Chapter 1

INTRODUCTION

Hepatitis C is one of the major forms of hepatitis caused by Hepatitis C virus (HCV). Its severity ranges from mild to chronic infection like cirrhosis and cancer. Each year 3-4 million people are infected with HCV worldwide, with an estimation of 130-170 million people at risk of developing cancer (WHO 2011 report). In Pakistan prevalence of HCV is 4.8% with more than 10 million suffering with chronic state. Genotype 3a (GT 3a) is the most common genotype with approximate 48% prevalence in Pakistan (Waheed *et al.,* 2009).

Current therapies for HCV infection primarily include interferon α. INF α is a water soluble glycoprotein of weight 19,000 Daltons (SWISSPROT) and is one of the important immunomodulatory cytokine primarily secreted by leukocytes in response to viral activity. Interferon alpha belongs to a family of thirteen homologous genes. Genes for interferon alpha are present on human chromosome 9p22 in the form of a cluster (Pestka *et al.,* 2004). Recombinant interferon alpha is produced by expressing it in E coli (Rubinstein *et al.,* 1979; Nagata *et al.,* 1980).

Recombinant interferon therapy has been in use since 1991 FDA approval for non A non B hepatitis. Under standard therapy $3x10^6$ units are injected intra subcutaneously. About half of the patients on interferon therapy are unable to respond to this standard interferon therapy (Farooqi *et al.,* 2011). However; with 10 million

units per injections only about 20% of the non-responders are able to show some response to the therapy. Yet a huge amount of people show substandard response. Combining ribavirin with IFN therapy increases the proportion of patients demonstrating immediate clinical improvement but permanent resolution of viral infection remains disappointingly low (Davis *et al.*, 1998; McHutchinson *et al.*, 1998; Poynard *et al.*, 1998; Reichard *et al.*, 1998; Andreone *et al.*, 1999; Pol *et al.*, 1999).

Many factors of both host and viral origin, contribute towards host response to the interferon therapy (Asahina *et al.*, 2005; Elhefnawi *et al.*, 2010). Unfortunately these factors are not adequate enough for reliable prediction of therapeutic outcomes. Age is one of the most important factors in generation of immune response against the infection. Viral genotype along with viral copy number play a critical role in determining the outcome of the interferon therapy while on the other hand genetic factors of host itself contribute greatly to the level of Immune response generated by the host against the infectious particle. Cytokines play a very critical role in determining these immune responses in individuals.

Cytokines are small glycoproteins that create a communication bridge between the cells. Cytokines are pleiotropic molecules that exhibit vast variety of functions, both locally and systematically, including acute and chronic inflammatory reactions. The synergic and antagonistic effects of cytokines influence the positive and negative regulation of the cells. Levels of cytokines greatly influence the host immune response to a foreign particle (Kuby Immunology, 1997). Single nucleotide polymorphisms in the promoter region of these cytokine genes is accountable for flexibility in expression

levels of cytokines and thus the polymorphisms are associated with disease susceptibility and immune incompetency. These polymorphisms are closely associated with various ethnic groups around the globe (Reich *et al.,* 2001).

Interleukin 10 (IL-10) is a highly pleiotropic cytokine having both immunosuppressive and immunemodulatory functions. IL-10 was first reported by Fiorentino in 1989 as cytokine synthesis inhibitory factor (CSIF). The Human IL-10 gene is 4849 bp long and consists of 5 exons, mapped on position q31-q32 on chromosome 1 (Eskadale *et al.,* 1997). IL-10 is encoded as 18 kDa peptide of 160 amino acid length that dimerizes to form a 36 kDa polyprotein to form a functional molecule (Chen *et al.,* 1991; Tan *et al.,* 1993). IL-10 is characterized as Th2 cytokine that inhibits the secretion of Th1 cytokines (Fiorentino *et al.,* 1989).

Several single nucleotide polymorphisms (SNPs) are associated with IL-10 promoter region. These SNPs are responsible for variable expression levels of IL-10 gene in the cells. Among these polymorphisms, SNPs at positions -1082, -819 and -512 are studied in detailed (Kube *et al.,* 1995), and play a very important role in regulating the expression levels of the gene (Bidwell *et al.,* 1999**).** These polymorphisms show very strong linkage disequilibrium, and three major haplotypes GCC, ACC and ATC appear to segregate in populations (Turner *et al.,* 1997).

The establishment of chronic HCV infection and poor responsiveness to current therapeutic regimens (IFN-α and ribavirin) may be associated with impaired virus-specific T cell responses, and enhanced levels of immunosuppressive cytokines

like IL-10 (Dolganiuc *et al.*, 2003). IL-10 down regulates monocytes and macrophages by both translational and post translational inhibition of broad range of pro inflammatory cytokines (De Waal Malefyt *et al.*, 1991). As IL-10 inhibits vast variety of cytokines it is suggested that it might have a role to play in determining the outcome of interferon therapy.

Aims and Objectives:-

- To investigate the IL10 promoter polymorphism distribution among responder and non-responder of interferon therapy for HCV infection particularly against genotype 3a (GT 3a).
- To infer the association of IL-10 polymorphism in responsiveness or failure to interferon therapy in the studied group.

Chapter 2

LITERATURE REVIEW

2.1 Interferon

Issaac and Lindenmann (1957) reported interferon for the first time while investigating the mechanism responsible for viral interference in an egg inoculated with heat denatured virus and subsequently challenged with live virus. They found that certain cytokines are responsible for inhibition of virus replication and thus, named them interferon. The Interferon family is a large heterogeneous group of cytokines that has antiviral properties. Interferons are divided into two types, denoted as type I (INF alpha, INF-beta, INF-omega) and type II (interferon gamma) (Allen and Diaz, 1994). Interferon alpha was the first ever biopharmaceutical product that was approved by FDA as a therapeutic.

Genome sequencing has revealed that there are 13 functional interferon alpha genes, located at small arm of the chromosome 9 (Pestka *et al.,* 2004). Interferon genes are intron less and show high degree of homology. The interferon alpha proteins consist of homologous group of 12 subtypes whereas subtype a1 and a13 are identical. These monomeric proteins consist of 166 amino acids with 75-95% homology at amino acid level (Kontsek *et al.,* 1994). Presence of high homology and same gene structure in all the interferon alpha subtype, suggest that these genes have evolved from a common ancestral gene by successive duplication.

2.1.1 Functions of Interferons:-

Interferon acts as an intracellular messenger molecule that forms a regulatory network in cells. It has pleiotropic physiological activity depending on the target cells. Type I interferon e.g. INF alpha acts as a potent antiviral agent in innate immune system. All nucleated cells can produce interferon and can induce antiviral state in neighboring cells (Levy *et al.,* 2001). With evolution, pathogens have developed several mechanisms to escape interferon mediated anti pathogen state. As a regulatory element, cells have devised several mechanisms to inhibit these pathways (Cebulla *et al.,* 1999; Goodbourn *et al.,* 2000). Most of the pathogens can up regulate these mechanisms for their own advantage. In addition to antiviral properties interferon also has anti-proliferative potential. The anti-proliferative property of interferons ranges from extremely sensitive to resistant (Melamed *et al.,* 1993). Interferons also show immuno-modulatory and cytotoxic properties. Moreover, these proteins induce proliferation of natural killer (NK) cells indirectly via IL-15 secretion and increase their cytotoxicity by increasing perforin levels (Reiter *et al.,* 1993; Biron *et al.,* 1999; Fawaz *et al.,* 1999; Gosselin *et al.,* 1999). In both infected and transformed cells, interferons have been shown to induce apoptosis; however, their mode of action is still poorly understood (Tanaka *et al.,* 1998).

