and Sindh) of Pakistan.

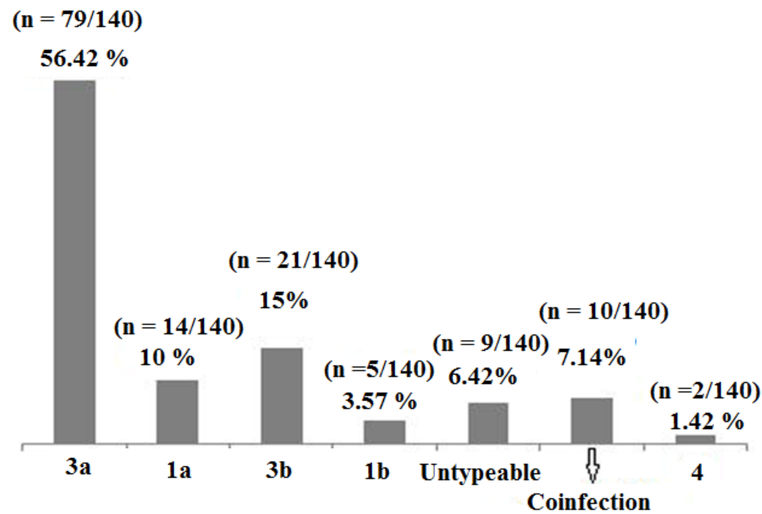


Figure 4.2: Percentage distributions of HCV genotypes in present study. The frequency of HCV patients for genotypes; 3a, 1a, 3b, 1b untypeable, coinfection and 4 were 79 (56.4%), 14 (10%), 21 (15%), 5 (3.5%), 9 (6.42%), 10 (7.1%), 2 (1.4%) respectively. Regarding 10 co-infected patients, two patients were infected with genotype 3a plus 1b, one patient with 3a plus 1a while six patients were infected with 3a plus 3b.

4.2.2 Treatment Response Rates of Different HCV Genotypes

Owing to its RNA genome, HCV has acquired the capability of genetic variability. Within all chronic HCV patients, there are evolved slightly different genetic variants of HCV known as quasi-species. Genotypes represent major genetic differences while quasi-species represent minor genetic differences within individuals (Bukh *et al.*, 1995). HCV quasi-species are spontaneously evolved over the time in response to the host immune pressure and treatment response. It is well established that HCV quasi-species are stable and tend to increase in treatment non-responders while in treatment responders, the number of quasi-species decreases (Puig-Basagoiti *et al.*, 2005).

Since the past decade the gold standard treatment of HCV infection is interferon plus ribavirin. Response to interferon based therapy of HCV infection is mostly dependent on HCV genotype. Response rate to combinational therapy of HCV infection for HCV genotype 2 and 3 is approximately 80%, while for HCV genotype 1 the treatment response rate is 40% - 52% (Zeuzem *et al.*, 2006). In Pakistani population also, treatment response to HCV genotype 1 and 4 is poor while response rate for genotype 3 is high (Idrees *et al*., 2008).

In the current study collectively 140 HCV patients from Swat (KPK) and Gambatt (Sindh) were genotyped for HCV infection. Out of these 140 patients 115 patients were SVRs and 25 were NRs. The response rate to combinational therapy of HCV infection is shown in Figure 4.3.

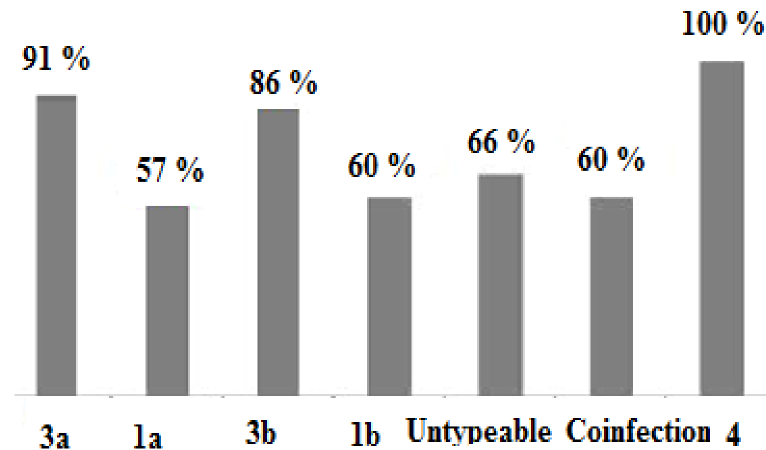


Figure 4.3: Percentage response rates of different HCV genotypes to interferon plus ribavirin treatment. The response rate to interferon based therapy of HCV infection was maximum for HCV genotype 3 and 4. However, there were only two patients of HCV genotype 4. Therefore, its response rate to therapy is not significant. Most of the patients were infected with HCV genotype 3 and they showed high response rate to HCV infection i.e. 91% and 86% for genotype 3a and 3b respectively. Response rate to HCV co-infection was also high (60%). The most possible reason may be the involvement of HCV genotype 3 with other HCV genotypes. In the current study, response rate to HCV genotype 1 was minimum (57%).

4.3 HOST GENETIC VARIATIONS AND COMBINATIONAL THERAPY OF HCV INFECTION

Since the discovery of HCV infection in 1989, there is still a continuous effort for the discovery of new HCV treatment options. In almost all these treatment options of HCV infection, there is still a significant involvement of interferon. The antiviral activity of interferon is also dependent on viral and host genetic variations. Therefore, the aim of the study was to search for important host SNPs that may significantly predict success

rate of combinational therapy of HCV infection in Pakistani population. The association of; IL28B rs12979860 and rs8099917, TGF-β1 functional polymorphism at codon 10 T/C (rs1982073) and codon 25 G/C (rs1800471), OAS1 genetic mutation at rs10774671, IL18 promoter variants, -607C/A (rs1946518) and -137G/C (rs187238), OPN promoter polymorphism, -442 C/T (rs11730582), a single nucleotide variant in intron 6 of GALNT8 rs10849138 were considered with combinational therapy of HCV infection in the current study.

4.4 ANALYSIS OF IL28B rs12979860 POLYMORPHISM BY ALLELE SPECIFIC POLYMERASE CHAIN REACTION

IL28B also known as interferon λ (IFN- λ3) belongs to type III IFNs (Imran *et al.,* 2013a). Antiviral activity of IFN-λ is less than IFN-α but it possesses a critical role in fighting against viruses. The two main SNPs identified as the strongest predictor of spontaneous clearance and treatment responders to interferon based therapy of HCV infection were rs12979860 (3 kb upstream of the IL28B gene) and rs8099917 (8 kb upstream of IL28B gene) (Ank *et al.*, 2006; Coccia *et al.*, 2004; Thomas *et al.*, 2009).

To study the significance of IL28B rs12979860 in combinational therapy of HCV infection in the current study, allele specific polymerase chain reaction (AS-PCR) was performed. Initially, DNA was extracted from the blood samples of both healthy controls and chronic HCV patients. The quality of DNA was testified both by running DNA on 1% agarose gel and also by Nanodrop. The extracted DNA was amplified by a set of two primers that were allele specific in two separate PCR tubes. One set of primer was forward primer 1 and reverse primer (FP1+RP) while the other set of primers was forward primer 2 and reverse primer (FP2+RP). The reverse primer of both sets was

same. In forward primer 1 and forward primer 2 there was only a single nucleotide difference at 3′-end. PCR amplified product was run on 2% agarose gel and visualized under UV light by gel documentation system (Figure 4.4).

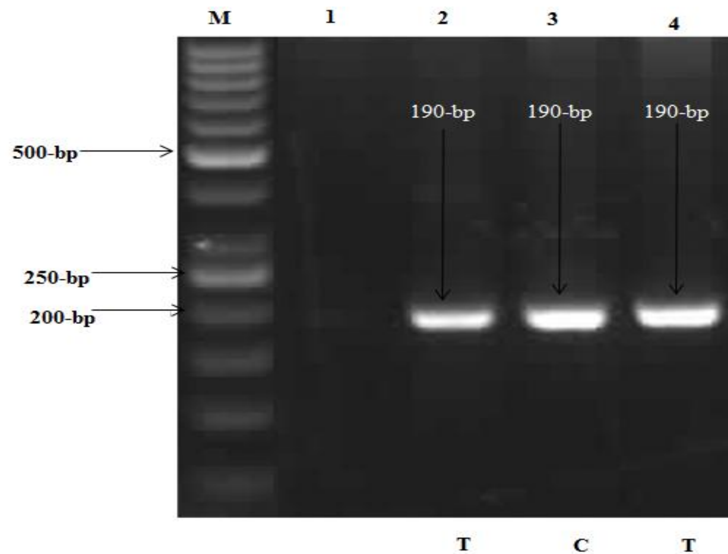


Figure 4.4: Detection of IL28B rs12979860 genetic variation by AS-PCR. Digital print out of agarose gel showing AS-PCR amplification of IL28B rs12979860 polymorphism of two individuals. The first individual represented by lane 1 and 2 is homozygous for T allele (product size 190bp). The second individual represented by lane 3 and 4 is heterozygous.

4.4.1 Analysis of IL28B rs12979860 Polymorphism by Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP-PCR)

To check out the consistency of results obtained by AS-PCR amplification of IL28B rs12979860, RFLP-PCR analysis was also performed. The extracted DNA was subjected to PCR amplification by a single set of primers. The PCR product was digested with BstU-I restriction endonuclease (New England Bio-labs, Hitchin, UK) for 4 hours. The 10 µL digested product was run on 3% agarose gel along with 100-bp ladder and

visualized under UV light of gel documentation system as shown in the Figure 4.5. The digested product was represented by products of 109-bp and 39-bp. As the product size of 39-bp is smaller, so therefore it was not shown and only 109-bp product was visualized. If the digestion not occurred by restriction enzyme, then a single band of 139-bp was shown. On the other hand, if the individual was heterozygous then all the three products (139, 109, 30bp) were observed.

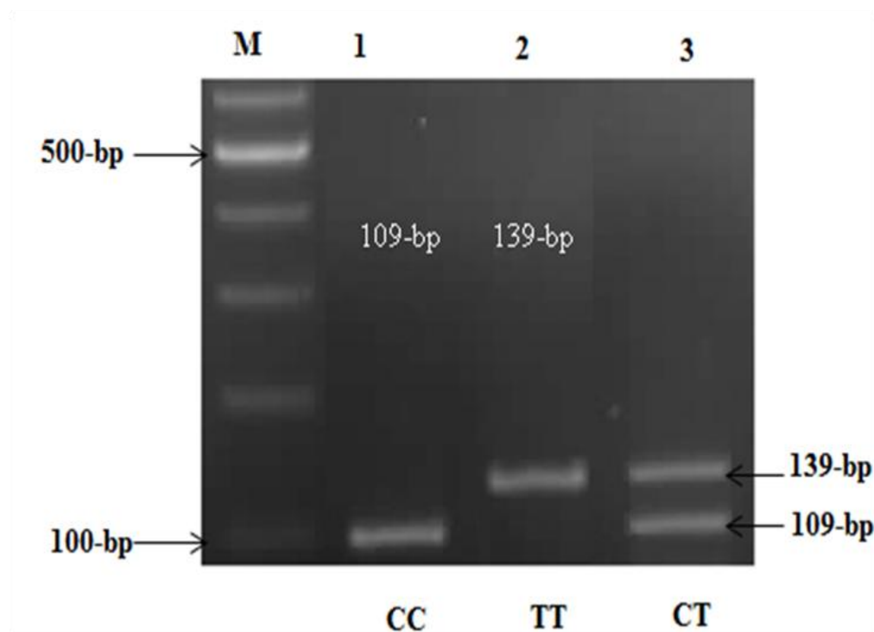


Figure 4.5: Detection of IL28B rs12979860 genetic variation by RFLP-PCR. Digital print out of agarose gel representing RFLP-PCR amplification of IL28B rs12979860 polymorphism in three individuals. Lane 1 represents homozygous CC individual (product size: 109bp). Lane 2 represents homozygous TT individual (product size: 139bp). Lane 3 represents heterozygous CT individual (product size: 109bp + 139bp).

4.4.2 Distribution of IL28B rs12979860 genotypes in healthy controls and chronic HCV patients

The distribution of IL28B rs12979860 CC, CT and TT genotypes in healthy controls and chronic HCV patients was; 48 (40%), 58 (48%), 14 (12%) vs 51 (36%), 68 (49%), 21 (15%) respectively. These Figures showed that there is no significant association of any IL28B rs12979860 genotype with the spontaneous clearance of HCV infection.

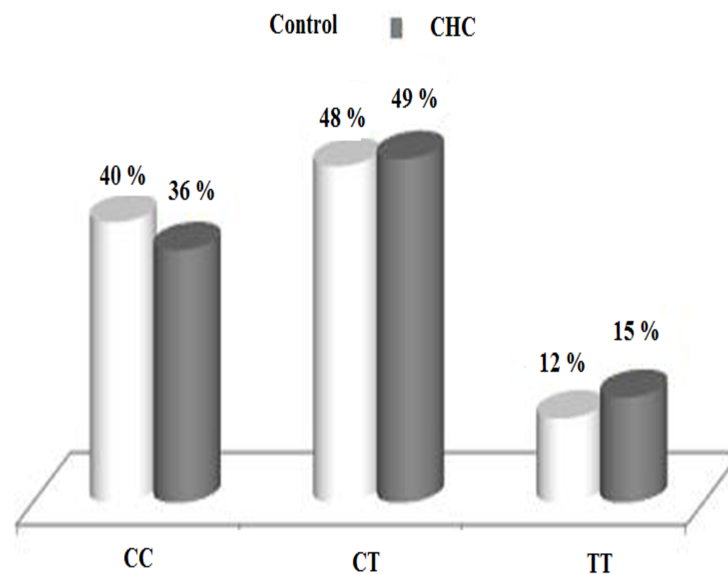


Figure 4.6: Percentage distribution of IL28B rs12979860 genotypes in healthy controls and chronic hepatitis C patients. The prevalence of IL28B rs12979860 genotypes; CC, CT and TT in healthy controls was 40%, 48%, and 12% respectively while their distribution in chronic HCV patients was 36%, 49% and 15% respectively.

4.4.3 Association of IL28B rs12979860 Polymorphism with Interferon Based Therapy

Chronic HCV patients were categorized into two groups on the basis of response to interferon based therapy of HCV infection. The SVR group was comprised of 115 chronic HCV patients, while in NR group there were 25 chronic HCV patients. The distribution of CC, CT and TT genotypes in SVR and NR groups was; 47 (41%), 53 (46%), 15 (13%) and 4 (16%), 15 (60%), 6 (24%) respectively. Pearson chi square analysis by SPSS software version 13 showed that the P values were; 0.019, 0.207 and 0.164 for IL28B rs12979860CC, CT and TT genotypes. These analysis demonstrated that patients possessing CC genotype at rs12979860 were positively associated with the treatment response (P = 0.019). The other two genotypes i.e. CT and TT were not statistically noteworthy in SVRs and NRs (Figure 4.7).

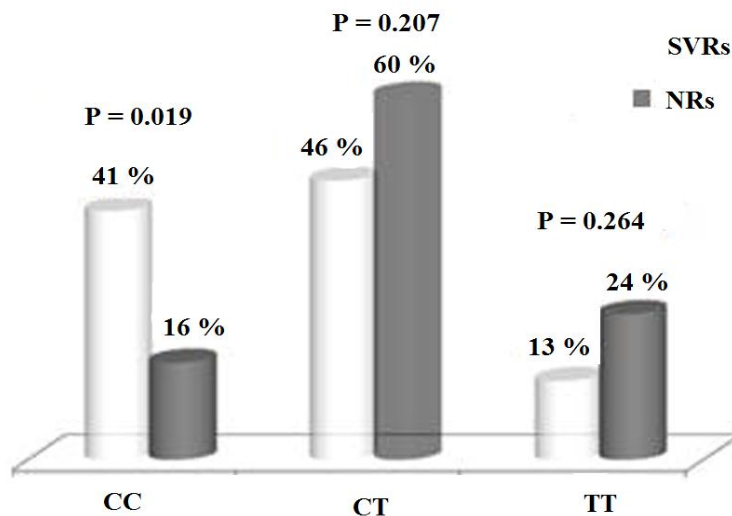


Figure 4.7: Percentage distributions and P-values of IL28B rs12979860 genotypes in SVRs and NRs. Pearson chi square test was applied to find the P-values. The P-values for IL28B rs12979860; CC, CT and TT were 0.019, 0.207 and 0.264 respectively.

