STUDY OF HLA-C AND KIR GENE VARIANTS IN HCV INFECTED PATIENTS AND THEIR PLAUSIBLE EFFECT ON TREATMENT RESPONSE

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS Industrial Biotechnology

Supervised by:

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Industrial Biotechnology

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST), Islamabad, Pakistan

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TH form

DECLARATION

I certify that this research work titled "**Study of HLA-C and KIR gene variants in HCV infected patients and their plausible effect on treatment response**" is my own work. This work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged/referred.

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ACKNOWLEDGEMENTS

All praises to ALLAH almighty who is the most beneficent the most merciful. I am grateful to ALLAH Almighty for giving me the strength to complete my Master's degree and for the strength that kept me standing in the most difficult circumstances and completing the degree.

I feel great pleasure and privileged to have this opportunity to express my gratitude to my supervisor **Dr. Amjad Ali**. I feel honored to work and learn under his kind supervision. I am thankful to my supervisor who gave me enthusiasm and esteemed inspiration, guidance and moral support throughout my research work.

I am thankful to Higher Education Commission (HEC) Pakistan for providing the funds to carry out this study. My thesis was the part of project funded by HEC under NRPU projects.

I am thankful to Dr. Ayesha Obaid for extending her valuable knowledge and input at every stage of my research work. Without her kind guidance, the study could not have been successfully conducted.

A very special thanks to Mr. Muhammad Shehroz, Ms. Tahreem Zaheer, Ms. Shifa Tariq, Ms. Nigham Sarfaraz, Ms. Tanzeela Raza, Ms. Arooma Jannat, Ms. Shama Shah, Ms. Maryum Arshad, Mr. Bilal Naveed, Mr. Syed Abdullah Tirmizi and Mr. Nimat Ullah for their precious time and helping me in my lab work and encouraging me.

The years 2016-2018 were an amazing experience for me and for that I owe huge thanks to many people for their contribution in making this entire time unforgettable. I would like to say thank you to Mehk Arshad, Nigham Sarfaraz, Maheen Javed, Sahar Shafait and Hafiza Noor Ul Ain for encouraging me and helping me at every step. Maryum Arshad and Hafiza Noor Ul Ain, I owe you people a massive thank you for always motivating me and for all the conversations that gave me the positive energy. Thank you for being always there for me. You made my stay at NUST much easier and so memorable. I would like to extend my gratification to Sidra Rasheed and Hina Kanwal for their moral support.

I wish to articulate my profound appreciation to my caring parents, Mr. Sikandar Shahzad and Mrs. Mumtaz Malik, for providing me this opportunity to study at one of the best institute. Without their support this journey was impossible. I am thankful to

my brothers M. Shahroz Sikandar and M. Maaz Malik for their constant motivation and moral support. This achievement merely belongs to my family, thank you for always believing in me, instilling self-belief and confidence, thank you for always motivating me to be the best of myself.

Thank you.

Dedicated to my Baba & Mama

My brothers M. Shahroz Sikandar and M. Maaz Malik

Who gave me the opportunity to study at one of the best institute and supported me at every step of my life

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LIST OF ABBREVIATIONS

3'UTR	3'-Unstranslated Region
5'-UTR	5'-Untranslated Region
C	Core
CD4+	Cluster of Differentiation 4
DAAs	Direct Acting Antivirals
DNA	Deoxy Ribose Nucleic Acid
E1	Envelop Glycoprotein 1
E2	Envelop Glycoprotein 1
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IFN- γ	Interferon-gamma
IL-10	Interleukin 10
IL-2	Interleukin 2
IL28B	Interleukin 28B
Kb	Kilo bases
KDa	Kilo Dalton
NANBHV	Non-A non-B Hepatitis virus
NK Cells	Natural Killer Cells
NS2	Non-structural protein 2
NS3	Non-structural protein 3
NS4A	Non-structural protein 4A
NS4B	Non-structural protein 4B
NS5A	Non-structural protein 5A
NS5B	Non-structural protein 5B
NSVR	Non- Sustained Virological response
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction

PEG-IFN	Pegylated interferon
RBV	Ribavirin
RNA	Ribose Nucleic Acid
SNPs	Single Nucleotide Polymorphisms
SVR	Sustained Virological Response
WHO	World Health Organization

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Abstract

Hepatitis C Virus, a major etiologic cause of acute and chronic hepatitis, evades the host immune system and infects millions of people, leads to severe morbidity and mortality. It has been investigated that Human Leukocyte Antigen-C and Killer Immunoglobulin-like Receptor association play a vital role in either progression or clearance of HCV. These receptors specific for Major histocompatibility class I molecules located on their target cells regulate the natural killer cell production. Treatment outcome differs from individual to individual as the breadth of the genetic heterogeneity of KIR and HLA is great, due to viral as well as host factors. They are the major factors affecting the rate of treatment and found to be a key predictor for treatment outcome. Therefore, the current study was aimed to investigate the prognostic host factors to predict the treatment response to PEG IFN/RBV therapy in association with KIR/HLA-C gene variants in HCV infected patients. 102 HCV patients were included in the study and their KIR and HLA was genotyped. 30 healthy volunteers negative for HCV were kept as controls. In this study, we found homozygous C2C2 is associated with failure of treatment (P-value 0.0213). KIR2DL1+C2 carriers also indicated disease progression. Our results suggest that the presence of certain activating KIR receptors could protect the patient by enhancing the immune response against virus. Also it was found that there was a significant correlation between the type of KIR and HLA of individual and HCV infection ($P<0.05$). Finally, it can be concluded that particular HLA-C variant in combination with specific KIRs might effect and thus alters the treatment outcome to some extent.

Chapter 1

Introduction

Hepatitis C Virus is globally prevalent pathogen causing Hepatitis C disease, which has been known since 1989, leading to cirrhosis, hepatocellular carcinoma and liver transplant (Rosenthal et al., 2018). HCV, a ssRNA virus, belongs to family Flaviviridae (Nie et al., 2015). Family Flaviviridae includes human pathogens, they are arthropod borne i.e dengue virus, yellow fever virus and West Nile virus (Dubuisson & Cosset, 2014). HCV contain a single ORF, genome size is 9.6 kb in length having 5' and 3' non-coding regions (Nie et al., 2015). HCV genome consist of three structural and seven non-structural proteins out of approximately 9000 nucleotides (D. Wyles & Gutierrez, 2014). Its natural hosts are humans, but experimentally also chimpanzees can be infected. It is hepatocyte tropic and has only been shown to robustly complete its life cycle in liver cells. According to WHO, Report 2018, 71 million people globally are infected with chronic HCV infection and about 399 000 patients die every year due to HCV, (WHO 2018). HCV is a blood-borne infection, transmitted by unsterilized needles used in drug intake or medicinal purposes, blood transfusions, maternal transmission, sexually and during organ transplantation (T. Yen, Keeffe, & Ahmed, 2003). There is a significant variation in global HCV prevalence i.e low prevalence in the Western world and higher in the Middle East and Africa, Denmark counts for less than 0.5% of the population, Egypt marks 15-20% of HCV infection (Hajarizadeh, Grebely, & Dore, 2013; T. Yen et al., 2003). HCV exhibit a high genetic diversity, it exists in seven major genotypes and 86 subtypes. Major genotypes exhibit 30% differences at nucleotide level while the subtypes show 15-25% differences at the nucleotide level (Goldberg et al., 2017b). No vaccine against HCV is known till date, but present studies on HCV lifecycle has enabled to develop effective treatments including direct-acting antivirals (DAAs), however, resistance against DAAs have also been reported in HCV patients (Pawlotsky, 2016; D. L. Wyles, 2012). Virus-host interaction is the key factor to predict the treatment outcomes. The treatment is affected by both viral and host factors. In case of viral factors, a core protein, p7, NS2, NS3 and