2.1.2 Interferon Signaling Pathway

Mechanism responsible for interferon expression is still tattered, yet a positive feedback loop is proposed to be important. Molecular mechanism of interferon activity is a two-step process. In the first step, viral infection causes phosphorylation of interferon regulatory factor (IRF) 3.These IRF-3 are then dimerised and transported inside the nucleus. Later, it binds to specific response elements resulting in transcription of INF4-alpha and interferon beta. Whilst, in the second step the IFN4-alpha and INF-beta signal through interferon receptor alpha complex (INFAR). INFAR complex activates the receptor associated janus kinases and Tyrosine kinase receptor 2 by phosphorylation (Gauzzi *et al.,* 1996). These activated kinases recruit several intracellular proteins. One of these proteins is STAT protein family.Upon activation STAT1 and STAT2 form a dimer and translocate the dimerised complex to the nucleus where they affect the expression levels of several proteins e.g. IRF7. IRF7 dimerizes with IRF3 and induces the expression of, if not all, but most of the interferons. Different viruses induce expression of different INF-alpha subtypes and are regulated by a different set of transcriptional factors (Marie *et al.,* 1998; Lin *et al.,* 2000).

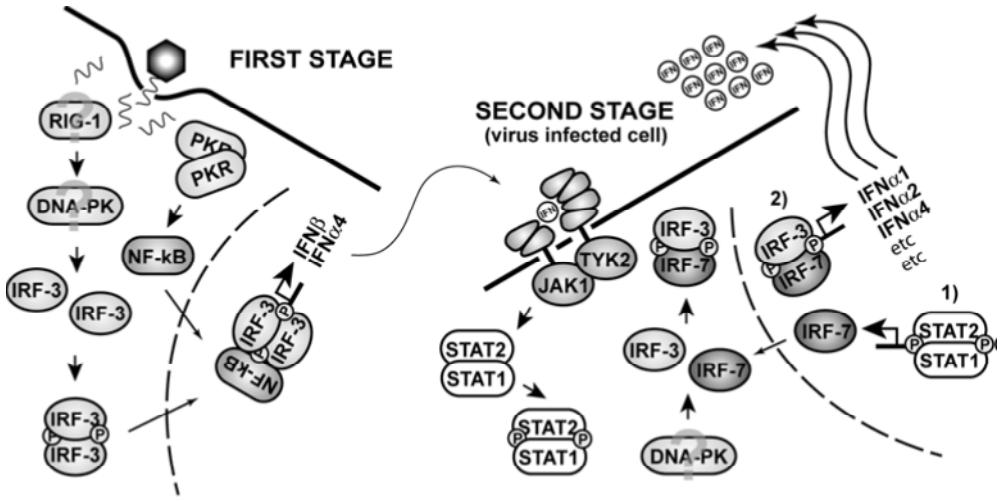

Figure 2.1 Diagrammatic representation of two step mechanism model of interferon action. In the first step virus causes activation of IRF3 which produces INF-beta and INF-alpha-4 while in second step these INF affect neighboring cells and cause release of rest of INF. Courtesy Carlson Lannert

T helper type -1 (Th1) responses are essential in chronic state viral infections (Rossol *et al.,* 1997). A shift of Th1 type immune response in HCV and HBV is correlated with viral clearance in interferon responsive patients (Lohr *et al.,* 1995; Cacciarelli *et al.,* 1996; Rossol *et al.,* 1997; Piazzolla *et al.,* 2001). Th1 are produced *via* action of interferon mediated polarization of CD4+ Th0 cells. Reports confirm that interferon alpha induces Th1 response in peripheral blood mononuclear cells in HCV and HBV patients (Rogge *et al.,* 1997, 1998). Although INF-alpha increases Th1 derived responses, yet mechanisms of Th1 driven responses in responders is unclear, but not in non-responders. These Th1 cells further effect cytotoxic T cells and B cell antibody class switching (Kuby Immunology 1997). The efficacy of viral clearance

depends on these interferon mediated Th1 polarization. Thus, we can say interferon mediated Th1 stimulation of CD4+ cells is strongly linked with host ability to clear infection.

2.2. Hepatitis C.

Hepatitis C is the major form of hepatitis present worldwide. About 200 million people are suffering with Hepatitis C. Hepatitis leads to chronic infection in 50-80% of individuals. According to WHO report (2004) the annual death toll due to hepatocellular carcinoma caused by Hepatitis C virus and cirrhosis was 308,000 and 785,000 respectively. Pakistan is a very low socio-economic country where health and education standards are underprivileged. People are not aware of it and its modes of transmission; therefore the relative incidence rate of HCV is higher in Pakistan than the rest of the world. According to recent findings the prevalence of HCV in Pakistan is around 4.95% (Waheed *et al.,* 2009).

Hepatitis C is caused by Hepatitis C virus (HCV). HCV was first discovered in 1989 as causative agent of non A- non B Hepatitis (Choo *et al.,* 1989). It is a positive stranded virus belonging to family *Flaviviridae*. HCV genome is ~9kb long encoding ten genes: 3 structural (core E1 and E2), a p7 protein, and 6 nonstructural proteins (NS2, NS3 NS4A/B, and NS5A/B).There is one open reading frame (ORF) that encodes a 3000 amino acid long polypeptide chain which is eventually cleaved by host and viral proteases. HCV is sub divided into six genotypes on the basis of nucleic acid.

Genotype 3a (GT 3a) is the most prevalent genotype in Pakistan with approximate prevalence of 48% (Waheed *et al.*, 2009).

2.2. Interferon Therapy

Interferon alpha is the first ever FDA approved biopharmaceutical product. Interferons were approved as antiviral therapy for non A non B hepatitis in 1991. In pilot scale study, interferon was found to be very effective against viral infects as well as restoring the normal physiology of the tissue (Hoofnagle *et al.*, 1986). Interferon therapy reduces the risk of cirrhosis and hepatocellular carcinoma in HCV in infected patients (Imai *et al.,* 1998). Under standard interferon therapy 3×10^6 units are injected subcutaneously for 24 or 48 weeks. About half of the patients receiving interferon therapy do not show sustained virological response i.e. clearance of virus to undetectable level post therapy (Farooqi *et al.*, 2008). High dose of 10 million units' per injection showed better responses in HCV patients but individuals showed severe adverse reactions (Vrolijk *et al.*, 2003).

2.2.1. Factor Effecting Interferon Therapy

Numbers of factors are involved in host response to interferon therapy. These factors can be of both, viral or host origins e.g. age, sex, ethnicity, viral load and genotype (Asahina *et al.,* 2005; Elhefnawi *et al.,* 2010). Therefore, prediction and management of resistance to antiviral therapy remains a serious concern in chronic Hepatitis C cases (Jain and Zoellner, 2010). People who do not respond properly to interferon therapy constitute a heterogeneous group. Patients show wide range of

responses from no effect on viremia or alanine aminotransferase (ALT) levels However, some patients displayed an initial response followed by reactivation or relapse after discontinuation (Su *et al.,* 2008). Host genetic factors play a huge role in determining the outcome of the therapy. Interferons induce wide range of innate immune response genes like Mx1, PKR and -5 oligoadynylatesynthetase 1 (OAS-1) (Knapp *et al.,* 2003). In addition to antiviral state activation, interferons also induce different kinds of cytokines. Cytokines are small immune modulatory molecules that determine the host immune level. There are more than 30 cytokines reported that are classified on bases to the class they belong (Kuby Immunology, 1997). Levels of cytokine in blood have been associated with chronic and acute infections or resistance or susceptibility (Abbas *et al.,* 1996). There are two types of T helper (Th) cells depending on the cytokines they secrete upon their activation and proliferation i.e. Th1 and Th2. Activated Th1 cells produce cytokines like interleukin-2 (IL-2), interferon-γ (IFN- γ), tumor necrosis factor- α (TNF- α), and TNF-β while Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 (Mosmann and Sad, 1996). Th1 responses are an essential component for viral clearance and any disturbance in this arm of the immune system results in persistent infection. IL-10 is an important Th2 induced mediator that negatively down regulates Th1 responses. Expression levels of IL-10 are highly significant in determining the outcome of viral infections i.e. persistent or clearance. Thus Th1 are strong predictive markers of interferon therapy efficacy in HCV patients and IL-10 inhibits Th1 response, so its levels can be an important candidate for determining host response against the infectious agent.