4.5 ASSOCIATION OF IL28B rs8099917 WITH COMBINATIONAL THERAPY OF HCV INFECTION

The IL28B rs8099917 polymorphism is also known to possess a role in interferon based therapy of HCV infection. This SNP is determined to mainly dependent on ethnicity also. For IL28B rs8099917, treatment favorable allele is "T" and treatment unfavorable allele is "G". Moreover, IL28B rs8099917 polymorphism have an impact on the mRNA expression of IL28B describing its possible role in the regulation of intra-hepatic expression of interferon stimulated genes (ISGs) (Suppiah *et al.*, 2009; Tanaka *et al.*, 2009). The polymorphism of IL28B rs8099917 in the current study was determined by RFLP-PCR method. Initially, genomic DNA was extracted from blood by kit method. The DNA was amplified by PCR. The amplified product was digested by BseMI (BsrDI) restriction endonuclease (Fermentas, Vilnius, Lithuania) for four hours. 10 µL of digested product was analyzed on 3% agarose gel along with 100-bp ladder (Fermentas, Vilnius, Lithuania) and observed under UV light of the Wealtec gel documentation system as shown in Figure 4.5. The digested product generated two products indicated homozygous GG subject while in case of heterozygous subject there were produced three products. The un-digested RFLP-PCR product showed homozygous TT subject.

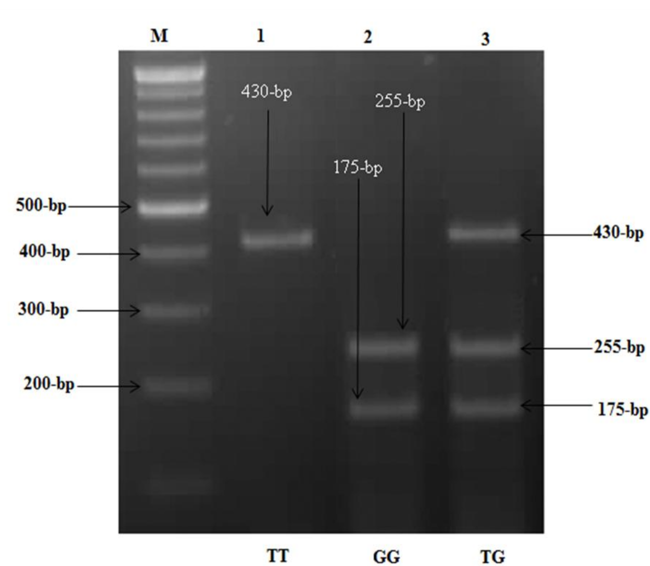


Figure 4.8: Detection of IL28B rs8099917 genetic variation by RFLP-PCR. Amplification of IL28B rs8099917 polymorphism in three different individuals. Lane 1 represents homozygous TT individual (band size 430bp). Lane 2 represents homozygous GG individual (band size 175bp + 255bp). Lane 3 represents heterozygous TG individual (band size 430bp+255bp + 175bp).

4.5.1 Distribution of IL28B rs8099917 Polymorphism in Chronic HCV Patients and Healthy Controls

The distribution of IL28B rs8099917 TT, TG and GG genotypes in healthy controls and chronic hepatitis C (CHC) patients was; 39 (32%), 64 (53%), 17 (14%) and 47 (34%), 68 (48%), 25 (18%) respectively. These Figures showed that there is no significant differences in the distribution of any genotype in healthy controls and CHC patints. Thus, IL28B rs8099917 genotypes were not linked with the the spontenous clearance of HCV (Figure 4.9).

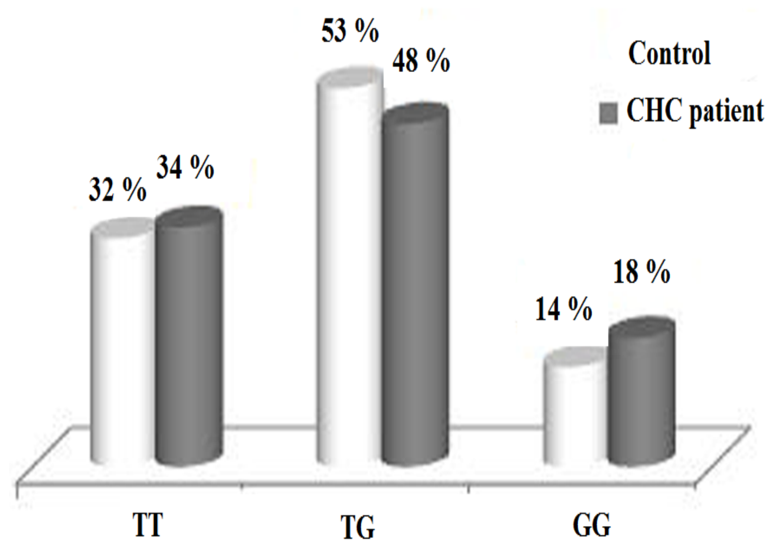


Figure 4.9: Percentage frequencies of IL28B rs8099917 genotypes in healthy controls and chronic hepatitis C patients. The prevalence of IL28B rs8099917 genotypes TT, TG GG in healthy controls was; 32%, 53%, 14% while in chronic HCV patients it was 34%, 48%, 18% respectively.

4.5.2 Distribution of IL28B rs8099917 Polymorphism in SVR and NR Patinets

To find the effect of interferon based therapy on HCV infection, the prevalance of of IL28B rs8099917 in SVR and NR groups was analyzed by Pearson chi square test. The distribution of IL28B rs8099917TT, TG and GG genotypes in SVR and NR groups was; 41 (36%), 55 (48%), 19 (16%) and 6 (24%), 13 (52%), 6 (24%) respectively. The P values for all the three genotypes showed no significance (Figure 4.10).

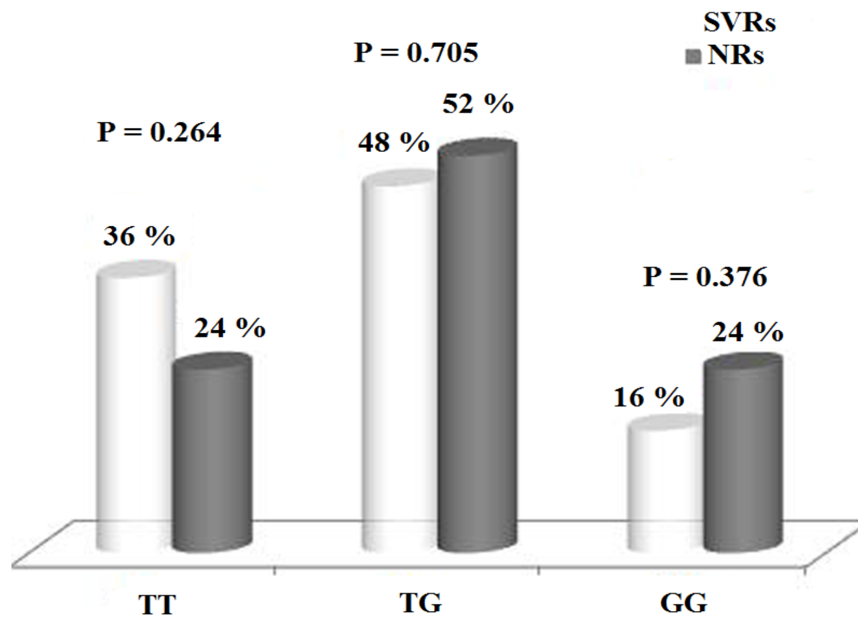


Figure 4.10: Percentage frequencies and P-values of IL28B rs8099917 genotypes in SVRs and NRs. P-values were find by Pearson chi square test. P-values for IL28B rs8099917 genotypes; TT, TG and GG were, 0.264, 0.705 and 0.376 respectively.

4.6 TRANSFORMING GROWTH FACTOR (TGF)-β

Transforming growth factor (TGF)-β is anti-inflammatory cytokine that suppresses the development and cytotoxicity of natural killer (NK) cells. It blocks the production of IFN-γ and IL12. SNPs of TGF-β are associated with variable production of this cytokine (Grainger *et al.*, 1999), liver cirrhosis (Okumoto *et al.*, 2004), and natural clearance of virus (Kimura *et al.*, 2006). We studied the correlation of TGF-β functional polymorphism at codon 10 and 25 with the natural clearance and treatment response of HCV infection in 140 chronic HCV patients and 120 healthy controls.

4.6.1 Detection of TGFβ Codon 10 Polymorphism

The correlation of TGF-β functional polymorphism at codon 10 with interferon based therapy of HCV infection in Pakistani patients was investigated by AS-PCR. DNA was extracted from the whole blood and amplified for the desired gene by AS-PCR. The amplified product was run on 2% agarose gel and observed under gel documentation system as shown in Figure 4.11.

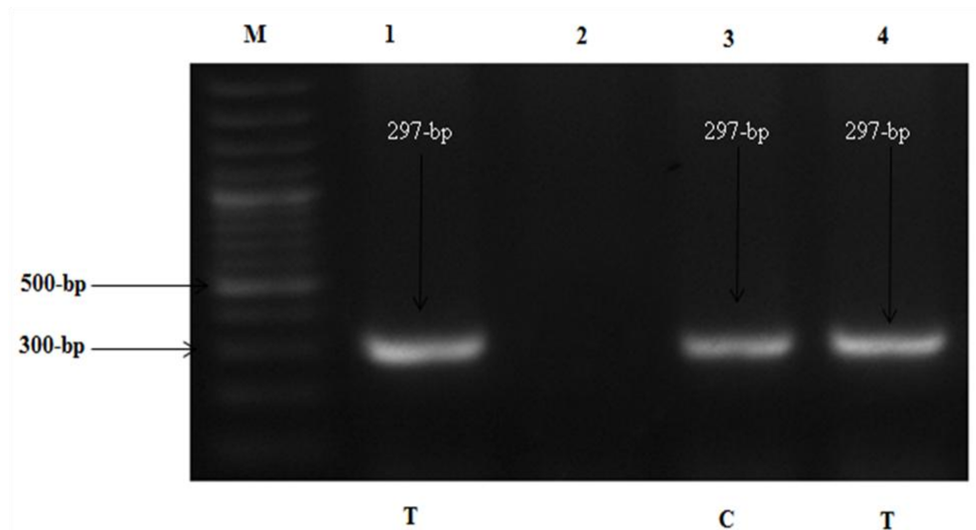


Figure 4.11: AS-PCR amplification of TGFβ codon 10 amplification. Lane M represents 100-bp ladder. Lane 1 and 2 represent homozygous individual for TT genotype at TGFβ codon 10 functional polymorphism. Lane 3 and 4 represent heterozygous individual for TGFβ codon 10 polymorphism.

4.6.2 Distribution of TGFβ codon 10 polymorphism in healthy controls and chronic hepatitis C patients.

The distribution of TGFβ codon 10 genotypes; TT, CT, CC in 120 healthy controls and 140 chronic HCV patients was 34 (28.30%), 66 (55%), 20 (16.60%) and 46 (32.80%), 74 (52.80%), 20 (14.20%) respectively. There were no significant variations in distribution of any TGFβ codon 10 genotypes in SVRs and NRs group. Thus, the results suggest that there is no association of TGFβ codon 10 polymorphism with the natural clearance of HCV infection (Figure 4.12).

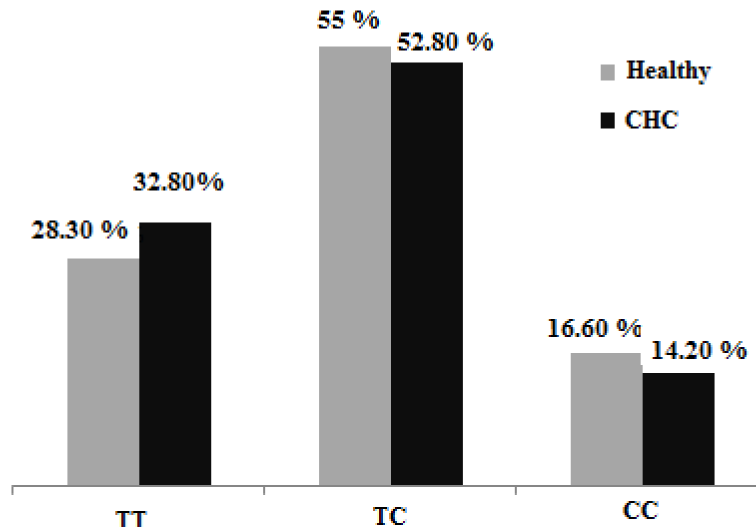


Figure 4.12: Distribution comparison of TGFβ polymorphism at codon 10 in healthy controls and chronic hepatitis C patients. The percentage distribution of TGFβ codon 10 genotypes TT, TC and CC in healthy controls was; 28.30%, 55%, and 16.60% respectively. In chronic HCV patients, the prevalence of TGFβ codon 10 genotypes; TT, TC and CC was; 32.80%, 52.80% and 14.20% respectively.

4.6.3 Effect of TGFβ codon 10 polymorphism on interferon based therapy of HCV infection.

Our results suggest that the distribution of TGFβ codon 10 genotypes; TT, CT and CC in SVRs and NRs group was; 38 (33%), 62 (54%), 15 (13%) and 8 (32%), 12 (48%), 5 (20%) respectively. Furthermore, Pearson chi square test showed that TGFβ codon 10 genotypes have no association with the treatment response of HCV infection as the P values for all genotypes were greater than 0.05 (Figure 4.13).

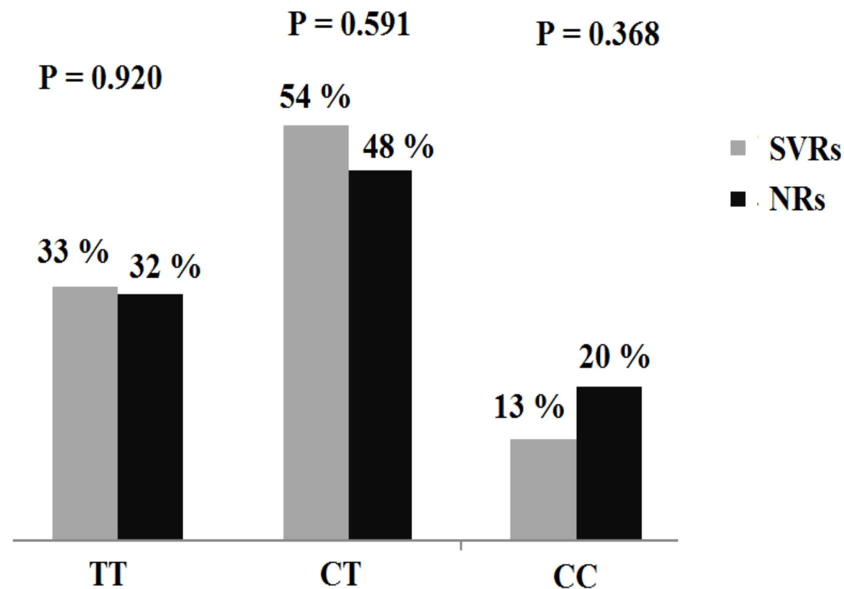


Figure 4.13: Percentage frequencies and P-values of TGFβ codon 10 polymorphism in SVRs and NRs. The percentage distribution of TGFβ codon 10 genotypes; TT, CT and CC in 115 SVR patients was 33%, 54%, and 13% respectively. The prevalence of TGFβ codon 10 polymorphism genotypes; TT, CT and CC, in 25 NR patients was 32%, 48%, and 20% respectively.

4.7 AMPLIFICATION OF TGFβ CODON 25 POLYMORPHISM

Initially, DNA was extracted from the whole blood of 120 healthy control and 140 chronic HCV patients. Genomic DNA was amplified by a set of allele specific primers. Amplified PCR product was run on 2% agarose gel and observed under UV light of gel documentation system (Figure 4.14).