NS5, viral load and most important is viral genotype. Among host factors, age, gender, ethnicity, social life, environmental factors and single nucleotide polymorphism in various genes are the vital influencing factors (Bengsch, Thimme, & Blum, 2009). There is a need to investigate prognostic host gene factor targets to predict treatment outcome to PEG IFN/RBV treatment in combination with HLA and KIR gene variants in HCV infected patients. In current study, we selected host HLA-C and KIR gene variants and analyzed their effect on the patients, who were given PEG IFN/RBV therapy. There exist a highly diversified association between KIR and HLA-A,-B and –C, difference in their interaction ultimately cause a variation in immune response of every single individual (Valiante et al., 1997; Carlos Vilches & Parham, 2002). For KIR receptors to recognize specific HLA C molecule, HLA-C allotypes have been classified into two groups on the basis of sequence dimorphism in α1 domain specifically at position 80 (Colonna, Borsellino, Falco, Ferrara, & Strominger, 1993; Moretta et al., 1993). The particularity of C1 epitope is specified by the presence of Asp residue at position 80 on the other hand for C2 allotype, Lysine residue is present at position 80 (Robinson, Mistry, McWilliam, Lopez, & Marsh, 2009; Uhrberg, Parham, & Wernet, 2002). Single amino acid substitution in KIR receptors result in the drastic change in epitope specificity or shift its interaction from C-1 to C-2 allotype (Roberto Biassoni et al., 1997; Winter, Gumperz, Parham, Long, & Wagtmann, 1998; Winter & Long, 1997). Winter and Long in their study reported that a single substitution at residue 44 swapped the specificity of KIR2DL1 from C-2 to C-1 and that of KIR2DL3 from C1 to C-2 allotype (Carlos Vilches & Parham, 2002). KIR Receptors regulate the production of cytokines by NK production (Caligiuri, 2008; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). Clonally distributed inhibitory receptors mediate the activity of cytokines; these receptors are highly specific for MHC class 1. Their interaction results in inhibition of cell lysis by NK cells (Ciccone et al., 1992; Litwin, Gumperz, Parham, Phillips, & Lanier, 1994; Moretta et al., 1994). Inhibitory receptors transduce the inhibition signals by inhibiting the production of NK cells(Jost & Altfeld, 2013; Morvan & Lanier, 2016). KIRs are classified on the basis of number of extracellular domains and length (long or short) of cytoplasmic tail. Among the inhibitory receptors, KIR inhibitory receptors are considered as the most potential one. They recognize MHC I molecules (Vivier & Anfossi, 2004). KIR2DL1 consists of two extracellular domains and one long cytoplasmic tail, these receptors recognize

HLA-Cw4 (Lanier, 2005). Basically, inhibitory receptors abolish the signals of activating receptors by dephosphorylation of proteins of activating receptors. Inhibitory Receptors require two phosphatases SHP-1 or SHP-2, they bind to inhibitory receptors after identifying the ligand. Some Inhibitory Receptors need only one phosphatase instead of both (Yusa & Campbell, 2003; Yusa, Catina, & Campbell, 2002). Killer Immunoglobulin like Receptor, harboring two Immunoglobulin domains and short cytoplasmic tail 2, are glycoproteins produced by NK cells. They are also known as NKAT5, cl-49, CD158J/b, or 183ActI. KIR2DS2 encodes a protein of 304 amino acids. They are mapped to chromosome 19q13.4. The cytoplasmic tail consists of 39 amino acids. These KIR genes show polymorphism and thus are divided into haplotypes A and B. KIR2DS2 belongs to haplotype B. They regulate the immune response against viral infections (Bottino et al., 1996; Wagtmann et al., 1995). KIR2DS2 is an activating receptor they trigger NK cell production and enhance cytokine activity. The association between KIR2DS2 and HCV clearance or progression is not consensual. Better understanding about the association between viral and host factors and their effect on treatment outcomes may help in improving the treatment options and efficacy as well as predict the infection susceptibility. These receptors control the immune response by activating or inhibiting the NK cell activation.

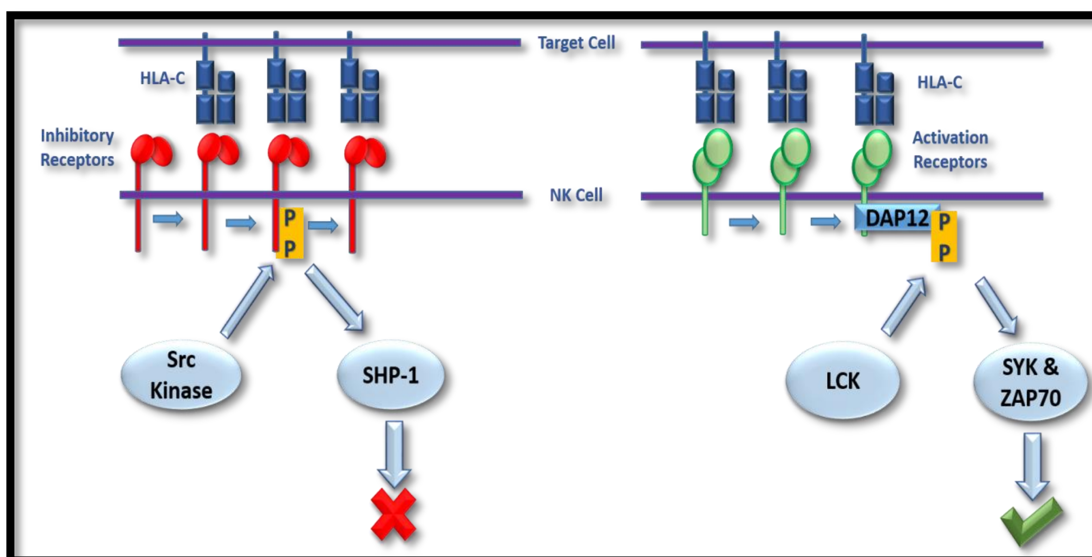

Figure 1.1 Signaling and expression of HLA and KIR genes in immune response

Basically, when a viral pathogen enters the body, the specific types of cells called Antigen presenting cells (APCs) engulf the viral pathogens through the process of phagocytosis. They convert the pathogen into small particles, called peptide; these peptides are then loaded onto the HLA, specifically C1 in case of viruses. These HLA-C1 ligand molecules on binding to their specific receptor molecules (activation or inhibition) regulate the immune response. These responses differ from individual to individual, according to the type of KIR haplotype and HLA genotype of an individual. When HLA loaded with peptide binds to Inhibitory receptor, Kinase from Src family phosphorylates the immunoreceptor tyrosine based inhibition motif which results in activation of SHP (Src-homology domain bearing tyrosine-phosphatase) and this inhibits the proteins which are involved in intracellular activation cascade (Figure 1). Similarly, in case of activation receptors on binding to specific ligand HLA molecule, the Lymphocyte-specific protein tyrosine kinase (Lck) phosphorylates the DAP-12 molecule attached to the cytoplasmic tail of the activation receptor. This phosphorylation results in activation of Syk (spleen tyrosine kinase) and ZAP70 (Zeta-chain associated protein-kinase). These kinases activate the proteins involved in intracellular cascade (Figure 1.1). This is how HLA and KIR molecules control the immune response. The therapy can be predicted on the basis of the type of KIR and HLA an individual express. Host KIR and HLA gene variants should be investigated to predict the treatment outcome.

Chapter 2

Literature Review

2.1 Faviviridae Classification

Flaviviridae is classified into four genra namely, Flavivirus, pegivirus, Pestivirus and Hepacivirus. Viruses belonging to these genera's are responsible for deadly disease. Pestivirus includes bovine viral diarrhea virus, (M. J. Alter & Margolis, 1998) Swine fever virus and border-disease virus. Flavivirus comprise of Japanese Encephalitis, yellow fever virus, dengue virus and Tick-borne encephalitis. Pegivirus covers GB viruses, i.e GBV A, C AND D. Hepatitis C virus belongs to genus Haepacivirus (Stapleton, Foung, Muerhoff, Bukh, & Simmonds, 2011). HCV has been classified into 7 genotypes and 87 subtypes (Goldberg et al., 2017a) (M. J. Alter & Margolis, 1998).