2.3. Interleukin-10

Interleukin 10 is a pleiotropic glycoprotein belonging to cytokine family that has both immunoregulatory and immunosuppressive functions. IL-10 was first discovered by Fiorentino et al., in 1989 as a cytokine synthesis inhibitory factor (CSIF). It acts as an inhibitor of macrophages by down regulating many cytokines and natural killer cells and Tcells (Fiorentino *et al.,* 1991). Later in 1994 Ho and Moore reported that IL-10 stimulates antibody production by activating B cells. Interleukin 10 is expressed as an 18 kDa polypeptide chain of 160 amino acids that dimerizes to form a 36 kDa homodimer to become a biologically active cytokine (Tan *et al.,* 1993).

Interleukin 10 transduces signals via a heterodimer receptor known as IL-10R. IL-10/IL-10R interaction activates Jak kinase which leads to phosphorylation of STAT transcriptional factors (Finbloom and Winestock, 1995). The exact molecular mechanism of IL-10 mediated immunomodulation via STAT protein is still not well established. Some data suggests that like macrophage model, SOCS proteins are the target genes by IL-10 activated STAT proteins (Matsumoto *et al.,* 2003). Although SOCS genes down regulate STAT pathway by negative feedback mechanism (Ding *et al.,* 2003). However, there are reports that suggest IL-10 mediated STAT3 activated SOCS proteins negatively regulate cytokine gene expression in immune cells thus regulating the immune response (Alexander *et al.,* 2002). But their role as mediators of IL-10 activates is not yet well established.

Nuclear factor kappa Beta (NF_KB) is a well known nuclear transcriptional factor that regulates many cytokine genes (Hanada and Yoshimura, 2002). The effect of IL-10 on NFkB depends on the type of the target cell it acts on. NFkB is inhibited in macrophages and CD4+ T cells (Moore *et al.*, 2001) while it is activated in CD8+ cells (Hurme *et al.*, 1994). Recently, it has been reported that IL-10 inhibits CD28 mediated co-stimulatory signal for T cell activation. Association of IL-10 with CD28 is similar to the association of CTLA-4 (a negative regulator of T cell) and TCR complex. IL-10 has a selective inhibition of CD28+ T cells as experiments suggested that CD45RO+ (activated T cells) are not affected by IL-10 stimulation (Akdis and Blaser, 2001). Hence, it can be concluded that IL-10 down regulated adaptive immunity by acting on naive T cells rather than activated T cells.

Moore et al., (1990) first reported the genomic structure of IL-10 in mouse and human. Both the sequences showed more than 80% of homology in nucleotide sequence. The human IL-10 gene is of 4894 bps comprising of 5 exons. Interleukin 10 gene is present on position q31-32 on chromosome 1 (Eskadale *et al.*, 1998). The level of gene expression is associated with binding affinity of transcriptional factors to the 5' non coding sequence. The data suggest that interindividual differences in cytokine levels are due to differences in 5' flanking non coding sequence of the gene. These genetic variations in the 5' regions control the cytokine levels at the transcriptional levels (Bienvenu *et al.*, 2005). Genetic variations in the 5' flanking region are associated with 75% of Interindividual variation of IL-10 secretions (Westendrop *et al.*, 1997). Platzer et al., (1995) reported the presence of sequence patterns in the 5'

promoter region of the genes associated with the transcriptional regulation of the gene. Cloning and sequencing of promoter region of IL-10 gene showed that a typical TATA box is present 97 bps upstream of the ATG start codon. Other than the TATA sequences three additional sequences were identified, that were involved in regulation of IL-10 gene. Two positively regulating elements located near TATA box and other between -1100/-900 and a negatively regulating element present between -750/-350 downstream of the transcription initiation site (Kube *et al.,* 1995).

SNPs and microsatellite repeats in the promoter or enhancer regions can alter the gene expression levels in the individuals. These genetic disparities result in Interindividual variations in cytokine production. These differences in cytokine levels can allow a certain degree of flexibility in the immune response. Several single nucleotide polymorphisms and a pair of microsatellite are located in promoter region of the IL-10 gene. Many SNPs have effect on transcriptional activity of promoter region.

Among all the SNPs identified in the promoter region of IL-10 gene, SNPs at -1082, -819 and -592 are studied in detail. The -1082 polymorphism is a substitution mutation of G to A in putative ETS transcriptional factor-binding site. Deletion in this region significantly alters the activity of the promoter. Polymorphism at -819 is a C to T substitution and lies in proximity of positive regulatory element. The SNP -592is present between a putative STAT3 binding site and negatively regulating element (Kube *et al.,* 1995). All these three SNPs have showed a strong linkage disequilibrium and three haplotypes (GCC, ACC, and ATA) segregate independently in most

populations (Turner *et al.,* 1997). Several in-vitro experiments have shown the association of haplotypes with IL-10 production in immune cells (Eskdale *et al.,* 1998; Eskdale *et al.,* 1999; Gibson *et al.,* 2001; Turner *et al.,* 1997). Several different methodologies were adopted in order to prove the effect of polymorphism in promoter region. These methods include stimulation of whole blood culture (Crawley *et al.,*1999), peripheral blood mononuclear cells (PBMC) culture (Eskdale *et al.,* 1998; Edwards-Smith *et al.,* 1999) or transfection studies (Crawley *et al.,* 1999) with known stimulants like Lipopolysaccharides or concavalin A (Con A) (Crawley *et al.,* 1999; Gibson *et al.,* 2001; Turner *et al.,* 1997). *In-vitro* studies showed that genotype GCC/GCC was linked with high IL-10 levels and ATA/ATA was lowest IL-10 producers as compare to other genotypes. Thus it is quite evident that polymorphisms in promoter/enhancer regions are associated with altered gene expression in the individuals. Several reports on IL-10 promoter genotype/haplotype frequencies in different populations have demonstrated that the distribution of these polymorphisms varies according to ethnicity (Eskdale *et al.,* 1998; MacKay *et al.,* 2003; Meenagh *et al.,* 2002; Mok *et al.,* 1998; Padyukov *et al.,* 2002; Reynard *et al.,* 2000).

2.4. IL-10/INF paradigm and Th1/Th2 regulation.

Cytokines regulate immune responses by regulating levels of T cells. If Th1 cells are in excess, the cell mediated immunity evades, whereas if Th2 responses outnumber, humoral arm becomes effective. This is a very effective counter regulation mechanism between these T cell populations. Anomaly in this regulation may result in serious consequences like autoimmunity or infection susceptibility (Kuby

Immunology 1997). IL-10 and interferons are a very important part of this Th1/Th2 regulation mechanism. The balance between the relative amount of IL-10 and interferon may influence the type of responses generated. Interferons stimulate Th1 phenotypes while suppressing Th2 phenotype. Il-10 has been reported as inhibitor of Th1 responses and activates Th2 responses (Romagnani *et al.*, 1991). HCV NS4 is reported to inhibit Th1 response via over expressing IL-10 (Brady *et al.*, 2003). Over expression of IL-10 in HCV patients can be a possible reason of failure of interferon therapy in viral Hepatitis patients.