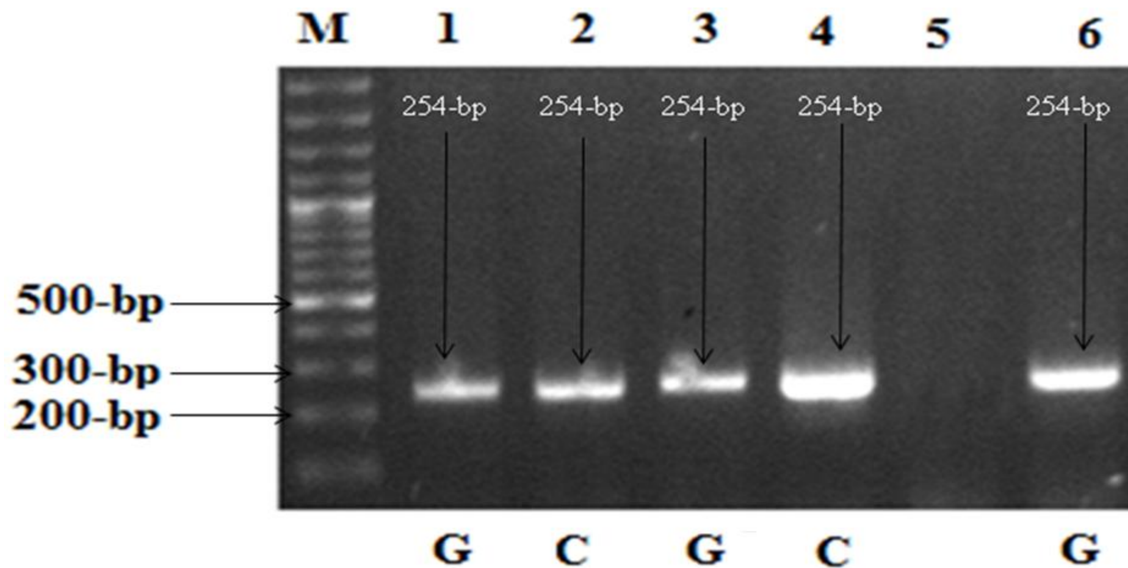


Figure 4.14: Agarose gel representing AS-PCR amplification of TGFβ codon 25 of three individuals. Lane M represents 100-bp ladder. Lane 1, 2, 3 and 4 represent two heterozygous individual while lane 5 and 6 are representing homozygous individual. TGFβ codon 25 amplification was performed for 140 chronic HCV patients and 120 healthy controls.

4.7.1 Distribution of TGFβ codon 25 Polymorphism in Healthy Controls and CHC Patients

The prevalence of TGFβ codon 25 genotypes GG, GC and CC in healthy controls and chronic HCV patients was; 55 (45.80%), 48 (40%), 17 (14.10%) and 69 (49.20%), 53 (37.80%), 18 (12.80%) respectively as shown in Figure 4.15. Pearson chi square test showed that the P values for GG, GC and CC genotypes were all greater than 0.05 and insignificant. The results suggest that there is no role of any TGFβ codon 25 genotypes in the natural clearance of HCV infection.

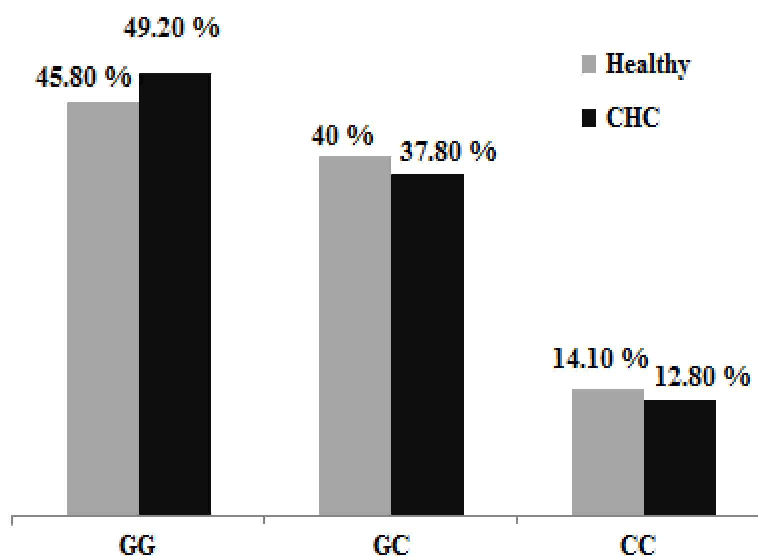


Figure 4.15: Distribution variations of TGFβ polymorphism at codon 25 in healthy controls and chronic hepatitis C patients. The prevalence of TGFβ polymorphism at codon 25 in healthy control was; 45.80%, 40%, and 14.10% for genotypes, GG, GC and CC respectively. The distribution of TGFβ polymorphism at codon 25 in chronic HCV patients was; 49.20%, 37.80%, and 12.80% for TGFβ codon 25 genotypes, GG, GC and CC respectively.

4.7.2 Association of TGFβ codon 25 Polymorphism with Treatment Response of HCV Infection

The distribution of TGFβ codon 25 genotypes; GG, GC, CC in SVRs and NRs group was; 59 (51%), 44 (38%), 12 (36%) and 10 (40%), 9 (36%), 6 (24%) respectively (Figure 4.16). Pearson chi square analysis showed that P values for all the genotypes were greater than 0.05. It can be inferred that there is no association of TGFβ codon 25 polymorphism with interferon based therapy of HCV infection in the current study.

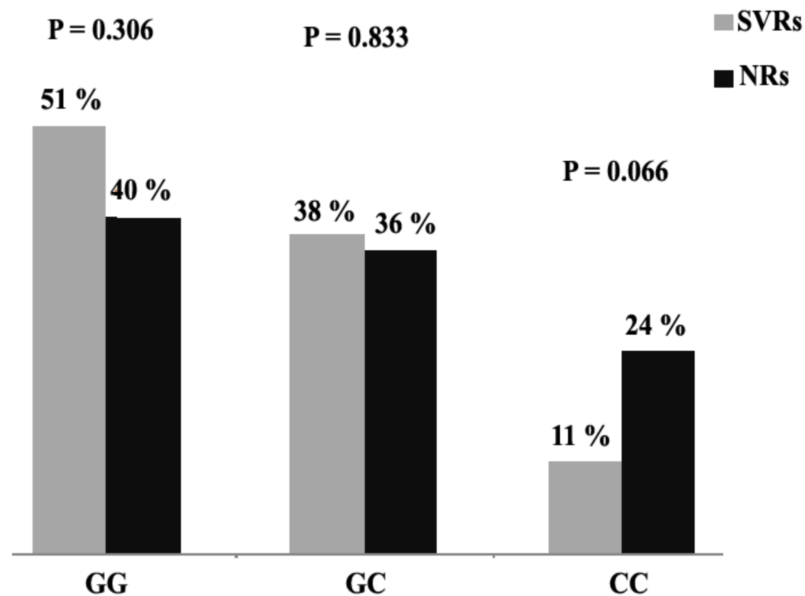


Figure 4.16: Percentage frequencies and P-values of TGFβ codon 25 polymorphism in SVRs and NRs. The prevalence of TGFβ codon 25 genetic variations was demonstrated for 115 SVR chronic HCV patients and 25 non-responder chronic HCV patients. The P-values were calculated by Pearson chi square analysis. The P values for TGFβ codon 25 genotypes GG, GC and CC were, 0.306, 0.833 and 0.066 respectively.

4.8 DETECTION OF OAS1 -442 GENE POLYMORPHISM

OAS1 gene has antiviral activity but there are a lot of variations in its sequence. Approximately, 36 SNPs have been noted in OAS1 gene. The strongest SNP of OAS1 antiviral activity is present at exon 7 SAS (rs10774671). This genetic variation is also suggested to have a significant role in type 1 diabetes (Bonnevie-Nielsen *et al.*, 2000). The normal splicing at exon 7 of OAS1 gene is achieved by the presence of AG sequence at this site. The "G" allele at this position retains the splice site and confers higher

enzymatic activity, (p46) whereas the presence of "A" allele at this postion (AA at acceptor site) ablates the splice site and confers lower enzymatic activity (p48 and p52) to OAS1 gene (Bonnevie-Nielsen *et al.*, 2005; King *et al.*, 2002). Thus, the genetic variation at exon 7 SAS of OAS1 renders it the characteristic of an excellent candidate gene that can significantly predicts the response to IFN based therapy in HCV or HBV patients (King *et al.*, 2002; Suzuki *et al.*, 2004).

The role of this important antiviral gene was analyzed by RFLP-PCR. Firstly, the genomic DNA was extracted and then amplified by a set of RFLP primers. 8 µL of the amplified product was digested by AluI enzyme in a total volume of 20 µL. 10 µL of the digested product was run on a 3% of agarose gel. The electrophoresed product was observed under UV light of Wealtec gel documentation system. The presence of AA genotype in a subject was confirmed by complete digestion of the amplified product into two fragments; 150 bp and 53 bp. The presence of GG genotype was shown by no digestion while heterozygous (AG) condition at the exon 7 SAS of OAS 1 gene was demonstrated by three fragments of; 203-bp, 150-bp and 53-bp (Figure 4.17).

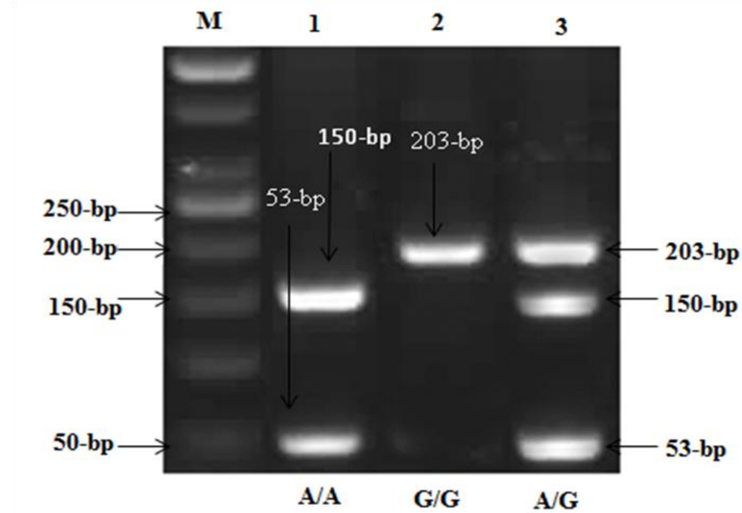


Figure 4.17: Electrophoresis patterns of OAS1 gene of three different individuals by RFLP-PCR. Lane 1, 150bp and 53bp products represent homozygous allele "A". 203bp product in lane 2 represents homozygous "G" allele. Lane 3, 203bp 150bp and 53bp products represent heterozygous A/G alleles of OAS1 gene polymorphism.

4.8.1 Frequency of OAS1 Genotypes in Healthy Controls and Patients Group

One hundred and twenty healthy controls and one hundred forty chronic HCV patients from both sexes were enrolled in the current study to find the importance of genetic testing of OAS1 gene. The frequency of OAS1 gene polymorphism at exon 7 SAS was studied in healthy controls and chronic HCV patients. The prevalence of OAS1 genotypes GG, GA and AA in healthy controls was; 74 (62%), 40 (33%), and 6 (5%) respectively. The distribution of OAS1 genotypes GG, GA and AA in chronic HCV patients was; 97 (69%), 35 (25%) and 8 (6%) repectively as shown in Figure 4.18. The

results suggest that "G" allele (70%) tends to be more frequent than "A" allele (30%) in both control and infected individuals. The prevalence of all genotypes in both groups was comparable. Thus, there is no role of OAS1 SAS at exon 7 polymorphism in spontaneous clearance of HCV infection.

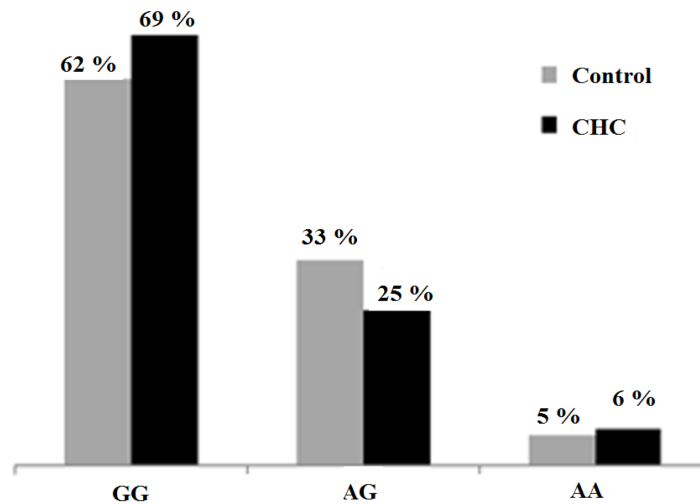


Figure 4.18: Comparison of percentage distributions of OAS1 gene in healthy controls and chronic hepatitis C patients. The prevalence of OAS1 genotypes GG, AG and AA in healthy controls was; 62%, 33%, and 5% respectively. The occurrence of OAS1 genotypes GG, AG and AA in chronic HCV patients was; 69%, 25%, and 6% respectively.

4.8.2 Distribution of OAS1 Genotypes in SVR and NR Groups

The group of chronic HCV patient was further classified into SVR and NR to reveal any correlation of OAS1 polymorphism with interferon based therapy of HCV infection in Pakistani population. Pearson Chi square analysis was performed to compare the two groups. The prevalence of GG genotype was significantly higher in SVR group than NR group (N = 84 vs N = 13; P value: 0.039). The frequency of the other two genotypes of OAS1 gene i.e. AG and AA were almost similar in both SVR and NR

groups representing a non-significant P value (Figure 4.19). The high occurrence of GG in SVR group demonstrated that individuals possessing this genotype at exon 7 SAS of OAS1 gene are better responders to interferon based therapy of HCV infection.

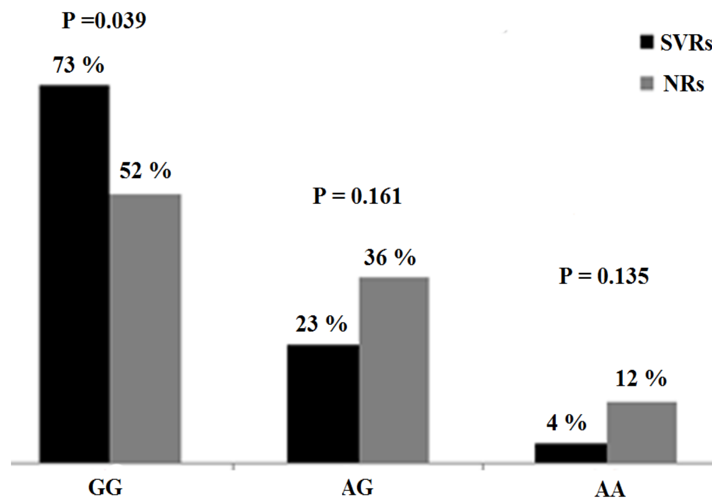


Figure 4.19: Comparison of P-values and percentage variations of OAS1 gene in responder and non-responder groups. Pearson chi square test was applied to find the P-values. The P-values for OAS1 exon 7 SAS genotypes; GG, AG and AA were 0.039, 0.161 and 0.138 respectively.

4.9 INTERLEUKIN-18 POLYMORPHISM

Interleukin 18 (IL18) is inflammatory cytokine that is mainly produced by immature dendritic cells, monocytes and macrophages. It induces the production of two important cytokines, TNF-α and IFN-γ (Zecchina *et al.*, 2001). Up-regulation of IL18 possesses a role in hepatic injury (Vecchiet *et al.*, 2005), and a weak treatment response to interferon therapy (Asakawa *et al.*, 2006). The two strong SNPs of IL18 that regulate its production are -607 C/A and -137 G/C. These SNPs are reported to influence

interferon based therapy of HCV (Manohar *et al.*, 2009), HBV (Kimura *et al.*, 2002; Yao and Tavis, 2005), and human immunodeficiency virus (HIV) infections (Segat *et al.*, 2006), hence selected for the current study.

4.9.1 Detection of IL18 -607 Single Nucleotide Polymorphism

Genomic DNA was extracted from the blood samples of 120 Healthy controls and 140 chronic HCV patients. The extracted DNA was amplified by amplification refractory mutation system polymerase chain reaction (ARMS-PCR). There were used two sets of primers. One set of primer amplified 300-bp product and was used as internal control to validate the PCR reaction. The other set of primer was allele specific for IL18 607 and amplified 196-bp product. The amplified product was run on 2% agarose gel and observed under UV light of gel documentation system to analyze genetic polymorphism of the studied subjects (Figure 4.20).