2.2 HCV Discovery and Progress

Hepatitis C virus history started in 1975, at that time the only causative agents of hepatitis were hepatitis A and B and serological tests were also available only for these types. Blood screening was done for Hepatitis A virus and as well as for HBV, both were non –positive, this resulted in the discovery of another causative agent other than HAV and HBV (Feinstone, Kapikian, Purcell, Alter, & Holland, 1975). This non-A and non-B hepatitis causative agent (NANBH) was tested with the available equipment of that time and proved to be small-sized and enveloped particle, in 1983 (Bradley et al., 1983). Finally over a decade of research, Choo et al in 1989, by screening the cDNA library from infected chimpanzee plasma, they isolated the very first HCV clone. Then after its identification, further studies were carried out for confirmation and construction of cell culture model (Choo et al., 1989). Viral RNA was extracted from the liver of patient, who was infected with this NANBHV and full length genome was cloned from viral RNA and then sub-genomic replicons were created. This enabled the researchers to study the replication and translation of virus (Lohmann et al., 1999). HCV pseudoparticles (HCVpp) were discovered in 2003, HCVpp are recombinant viral particles, containing HCV glycoproteins (Bartosch, Dubuisson, & Cosset, 2003). These

glycoproteins helped in understanding the process of viral entry in the host body. Lack of viral culture systems delayed the detailed study of HCV life cycle. But in 2005 Wakita et al presented full-length clone of JFH1 genotype 2a strain that replicated in cell culture. They made genome replication in cell culture, which provided a gateway towards the studying of viral life cycle (Wakita et al., 2005).

2.3 Global Distribution of HCV Genotype

Hepatitis C virus exhibits genotypic diversity, includes 7 genotypes and 87 subtypes, global distribution of each genotype shows variation from region to region depending on the lifestyle of individuals. HCV genotypes and subtypes show variation at the nucleotide level (Schnell et al., 2018). Human migration from one region to another has contributed a lot to the diverse genotypic variation in HCV strains. For instance strain of West Africa were reported to be transferred to America by means of transatlantic slave trade. Among the six genotypes of HCV, the most prevalent is genotype 1, accounts for 46 % of total HCV infection(Schnell et al., 2018). Genotype 3 is the second most occurring HCV genotype represents 30% of HCV infections prevalent in the population of Europe, Asia and Australasia (Gower, Estes, Blach, Razavi-Shearer, & Razavi, 2014; Schnell et al., 2018). Whereas genotype 2 represents 9-13% of the HCV infection worldwide, geographically distribution includes Asia and West Africa. Incidence of genotype 4 have been reported in the Middle East, North Africa and Central and Eastern sub-Saharan Africa, representing 9-13% of HCV cases (Gower et al., 2014). Genotype 5 occurrence is reported in South Africa, genotype 6 and 7 have been reported in East, Southeast Asia (Schnell et al., 2018) and the Democratic Republic of Congo respectively (Murphy et al., 2015). In one study, examining about 60% of the world's countries, or 90% of the world's population, the most prevalent was genotype 1 accounting for 46.2% of cases, followed by genotype 3 accounting for 30.1% of cases globally (Messina et al., 2015).

2.3.1 Prevalence of HCV

The numbers of deaths caused by HCV reported in 2015 were 1.34 million; this figure is higher than the number of deaths caused by HIV and tuberculosis. According to a report of WHO, 2017, 177.5 million people are infected with Hepatitis C Virus, 71 million people are globally infected with chronic Hepatitis C virus, which on its later

stages will lead to cirrhosis or liver cancer or even death. It is estimated that 399 000 patients die from HCV per year (WHO, global hepatitis report 2017).

2.3.2 Prevalence of HCV in Pakistan

Hepatitis C virus has emerged as a major cause of liver diseases in Pakistan and a continuous increase in the number of infection has been reported. HCV sero-prevalence among adult population of Pakistan has been reported in 6.8 % of the total population while the active HCV infection in about 6% of population. Genotype 3a is found to be the most common one in Pakistani population, estimated as 61.3% and subtype 2a found to be second most common in Sindh and KPK i.e 11.3% and 17.3% respectively (Umer & Iqbal, 2016). It has been reported multiple times in multiple studies that HCV subtype 3a is most prevalent among Pakistani population (Hamid et al., 2004; Waheed, Shafi, Safi, & Qadri, 2009). A survey conducted by Aiman et al, in 2017, reported that the overall percentage of HCV prevalence in provinces of Pakistan is as 2.55% in Sindh, 25.77% in Balochistan, 6.07% in KPK, and 5.46% prevalent in Punjab (Arshad & Ashfaq, 2017).

2.4 HCV Genome Organization

HCV belongs to genus Hepacivirus, family Flaviviridae and is a hepatotropic Flavivirus (Robertson et al., 1998). HCV exhibit a high level of genetic diversity, having 7 genotypes which show less than 72% similarity at the nucleotide level (P. Simmonds et al., 1993) (Bukh, Miller, & Purcell, 1995; Robertson et al., 1998; Peter Simmonds, 2004). Hepatitis C Virus is an enveloped virus comprising of 9.6 kb with positive stranded RNA genome which contains a 5’ non-coding, an open reading frame and a 3’ non-coding region. This distinct solitary ORF contains polyprotein precursor of about 3000 amino acids in it that cleaves into proteins during and after translation process (Bartenschlager & Lohmann, 2000; Chen et al., 1992; Q. L. Choo et al., 1991; Kato et al., 1990; Rosenberg, 2001; Tellinghuisen & Rice, 2002). The proteins produced from the polyprotein in ORF gives rise to core, E1, E2, p7, NS2, NS3, NS4, NS4B, NS5A and NS5B shown in the figure below (Feld & Hoofnagle, 2005). Structural proteins include core, involved in the formation of nucleocapsid and E1 and E2, the two glycoproteins. Structural proteins are separated from non-structural one by means of p7, ion channel. The non-structural proteins include NS2-3, NS3, NS4A, NS4B, NS5A and NS5B (Penin, 2003; Reed & Rice, 2000).

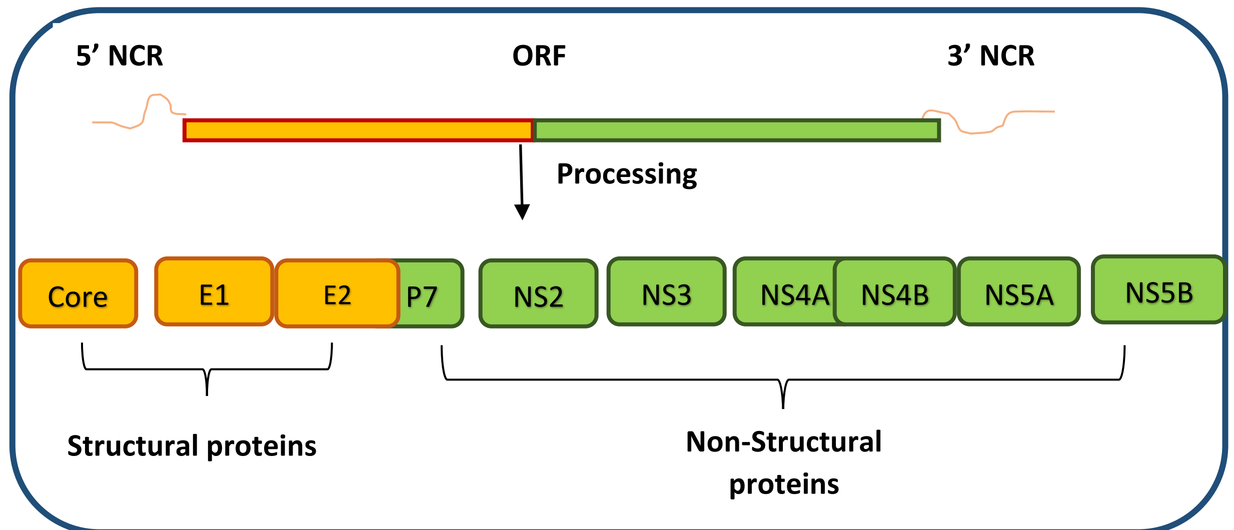

Figure 2.1 Genome organization of HCV (Duan et al., 2013)