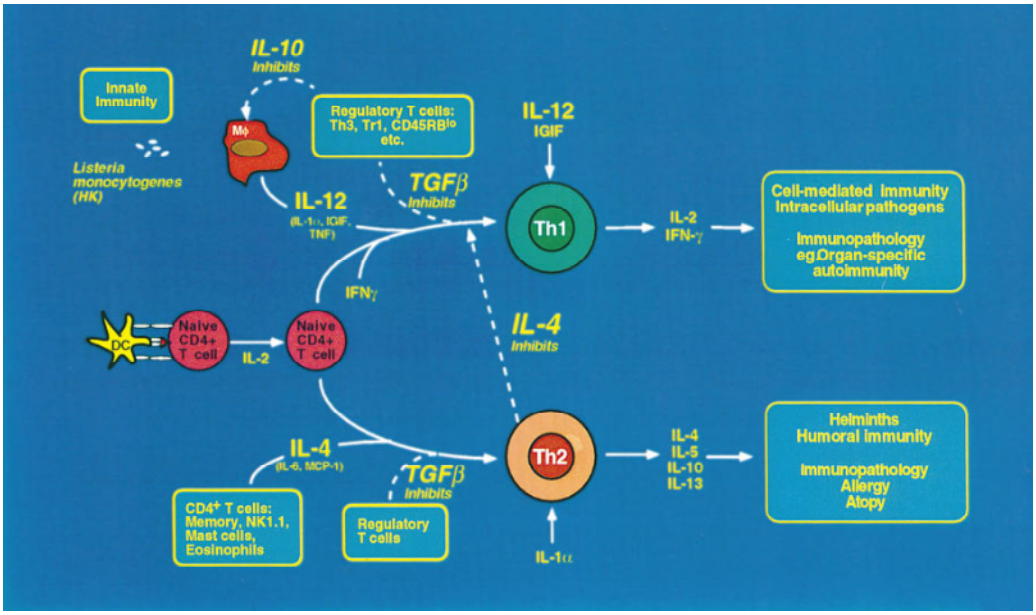

Figure 2.2 Diagrammatic illustration of CD4+ T cell regulation. IL-12 activated Th1 phenotype and IL- 4 induce Th2 subtype. Courtesy Garra 1998

2.5. Ethnicity and Cytokine Gene Polymorphisms.

Genetic variations and disease association studies are complicated by the well-recognized existence of important differences in human genetic characteristics and in disease risk associated with population origin (Reich *et al.,* 2001). Inheritance of polymorphic alleles is greatly influenced by racial backgrounds. For instance, the disparity in numbers of graft rejections in black and whites clearly suggests that genetic variation plays a vitalrole in outcomes in addition to poor economic backgrounds. Hoffmann et al. 2002 reported marked differences in frequencies of polymorphic alleles in cytokines genes from different origins. Studies suggest that there is a significant difference in allele frequencies of IL-10 polymorphisms in African populations as compare to Caucasian population. Afzal et al., (2011) reported presence of four allelic haplotypes (GCC, GTA, ACC, ATA) in Pakistani population, with genotype GCC/ATA 73.5% prevalent. The Genotype GTA is previously reported only in china (Mok *et al.*,, 1998).

2.6. Genetic Variations and Sustained Virological Response.

Genome wide association studies (GWAS) showed the polymorphisms near IL-28B gene is strongly associated with patient's response to interferon therapy (Clark *et al.,* 2011). Ghany et al., suggested that genotype C/C at polymorphic site, rs12979860 was a strong pretreatment marker for SVR (Ghany *et al.,* 2011). In another study association of IL-10 polymorphisms at -592 T to A was associated with outcome of interferon therapy in HBV patients (Wang *et al.,* 2011). Three SNPs

(rs3213545, rs1169279, and rs2859398), located in 2'-5'-oligoadenylate synthetase-like protein (OASL) was associated with SVR. SNP (rs2859398), located in the promoter region at position −2875bp in OASL was linked with SVR in Caucasian Americans, however no link was established in African American (Su *et al.*, 2008). MxA gene expression is highly associated with interferon response. Polymorphism at -123A and -88T is highly associated with interferon outcome. But Mohamed et al. found no link between polymorphism at -88 in Mx1 gene promoters and interferon therapy in HCV patients (Mohamed *et al.*, 2011). Chung et al. (2009) has reported no link between IL-10 polymorphism (-1082, -819, -592) and outcome of interferon therapy in Taiwanian population. Similar results were reported by Donaldson et al. (2002) and Dogra et al. (2011) in their study. Contradictory a very strong link between genotype ATA and initial interferon therapy outcome was reported in Australian population (p=0.01) (Edwards-Smith *et al.*, 1999). Similar findings were published by Yee et al. (2001) in which he reported association of polymorphism -819 and -592 with SVR in interferon and ribavirin combinational therapy.

Afzal et al., (2011) reported the association of IL-10 promoter polymorphism with HCV susceptibility. HCV nonstructural protein are reported to increase IL-10 production thus down regulating Th1 responses. Interferon shoots are given to restore normal antiviral responses. IL-10 promoter polymorphisms can be another factor associated with levels of IL-10 in blood. So, analysis of IL-10 polymorphism is essential to establish a link between IL-10 polymorphism and interferon therapy outcome.

Chapter 3

MATERIALS AND METHODS

3.1. Patient Enrolment.

PCR based confirmed patients infected with HCV genotype 3a (GT 3a) from ASAB diagnostic Lab and other private diagnostic labs were selected for this study. All patients were undergoing interferon therapy. Out of these 90 patients had shown Sustained Virological Response (SVR) and 45 were those who failed to show a significant response. SVR is taken as clearance of virus to undetectable levels. Patients with HBV or HIV co infections were excluded from the study. The patient consent form was essentially processed for this study and the study was approved by the ASAB, NUST ethical committees.

3.2. Sample Collection.

Blood Samples of 135 patients were drawn using 5 ml (BD 0.6mm X 25mm, 23 G X 1 TW) and were collected in EDTA vacutainers (BDTM vacutainer K3 EDTA). The samples were saved at 4º Celsius till the genomic DNA was extracted.

3.3. Genomic DNA Extraction.

Genomic DNA was extracted by using (Nucleospin DNA extraction kit, USA) according to the manufactures' protocol. Briefly; for each 200 µl of blood sample, 25 µl of proteinase K and 200 µl of buffer 3 (mixture of buffer 1 and buffer 2) was added

in a 1.5 ml micro centrifuge tube. The mixture was vortexed vigorously, and was incubated for 15 min at 70º C. After incubation 210 µl of pure chilled ethanol was added and was vortexed again. The mixture was then passed through silica columns for DNA binding using centrifugation at 11,000 g for 1 min and the supernatant was discarded. To wash out unbound residues 500 µl of wash buffer (BW) was added and centrifuged at 11,000 g for 1 minute. The flow through collected in the collection tube was discarded. The column was washed again by adding 600 microliters buffer 5 (BW 5). The column was centrifuged at 11,000 g for 1 min. The flow through was discarded and the columns was centrifuged at maximum speed for 3 minutes to eliminate any buffer residues. Finally elution buffer (100 µl; preheated at 70º) was added to the column. The column was incubated at room temperature for 1 minute. The column was then shifted to a fresh 1.5 ml microcentrifuge tube and was then centrifuged at maximum speed for one minute. The final elute was stored at -20 degree Celsius for further proceedings.