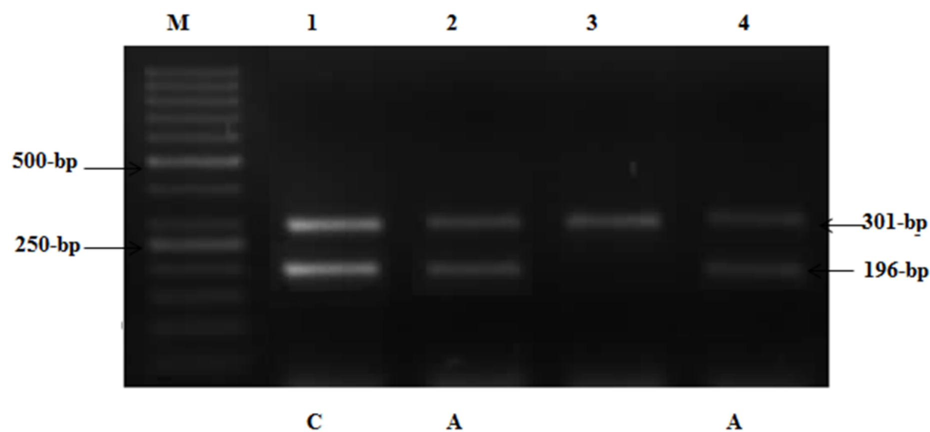


Figure 4.20: Demographic representation of IL18 607 ARMS-PCR amplified products. Lane M shows 100-bp ladder. Lane 1 and 2 show heterozygous individual while lane 3 and 4 represent homozygous individual for IL18 -607 polymorphism.

4.9.2 Distribution of IL18 -607 Genotypes in Healthy Controls and Chronic HCV patients

IL18 gene polymorphism was studied in 120 healthy individuals and 140 chronic HCV patients. Regarding, IL18 rs1946518 gene polymorphisms; there were total 87, 113 and 60 individuals for CC, CA and AA genotypes. All the individuals were divided into three groups, healthy controls, SVRs and NRs. The distributions of genotypes were according to Hardy-Weinberg principle in all three groups. The frequency of IL18 -607 genotypes; CC, CA, AA in healthy controls and patients was 32 (27%), 53 (44%), 35 (29%) and 45 (39%), 60 (43%), 25 (18%) respectively (Figure 4.21). These Figures showed that the distribution of IL18 -607 genotypes in the healthy controls and chronic HCV patients were comparable suggesting that this polymorphism possessed no role in spontaneous clearance of HCV infection in Pakistani patients.

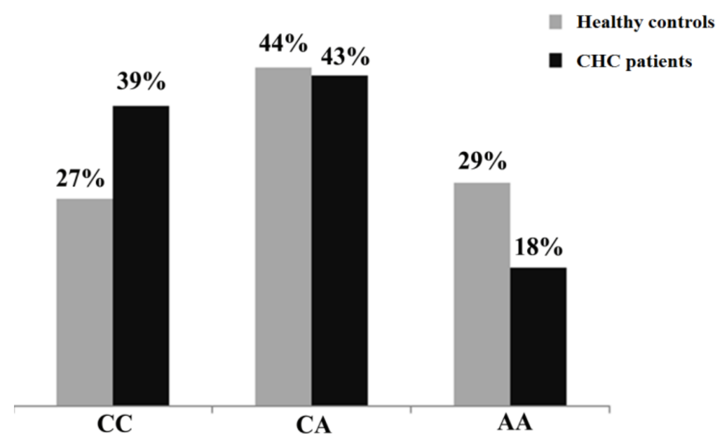


Figure 4.21: Percentage distribution of IL18 -607 polymorphism in healthy controls and chronic hepatitis C patients. The prevalence of IL18 -607 genotypes CC, CA and AA in healthy controls and chronic HCV patients was; 27%, 44%, 29% and 39%, 43%, 18% respectively.

4.9.3 Frequency of IL18 -607 genotypes in SVR and NR groups.

The distribution frequency of IL18 -607 genotypes; CC, CA, AA in treatment responders and non-responders group of chronic HCV patients was; 44 (38%), 47 (41%), 24 (21%) and 11 (44%), 13 (52%), 1 (4%). To explore the association of any genotype of IL18 -607 with interferon based therapy of HCV infection, Pearson chi square test was applied. The analysis showed that there were no significant differences in the two groups for the distribution of CC and CA genotypes. However, the distribution of AA genotypes in SVR group was significantly than NR group (P=0.046), (Figure 4.22). Thus, IL18 -607 SNP is positively associated with interferon based therapy of HCV infection in Pakistani population.

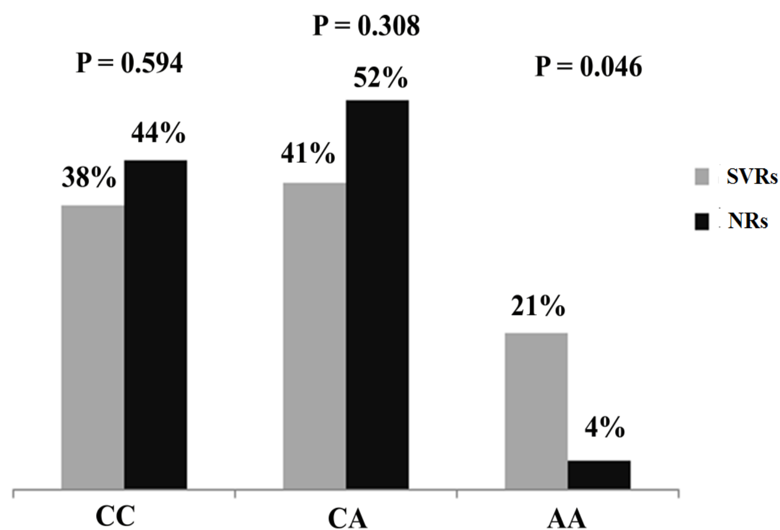


Figure 4.22: Percentage distribution and P-values of IL18 -607 in responder and non-responders. P-values for genetic variation in responder and non-responder groups were calculated by Pearson chi square test. The P-values for IL18 -607 genotypes; CC, CA and AA were 0.594, 0.308 and 0.046 respectively.

4.10 DETECTION OF IL18 -137 POLYMORPHISM

IL18 -137 SNP was identified by ARMS-PCR. Genomic DNA was extracted from the whole blood of 120 healthy controls and 140 chronic HCV patients. The extracted DNA was amplified by ARMS-PCR using two set of primers. One set of primer that amplified 446-bp product was used as internal control to validate the PCR reaction. the other set of primer was specific for the amplification of 261-bp product of IL18 -607 SNP. The amplified product was run on 2% agarose gel along with 50-bp ladder and observed by gel documentation system (Figure 4.23).

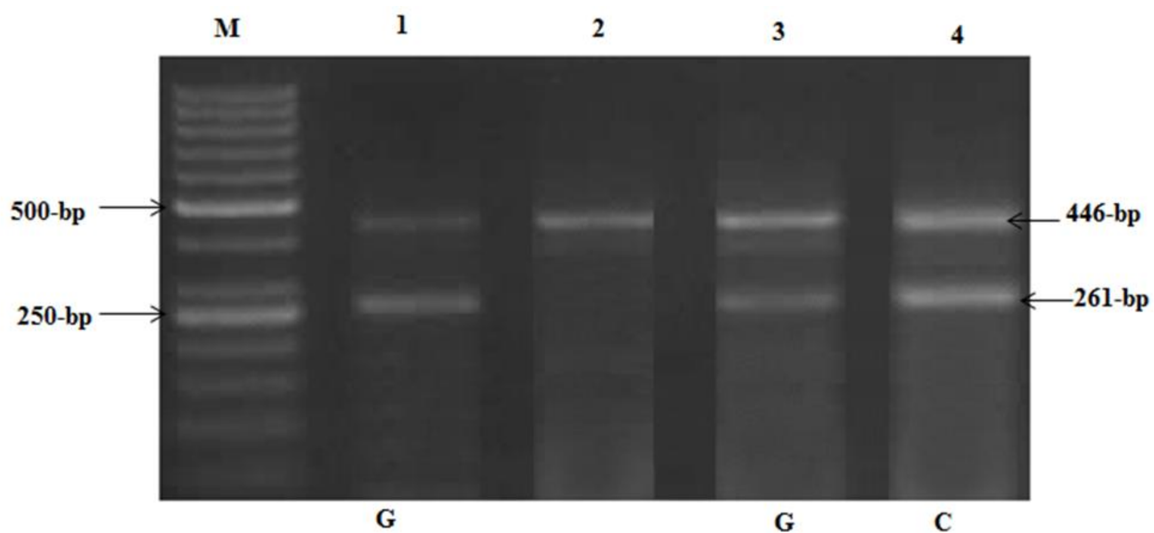


Figure 4.23: Digital print out of gel representing ARMS-PCR of IL18 -137. Lane M represents 50-bp marker. Lane 1 and 2 represent homozygous individual while lane 3 and 4 represent heterozygous individual for IL18 -137 polymorphism.

4.10.1 Distribution of IL18 -137 Genotypes in Healthy Controls and Chronic HCV Patients

The distribution of IL18 -607 genotypes; GG, GC, CC in healthy controls and chronic HCV patients was 43 (36%), 61 (51%), 16 (13%) and 57 (41%), 60 (50%), 13 (9%) respectively (Figure 4.24). The distribution of all the genotypes in the two groups was almost similar. Pearson chi square analysis of the data showed that the P values for all these genotypes were greater than 0.05 suggesting that there is no role of these genotypes in the natural clearance of HCV infection.

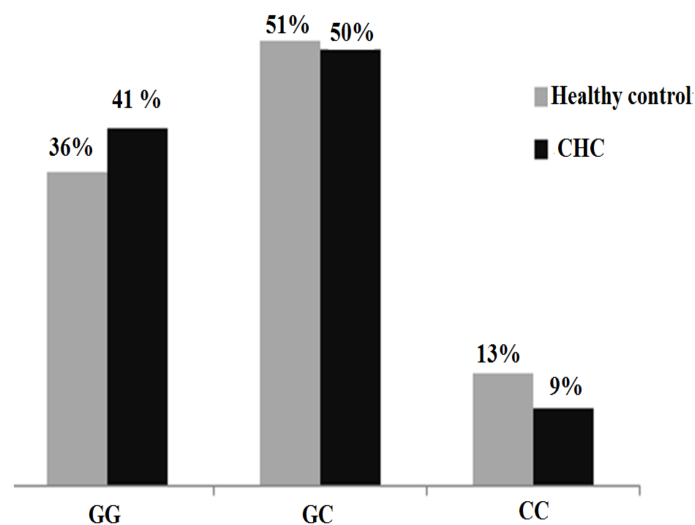


Figure 4.24: Percentage distribution of IL18 -137 polymorphism in healthy controls and chronic hepatitis C patients. The prevalence of IL18 -137 genotypes; GG, GC, CC in healthy controls and chronic HCV patients was 36%, 51%, 13% and 41%, 50%, 9% respectively. The prevalence of IL18 -137 genotypes were comparable in healthy controls and chronic HCV patients.

4.10.2 Distribution of IL18 -137 Genotypes in SVR and NR groups

To reveal any correlation of IL18 -137 genotypes with interferon based therapy of HCV infection, all patients were catagorized into SVR and NR group. The prevalence of IL18 -137 genotypes was observed in both groups. The results suggest that the distribution of IL18 -137 genotypes; GG, GC, CC in SVR and NR group was 48 (42%), 57 (49%), 10 (9%) and 9 (36%), 13 (52%), 3 (12%) respectively (Figure 4.25). Pearson chi square test was applied to find the influence of IL18 -137 genotypes on interferon based therapy of HCV infection. P values for all the genotypes were greater than 0.05. Thus**,** IL18 -137 polymorphism has no association with the interferon based therapy of HCV infection in the current study**.**

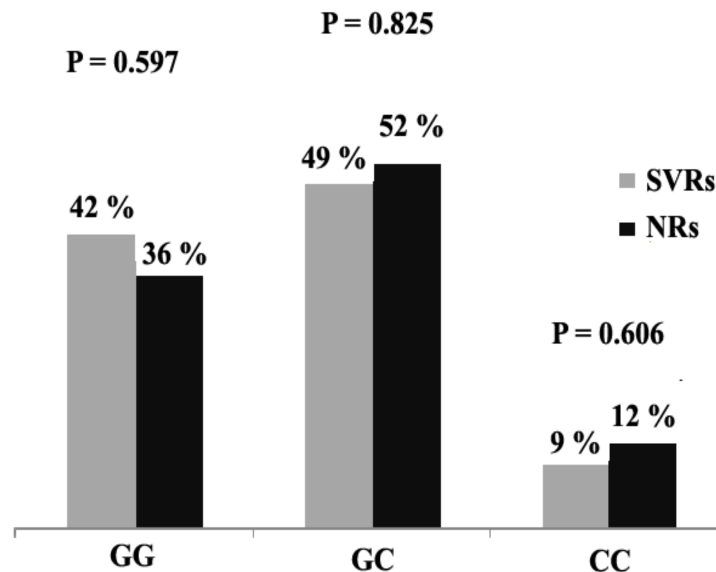


Figure 4.25: Percentage distribution and P-values of IL18 -137 in responders and non-responders. The P-values were calculated by Pearson chi square test. The P-values for IL18 -137 genotypes; GG, GC and CC in treatment responder and non-responder groups were 0.597, 0.825, and 0.606 respectively.

4.11 DETECTION OF OSTEOPONTIN -442 POLYMORPHISM

Osteopontin (OPN) is an extracullar matrix mainly produced by bone and kidney. It has a wide range of biological activities which involve cell adhesion, proliferation and migration (Giachelli and Steitz, 2000; Sodek *et al.*, 2000). Recently, the role of OPN promoter polymorphism in HCV infection has been investigated. It was found that elevated levels of OPN mRNA and protein expression were associated with hepatocellular carcinoma (Huang *et al.*, 2010). The influence of OPN promoter polymorphism -442 on natural clearance and interferon based therapy of HCV infection was noted in the current study. Genomic DNA was extracted from the whole blood of all subjects according to kit protocol. Extracted DNA was amplified by a set allele specific primers. The amplified product (442-bp) was run on 2% agarose gel with 50-bp ladder and observed under UV light of gel documentation system as shown in Figure 4.26.

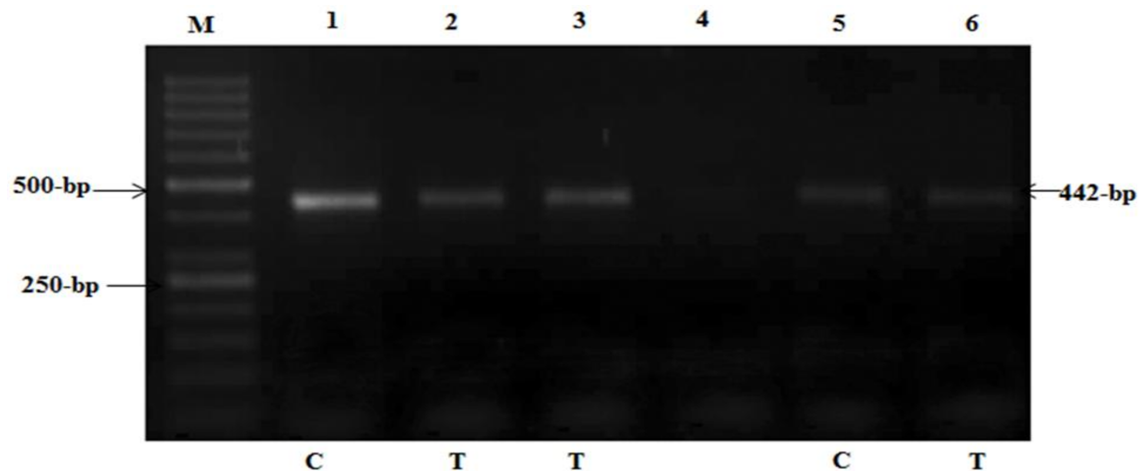


Figure 4.26: AS-PCR amplification of osteopontin -442 polymorphism of two subjects. Lane M specifies 50-bp ladder. Lane 1, 2 5 and 6 specify two heterozygous subjects while lane 4 and 5 specify homozygous subject for OPN -442 polymorphism.

4.11.1 Distribution of Osteopontin -442 Polymorphism in Healthy Controls and Chronic HCV Patients

The distribution of OPN -442 genotypes; CC, CT, TT in 120 healthy controls and 140 chronic HCV patients was 27 (22.5%), 52 (43.3%), 41 (34.1%) and 26 (18.5%), 66 (47.1%), 48 (34.2%) respectively (Figure 4.27). Pearson chi square analysis of the data showed that the P values of all the genotypes were greater than 0.05. Hence, there was no association of any OPN genotype with natural clearance of HCV infection in the present study.