2.5 Life Cycle of HCV

The whole mechanism of replication cycle is not fully understood, but the massive advancements on HCV have been made to date. Life cycle of HCV can be divided into several stages (figure 2.2), includes:

1. Binding and entry into host cell

2. Translation and processing

3. RNA Replication (packaging, assembly)

4. Release of virion.

2.5.1. Binding and Entry

Attachment of viral particle to host cell and then its entry has yet to be explained (Barth, Liang, & Baumert, 2006). The envelope glycoproteins i.e. E1 and E2 are necessary for infection, both plays a significant role in getting the virus inside the host cell on individual level. These glycoproteins represent the envelope glycoproteins of wild-type HCV and are essential for infection; each glycoprotein is involved in different steps of entry of the virus into host cell (Flint et al., 1999). Certain cell surface

molecules are essentially required, which facilitate the entry of the virus into the host cell. These surface molecules are expressed on hepatocytes, B Lymphocyte, dendritic cells, steroidogenic tissue, liver sinusoidal endothelial cells (Cormier et al., 2004). These surface molecules bind to E2 protein and protect E2 from the antibodies raised to inhibit the binding and entry. Envelope protein E2 attaches to human CD81, 25 kDa surface molecule, expressed on several hepatocytes and B-lymphocytes (Pileri et al., 1998). Other examples of these surface molecules may include, scavenger receptor class B type 1 (82 kDa), heparin sulfate and DC-SIGN and L-SIGN, members of C-type lectin family, have been reported to bind to HCV envelope protein E2 (Scarselli et al., 2002). After binding to host surface molecule, virus is endocytosed and membrane fusion takes place in endosome. The process of viral particle entry depends mainly on clathrin and pH.

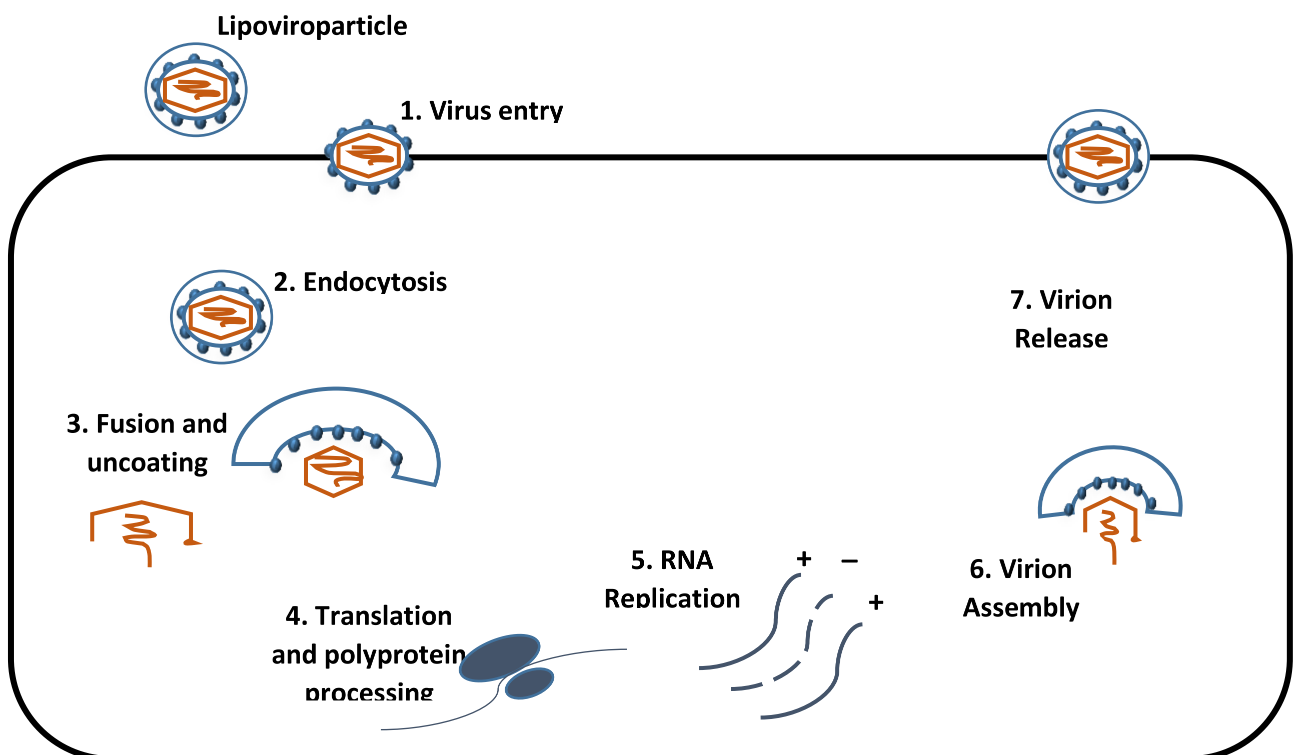

Figure 2.2 Life Cycle of HCV Adapted from (Duan et al., 2013)

2.5.2. Protein Translation and Processing

Once the virus has been internalized in the host cell, virus releases its RNA from capsid, and this initiates the process of protein translation. The Internal Ribosome Entry-Site (IRES), binds particularly to 40S ribosome and direct it to AUG start codon in viral mRNA and this results in the formation of 80S ribosomal subunit (Fraser & Doudna, 2007). Process of translation is followed by production of individual proteins from the cleavage of single large nascent polyprotein, cleavage is carried out by host proteases (figure 2.2). Four signal peptides are present in structural protein sequence, arranged in a tandem array. Host's peptidases cleave the polyprotein into mature proteins, i.e Core, two envelope glycoproteins proteins (E1, E2), and p7 (Wu, 2001).

2.5.3. RNA Replication

Host proteins for example cyclophilin, are involved in the regulation of viral replication process. RNA replication is carried out in non-structural viral proteins, bound to membrane. NS4B, is also involved in viral RNA replication (Gosert et al., 2003).

2.5.4. Packaging and Release of Virus

Basically, packaging of virions and release involves the process of budding of virions into endoplasmic reticulum following the secretory pathways. P7 has been reported in regulating the process of packaging and release of virions (Jones, Murray, Eastman, Tassello, & Rice, 2007).

2.6 HCV Genome Variability

The major hurdle in the study of HCV is the level of genetic diversity in HCV genome, which occurs between and within the genotypes. The main reason behind this genetic diversity seems to be that the viral RNA polymerase lacks proofreading function. HCV strains are divided into seven major genotypes 87 subtypes, on the basis of sequence similarity (Peter Simmonds et al., 1994). Nucleotide sequence difference between genotypes is found to be approximately 30-35% while that of within genotypes is 10-20% (Messina et al., 2015; Smith et al., 2014). One study which examined 90% of world's population, predicted that HCV genotype 1 being the most prevalent one and the second most prevalent being 3 accounting for 46.2% and 30.1% respectively (Messina et al., 2015). In addition to this, changes in genome occur at an individual level, this diversity is named as quasispecies. Along with Sanger Sequencing, next-

generation sequencing or pyrosequencing can be used for screening of multiple variants of HCV (Abdelrahman et al., 2015; Beerenwinkel & Zagordi, 2011). Viral genome diversity within an individual is the main hurdle in the development of vaccines and therapeutic drugs.