3.4. Genetic Analysis.

Polymorphisms at position -1082 G/A, -819 C/T, and -592 C/A were detected using Amplification Refractory Mutation System- Polymerase Chain Reaction (ARMS-PCR) (Perry *et al.*,1998). For each polymorphism, two separate reactions were performed. Each reaction contained allele specific primer and a generic antisense primer. To ensure a positive PCR, an internal control from human growth factor was amplified.

3.4.1. Primers.

Allele specific primers were used as explained by Perry et al., (1998). The list of primers is shown in table 3.1

Table: 3.1 Primers used for Genetic Analysis

Position	Primer	Sequence
-1082	Reverse primer	5'-CAGTGCCAACTGAGAATTTGG-3'
	Allele G primer	5'-CTACTAAGGCTTCTTTGGGAG-3'
	Allele A primer	5'-ACTACTAAGGCTTCTTTGGGAA-3'
-819/592	Reverse primer	5'-AGGATGTGTTCCAGGCTCCT-3'
	Allele T primer	5'-ACCCTTGTACAGGTGATGTAAT-3'
	Allele C primer	5'-CCCTTGTACAGGTGATGTAAC-3'
Internal control	Reverse	5'-GCCTTCCCAACCATTCECTTA-3'
	Forward	5'-TCACGGATTTCTGTTGTGTTTC-3

Note* IL-10 -819 polymorphism is in linkage disequilibrium with -592 polymorphism: allele C at -819 is always present when at position -592 is allele C and allele T at -819 is always present when at position -592 is allele A

3.4.2. Polymerase Chain Reaction Mixture:-

The reaction mixture of 20 microliters was prepared in Axygen 0.2 ml micro centrifuge tube. The ingredients of PCR mixture were taken from MBI fermentas UK.

Table 3.2: List and concentration of ingredients used for PCR

Ingredients	**Volume added (µl)**	**Concentration required**
10X Taq buffer	2	2X
MgCl2	2	
Taq	0.4	
Nuclease free water	5.6	---
dNTPs	2	0.2mM/microliters
DNA Template	4	75-100 ng
Reverse primer	1	0.5 picomole(pM)
Allele specific primer	1	0.5 pM
Reverse control	1	0.5 pM
Forward control	1	0.5 pM

Note* the reaction mixture was similar for both the reactions i.e. for SNP -1082 and SNP -819/592

The PCR was run on Dual block ESCO Swift® Maxpro thermo cycler. The reaction was optimized for 35 cycles. Initial denaturation was done at 95 degree Celsius for 5 minutes. Annealing was carried out at 58 degree Celsius for 45 sec for 35 cycles. Final elongation was programmed at 72 degree Celsius for 7 minutes (figure 3.1). PCR reaction was held on 4 degree Celsius till the reaction was removed from PCR and stored at -20 degree Celsius.

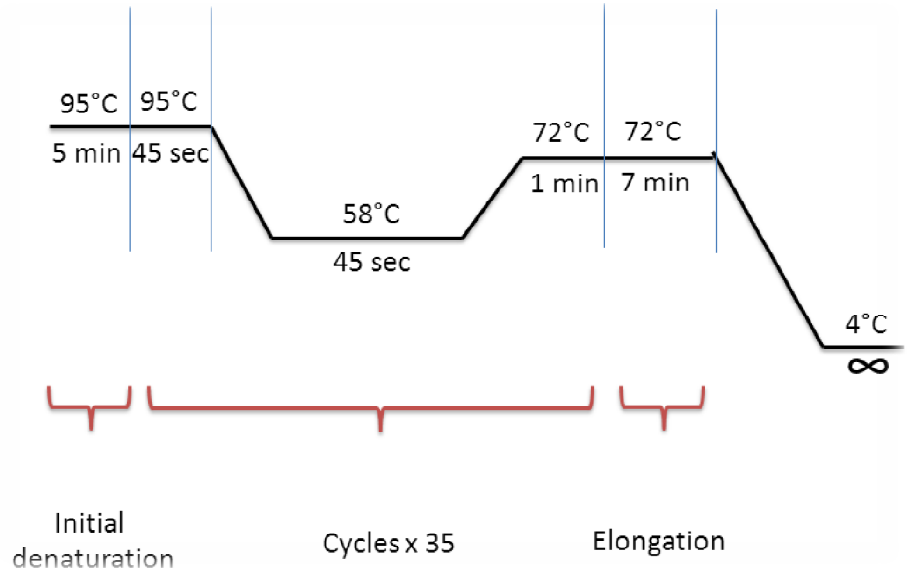

Figure 3.1 Schematic representation of PCR Programmed used for amplification

3.5. Gel Electrophoresis.

Agarose gel electrophoresis was employed for analyzing the DNA product of ARMS PCR. For this purpose 2 % (w/v) agarose gel was made using 1 gram agarose melted in 50ml 1X TAE (Tris-acetate, EDTA) in microwave oven. 5 µl of ethidium bromide solution was added to make the final concentration 10 mg/ml. The amplified DNA was mixed with 6X loading dye (Farmantas) in 5:1 ratio to make final concentration of loading dye 1X. Each sample mixture was loaded on the gel (5 µl). Electrophoresis was carried out at 90V for 30 minutes in TAE buffer. The results were analyzed by Wealtech dolphin-Doc gel analysis system.

3.6. Statistical Analysis.

Statistical analysis was done using Study Result Software Version 1.0.4 (CreoStat HB Frolunda, Sweden). Fisher Exact test was used to compare distribution of polymorphism among the responder and non-responders. Probability value of less than 0.05 was considered as significant.

Chapter 4

RESULTS

Blood samples from individuals included in the study were collected and DNA was extracted using Kit (Nucleospin USA). Genotyping was done by ARMS PCR as explained by Perry et al., (1998). Two reactions were performed to identify the alleles for each sample. The products were run on 1.5 % gel heterozygous were positive for both reactions while homozygous were positive for one. (Figure 4.1 and Figure 4.2)