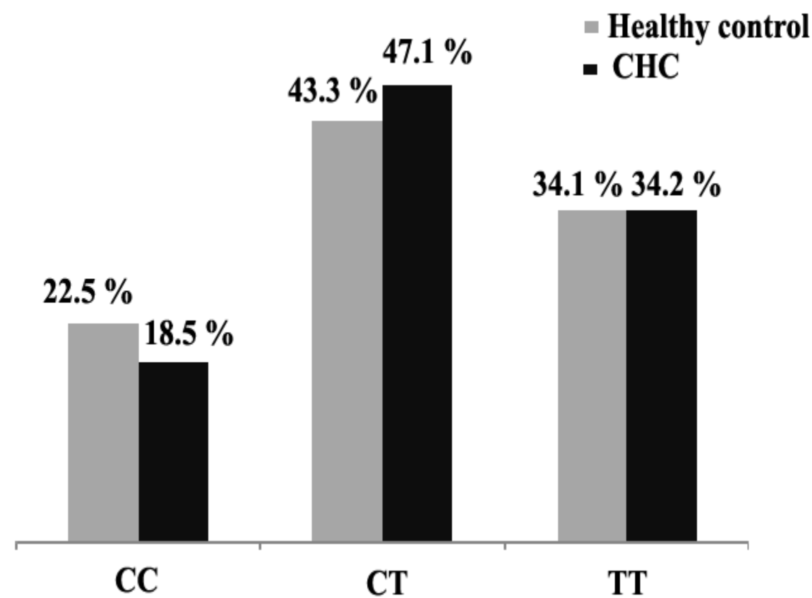


Figure 4.27: Variations in distribution of osteopontin -442 polymorphism in healthy controls and chronic hepatitis C patients. The frequency of OPN -442 genotypes; CC, CT and TT in healthy controls was 22.5%, 43.3%, and 34.1% respectively. The distribution of osteopontin -442 genotypes; CC, CT and TT in chronic HCV patients was 18.5%, 47.1%, and 34.2% respectively.

4.11.2 Distribution of Osteopontin -442 Polymorphism in SVRs and NRs

The current results suggest that the frequency of OPN -442 genotypes; CC, CT, TT in responder and non- responder group was 20 (18%), 51 (44%), 44 (38%) and 6 (24%), 15 (60%), 4 (16%) respectively. The distribution of all the genotypes in both groups was according to Hardy-weinberg equilibrium. To explore the influence of OPN polymorphism on interferon therapy of HCV infection, Pearson chi square test was applied. There were no significant differences in distributions of OPN GG and CG genotypes. However, the P value for OPN CC genotype was significant i.e. 0.034 (Figure: 4.28). Thus, the results suggest that OPN TT genotype was positively associated with interferon based therapy of HCV infection in Pakistani population.

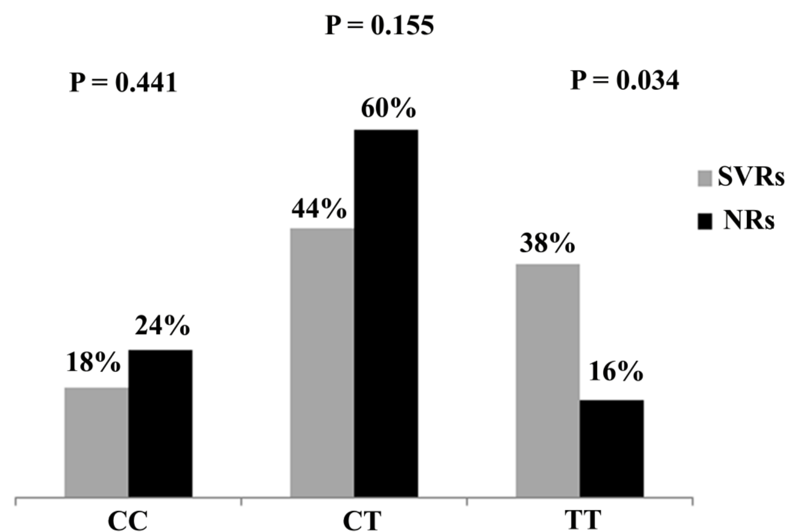


Figure 4.28: Comparison of percentage variation of osteopontin -442 polymorphism in responders and non-responders. The P values were calculated by Pearson chi square analysis. The P values in SVR and NR group for OPN -442 genotypes; CC, CT and TT were 0.441, 0.155 and 0.034 respectively.

4.12 DETECTION OF GALNT8 GENE POLYMORPHISM

Probable polypeptide N-acetylgalactosaminyltransferase 8 (GALNT8) catalyzes the initial reaction in O-linked oligosaccharide biosynthesis. SNP in intron 6 of this enzyme at position, rs10849138 is shown to be involved in response to interferon based therapy of HCV infection in the Japanese population (Nakano *et al.*, 2013). The association of genetic variation of GALNT8 (rs10849138) in healthy controls and chronic HCV patients with natural clearance and treatment outcomes of HCV infection was considered in Pakistani population.

Genomic DNA was extracted from the whole blood of all studied subjects. The extracted DNA was amplified by a set of allele specific primer. The amplified product (340-bp) was run on 2% agarose gel along with 100-bp ladder and observed under UV light of gel documentation system (Figure 4.29).

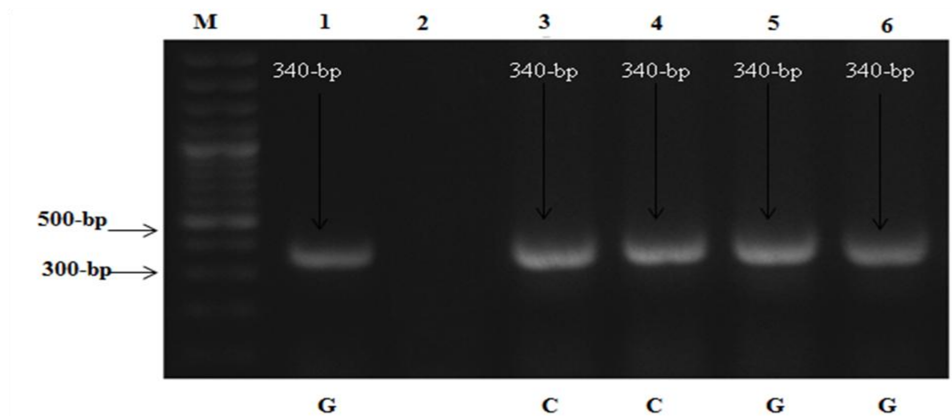


Figure 4.29: Agarose gel represting AS-PCR amplification of GALNT8 gene for three individuals. Lane M demonstrates 50-bp ladder. Lane 1 and 2 demonstrate homozygous individual while lane 3, 4, 5 and 6 demonstrate heterozygous individual for GALNT8 polymorphism.

4.12.1 Distribution of GALNT8 Polymorphism in Healthy and Patient Groups

The distribution of GALNT8 genotypes in healthy controls and patients followed Hardy-Weinberg equilibrium. The frequency of GALNT8 genotypes; CC, GC, GG in 120 healthy controls and 140 chronic HCV patients was 31 (25.8%), 64 (53.3%), 25 (20.8%) and 32 (22.8%), 59 (49.2%), 39 (27.8%) respectively (Figure 4.30). There were no significant differences in prevalence of GALNT8 polymorphism in healthy controls and chronic HCV patients. Pearson chi square test showed that P value of all genotypes were greater than 0.05. Thus, GALNT8 genotypes have no role in natural clearance of HCV infection.

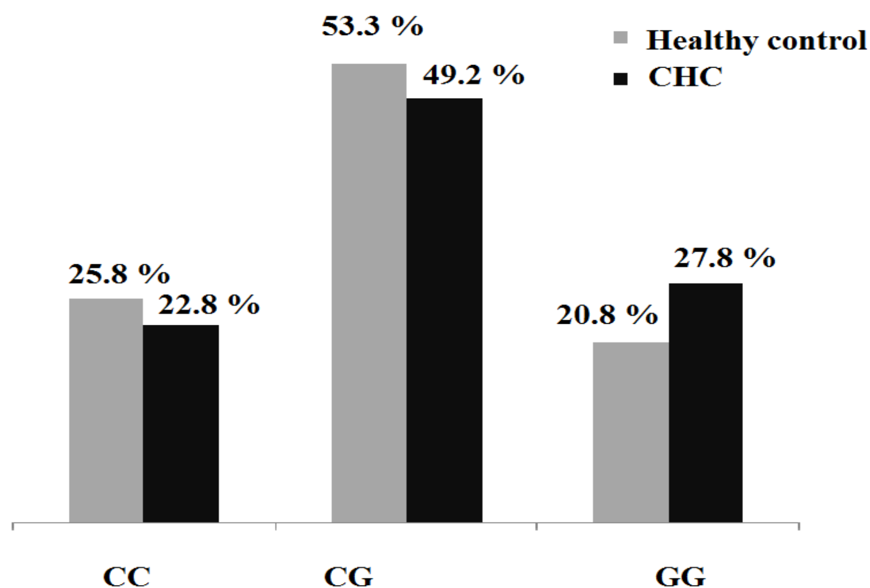


Figure 4.30: Percentage variation in prevalence of GALNT8 polymorphism in healthy individuals and chronic HCV patients. The occurrence of GALNT8 genotypes; CC, CG and GG in healthy controls was 25.8%, 53.3% and 20.8% respectively. The distribution of GALNT8 genotypes; CC, CG and GG in chronic HCV patients was 22.8%, 49.2% and 27.8% respectively.

4.12.2 Distribution of GALNT8 polymorphism in SVR and NR groups

To explore any correlation of GALNT8 gene polymorphism with interferon therapy of HCV infection, the prevalence of GALNT8 genotypes were studied in both responders and non-responders group. Our results suggest that prevalence of GALNT8 genotypes; CC, GC, GG in responders group and non responders group was; 25 (21%), 57 (50%), 33 (29%) and 7 (28%), 12 (48%), 6 (24%) respectively. Pearson chi square test showed that P-values for CC, GC and GG genotypes of GALNT8 genotypes were; 0.499, 0.887 and 0.635 respectively (Figure 4.31). These values showed that there was no association of any genotype of GALNT8 with interferon based therapy of HCV infection in the current study.

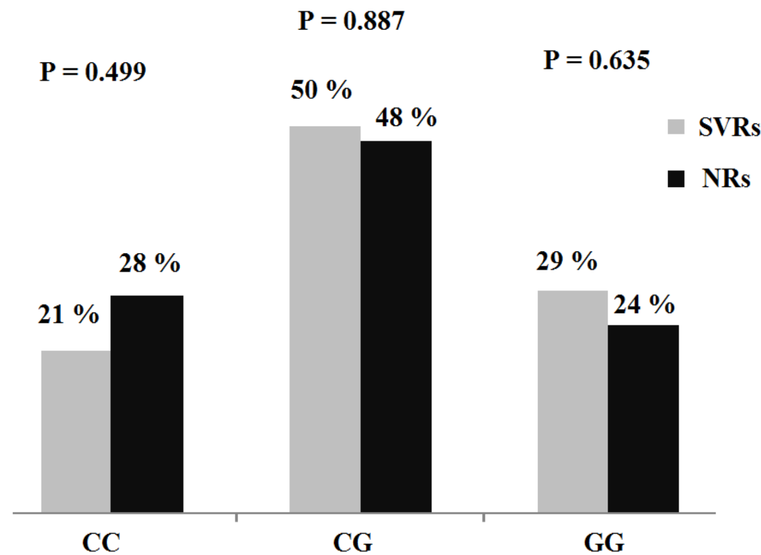


Figure 4.31: Percentage distribution of GALNT8 Intron 6 polymorphism in SVRs and NRs. The P-values for treatment responder and non-responder groups were calculated by Pearson chi square test. The P-values for GALNT8 genotypes CC, CG and GG were; 0.499, 0.887 and 0.635 respectively.

Table 4.2: Statiscal analysis of different genestic variations in response to interferon based therapy of HCV infection.

Genotype	Total (260)	Control (120)	SVR (115)	NR (25)	*P-value
IL28B rs12979860 genotype					
CC	99 (38.07%)	48	47	4	**0.019**
CT	126 (48.4%)	58	53	15	0.207
TT	35 (13.4%)	14	15	6	0.164
IL28B rs8099917 genotype					
TT	86 (33%)	39	41	6	0.264
TG	132 (50.7%)	64	55	13	0.705
GG	42 (16.1%)	17	19	6	0.376
TGFβ polymorphism at codon 10					
TT	80 (30.6%)	34	38	8	0.920
CT	140 (53.8%)	66	62	12	0.591
CC	40 (15.3%)	20	15	5	0.368
TGFβ polymorphism at codon 25					
GG	124 (47.6%)	55	59	10	0.306
GC	101 (38.8%)	48	44	9	0.833
CC	35 (13.4%)	17	12	6	0.066
OAS1 polymorphism at, rs10774671					
GG	125 (48%)	74	84	13	**0.039**

AG	108 (41.5%)	40	26	9	0.161
AA	27 (10.3%)	6	5	3	0.135
IL18 -607 polymorphism					
CC	87 (33.4%)	32	44	11	0.594
CA	113 (43.4%)	53	47	13	0.308
AA	60 (23%)	35	24	1	**0.046**
IL18 -137 polymorphism					
GG	100 (38.4%)	43	48	9	0.597
GC	131 (50.3%)	61	57	13	0.825
CC	29 (11.1%)	16	10	3	0.606
Osteopontin -442 polymorphism					
CC	53 (20.5%)	27	20	6	0.441
CT	118 (45.3%)	52	51	15	0.155
TT	89 (34.2 %)	41	44	4	**0.034**
GALNT8 polymorphism in intron 6 at rs10849138					
CC	63 (24.2%)	31	25	7	0.499
GC	133 (51.1%)	64	57	12	0.887
GG	64 (24.6%)	25	33	6	0.635

Chapter 5

DISCUSSION

Pakistan is the sixth largest country of the world by population that is situated in South Asia. It has four provinces. KPK and Sindh are the two provinces that were selected for blood sample collection of chronic HCV infected patients. In Pakistan more than 10 million people are infected by HCV that comprise almost 6% of the total population (Idrees *et al.,* 2008). Interferon based therapy of HCV infection is well known as a standard of care for HCV infection worldwide. However, interferon based therapy of HCV infection is associated with both serious side effects and cost effects. It has always been an exigent task to envisage the possible outcomes of treatment in HCV infected patients. Therefore, precise prediction of patient's response to therapy is becoming important both from patient point of care and treatment cost as well.

Initially, only baseline viral load of and HCV genotypes were considered as potential predictive markers in determining the treatment outcomes and duration of HCV therapy (Fried, 2002; Ghany *et al.,* 2009, Mccaughan *et al.,* 2007; Zeuzem *et al.,* 2009). Recently, the discovery of IL28B SNPs and their role in interferon based therapy of HCV infection result in their addition to the treatment guidelines by European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASL) (Ghany *et al.*, 2009). The distribution of SNPs and their potential roles in natural clearance and treatment outcomes of a disease varies from population to population (Rauch *et al.,* 2010; Mchutchison, 2011). Therefore, the present study was

designed to evaluate the potential role of HCV genotypes, baseline viral titer and host SNPs in interferon based therapy of HCV infection.

The results suggest that the most common genotype of IL28B rs12979860 was; CT (n = 99; 38.07%) followed by CC (n = 126; 48.4%) and TT (n = 35; 13.4%) in the present study. The prevalence of IL28B rs12979860 genotypes in healthy controls and patients group was almost similar. The presence of IL28B rs12979860CC genotype was favorably associated with interferon based therapy of HCV infection. The percentage of patients possessing successful fate of treatment was higher in patients with IL28B rs12979860CC genotype than TT or CT genotype at this position (P value = 0.019). The published data from different ethnic groups throughout the world has established the critical role of IL28B rs12979860 polymorphism in the treatment outcomes of HCV infection (Rauch *et al.,* 2010; Mchutchison, 2011). A noteworthy difference in response rate to interferon based therapy of HCV infection in African-Americans and European individuals has been reported. One of the major reasons for this difference in response rate is attributed to unequal distribution of IL28B rs12979860 genotypes in these cohorts. The prevalence of IL28B rs12979860CC was more common in individuals from European ancestry than individuals from African-Americans. Therefore, Europeans are better responders to interferon based therapy of HCV infection. Moreover, it was further found that individuals from East Asian ancestry were even better responders to interferon based therapy than European ancestor individuals (Yan *et al.,* 2008). Iran from the Middle East and India from the South Asia, are the two close neighbor countries of Pakistan. In these two countries HCV genotype 3 is one of the most prevalent genotype. The reports from these two countries also demonstrated the high prevalence of IL28B

rs12979860CC in their population. Furthermore, IL28B rs12979860CC genotype was also demonstrated to hold a positive association with interferon based therapy of HCV infection, supporting the current results demonstrating the high prevalence and favorable role of IL28B rs12979860CC in interferon based therapy of HCV infection (Sharafi *et al.,* 2012; Sivaprasad *et al.,* 2012).