2.6.1 Immune Responses

2.6.1a Innate Immune Response

HCV has the capability of persistence by evading the human immune response. The innate immune system recognizes viral pathogen as non-self by pattern-recognition receptors by identifying the pathogen-associated molecular patterns (PAMP's) which are conserved in many microbes. PAMP's includes glycoproteins, proteoglycans, nucleic acid motifs or lipopolysaccharides (Akira, Uematsu, & Takeuchi, 2006). Recognition of non-self leads to activation of an antiviral mechanism by signaling the proteins. Retinoic acid inducible gene I (RIG-I)-like receptor (RIR), melanoma differentiation-associated proteins 5 (MDA-5), endosomal sensor toll-like receptor 3 (TLR3), TLR7, Protein Kinase R (PKR), proinflammatory cytokines and interferons, being part of innate immune mechanism play vital role in recognition of HCV. These components of innate immune system activate the kinases which leads to phosphorylation of signaling entities activates type I and III interferon and protein synthesis (Horner & Gale Jr, 2013; Jensen & Thomsen, 2012). RIG-I and MDA-5 attache to poly-uridine rich part of 3' UTR and their replication intermediate in HCV genome. TLR7 detects the ssRNA oligonucleotides, TLR3 senses dsRNA, PKR attaches to dsRNA (Chattergoon et al., 2014; Horner & Gale Jr, 2013; Jensen & Thomsen, 2012; Saito, Owen, Jiang, Marcotrigiano, & Gale Jr, 2008). The interferon inhibit replication of virus in infected cells and alarms the neighboring healthy cells to enter an antiviral condition (Li & Lemon, 2013). Innate immune response through co-stimulatory compounds, antigen presenting and cytokines activates the adaptive immune response as well.

2.6.2 Adaptive Immune Response

2.6.2a Humoral Immunity

The reason for delayed antibody production and their neutralizing efficacy is not known, immune system takes 7-10 weeks for producing anti-HCV antibodies after its

exposure and recognition while in case of other viruses' immune system responds in 5-7 days (Netski et al., 2005). The genetically humanized mice and chimeric mice were protected from established HCV infection, this clearing of infection by antibody activity is demonstrated through eight evidences (De Jong et al., 2014; Law et al., 2008). During acute phase of HCV, neutralizing antibodies resulted in clearance of HCV (Pestka et al.,2007). There is a possibility of viral clearance without any contribution of antibodies and cross-neutralizing antibodies may not provide protection against heterologous virus challenge (Barth et al., 2011; Cooper et al., 1999; Post et al., 2004).

2.6.2b Cellular Immunity

Cellular immune response helps in clearing the HCV, most importantly virus-specific CD8 T cells facilitates this clearance. CD 8 T cells recognize motif of 8-10 amino acids on antigen presenting cells (APC) attached to MHC I molecules. CD 8 T (virus-specific) cells are cytolytic in nature, once the pathogen is recognized via human leukocyte antigen (HLA) they give rise to interferon- γ, granzymes, perforin and Fas-FasL interaction. These CD 8 cells are produced within 6-8 weeks after infection (Cox et al., 2005; Thimme et al., 2002). This delayed response is due to delayed CD8 T cells priming (Shin et al., 2011). Immediate clearance of HCV is dependent upon immediate response by CD8 T cells and robust production of cytokines followed by epitope recognition (Abdel-Hakeem, Bédard, Murphy, Bruneau, & Shoukry, 2014; Badr et al., 2008; Ciuffreda et al., 2008; Lauer et al., 2004; Osburn et al., 2010). CD4 T cells, during early infection, attacks the epitopes generally regardless of symptoms of infection while at chronic state number of CD4 T cell responses and time of symptoms of infection shows inverse relation. Immediate clearance of HCV is dependent on the vigorous response of CD4 T cells. HCV progression from acute to chronic is not due to loss of general or broad CD4 T cell response but is the result of different mechanisms that cause contraction of responses (zur Wiesch et al., 2012). HCV RNA level was lower once CD4 T cell depletion occurred as compared to the primary infection stage, this study indicates that CD4 T cells are not the prime effector cells for clearing of HCV (Grakoui et al., 2003).

2.7 Viral Escape of Immune System

Immune response, in the case of HCV infection, is unproductive and leads to chronic infection in 75% of patients (Kellner, Derer, Valerius, & Peipp, 2014). Humoral and cellular immunity pressure during chronic HCV infection results in the selection of escape variants and continued viremia (Von Hahn et al., 2007). As RNA dependent RNA polymerase lacks the proofreading mechanism and results in the production of variants that have the ability to escape the immune response.

2.8 Genetic Association with HCV

Genetic factors are highly associated with protective function in a number of diseases. For instance, deletion of CCR5 receptor results in resistance to HIV infection (Steinberger, Andris-Widhopf, Bühler, Torbett, & Barbas, 2000). Similarly, in case of HCV, many genetic factors are associated with infection outcome. A single nucleotide polymorphism (SNP) in a gene that encodes type III interferon i.e. IL28B is associated with immediate resolution of HCV (Askar et al., 2009). Human Leukocyte Antigen (HLA) alleles of class I and II are significantly associated with HCV clearance. Among class I and II the most significant alleles includes; MHC class I alleles HLA-A*11:01, HLA-A*03, and HLA-Cw*01:02 and MHC class II alleles HLA-DRB1*03 and HLA DQB1*03:01 (Mina et al., 2015; Schmidt, Thimme, & Neumann-Haefelin, 2011; Thio et al., 2001).

2.9 HLA/KIR Genetic Variation and Association with HCV

In case of HCV, innate immune response specifically NK cells exhibit the most effector response and play important role in either viral control or, on contrary, viral infection persistence. In addition to these immune responses, a number of host gene factors are also associated with disease response (Jamil & Khakoo, 2011; Long & Rajagopalan, 2000). The function of NK cells majorly depends on germline encoded receptors, which identify and kill the infected cells (Vivier et al., 2008). These receptors are able to differentiate between self and non-self ligands and host molecules. NK cell receptors have three major families, namely: Killer Immunoglobulin like Receptors, CD94/NKG2 C-type lectin like receptors and Natural Cytotoxicity Receptors (NCR) (Lanier, 2005; Ljunggren & Kärre, 1990; Parham & Moffett, 2013). These receptors

bind to their specific ligands expressed on MHC. NK cells are either inhibitory or activating in nature, depending upon the type of Immune receptor tyrosine motif attached to the intracellular tail of receptor. This can be either Immunoreceptor tyrosine-based Inhibitory Motif (ITIM) or Immunoreceptor Tyrosine-base Activating Motif (ITAM) (Horowitz, Stegmann, & Riley, 2012). Inhibitory signals are stronger than the activating signals, this phenomenon is termed as 'tolerance' (Liao, Bix, Zijlstra, Jaenisch, & Raulet, 1991).Study of interaction between HLA Class 1 (HLA C1) and NK receptors is very essential in order to understand the mechanism that how NK are involved in order to limit the viral replication as well as the mechanism through which viral cells escape the immune response. HCV core proteins trigger expression of p53 which in turn leads to enhanced production of TAP1, this results in improved cell surface expression of HLA C1 on infected HCV cells (Herzer et al., 2003). This enhanced expression of HLA C1 is reduced or weakened by HCV, in case of ligands for inhibitory NK cell receptors. Presence of specific KIR/HLA compound genotype contributes a lot towards the clearance of HCV infection. Genetic variation HLA and KIR is due to the phenomenon of natural selection (Ahlenstiel, Martin, Gao, Carrington, & Rehermann, 2008; Salim I Khakoo et al., 2004).