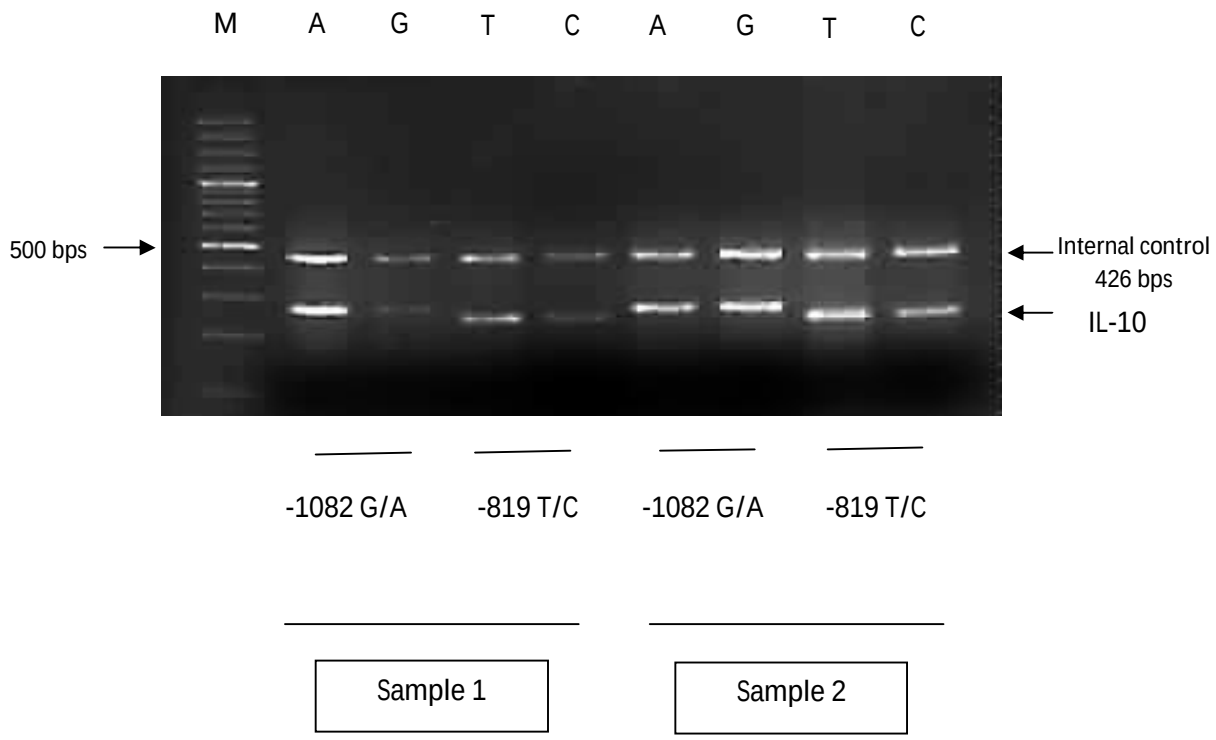

Figure 4.1 Representative gel for sample heterozygous for both alleles products are run on 1.5% agarose gel lane 1 contain 100 kb ladder marker while in Lane 2-9 different SNP products were run

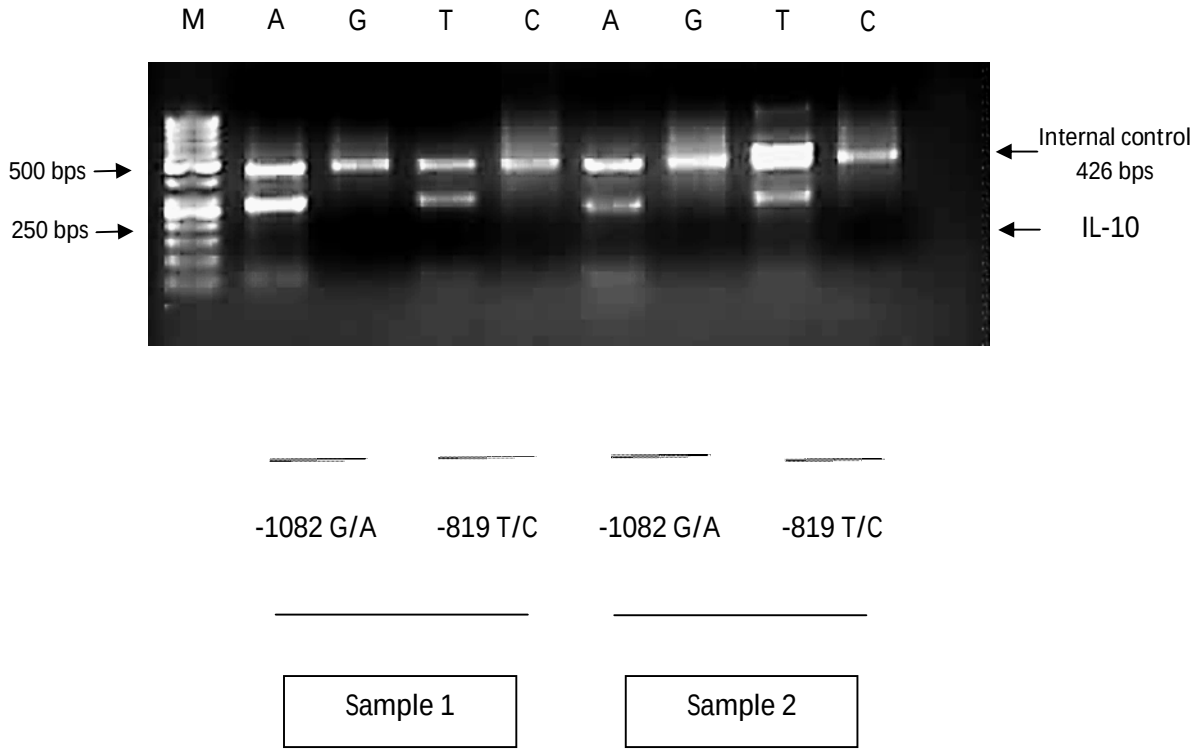

Figure 4.2 Representative gel for sample homozygous for A and T alleles. In Lane 1 50bps ladder is run while in Lane 2-9 contain different SNPs.

Table 4.1. Comparisons of baseline characteristics between healthy subjects, chronic hepatitis C patients with and without sustained virologic response to pegylated interferon plus ribavirin therapy.

	Healthy Control (N=90)	SVR (N=95)	Non-SVR (N=45)
Gender (M/F)	50/40 (55.5/45.5)	50/45	27/18
Age (Years)	45 ± 10.3	40 ± 15.1	50 ± 8.9
Pretreatment serum ALT level (IU/L, mean±S.D.)	31.2 ± 18.4	61.5± 29.9	54.5±17.5
Pretreatment serum HCV RNA level (log copies/mL, mean±S.D.)		5.1±0.88	6.04±0.67

Table 4.2. Comparisons of IL-10 genotype/allele frequency in healthy controls and chronic hepatitis C patients

IL-10 locus	Healthy Subjects (N=90)	HCV Patients (N=140)	P	OR (95% CI)
-1082 G/A				
G/G (high)	15 (16.7)	45 (32.1)	0.02	0.4 (0.2-0.8)
G/A (Intermediate)	65 (72.2)	80 (57.1)	0.03	1.95 (1.1-3.4)
A/A (Low)	10 (11.1)	15 (10.7)	1	
Allele Frequency				
G (High)	81 (45)	145 (51.8)	0.18	NS
A (Low)	99 (55)	135 (48.2)		
819C/T (592C/A)				
T/T (A/A)	15 (16.7)	24 (17.1)	1	NS
C/T (C/A)	68 (75.6)	101 (72.1)	0.7	
C/C (C/C)	7 (7.7)	15 (10.7)	0.5	
Allele frequency				
T(A)	103 (57.2)	151 (53.9)	0.5	NS
C(C)	77 (42.8)	129 (46.1)		