The present study revealed that regarding IL28B rs8099917 polymorphism, the most common genotype was TG (n = 132; 50.7%) followed by TT (n = 86; 33%) and GG (n = 42; 16.1%). The distribution of IL28B rs8099917 genotypes in healthy control and patient groups was similar. The prevalence of IL28B rs8099917 polymorphism in sustained virological responders (SVRs) and non-responders (NRs) was also comparable. Worldwide studies have shown a positive association of IL28B rs8099917TT genotype with interferon based therapy of HCV infection. These studies were mostly conducted against HCV genotype 1 and 4, and less data is available for genotype 3 (Antaki *et al.,* 2013; Fischer *et al.,* 2012). The reported data suggest that the treatment response rate of IL28B rs8099917 of HCV infection is mostly dependent on HCV genotype (Jia *et al.,* 2012). The current results showed no association between rs8099917TT and treatment response of HCV infection. The most probable reason for this lack of association may be attributed to the high prevalence of HCV genotype 3 in the current study (71.4%). A report from Moghaddam *et al.,* (2011) presented that IL28B rs8099917TT is initially associated with enhanced virological response against genotype 3 but later on the response was not sustained. Finally, there was no association between IL28B rs8099917TT and the treatment response to HCV genotype 3 (Moghaddam *et al.,* 2011). Another study also demonstrated a lack of association between IL28B rs8099917TT and

the treatment response of HCV genotype 3, but the same study showed an association between IL28B rs12979860CC with the response to interferon based therapy against HCV genotype 3 (Sarrazin e*t al.,* 2010).

Transforming growth factor beta (TGFβ) is an important cytokine that controls cell proliferation and differentiation. Its critical role has been suggested in various diseases such as diabetes, heart disease, marfan syndrome and AIDS (Epstein *et al.*, 2000). Accelerated production of TGFβ is associated with enhanced liver fibrosis (Kimura *et al.,* 2006). HCV core protein is reported to stimulate the production of TGFβ (Taniguchi *et al.,* 2004). Genetic variations at codon 10 and 25 of TGFβ are shown to play a significant role in accelerated liver fibrosis. (Gewaltig *et al.,* 2002; Osterreicher *et al.,* 2005).

Codon 10 polymorphism of TGFβ was reported to have a role in export efficiency of newly synthesized protein (Grainger *et al.,* 1999). Moreover, codon 25 polymorphism of TGFβ was shown to play a role in graft rejection after liver transplantation (Eurich *et al.,* 2011). It was proposed that these two SNPs of TGFβ may have a role in treatment outcomes of interferon based therapy of HCV infection. However, no association was found for both genotypes of TGFβ polymorphisms with treatment outcomes of HCV infection. The current results suggest that there were no significant differences in the distribution of both genotypes of TGFβ polymorphisms in healthy controls and patients group. The distribution of TGFβ genotypes between responder and non-responder groups was also comparable. TGFβ genotypes at codon 10 and 25 showed no association with interferon based therapy of HCV infection.

2'-5' oligoadenylate synthetase 1 (OAS1) gene is critically involved in the interferon signaling pathway. Its homolog in mice is reported for predisposition against *flaviviruses* (Mashimo *et al.,* 2008). Findings of current study have shown a significant association between polymorphism at exon 7 SAS and interferon based therapy of HCV infection. Previously, it was demonstrated by El-Awady *et al.,* (2011) that there is an important association between OAS1 polymorphism and the treatment outcomes of HCV infection. The present results suggest that patients possessing OAS1GG genotype at exon 7 SAS showed more sustained virological response than patients possessing either GA or AA genotypes (P value = 0.039 vs 0.161 and 0.135 respectively). Thus, patients possessing "G" allele at this particular position of OAS1 gene seem to confer better treatment response to HCV infection. Previous studies reported that "G" allele exon 7 SAS of OAS1 enzyme provides better functional capabilities. The current findings confirm the earlier reports that "G" allele at exon 7 SAS of OAS1 gene is linked with enhanced enzyme activity. On the contrary, "A" allele at this position provides higher molecular weight but a lower enzyme activity (El Awady *et al.,* 2011).

IL18 is a pro-inflammatory cytokine that encompasses six exons with five introns on chromosome 11q22.2-q22.3 (Kruse *et al.,* 2003). The results suggest that promoter polymorphism of IL18 -607 is significantly involved in the outcomes of interferon based therapy of HCV infection. This is in agreement with previous studies that revealed a significant association of promoter polymorphisms of IL18 -607 with natural clearance and treatment outcomes of various infectious diseases, (Sivalingam *et al.,* 2003; Gracie *et al.,* 2005) and atopic eczema (Novak *et al.,* 2005; Kretowski *et al.,* 2002; Ide *et al.,* 2004)**.** The two promoter polymorphisms of IL18 at position -607 and -137 were

considered for their association with HCV pathogensis and treatment outcomes. IL18 -607 showed a significant association with interferon based therapy of HCV infection. The current results suggest that individuals possessing AA genotype for IL18 -607 have a higher rate of successful treatment outcomes than individuals possessing CC and CA genotypes. A study from Egypt has shown that the prevalence of IL18 -607AA genotype was higher in healthy controls than patients group suggesting a protective role of this genotype against HCV infection (Farid *et al.,* 2013). Another report from India has shown that IL18 -607AA genotype is associated with milder liver disease in case of chronic HCV patients (Manohar *et al.,* 2009). In the current a significant difference in the distribution of -607AA genotype in healthy control and patient groups was found (P = 0.031). Therefore, according to the current results the prevalence of IL18 -607AA was significantly higher in SVRs group than NRs group (P = 0.046). Thus, individuals with IL18 -607AA genotype were better responders to combinational therapy of HCV infection than individuals possessing other genotypes.

Regarding IL18 -137 polymorphism, the distribution of IL18 -137 genotypes in healthy control and patient groups was comparable. Moreover, the distribution of IL18 -137 genotypes in treatment responder and non-responder groups was also similar. Thus, the current results reveal that IL18 -137 genetic variations possesses neither a role in spontaneous clearance of HCV infection nor in treatment response.

Osteopontin (OPN) also known as sialoprotein is positioned on chromosome 4 long arm region 13, i.e. 4q13. It comprises of 5 kilobase region with seven exons. SNP at OPN -442 was found to have a role in hepatitis activity in chronic HCV patients (Mochida *et al.,* 2004). The current results showed that chronic HCV patients with OPN -

442TT genotypes have a more success rate of combinational therapy than patients possessing either CC or CT genotypes (P = 0.034). Thus, OPN -442TT genotype have a positive association with the combinational therapy of HCV infection. A study of Naito *et al.,* (2005) has shown that OPN polymorphism at -442 can be used as a predictive marker for interferon based therapy of HCV infection. The study also showed that SVR rate was significantly higher in patients possessing OPN -442TT genotype than any other genotype at this position. The results show that there were no significant differences in the distributions of OPN -442 genotypes in healthy control and patient groups. Thus, OPN -442TT polymorphism appears to have no role in spontaneous clearance of HCV infection.

The association of GALNT8 SNP at rs10849138 with interferon based therapy of HCV infection in chronic HCV Pakistani patients was also investigated in the current study. The distributions of GALNT8 rs10849138 genotypes in all studied groups i.e. healthy controls, chronic HCV patients, treatment responders and non-responder were comparable. The current results propose no significant association of any genotype of GALNT8 rs10849138 with the natural clearance and interferon based therapy of HCV infection. A recent study of Nakano *et al.,* (2013) illustrated an association of GALNT8 rs2286580 with interferon therapy of HCV infection in the Japanese population. However, the results were not repeated in patients receiving peg-interferon and ribavirin treatment for HCV infection. The association was at maximum point for GALNT8 rs10849138 and combinational therapy of HCV infection.

Regarding HCV-related factors, baseline viral titer and HCV genotypes are considered as an independent predictor of SVR but there are suggested several cut-off

values for it, depending upon the kit used for viral quantification (Kau *et al.,* 2008). Although, there is standardization of viral load to IU/ml, but still it is not possible to determine the absolute cut-offs for low and high viral titers. The main reason is that there are several commercially available HCV RNA quantification kits that differently measures HCV RNA concentrations of the same patient. So therefore, each kit has its own cut-offs of low and high viral concentrations (Chevaliez e*t al.,* 2007; Michelin *et al.,* 2007; Sarrazin *et al.,* 2006). The median baseline viral titer of SVR group in the current study was; 1.12×10^5 IU/ml while for NR group, it was 5.64×10^5 IU/ml. The results suggest that although NR group has a high viral titer, but it was statistically not important ($P = 0.241$). Generally the low baseline viral titer is considered to be associated with high SVR rate (Ismail *et al.,* 2013).

There is a significant influence of HCV genotypes on disease severity and response to interferon based therapy of HCV infection. Information about HCV genotypes is very helpful in the determination of treatment duration and options. Generally, patients with HCV genotype 3 are comparatively easy to treat, taking less treatment duration and getting a higher rate of SVR. According to the current results, HCV subtype 3a was the most common genotype (56.4%). The response rate to interferon plus ribavirin treatment of HCV genotype 3 was 91%. These results are in parallel with the previous reports showing that HCV genotype 3 is the most prevalent genotype in Pakistan with maximum response rate to interferon based therapy (Idrees *et al.,* 2008; Ahmad *et al.,* 2012). The second most prevalent HCV genotype was genotype 1 (13.5%). Treatment response rate to HCV genotype 1 in the present study was 58.5%. Worldwide studies have shown that the treatment response rate to HCV genotype 1 is

almost 50%. Nine patients in the current study were coinfected. The co-infected patients were of genotype 1 and 3 with treatment response rate of 60%. Genotype 4 was the least prevalent, only in two patients out of 140. Nine chronic HCV patients were not genotyped by the present method of genotyping. Previously a study of Safi *et al.,* (2012) also noticed two untypable HCV genotypes from KPK. The mystery of these untypable genotypes can be solved by cloning and sequencing of genomic portion of these HCV samples. In conclusion, the current study confirms the previous findings that the prevalence of HCV genotype 3 was high in chronically HCV infected Pakistani patients with a very good response to interferon based therapy of HCV infection. Other HCV genotypes, 1 and 2 were less prevalent.

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), also known as serum glutamic pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase, (SGOT) respectively are transaminases. The elevated levels of ALT and AST are the markers of hepatic injury, heart injury or muscle disease. Therefore, these enzymes are not liver specific and cannot be used as markers of progression of liver fibrosis. In the current study, the baseline level of ALT for responder group was; mean±SD: 66.3±33, while for non-responder group it was, mean±SD: 90.3±33.6. The P value of independent T test was 0.001. Regarding AST, no significant difference in baseline AST levels of responder and non-responder groups was found (72.6±42 vs 82.5±30; P value = 0.267). Various studies have shown conflicting results about the association of baseline levels ALT and AST with interferon based therapy of HCV infection (Fried, 2002; Manolakopoulos *et al.,* 2006; Shiffman *et al.,* 2007).

Alkaline phosphatase is another important liver enzyme which shows liver injury, but it is also not specific for hepatic injury. A non-significant difference in baseline ALP of responders and non-responder groups was found (311.5.6±143.1 vs 340 ±105.1; P value = 0.347). Bilirubin is mainly produced by break down of hemoglobin. Its level is also elevated in chronic HCV patients. There were found no significant differences in the bilirubin levels of responder and non-responder groups. (1.3±0.87 vs 1.4 ±0.86; P value = 0.420).

It is established that young age has an important role in achieving SVR in interferon based therapy of HCV infection. Large prospective reports have shown that patients younger than forty years are better responders to interferon therapy of HCV infection (Poynard *et al.,* 2000; Manns, 2001; Fried, 2002; Shiffman *et al.,* 2007). In the current study, the mean±SD of the SVR group was 36.28±12.12. The mean±SD for NRs group was 37.32±8.24. Thus, mean±SD age of NR group was higher than individual of SVR group but independent T test on the data suggest that the difference was not statistically significant (P value = 0.608). The summary of the entire study is shown in figure 5.1.

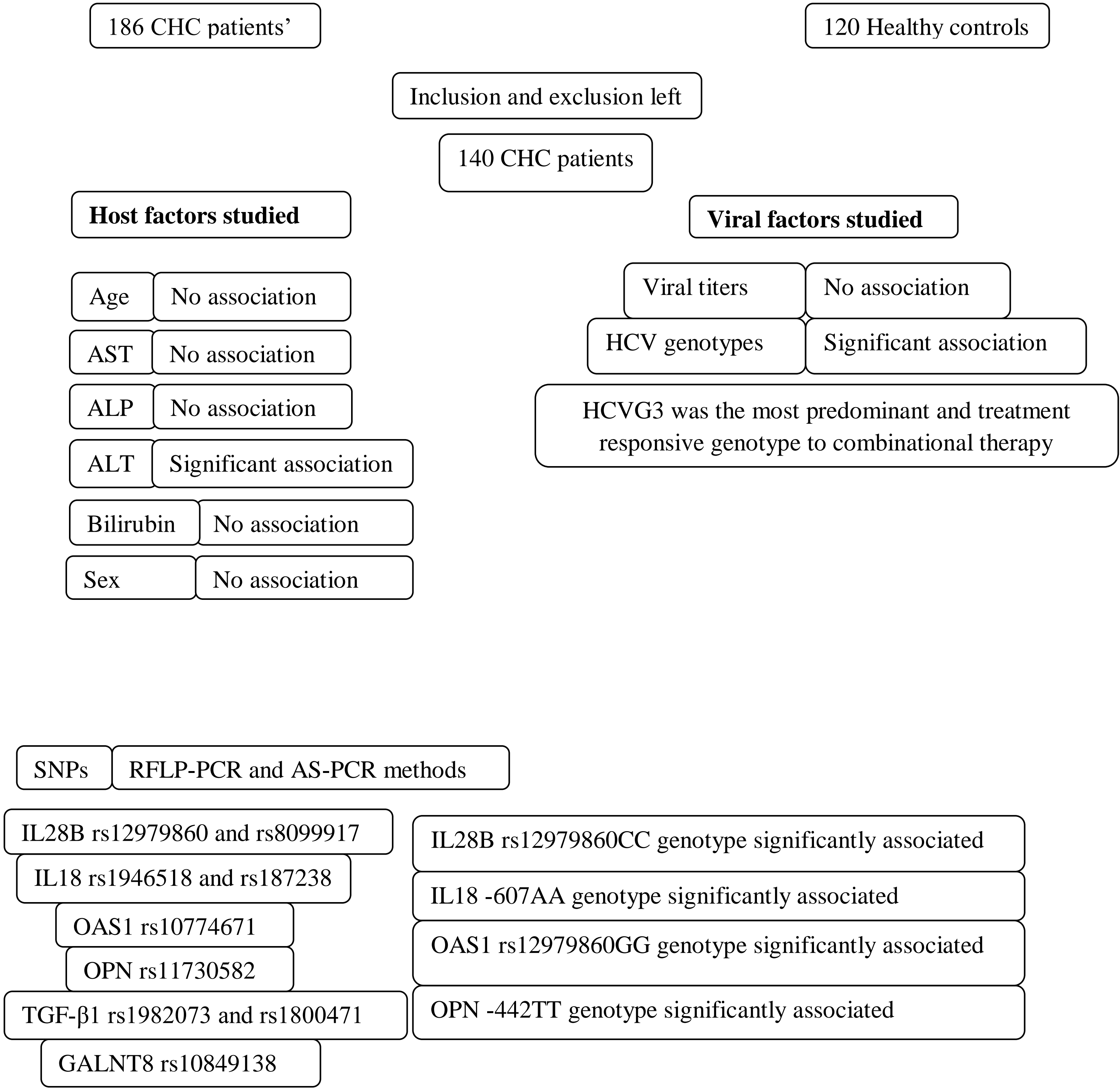


Figure 5.1. Schematic representation of the study summarizing the results and conclusions.