2.9.1 Killer Cell Immunoglobulin like Receptors (KIRs)

KIRs are glycoprotein in nature and reside on the surface of NK cells; they are important part of innate immune system and exhibit response against pathogen (G. Alter et al., 2011; Ashouri, Farjadian, Reed, Ghaderi, & Rajalingam, 2009; Schleinitz et al., 2006). A plethora of receptors that modulate the mechanism of natural killer cells of innate immune response is the Killer Cell Immunoglobulin-like Receptors (KIRs). These receptors exhibit polymorphism which alter their function and cell-surface expressions (Bari et al., 2009; Carr, Pando, & Parham, 2005; Frazier, Steiner, Hou, Dakshanamurthy, & Hurley, 2013; Moesta et al., 2008; VandenBussche, Dakshanamurthy, Posch, & Hurley, 2006). KIR genes can be detected via Sequence Specific Primers (SSP) Polymerase Chain reaction (PCR) (PCR-SSP). The main hurdles include that molecular determinants of operational strength is still unpredictable and due to homologous nature of KIR genes, they exhibit low specificity (Gomez-Lozano & Vilches, 2004; Rajalingam, Gardiner, Canavez, Vilches, & Parham, 2001; Uhrberg et al., 1997; C Vilches, Castano, Gomez-Lozano, & Estefania, 2007; C

Vilches, Pando, Rajalingam, Gardiner, & Parham, 2000). Till dte total 16 KIR genes have been reported, out of these 16, 2 are pseudogenes namely, KIR 2DP1 and 3DP1, these receptors genes are mapped to chromosome 19q13.4 (Wilson et al., 2000). Out of these 14 KIR genes six are activating in nature while seven are inhibitory and only one, KIR2DL4 exhibit both activating as well as inhibitory strengths (Ashouri et al., 2009; Bao et al., 2013; Faure & Long, 2002). These KIR genes have been separated into two haplotype groups 'haplotype A & B'. Group A includes 2DL1, 2DL3-4, 3DL1-3, 2DS4, 2DP1 and 3DP1, KIR2DS4 the only activating receptor in group A, while group B contains KIR genes and its alleles, which exhibit genetic variation, these genes are not included in group A especially, KIR2DS1-3, 2DS5, 2DL2, 2DL5 and 3DS1. Group B contains more activating genes as compare to group A. All these genes are found in almost every population but their frequencies show variation (Ashouri et al., 2009; Parham, 2005). Number of individuals carry two haplotypes in varying manner e.g it can be, two copies of A haplotype i.e AA genotype, one copy of A haplotype and one of B haplotype (AB genotype), both copies of B haplotype(BB genotype), this variation is based on presence or absence of C4 and T4 gene clusters (C4 is the component of the complement system and T4 is binding protein of bacteriophage), (C4, KIR2DS2-2DL2-2DS3-2DL5; T4, KIR3DS1-2DL5-2DS5-2DS1). Further classification is done on the basis of presence or absence of C4 and T4 gene clusters in an individual. This classification has four subsets namely, CxT4, CxTx, C4Tx and C4T4. CxT4 indicates the absence of C4 and the presence of T4, CxTx shows absence of both C4 and T4 while C4T4 indicates the presence of both C4 and T4 and C4Tx shows presence of C4 and absence of T4 (Ashouri et al., 2009; McQueen et al., 2007; Single et al., 2007). In this study, we included KIR2DL1, KIR2DL2 and KIR2DS2.

2.9.2 Inhibitory Receptor KIR2DL1

Inhibitory Receptors downregulate the cytotoxicity of Natural Killer cells, as they promote the inhibition of cytokines. First crystal structure of KIR2DL1 was reported in 2001, bound to its ligand HLA-Cw4, specificity is regulated by lysine residue 80 of HLA-Cw4, they form a dimeric complex (Fan, Long, & Wiley, 2001). Among the inhibitory receptors, KIR inhibitory receptors are considered as the most potential one. They recognize MHC I molecules (Vivier & Anfossi, 2004). Inhibitory receptors are extremely polymorphic in nature e.g KIR2DL1 have 25 variants, defined till date

(Hilton et al., 2015). Their mechanism of signal transduction is based on the presence of Immunoreceptor tyrosine-based inhibitory motif (ITIM) present in cytoplasmic tail. Tyrosine entities are phosphorylated which results in recruitment of Src homology 2 (SH2) domain-containing protein tyrosine phosphatases e.g. SHP-2, SHP-1 or SHIP and generate inhibition signals (Barford & Neel, 1998; Burshtyn et al., 1996; Olcese et al., 1996). These SHP-1 and SHP-2 aids in abolishing the activating signals via dephosphorylating of protein tyrosine kinases. Some studies have also shown that ITIM can mediate the activating signals instead of the inhibitory signals and ITAM mediates the inhibition (Barrow & Trowsdale, 2006). KIR receptors show structural diversity, with longer cytoplasmic tail they exhibit the properties of inhibition by delivering negative signals when comes in contact with MHC molecules (Burshtyn et al., 1996; Colonna & Samaridis, 1995; Wagtmann et al., 1995). Mutations in KIR2DL1 and KIR2DL2 carry the sequences which have no effect regarding the HLA specificity, on the other hand, these mutations helped in enhancing the binding properties. From literature, it has been reviewed that few residues of KIR2DL1 (SRMT) when transferred into KIR2DL2 gene, these changes helped in improved binding capabilities. Structurally KIR2DL1 depicts strong binding affinities with MHC I molecules, even when its specific residues are introduced into KIR2DL2 genes e.g. residues 67-70 or 150-151 these residues enhance the binding properties(Winter & Long, 1997). A similar study was conducted on individuals from Africa and South Asia, they concluded that mutations in 2DL1 and 2DS1 resulted in 2DLv (2DL1*004) and this recombinant allele exhibit inhibitory effects and identify the same epitope as KIR2DL1. This study also revealed the fact that out of 608 populations only 13 individuals lacked KIR2DL1, these results indicate that KIR2DL1 is the frequent receptor but cannot be used as a marker for haplotype A (Norman et al., 2002; Shilling, Lienert-Weidenbach, Valiante, Uhrberg, & Parham, 1998). In 2003, Zhang *et al* also concluded in their study of the diversified allelic frequency of KIR genes that KIR2DL1 is the most frequent inhibitory receptors (Zhang et al., 2003). Khakoo *et al* in 2004 in his study hypothesized that the interaction between KIR2DL1 and HLA-C2 is stronger than the interaction between KIR2DL3 and its ligand HLAC-1, which prevents activation of NK cells and ultimately less production of cytokines and thus provide no protection from virus (Salim I Khakoo et al., 2004). In another study, genomic DNA from 100 randomly selected African Americans was amplified and their KIR2DL1 alleles were

identified by DNA sequencing. 97% of the individual carried KIR2DL1 locus, KIR2DL1*00302 being the most frequent account for 68% and KIR2DL1*00401, *00303, *006, *007 and *002 was also found in many individuals. In this study, 11 new alleles for KIR2DL1 were reported (L Hou, Chen, Jiang, Ng, & Hurley, 2010). Wang et al in 2011 investigated KIR gene frequency and haplotypes, their population size was vast and included individual from different geographical regions. This showed that genetic variation resulted in functional variations. They studied 15 KIR genes, among these 15 genes KIR2DL1 was among the one which exhibited a relationship between previous populations (H.-d. Wang et al., 2012). KIR2DL1 characteristics and allelic frequency was also checked in Chinese Han population, they concluded among no. of alleles the most frequent one is the KIR2DL1*00302. They suggested through genotyping that KIR genes would be helpful in the prediction of stem cell transplantation and donor selection (M. Wang et al., 2013).