*NS = not significant *P = probability

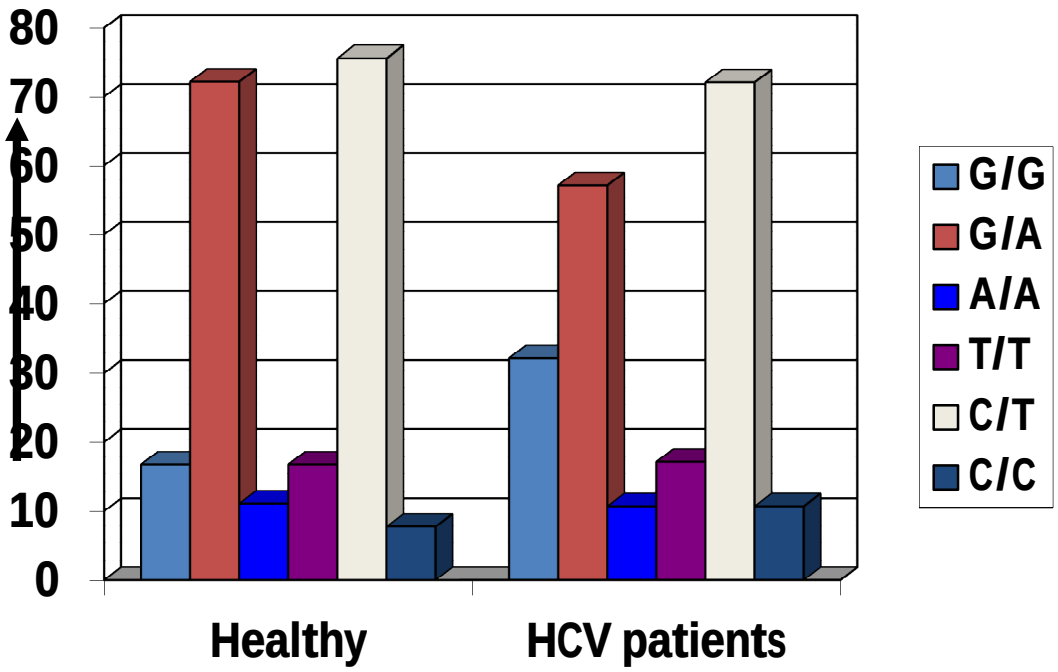

Figure 4.3 Graphical representation of percentage frequency of IL-10 genotype in HCV pateints and healthy controls. The genotype G/G at position -1082 was significantly associated (p = 0.02) with HCV susptibility in genotype 3a. whereas G/A was higher in controls (p = 0.03)

Table 4.3.Comparisons of IL-10 genotype/allele frequency in chronic hepatitis C patients with and without sustained virologic response to pegylated interferon plus ribavirin therapy

IL-10 locus	SVR (N=95)	Non-SVR (N=45)	P
-1082 G/A			
G/G (high)	30 (31.7)	15 (33.3)	0.86
G/A (Intermediate)	55 (57.8)	26 (55.5)	1
A/A (Low)	10 (10.5)	5 (11.1)	1
Allele Frequency			
G (High)	99 (52.1)	49 (54.4)	NS
A (Low)	91 (47.9)	41 (45.6)	
819C/T (592C/A)			
T/T (A/A)	21 (22.1)	7 (15.6)	NS
C/T (C/A)	65 (68.4)	33 (73.3)	
C/C (C/C)	9 (9.5)	5 (11.1)	
Allele frequency			
T(A)	109 (57.4)	21 (46.7)	NS
C(C)	81 (42.6)	24 (53.3)	

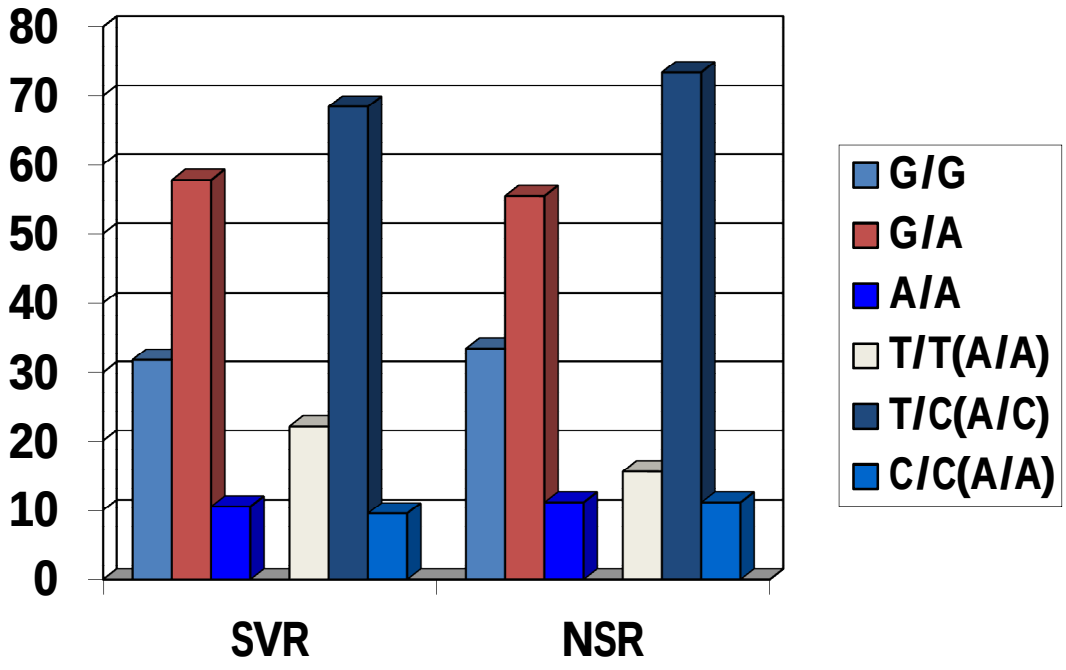

Figure 4.4 graphical representation of percentage frequency of IL-10 genotype in patients with sustain virological response with patients who showed non sustain respond. No significant association was found between IL-10 genotype and sustain responses

Table 4.4 Comparisons of IL-10 haplotypes frequency in chronic hepatitis C 3a patients and healthy adults

IL-10 Haplotypes	Healthy Subjects (N=90)	HCV Patients (N=140)	P	OR (95% CI)
GCC (high)	80 (44.4)	115 (41.8)	0.49	
GTA (high)	20 (11.1)	52 (18.6)	0.03	0.55 (0.3-1)
ACC (intermediate)	5 (2.8)	8 (3.6)	1	
ATA (low)	75 (41.7)	105 (37.5)	0.38	

Table 4.5 Comparisons of IL-10 haplotypes frequency in chronic hepatitis C patients with and without sustained virologic response to pegylated interferon plus ribavirin therapy

IL-10 locus	SVR (N=95)	Non-SVR (N=45)	P	OR (95% CI)
GCC (high)	75 (39.5)	39 (43.3)	0.54	
GTA (high)	37 (19.5)	16 (17.7)	0.76	
ACC (intermediate)	6 (3.1)	4 (4.4)	1	
ATA (low)	72 (37.9)	31 (34.6)	0.9	

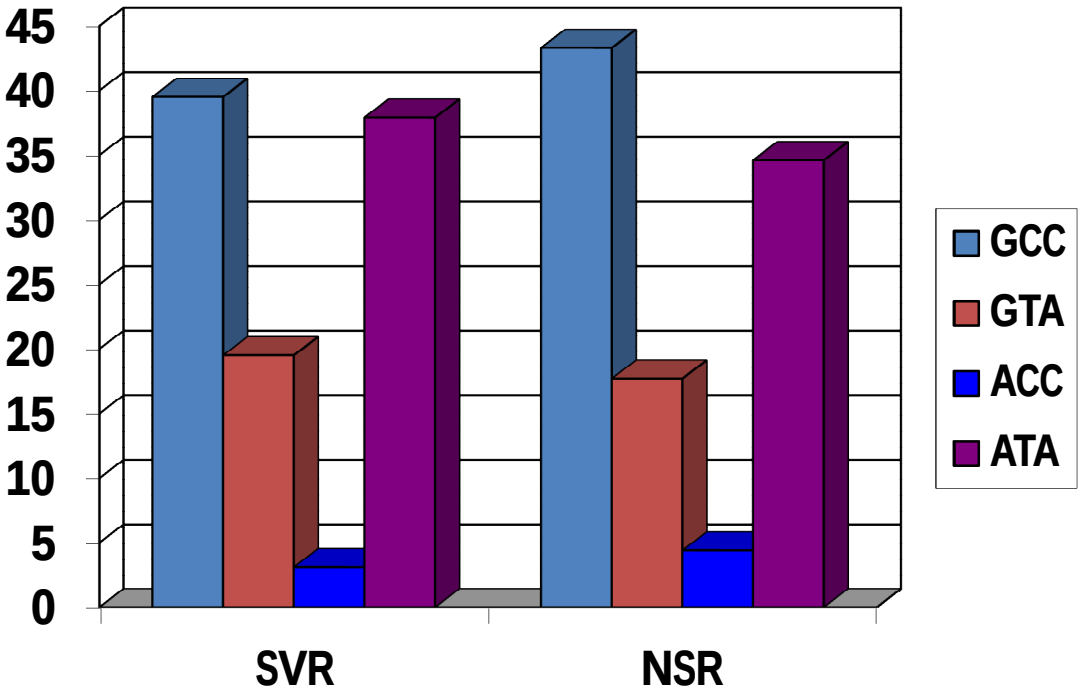

Figure 4.5 Graphical representation of percentage frequency of IL-10 haplotype in responders and non responders.