In conclusion, these findings constitute the evidence that host SNPs; IL28B rs12979860CC genotype, OAS1 rs10774671GG genotype, IL18 -607AA genotype (rs1946518), osteopontin -442 CC genotype (rs11730582), low ALT level and HCV genotype 3 are all positively associated with treatment outcomes and may be used as predictive markers of interferon based therapy in chronic HCV Pakistani patients.

Chapter 6

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Appendix I: Supplementary material of patients and healthy controls history

Table representing history of SVR patients

S/N	Age	Gender	Base line viral load	HCV genotype	IL18 - 607	IL18 -137	OPN - 442	TGF β Codon 10	TGF β Codon 25	GALNT8
1	35	M	210733	3a	CC	GG	CC	CT	GG	GG
2	40	M	458348	3b	CC	GG	CC	CT	GG	GG
3	25	M	4384309	3a	CA	GG	TT	CT	GG	GG
4	28	M	484308	3b	AA	GC	TT	CC	GC	GC
5	26	M	483489	3a	CA	GC	TT	CC	GC	CC
6	47	M	848,942	3b	CC	GC	CC	CT	CC	GC
7	35	F	48,498	3a	CC	GC	TT	CC	GG	GC
8	22	M	73228	3b	CA	CC	CC	CT	CC	CC
9	41	M	57439	3a+1b	CA	CC	CT	CT	CC	CC
10	40	F	905,501	3a	CC	GC	CT	TT	GC	GC
11	44	M	407,570	3a	CA	GC	TT	CT	GG	GC
12	22	M	310002	3a	CC	GG	CC	CC	GG	GG
13	29	M	331057	3a	CC	GC	CC	TT	GC	GC
14	42	M	678342	1a+3a	CA	GC	TT	CT	GC	GC
15	33	F	578347	3a	CC	GG	CC	CT	GG	GG
16	53	M	457825	3b	CA	GG	CT	CC	GG	CC
17	48	F	748785	3a	CC	GG	TT	TT	GG	GG
18	31	M	573429	3b	CA	GC	TT	TT	GC	CC
19	45	F	67487	3a+1b	CC	GG	CC	CT	GG	CC
20	41	F	547831	3a	CC	GG	CT	TT	GG	GG
21	47	M	584373	3a	CA	GC	TT	TT	GC	GC
22	44	M	678489	3b	CC	GC	CT	TT	GC	GC
23	45	F	473473	3a	CA	GG	TT	CT	GG	GG
24	41	F	474327	3b	CC	GC	TT	TT	GC	GC
25	48	F	48482	3a	CC	GC	TT	CT	GC	GC
26	59	M	55001	3a	CC	GG	CC	TT	GG	GG
27	46	F	902139	3a	CC	GC	TT	CT	GC	GC
28	30	F	834874	3a	CA	GC	TT	TT	GC	GC
29	21	F	484398	1a	AA	GG	CT	CT	GG	GG
30	48	F	684590	3a	CC	GC	TT	TT	GG	GC
31	22	F	43832	4	AA	GC	TT	TT	GG	GC
32	56	M	498483	3a	CC	GG	CT	CT	GG	GG
33	44	M	548,741	3a	AA	GG	CT	CT	GG	GG
34	34	M	5,473,847	3a	AA	GG	CT	CT	GG	GG
35	34	F	458735	1a+3a	AA	GC	TT	CT	GC	GC
36	62	F	474387	3a	CA	GG	CT	CT	GG	GG

37	29	F	487487	3a	CC	GG	CT	CT	GG	CC
38	33	M	73488	3a	CC	GC	TT	CT	GC	GC
39	67	M	85732	1a	CA	GC	TT	CT	GC	GC
40	29	F	573899	3b	CC	GG	CT	CT	GG	CC
41	53	M	489458	3a	CC	GC	CT	CT	GC	GC
42	45	M	94843	3a	AA	GC	CT	CT	GG	GC
43	57	F	75487	3a	CC	GG	TT	CT	GG	GG
44	55	M	484589	3a	CA	GC	CC	CT	GC	GC
45	34	M	458734	3b	CC	GG	CT	CT	GG	GG
46	30	M	20875	3a	CA	GC	CT	CT	GC	GC
47	56	M	995433	3a	AA	GC	TT	CT	GC	GC
48	50	M	67342	3a	CA	GG	CC	CT	GG	GG
49	34	M	324579	untypea	CC	GG	CT	CT	GG	GG
50	26	M	984242	3a	CA	GG	CT	CT	GG	GG
51	40	F	558403	untypea	AA	GC	TT	TT	GC	GC
52	37	M	584853	3a	CA	GG	TT	CT	GG	CC
53	33	M	98824	3a	CC	GG	TT	CT	GG	CC
54	29	M	530248	3b	CA	GC	TT	TT	GC	GC
55	28	M	32458	3a	CC	GC	CT	CT	GC	CC
56	27	F	487483	3a	CA	GG	TT	CT	GG	GG
57	29	M	848438	3a	CC	GC	TT	TT	GC	GC
58	44	M	484892	3a	CA	GC	CT	CT	GG	GC
59	59	M	585433	3a	AA	GC	CT	TT	CC	GC
60	54	M	583442	3a	CA	GC	CC	TT	GC	CC
61	22	M	78965	3b	CC	GC	TT	TT	GG	GC
62	30	F	45687	3b	AA	GC	CT	CT	GC	GC
63	22	F	46698	3a	CA	CC	CT	CT	CC	CC
64	25	M	36697	1b	CC	GC	CC	CT	GC	GC
65	22	F	145691	3a	CC	GG	CT	TT	GG	GG
66	25	M	65894	3a	CA	GC	TT	CT	GC	GC
67	45	M	45879	1b	CC	GC	CT	CT	GC	CC
68	60	F	546487	3a	CA	GG	CT	TT	GG	CC
69	20	F	4578	3a	CC	GC	CT	CT	GC	GC
70	23	M	45657	3a	CA	GC	CC	CC	GC	GC
71	26	M	45645	3a	CC	GC	CT	TT	GC	GC
72	23	M	224567	3b	AA	GG	CT	TT	GG	GG
73	22	F	77586	1b	CA	GG	CT	CT	GG	GG
74	25	F	45649	3a	CC	CC	CC	CC	CC	CC
75	30	M	65784	3a	CA	GG	CT	TT	GG	GG
76	19	M	89751	3a	CA	GG	CT	CC	GG	GG
77	35	M	98745	3b	CC	GC	CT	CT	GC	GC
78	60	M	11456	3a	CC	GC	CT	CT	CC	GC
79	50	M	204597	3a	CA	CC	TT	CT	GC	GC
80	20	M	77546	untypea	AA	GG	CT	TT	GG	GG

81	21	M	55449	3a	CA	GC	CT	CC	GG	GC
82	22	M	84454	3a	CC	GC	CC	CC	GG	GC
83	30	M	76549	3a	CA	GG	CT	TT	GG	CC
84	35	F	54897	untypea	CA	GG	CT	CC	GG	GG
85	28	M	644581	3b	CC	GG	TT	CT	GG	CC
86	25	F	445654	3a	CA	GC	CC	CT	GC	GC
87	35	M	546989	untypea	CA	GG	CT	CC	GG	GG
88	22	F	87546	3a	AA	GG	TT	CT	GG	GG
89	40	F	24568	3b	CA	CC	TT	CC	GG	GC
90	22	F	55467	3a	AA	CC	CT	TT	GC	GC
91	45	M	885746	3a	CC	GG	TT	TT	GG	GG
92	41	M	98745	3a	CA	GG	TT	CT	GG	CC
93	35	M	65897	untypea	AA	GG	CT	CC	GC	GC
94	40	M	21465	3a	CA	GC	CT	CT	GC	GC
95	25	F	5462	1a	CA	GG	CT	CT	GG	GG
96	37	F	44576	3b	AA	GG	CT	CT	GG	GG
97	28	M	111249	3a	CA	GC	CC	TT	GC	GC
98	32	M	44758	3a	AA	GG	TT	CT	GG	GG
99	26	M	68754	1a	CA	GC	TT	TT	GC	CC
100	30	F	20457	3a	CA	CC	TT	TT	GC	GC
101	35	F	980451	3a	CC	GC	CT	TT	CC	GC
102	28	M	55471	3a+3b	AA	GG	CT	CT	GG	CC
103	27	F	498754	3a	CA	GG	CT	TT	GG	GC
104	58	F	44571	1a	CA	GC	CT	CT	CC	GC
105	50	M	98745	3a	CC	GC	TT	TT	GC	CC
106	45	F	45475	3a	CA	GC	TT	CT	GG	GC
107	35	F	98741	4	AA	GC	CC	TT	GC	GC
108	23	M	114576	3a	CA	CC	CT	CT	CC	CC
109	25	M	45769	1a	AA	GC	TT	CC	GG	GC
110	52	F	58425	1a	AA	GC	CT	TT	GG	CC
111	25	M	668745	3a	CA	GC	TT	TT	GC	GC
112	20	M	78956	3a	AA	GG	TT	CT	CC	GC
113	45	M	75698	1a	CC	GC	CT	TT	GC	GC
114	65	F	58461	3a+3b	AA	CC	TT	CT	CC	CC
115	31	M	87549	3a	CC	GG	CC	TT	GG	GC

Table representing history of SVR patients

S/N	SGPT	Bilirubin	SGOT	Alkaline phosphatase	OAS1 genotype	IL28B rs12979860	IL28B rs8099917
1	51	0.7	45	233	GG	CC	GG
2	59	3.7	12	184	AA	CC	TT
3	40	0.3	100	220	AG	CT	TG
4	107	0.3	95	233	GG	CC	TG
5	122	0.6	56	273	AA	CT	GG
6	68	1.1	115	250	GG	CC	TG
7	72	0.8	151	253	AG	CT	GG
8	33	0.6	95	174	GG	CT	TG
9	53	0.3	27	233	AG	TT	TT
10	37	0.4	133	74	GG	CT	TG
11	137	1.1	100	377	AA	CC	GG
12	90	0.5	55	212	AG	CT	TT
13	29	0.6	41	133	AG	CT	GG
14	53	0.6	30	300	AG	CC	TT
15	42	0.7	50	467	GG	CC	TG
16	43	0.4	65	248	AA	CC	TG
17	78	0.6	71	288	AG	CC	TT
18	64	0.7	45	148	AA	CC	TT
19	74	1.6	75	101	GG	CT	TG
20	97	0.3	23	200	AG	CT	TG
21	38	2	20	222	GG	CC	GG
22	31	2.5	23	432	AA	CC	TT
23	23	2	25	300	AG	CT	GG
24	40	0.5	56	434	GG	CC	TT
25	76	0.9	44	285	AG	CT	GG
26	18	3	35	180	AG	CT	TG
27	32	0.5	66	201	GG	TT	TG
28	74	0.5	75	284	GG	CC	TG
29	56	0.6	55	172	GG	CT	TG
30	70	1	90	223	AG	CT	TG
31	67	0.3	10	615	AG	TT	TG
32	27	1	85	574	AG	CC	TG
33	70	2	24	300	AG	CT	TG
34	30	1.1	45	224	AG	CC	TG
35	50	0.9	60	304	AG	CC	TT
36	88	0.8	55	243	GG	CT	TT
37	59	1.5	50	430	AG	CT	TT

38	65	0.7	70	255	AG	TT	TT
39	61	0.7	75	180	GG	TT	TT
40	69	1.5	110	301	GG	CC	GG
41	23	0.9	15	211	GG	CC	TG
42	20	2	55	225	GG	CT	TG
43	47	0.4	76	489	AG	TT	TG
44	56	0.6	47	278	AG	CC	TG
45	38	2.4	78	173	GG	CC	TT
46	67	0.7	95	516	AG	TT	TT
47	45	0.7	50	638	AG	CT	GG
48	98	0.8	60	152	AG	TT	TT
49	81	2.5	65	435	GG	CC	GG
50	97	3	78	150	AG	TT	TG
51	50	0.9	74	201	AG	CC	GG
52	98	2.5	45	500	AG	CC	TT
53	50	0.5	24	223	AG	TT	TG
54	21	1.6	131	399	GG	CC	GG
55	160	0.5	66	95	GG	TT	TT
56	42	1.3	80	227	GG	CT	TG
57	126	1.5	40	235	AA	CT	TG
58	70	1.2	25	231	AG	CT	TG
59	18	0.6	45	227	AG	CC	TG
60	51	1	66	344	GG	CT	TT
61	23	0.9	55	114	GG	CC	TT
62	65	0.78	182	300	GG	CT	GG
63	39	2.5	45	360	AG	CC	TT
64	51	1	80	260	AG	TT	TT
65	73	0.9	130	400	AG	CC	TG
66	99	0.7	68	126	GG	TT	TG
67	72	1.7	80	134	AG	CT	TG
68	48	2	55	731	AA	CC	GG
69	66	2.5	78	93	AG	TT	TT
70	25	0.5	200	400	AG	CT	TT
71	67	2.3	89	610	AA	CC	GG
72	56	4	30	369	GG	CC	GG
73	98	3.3	90	150	GG	CT	TG
74	56	1.5	87	230	GG	CT	TG
75	78	0.9	37	430	AA	CT	TG
76	89	2.5	138	320	AG	CT	TG
77	45	2.3	35	431	AG	CT	TG
78	70	1.7	39	98	GG	CT	TT
79	30	0.2	67	330	GG	CC	TT
80	38	2.1	100	540	AG	CT	TT
81	57	1.1	53	700	AG	TT	TT

82	93	0.97	63	660	GG	CT	TG
83	107	0.5	39	340	GG	CC	TG
84	65	1.5	89	234	GG	CT	TG
85	69	1.1	31	360	AG	CC	TT
86	35	1.7	124	479	AG	CC	TG
87	55	0.8	128	312	AG	CC	TG
88	89	0.6	38	287	GG	CC	TT
89	39	2.89	199	345	GG	CT	TG
90	105	2.45	62	287	GG	CC	TG
91	80	1.3	89	519	GG	CC	TT
92	29	0.1	32	402	GG	CT	TT
93	44	0.7	85	303	AA	CT	TG
94	78	1.3	75	520	GG	CT	TG
95	56	1.8	54	89	AA	CT	TT
96	231	1.4	79	431	GG	CT	TG
97	104	2.4	42	238	GG	CC	TG
98	37	0.4	63	342	GG	CC	TG
99	89	0.9	213	439	AG	CT	TT
100	27	2.1	231	234	AG	CT	TT
101	79	0.5	54	340	AG	CC	TT
102	88	2.4	76	489	AA	CT	TT
103	96	0.8	49	320	AG	CT	TG
104	82	1.8	129	210	AG	CT	TG
105	83	1.3	143	265	AG	CT	TG
106	63	0.67	63	326	AA	CC	GG
107	130	0.8	78	279	GG	CT	TG
108	91	3.4	86	519	GG	CC	TT
109	52	2	29	345	GG	CT	TG
110	123	1.34	93	620	AG	CT	TG
111	142	2.9	46	320	AG	CC	TT
112	42	3.5	86	290	AA	CT	TT
113	99	2	145	134	AG	CT	TG
114	56	1.8	59	320	AG	CT	TT
115	78	1.7	70	390	GG	CC	GG

Appendix II: Table representing history of Non-SVR patients

S/N	Age	Gender	Base line viral load	HCV genotype	Viral load after six months therapy	IL18 -607	IL18 ,137 genotype	OPN -442	TGF β Codon 10	TGF β Codon 25	GALNT 8
1	28	F	689457	3a+3b	456789	CC	GG	CT	CT	GG	GC
2	36	F	897456	1a	759743	CC	GG	CC	CC	CC	GG
3	38	M	968751	3a	1193832	AA	GG	CT	CT	GG	CC
4	39	M	502347	3b	434409	CA	GC	CC	TT	CC	GC
5	29	F	125984	1a	393481	CA	GC	CT	TT	GC	GC
6	31	F	158764	1a	232841	CA	GC	CT	CC	CC	GG
7	23	F	565946	untypea	753439	CC	GC	CT	TT	GC	GC
8	40	M	302598	1a	245389	CA	CC	CT	CC	CC	CC
9	37	F	542568	3a+1b	534892	CA	CC	CT	CT	CC	CC
10	35	M	984561	3b	288232	CC	GC	TT	CT	GC	GC
11	44	F	654879	3a	489284	CA	GC	CT	CC	GC	CC
12	46	M	654791	3a	7329382	CC	GG	CC	TT	GG	GG
13	51	F	456258	3a+1b	428210	CC	GC	TT	CC	GC	GC
14	38	M	958923	3a	739292	CA	GC	CT	CT	GG	GC
15	51	M	532659	1a+3a	392398	CC	GG	CT	TT	GG	CC
16	32	F	568974	3b	4447221	CA	CC	CC	CT	GG	CC
17	35	F	648991	1a	482387	CA	GG	CT	CT	GG	GG
18	41	F	987549	3a	487238	CA	GC	TT	TT	CC	GC
19	45	F	658457	untypea	848721	CC	GG	CT	CT	GG	CC
20	52	M	56644	untypea	4889322	CC	GG	CC	CT	GG	GG
21	30	M	145578	3a	3489321	CA	GC	CC	TT	GC	GC
22	27	F	564551	1a	4389230	CC	GC	TT	CT	GC	GC
23	25	M	44567	3a	39221	CA	GG	CT	CT	GG	GG
24	34	F	455987	1b	438921	CC	GC	CT	TT	GC	GC
25	46	M	464556	1b	489221	CA	GC	CT	CT	GC	GC