In a study, Hilton and Norman concluded that there exists a very dominant interaction between C2 epitope of HLA-C and KIR2DL1. From their study, they confirmed that they show genetic diversity and concluded that the population of southern Africa represents their forefathers as their KIR and HLA genes reflect their genetic qualities. Although the allelic frequencies differ among neighboring areas and due to point mutation different alleles arose through gene flow. They justified that mutation of KIR2DL1*001 resulted in KIR2DL1*022 and this resulted in a gain of identification of C1 instead of C2. Their study also enlightens the mutations in KIR2DL1*012 which mutated to KIR2DL1*026, this mutation results in early termination of translation (Hilton et al., 2015). KIR2DL1, found to be one of the most polymorphic gene and Caiyong et al found in their study that these genes are helpful in forensic and phylogenetic studies (Yin et al., 2017). Recently in December 18, the study was conducted to determine the role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. KIR and HLA play an important role in determining the compatibility of donor and recipient, that either the grafting is possible or not. KIR2DL1 identifies an epitope shared by the reciprocal group 2 HLA-C allotypes Cw1, 3, 7, and 8. This study involved the indirect immunofluorescence with primary monoclonal antibodies along with secondary fluorochrome-conjugated goat anti-mouse Ig and flow cytometry determined NK cell phenotypes, NK cell clones were

identified using an anti-CD16 MoAb and expression of group 2 HLA-C alleles (KIR2DL1) was determined with MoAbs GL183 (Heatley et al., 2018).

2.9.3 Activating Receptor KIR2DS2

Killer Immunoglobulin-like Receptor, with two Ig domains and short cytoplasmic tail 2, are glycoproteins produced by NK cells. In 1995, KIR2DS2 cDNA was isolated from the screening of Natural Killer cell cDNA library of KIR2DL3 probe (Wagtmann et al., 1995). This transmembrane protein was named as clone 49 (CL49). Then in 1996, Biassoni et al, isolated KI2DS2 cDNA by Real-Time PCR of cDNA expressing p50 instead of p58 (KIR2DL), they termed it as 183ACTI (R Biassoni et al., 1996). Unlike KIR2DL1, KIR2DS2 carry a charged Lysine residue in transmembrane and the tail lacks inhibitory motif. KIR2DS2 is thought to be highly homologous to KIR2DL3 and KIR2DL2 (Valés-Gómez, Reyburn, Erskine, & Strominger, 1998; Winter et al., 1998). The very first crystal structure of activating KIR2DS2 gene (CD158j) at 2.3Å was reported in 2003, by Xavier et al (Saulquin, Gastinel, & Vivier, 2003). Binding specificities were tested by use of tetrameric probes (Altman et al., 1996). KIR2DS2 exhibit low level of binding to HLA-C, this pattern of binding allows the receptor-ligand binding better production of NK cells and cytokine activity (Stewart et al., 2005). Low level of binding of KIR2DS2 is due to the displacement of a glutamine residue at position 71, which hinders or protect from creation of H-bond with the nitrogen of peptide at residue 8 (Moesta et al., 2010). KIR2DS2 exhibit activation signals with DAP12 protein (DNAX-activation protein) of 12 kDa and KIR2DS2 Ab cross-linking results in phosphorylation of this protein entity (Lanier, Corliss, Wu, Leong, & Phillips, 1998). Recently, it has been reported that KIR2DS2 bind with HLA-A*1101, the same allele to which KI3DL2 and KIR2DS4 bind with, from HLA class I (Graef et al., 2009; Liu, Xiao, Ko, Shen, & Ren, 2014). A number of studies have indicated a weak interaction between KIR2DS2 and its ligand HLA-C1 molecule, similarly Moesta et al, also observed the very same pattern of binding between KIR2DS2 and HLA-C*16:01 and demonstrated that it transduce activating signals. Whereas Stewart et al, reported in his study that KIR2DS2 binds with low strength to EBV infected B cell and not to primary B cells from C-1 (not C-2) donor, this suggest the hypothesis that KIR2DS2 performs its function in inflammatory conditions (Moesta et al., 2010; Stewart et al., 2005). In another study, it has been observed that decreased

frequency of KIR2DS2 in patients who have cleared HCV infection (N. Paladino et al., 2007). In the same year another study carried out by Vasconcelos et al, they also reported that the absence of KIR2DS2 is associated with HCV infection clearance (N Paladino et al., 2007). KIR2DS2 is considered as very crucial factor that helps in achieving clearance of viral infection by boosting the NK cell activity. In 2009, a study by Medhat proposed that there is an association between lack of KIR2DS2 and treatment failure. They reported that lack of KIR2DS2 resulted in the failure of Peg/RBV treatment in recurrent HCV patients after LT (J.-H. Yen et al., 2001). KIR2DS2 show variation, even a single substitution creates a huge difference in their ligand specificity and swap from C-1 to C-2 MHC or vice versa. A similar study was done which revealed new alleles of KIR2DS2, KIR2DS2*006 which differs from KIR2DS2*00102 due to a single residue change and created asp from Asn, at codon 250. The very same codon is reported in KIR2DS2*005 allele (Artavanis-Tsakonas et al., 2003; LiHua Hou et al., 2009). The study conducted on the Korean population reported KIR2DS2 as the factor that helps in the clearance of HCV infection, at least in Korean population. They reported that KIR2DS2 enhance both innate as well as adaptive immune response against HCV (Askar et al., 2009). Nozawa et al, reported decreased SVR results in patients with KIR2DS2 and KIR2DL2 (Nozawa et al., 2013). The interaction between KIR2DS2 and HLA-C1 is associated with onset age of Hepatocellular Carcinoma (HCC) in male patients (Pan et al., 2013). Gardiner et al, in their research also reported that KIR2DS2 transduce activating signals and binds to HLA-C1 (Gardiner, 2015). KIR2DS2 & KIR2DL2 are associated with an increased risk of HCV infection only in males, this pattern is not observed in females, this is may be due to the genetic or hormonal factors (Kuśnierczyk et al., 2015).KIR2DS2 identify conserved region of HCV 2 RNA helicases by MHC class I. KIR2DS2 resulted in NK cell activation, when Naiyer et al, presented that conserved peptide LNSPSVAATL, it binds to HLA-C*0102. They reported that KIR2DS2 is capable of clearing HCV viral infection and dengue as well, in the context of HLA-C*0102 allele (Mohammed M. Naiyer et al., 2017). The association between KIR2DS2 and HCV clearance or progression is not at all consensual (Vasconcelos et al., 2013).

2.10 Objectives

The objectives of designed study were to investigate the host genetic factors that help in predicting the treatment, on the basis of certain host gene factors, before starting the treatment. In order to achieve this aim, we formulated these objectives:

1. To determine the frequency of Human Leukocyte Antigen-C and Killer Immunoglobulin Receptor gene variants in HCV diagnosed patients of Pakistani origin.

2. To predict the plausible effect of these variants on treatment response.

Chapter 3

Material and Methods

3.1 Sample Collection

Study approval was taken from Institution review board (IRB) as well hospital ethical board. Blood samples positive for Hepatitis C virus were collected in 5 ml EDTA tubes with 5ml syringes. Blood was collected from patients who had received PEGylated interferon and Ribavirin doses. Samples were collected from Khyber Pakhtunkhwa, province of Pakistan. Samples were stored at -20 °C.

3.1.1 Control Sample Collection

Blood samples from 30 healthy Volunteers were collected from National University of Sciences and Technology (NUST), H-12 Campus, Islamabad and processed as a control.