Chapter 5

DISCUSSION

Hepatitis C is a major problem in Pakistan. Everyone in twenty person is infected with HCV in Pakistan (Waheed *et al.,* 2010). Disease severity varies differently among HCV infected individuals ranging minimally from hepatocytic lesions to more severe conditions like liver cirrhosis and hepatocellular carcinoma (HCC) (Persico *et al.,* 1998). There are increasing evidences about the role of host genetic makeup and immune response in heterogeneity of disease outcomes (Bouzgarrou *et al.,* 2009). Due to unique genetic background 20 % of the HCV patients' clear virus naturally and among others 60-80 have got chronic infection (Rehermann and Nascimbeni, 2005). Similarly interferon therapy responses are different in infected subjects, depending both on viral genotype as well host genetic variations. Cytokines play a very important role in disease pathogenesis (Farooqi *et al.,* 2011). Among cytokines IL-10 is a member of Th2 cytokines with direct effect of infection outcomes. IL-10 polymorphic variants inheritance depends greatly on ethnicity (Afzal *et al.,* 2011 a) and its production varies inter individually depending on genetic makeup of the person (Afzal *et al.,* 2011 b). IL-10 level influences the Th1 and Th2 cytokine balance (Thio, 2008) and may have an effect on HCV pathogenesis. Keeping in view the importance of IL-10 in immune regulation, we have tried to investigate the potential role of IL-10 functional polymorphism on HCV infection

susceptibility and its effect on outcomes of standard therapy in 3a genotype infected patients.

There are many studies from different populations regarding the role of IL-10 polymorphic variants in HCV susceptibility, disease clearance and effect on interferon therapy. The results of most of studies are conflicting, in current study IL-10 -1082 GG and GA were shown associated with disease susceptibility and protection. Previous reports regarding homozygous G at -1082 from Pakistan (Afzal *et al.,* 2011 c), Italy (Lio *et al.,* 2003), USA (Vidigal *et al.,* 2002) and Germany (Reuss *et al.,* 2002) were in accordance with recent results while from Japan (Abe *et al.,* 2003), Tunisia (Bouzgarrou *et al.,* 2009), Italy (Persico *et al.,* 2006), China (Gao *et al.,* 2009) and the Caucasian population (Constantini *et al.,* 2002) were contradictory. In accordance with recent results, the 1082 GA genotype was shown to be significantly higher in healthy subjects from China (Gao *et al.,* 2009) and from Italy (Lio *et al.,* 2003). A high IL-10 producing haplotype GTA also showed significant different inheritance pattern among patients and healthy subjects similar to our previous report on same ethnic group. Contradictory to our results, IL-10 haplotypes were not distributed differently between healthy controls and HCV patients in different reports from Japan, Caucasian and Italy, the results may be differ due to different ethnicity of studied populations (Constantini *et al.,* 2002; Knapp *et al.,* 2003; Kusumoto *et al.,* 2006; Shin *et al.,* 2003; Mangia *et al.,* 2004).

Reports regarding IL-10 polymorphism association with persistent HCV infection and self-clearance showed that IL-10-1082 GG was associated with

persistent infection in African-American (Oleksyk *et al.,* 2005) and Caucasian (Knapp *et al.,* 2003) populations while not in European-American (Oleksyk*et al.,* 2005) and Japanese patients (Kusumoto *et al.,* 2006). Another study showed that IL-10-592 A and IL-10 -1082 GA were high in self-limiting recovered HCV patients in a Caucasian population (Knapp *et al.,* 2003). Current study on Pakistani Punjabi ethnic group showed that there is no significant association between IL-10 polymorphism and outcomes of interferon ribavirin therapy. Our results are in accordance with a study from Taiwan, which includes 143 HCV patients and 134 healthy adults. Chuang et al. investigated the same genotypes and haplotypes (−1082, −819 and −592) like us and there data also failed to show any significant association of IL-10 promoter gene polymorphisms in the response to combination therapy (Chung *et al.,* 2009). Similarly data from two other reports from Dogra et al., and Donaldson et al., *w*as unable to show any significant relationship between IL-10 genotypes and interferon therapy outcomes (Donaldson *et al.,* 2002; Dogra *et al.,* 2011). Contradictory to this Edwards-Smith et al., reported that IL-10 haplotype ATA positively correlated with initial response to standard interferon-alphamonotherapy in CH-C patients (Edwards-Smith *et al.,* 1999). Similarly another report by Yee et al., showed that homozygosity for −592A, −819T or the extended haplotype (108 bp)–(−2575T)– (−2763C)–(−1082A)–(−819T)–(−592A) was associated with SVR to standard interferon alfa-2b plus ribavirin (Yee *et al.,* 2001).

Factors, which effect treatment response other than IL-10 polymorphism, were age and pretreatment RNA level. Martinot-Peignoux et al., demonstrated that the low

baseline serum viral load is associated with a significantly higher probability of achieving sustained virological response following INF-based therapy and were supported by others (Aziz *et al.,* 2011; Martinot-Peignoux *et al.,* 1995; Davis *et al.,* 1998; Jensen *et al.,* 1999). Age is associated with effectiveness of immune response. Men and women with <40 year age responded more to the treatment as compared to men and women having age >40 years (Hayashi *et al.,* 1998; Aziz *et al.,* 2011).

The existence of conflicting data suggest that there are perhaps more than one factor that that regulate interferons and these factors can vary among various ethnic groups. There is a need of broader studies including multiple factors to properly evaluate the association of IL-10 polymorphisms in various ethnic groups

CONCLUSION

In a poor socio economic country like Pakistan with more than 10 million HCV infections, it is the need of the hour to look at the molecular level of infection. As about 4-5 % of population is infected with HCV of which 3a GT is the major causative GT, we tried to investigate the ethnic and GT based molecular marker for early prediction of infection susceptibility and therapy outcomes. According to this study IL-10 can play a protective role or may favor disease occurrence depending on the genetic background of the individual. But we were unable to find any co relation between IL-10 polymorphic variants and interferon therapy out comes may be due to small number of subjects. This study is a preliminary and is on small scale. We believe that our findings may stimulate some additional genetic studies on larger scale using these and new multi-locus analysis approaches for a deeper analysis of the epistatic interaction of the pro- and anti -inflammatory molecules toward hepatitis C progression. Better understanding of genetic factors that have effect on hepatitis c progression, pathogenesis will provide scientific basis for the development of new immunomodulatory treatments for chronic hepatitis C patients and will also help health care workers about the standard therapy effectiveness.

Chapter 6

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