Table representing history of Non-SVR patients

S/N	SGPT	Bilirubin	SGOT	Alkaline phosphatase	Viral load after six months therapy	OAS1 genotype	IL28B, rs12979860	IL28B, rs8099917
1	77	1.7	99	311	456789	AG	CT	TG
2	45	3.7	91	425	759743	AG	CT	TG
3	111	2.3	56	289	1193832	GG	CT	TT
4	122	0.9	95	278	434409	AG	TT	TG
5	67	0.8	115	273	393481	AA	CT	TG
6	131	1.1	95	516	232841	AG	CT	TT
7	66	0.8	87	538	753439	AG	CT	TG
8	93	2.6	93	190	245389	AG	CT	TG
9	123	1.3	145	535	534892	AA	CT	GG
10	149	0.4	58	250	288232	GG	TT	TG
11	152	3.1	81	280	489284	AG	CT	GG
12	59	1.5	70	505	7329382	AG	CT	TG
13	98	2.6	150	323	428210	GG	CT	GG
14	47	0.6	65	399	739292	AG	CC	TT
15	88	1.7	71	195	392398	AG	CT	GG
16	142	0.9	45	227	4447221	AA	TT	GG
17	117	0.6	75	235	482387	AA	CC	TG
18	93	0.9	93	239	487238	AG	TT	TT
19	89	2.6	40	327	848721	AG	CC	TT
20	81	0.5	43	344	4889322	AG	CT	TG
21	63	2	55	414	3489321	AA	TT	TG
22	46	2.5	56	380	4389230	AG	CT	GG
23	35	2	84	369	39221	AG	TT	TG
24	78	0.5	135	260	438921	GG	CT	TG
25	87	0.9	66	400	489221	AG	CC	TT

Appendix III: Tables representing features of healthy controls

S/N	Gender	Age	IL18, 607 genotype	IL18 ,137 genotype	OPN - 442	TGF β Codon 10	TGF β Codon 25	GALNT 8	OAS 1	IL28B rs 12979860	IL28B 8009917
1	M	28	CC	GC	CT	CT	GG	CC	AG	TT	TG
2	M	44	AA	CC	CC	TT	CC	GG	GG	CT	TG
3	M	30	CC	GC	TT	CT	GC	CC	AA	CC	TT
4	M	30	AA	GG	CT	CT	GG	CC	GG	CT	TG
5	F	25	CC	GG	CC	TT	CC	GC	GG	TT	TG
6	M	20	AA	GC	CT	CT	GG	GG	GG	CT	GG
7	M	40	CC	GC	TT	TT	CC	GC	GG	CT	TG
8	M	25	AA	GC	CC	TT	GG	GC	AG	TT	TT
9	M	23	CA	CC	TT	CT	CC	CC	GG	CT	TG
10	M	28	CA	GC	CC	TT	GC	GG	AA	CC	TG
11	F	23	CA	GC	CT	CT	GG	GC	GG	CT	TT
12	F	24	CA	GG	CC	TT	CC	GC	GG	TT	TG
13	M	29	CA	GC	TT	CT	GC	GG	GG	CT	TG
14	F	24	CC	CC	CC	CC	GG	GC	AG	CT	TG
15	M	25	CA	GC	TT	CT	GG	CC	AG	CT	GG
16	M	24	AA	GG	TT	CC	GG	GC	AG	CC	TT
17	M	24	CA	GG	CC	CT	GC	GG	AG	CT	TG
18	M	23	CA	GG	CT	CC	GC	GC	AG	TT	TG
19	M	23	CC	CC	TT	TT	GC	GC	AG	CT	TT
20	M	29	CC	GC	CC	CT	GG	GG	GG	CT	GG
21	F	24	CA	GC	TT	TT	GG	GG	AG	CC	TG
22	M	24	CC	GG	CC	TT	CC	GG	GG	CT	TG
23	M	31	CC	CC	TT	CT	GG	GC	AG	CC	TT
24	F	27	CC	GC	TT	TT	GG	GG	GG	CC	TG
25	M	35	CA	GC	TT	CC	GG	GC	AG	CC	TT
26	M	23	AA	GC	CT	CT	GC	GG	GG	CT	TG
27	F	21	AA	GC	CT	CT	GC	GC	GG	CT	TG
28	F	21	CA	GC	CC	TT	GG	GC	GG	CT	TG
29	F	21	CA	GC	CT	TT	CC	GC	GG	CT	TG
30	F	21	CA	GC	TT	CT	GC	GC	GG	CT	TT
31	F	21	AA	GC	CT	CC	GG	GC	GG	CT	TG
32	M	27	CA	CC	CT	TT	CC	CC	GG	CT	TG
33	M	21	AA	GC	CC	TT	GC	GC	AG	CT	TG
34	F	22	CC	CC	CT	TT	GG	CC	AG	TT	TT
35	F	22	CA	GC	CT	CT	GG	CC	AG	TT	TG
36	M	20	AA	GG	CT	CT	GG	GC	GG	TT	GG
37	M	18	CA	GG	CC	CT	GG	GC	GG	CT	TG
38	M	19	CA	GC	CT	CT	GC	CC	AG	CT	TG

39	F	18	CC	GG	TT	CT	CC	GC	GG	CT	TG
40	F	18	CA	GG	CT	TT	GC	CC	AG	CT	TG
41	M	18	AA	GG	TT	TT	GC	GC	GG	TT	TG
42	M	20	AA	GG	CT	CT	CC	GG	AG	CT	GG
43	F	19	AA	GC	TT	CT	GG	GC	AG	CT	TG
44	F	19	CA	CC	CT	TT	GC	GC	GG	CC	TG
45	F	20	CA	GC	CT	CT	GG	GC	GG	CT	TT
46	F	19	CC	GC	CC	TT	GC	CC	GG	TT	TG
47	F	18	CA	GC	CT	CT	GG	GC	GG	CT	TG
48	F	19	AA	GG	TT	CT	GG	GC	AG	CC	TT
49	F	20	CA	GG	CT	CT	GG	GG	GG	CT	TG
50	F	18	CA	GC	CT	CC	GC	CC	GG	CT	TG
51	F	19	AA	GC	CC	CT	GC	GC	AG	CT	TG
52	F	19	CA	GC	CT	CT	CC	CC	GG	CT	TG
53	F	19	CA	CC	CC	CC	GC	CC	GG	TT	TG
54	F	18	CA	GC	TT	TT	GG	GC	GG	CC	TG
55	F	19	AA	GC	TT	CT	GC	GC	GG	CT	TG
56	F	19	AA	CC	CT	CC	GG	GC	GG	CT	TT
57	M	19	CC	GC	CC	CT	GC	GC	AG	CT	TG
58	M	17	AA	GC	CT	TT	GG	CC	AG	CC	TT
59	M	18	CA	CC	CT	CT	CC	GC	AG	CT	TT
60	F	19	CC	GC	CC	CT	CC	GC	AG	CT	TG
61	F	18	CA	GG	TT	CT	GC	GC	GG	CC	TT
62	F	19	CA	GC	CT	CT	GC	CC	AG	CT	TG
63	F	18	CA	GC	CT	CC	GG	GC	AG	CT	TT
64	M	19	CC	GC	TT	CT	GG	CC	AG	CT	GG
65	F	19	AA	GC	TT	CC	GC	CC	AA	CC	TT
66	F	19	AA	CC	CC	CT	GG	GC	GG	CT	TG
67	M	47	CA	GC	TT	CT	GG	GC	AG	CT	TG
68	M	37	CA	GG	CC	CT	GG	GC	GG	CC	GG
69	M	48	CA	GG	TT	CC	GG	GG	AG	CT	TG
70	M	46	CC	CC	TT	CT	GG	CC	AG	CT	TG
71	M	34	CA	CC	TT	CT	GC	GC	GG	CC	TG
72	M	26	AA	GC	CT	CT	CC	GC	AG	CC	TT
73	F	20	CA	GG	TT	TT	GC	GG	GG	CT	TG
74	F	20	CA	GC	TT	CT	GG	GG	GG	CT	TG
75	F	21	CA	GG	CT	CT	GC	GC	GG	CT	TG
76	F	22	CC	GG	CC	CT	GC	CC	GG	CT	TG
77	F	22	AA	GC	CT	TT	GC	GC	AG	TT	TG
78	F	22	CA	GG	CT	CT	CC	GC	GG	TT	TG
79	F	23	CC	GC	TT	CC	GC	CC	GG	CT	TG
80	M	23	CA	GC	TT	CT	GG	GG	GG	CT	TG
81	F	22	AA	GC	CT	CT	GG	GC	GG	CT	TG
82	F	22	CA	GG	TT	CT	GG	GC	GG	CC	TG

83	F	20	CC	GG	CT	CC	GC	GG	AA	CT	TG
84	M	20	CA	GG	CT	CT	GC	GG	GG	CC	TG
85	M	21	AA	GC	TT	TT	GG	GG	GG	CT	TG
86	F	20	CC	GG	CT	TT	GG	GC	GG	CC	TG
87	M	21	CA	CC	CT	CT	GC	GG	GG	CT	TG
88	M	21	CA	GC	CT	TT	GC	GC	AG	CC	TG
89	M	24	CA	GG	CT	CT	GC	GG	GG	CC	TG
90	M	19	CA	GG	CT	CT	GG	GC	AA	CC	GG
91	M	20	CC	GC	TT	CT	CC	CC	GG	CT	TG
92	M	20	CA	GG	CT	CT	CC	GC	GG	CT	TG
93	F	19	AA	GC	CC	TT	GG	CC	GG	CC	TG
94	F	21	AA	GG	CC	CT	GG	CC	GG	CT	TG
95	F	21	CA	GC	CT	CT	GG	GC	AG	CT	TG
96	F	20	CA	GG	CC	CT	GG	GG	GG	CT	TT
97	M	21	AA	GG	CT	TT	GC	GC	AG	CT	TG
98	M	24	CC	GC	CT	CT	GC	GG	GG	CC	TG
99	M	29	AA	GG	CC	CC	GC	CC	AG	CT	TG
100	M	34	CC	GC	TT	CC	GC	GC	GG	CT	TG
101	F	39	AA	GG	TT	CC	GC	GC	GG	CC	TG
102	M	23	CC	GC	TT	CT	GG	GC	AA	CT	TT
103	M	29	AA	CC	CT	TT	GG	GC	GG	CT	TG
104	F	33	CA	GC	CT	CT	GC	GC	GG	CC	TG
105	F	25	CC	GC	TT	TT	GC	CC	GG	CT	TT
106	F	29	CC	GG	CC	CT	GC	CC	GG	CT	TT
107	F	20	CA	GC	TT	CC	GG	GC	AG	CT	TG
108	M	40	AA	GG	CT	TT	GC	CC	AG	CC	TG
109	F	44	CC	GC	TT	CT	GG	CC	GG	CT	TT
110	F	23	AA	GG	CT	CT	GG	GC	GG	CT	TG
111	M	45	AA	GC	CT	TT	GG	GG	GG	CT	TG
112	M	59	CC	GG	TT	CT	GG	GC	GG	CC	TG
113	M	45	CC	GG	CC	CC	GC	GG	GG	CC	TG
114	M	33	AA	GC	CT	CC	GC	CC	GG	CT	TG
115	M	29	CC	GG	CC	CT	GC	GC	GG	CC	TG
116	F	20	CA	GC	CT	TT	GG	GC	AG	CT	TT
117	F	29	AA	GG	CT	CT	GC	GC	AG	CT	TG
118	F	33	CA	GC	TT	CT	GG	GC	GG	CT	TT
119	M	40	CA	GG	CT	TT	GC	GC	GG	CT	TG
120	M	43	CC	GG	TT	CC	GG	CC	GG	CT	TT

Appendix IV: List of research and review papers

Title of research papers published in international journals from PhD dissertation

1. **Imran M,** Manzoor S, Khattak NM, Tariq M, Khalid M, Javed F, Bhatti S. Correlation of OAS1 gene polymorphism at exon 7 splice accepter site with interferon-based therapy of HCV infection in Pakistan. Viral Immunol. 2014 Apr; 27(3):105-11. doi: 10.1089/vim.2013.0107. Epub 2014 Mar 27. (IF: 1.7)
2. **Imran M,** Manzoor S, Parvaiz F. Predictive potential of IL18 -607and osteopontin -442 polymorphism in interferon plus ribavirin treatment of HCV infection in Pakistani population. Viral Immunol. 2014 Oct; 27(8):404-11. doi: 10.1089/vim.2014.0044. Epub 2014 Sep 8. (IF: 1.7)
3. **Imran M,** Manzoor S, Sikander Azam, Saleha Resham. Genetic variant of *IL28B* rs12979860, as predictive marker of interferon based therapy in Pakistani population. (Accepted in APMIS). (IF: 1.9)

Title of review paper published in international journals from PhD dissertation

1. **Imran M,** Manzoor S, Ashraf J, Khalid M, Tariq M, Khaliq HM, Azam S. Role of viral and host factors in interferon based therapy of hepatitis C virus infection. Virol J. 2013 Oct 1;10:299. doi: 10.1186/1743-422X-10-299. (IF: 2.09

Review articles

1. **Imran M**, Waheed Y, Manzoor S, Bilal M, Ashraf W, Ali M, Ashraf M. Interaction of Hepatitis C virus proteins with pattern recognition receptors. Virol J. 2012 Jun 22; 9:126. doi: 10.1186/1743-422X-9-126. (IF: 2.09)
2. **Imran M**, Manzoor S, Khattak NM, Khalid M, Ahmed QL, Parvaiz F, Tariq M, Ashraf J, Ashraf W, Azam S, Ashraf M. Current and future therapies for hepatitis C virus

infection: from viral proteins to host targets. Arch Virol. 2014 May; 159(5):831- 46. doi: 10.1007/s00705-013-1803-7. Epub 2013 Aug 25. (IF: 2.28)

Research group publications

1. Khalid M, Manzoor S, Muqddas Tariq, **Imran M,** Ashraf W, Ahmed QL & Javed F Investigation of biomarkers in peripheral blood cells of Pakistani chronic HCV patients of genotype 3a: an assessment of HCV induced pathogenesis. Future virology. Vol. 9, No. 3, Pages 275-282 , DOI 10.2217/fvl.14.4. (IF: 1.00)
2. Khalid M, Manzoor S, **Imran M**, Tariq M, Ashraf J, Ahmed QL, Ashraf W, Parvaiz F, Ashraf M. Development of murine models to study Hepatitis C virus induced liver pathogenesis Indian J Virol. 2013 Sep; 24(2):151-6. doi: 10.1007/s13337-013-0152-1. Epub 2013 Sep 14. (IF: 0.3)
3. Ashraf W, Manzoor S, Ashraf J, Ahmed QL, Khalid M, Tariq M, **Imran M**, Aziz H. Transcript Analysis of P2X receptors in PBMCs of Chronic HCV patients: an insight into antiviral treatment response and HCV induced pathogenesis Viral Immunol. 2013 Oct; 26(5):343-50. doi: 10.1089/vim.2013.0044. (IF: 1.7)
4. Ahmed QL, Manzoor S, Tariq M, Khalid M, Ashraf W, Parvaiz F, **Imran M**. Hepatitis C virus infection in vitro triggers endoplasmic reticulum stress and downregulates insulin receptor substrates 1 and 2 through upregulation of cytokine signaling suppressor 3. Acta Virol. 2014; 58(3):238-44. (IF: 1.03)