3.2 Genomic DNA Extraction from Whole Blood

750µl of blood sample was withdrawn in 1.5 ml of eppendorf tube and 750µl of Solution-A (Table 3.1) was added to it. To mix the solutions thoroughly, eppenfof tube was inverted 4-6 times and kept at room temperature for 20 minutes. The mixture was centrifuged at 13000 rpm for 1 minute in centrifuge machine (Spectrafuge ™24D Digital-Microcentrifuge, Labnet International-Inc.). After Centrifugation of mixture, the supernatant was discarded whereas, nuclear pellet settled at the bottom was re-suspended (by beating the tube) in 400µl of solution A. Mixture was centrifuged again at 13000 rpm for 60 seconds. For smaller pellet, the mixture was again re-suspended in 200µl of solution-A (optional). The supernatant was discarded again and the pellet was re-suspended in 400µl of Solution B (Table 3.1), 15µl of 20% SDS and 5µl of Proteinase K mixture was incubated overnight at 37 °C. Following the incubation, 500µl of an equal volume of a fresh mixture containing 250µl of solution C (Table 3.1) and 250µl of solution D (Table 3.1) was added. Mixture was centrifuged for 10 minutes at 13000 rpm. Following centrifugation, an upper aqueous phase was collected in new

the new Eppendorf tube. 500µl of solution D (Table 3.1) was added to the tube, centrifuged the mixture for 10 minutes at 13000 rpm. Upper aqueous phase was recollected in new eppendorf. By adding 55µl of sodium acetate (3M, pH 6) and 500µl of chilled isopropanol DNA was precipitated, tube was inverted several times. Mixture was centrifuged the for 10 minutes at 13000 rpm, the supernatant was discarded and 200µl of chilled ethanol was added and centrifuged for 7 minutes at 13000 rpm, later on ethanol was discarded. This step was repeated to wash the pellet properly. DNA pellet was dried in an incubator for 45 minutes at 37 °C. Precipitated DNA was then dissolved in 70µl of Nuclease-free water and was stored in refrigerator at -20 °C.

Table 3.1 Composition of DNA extraction solutions

Solution A	Solution B	Solution C	Solution D
0.32M Sucrose 10mM Tris (pH 7.5) 5Mm $MgCl_2$ Autoclave & add TritonX 100 1% v/v	10Mm Tris (pH 7.5) 5Mm $MgCl_2$ Autoclave & add TritonX 100 1% v/v 10 mM Tris(pH 7.5) 400Mm NaCl 2mM EDTA (pH 8)	Phenol	Chloroform & Iso-amyl-alcohol (24:1)

3.2.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was done to examine the DNA product. 0.8% agarose gel was prepared in 50ml of 1X TAE buffer. To stain the DNA product 5µl of ethidium bromide (0.5µg/ml final concentration) was added to the gel. A DNA sample was loaded on a gel along with bromophenol blue. Electrophoresis was carried out at 100 volts for 30 minutes in 1X TAE buffer. Bands that appeared on gel were visualized using UV Transilluminator (Biometra, Goettingen, Germany).

3.2.2 DNA Quantification

Obtained DNA was quantified at 260nm using Nano drop (Titertek Berthold) than dilutions were prepared at 30ng/µL for PCR amplification.

3.3 Polymerase Chain Reaction (PCR) Amplification for Detection of Gene Variants

3.3.1 Primer Designing

For PCR amplification of KIR2DL1, KIR2DS2 and HLA-C sequence specific primers were designed by retrieving the specific human genes' sequence from NCBI nucleotide database. List of primers and their specific PCR product size along with melting temperature is given in (table 3.2).

Table 3.2 Primers information

Primer	**Sequence**	**Product Size**
KIR2DL1 Forward primer 1	5'-GTTGGTCAGATGTCATGTTTGAA-3'	146
Reverse primer 1	5'-GGTCCCTGCCAGGTCTTGCG-3'	
Forward primer 2	5'-TGGACCAAGAGTCTGCAGGA-3'	330
Reverse Primer 2	5'-TGTTGTCTCCCTAGAAGACG-3'	
KIR2DS2 Forward primer 1	TTCTGCACAGAGAGGGGAAGTA	175
Reverse primer 1	GGGTCACTGGGAGCTGACAA	
Forward primer 2	CGGGCCCCACGGTTT	240
Reverse Primer 2	GGTCACTCGAGTTTGACCACTCA	
HLA-C C1/C2 Forward Primer	GAGGTGCCCGCCCGGCGA	332
C1 Reverse Primer	CGCGCAGGTTCCGCAGGC	332
C2 Reverse primer	CGCGCAGTTTCCGCAGGT	332

3.3.2 PCR Amplification for Detection of KIR2DL1

Allele sequence specific primers were designed for detection and calculation of frequency of KIR2DL1 among the HCV patients particularly in KPK.

Table 3.3 Primer information for KIR2DL1

Primer	**Sequence**	**Product Size**
Forward primer 1	5'-GTTGGTCAGATGTCATGTTTGAA-3'	146
Reverse primer 1	5'-GGTCCCTGCCAGGTCTTGCG-3'	
Forward primer 2	5'-TGGACCAAGAGTCTGCAGGA-3'	330
Reverse Primer 2	5'-TGTTGTCTCCCTAGAAGACG-3'	

PCR reaction was carried out at following conditions;

10X Taq Buffer---1.25 µl

2.5 mM $MgCl_2$---1.5 µl

2 mM dNTPs---1.25 µl

Forward Primer--1 µl

Reverse Primer---1 µl

Nuclease Free Water------------------------------------4.3 µl

Taq Polymerase--0.2 µl

DNA Template---2 µl

Total Volume---12.5 µl

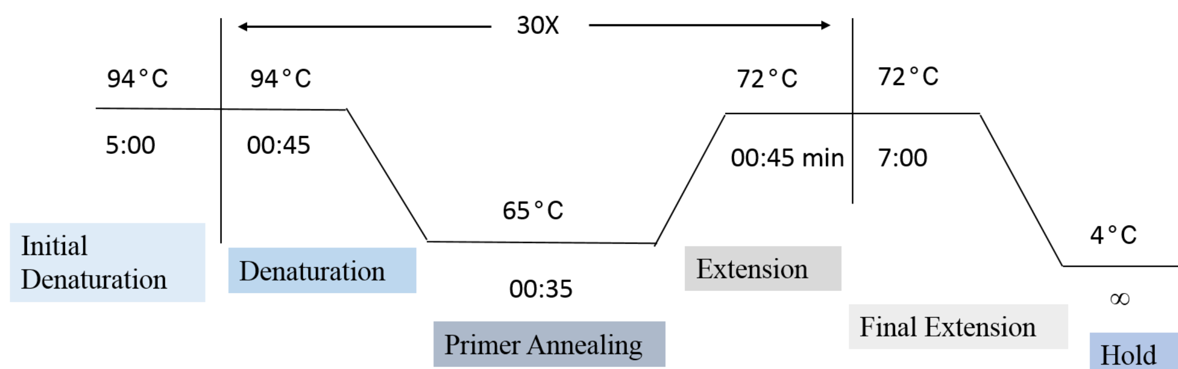

Figure 3.1 Diagrammatic presentation of PCR profile for amplification of KIR2DL1

The amplified PCR products were analyzed in 2.5% TAE-agarose gel for 30 minutes at 100 volts. As a reference 50bp DNA ladder (Thermoscientific) was used.

3.3.3 PCR Amplification for Detection of KIR2DS2

Allele specific primers were designed for detection and calculation of frequency of KIR2DL2 in patients diagnosed with HCV in KPK.

Table 3.4 Primer information for KIR2DS2

Primer	Sequence	Product Size
Forward primer 1	TTCTGCACAGAGAGGGGAAGTA	175
Reverse primer 1	GGGTCACTGGGAGCTGACAA	
Forward primer 2	CGGGCCCCACGGTTT	240
Reverse Primer 2	GGTCACTCGAGTTTGACCACTCA	

PCR reaction was carried at following conditions:

10X Taq Buffer--1.25 µl

2.5 mM $MgCl_2$--1.5 µl

2 mM dNTPs--1.25 µl

Forward Primer---1 µl

Reverse Primer--1 µl

Nuclease Free Water----------------------------------4.3 µl

Taq Polymerase---0.2 µl

DNA Template---2 µl

Total Volume--12.5 µl

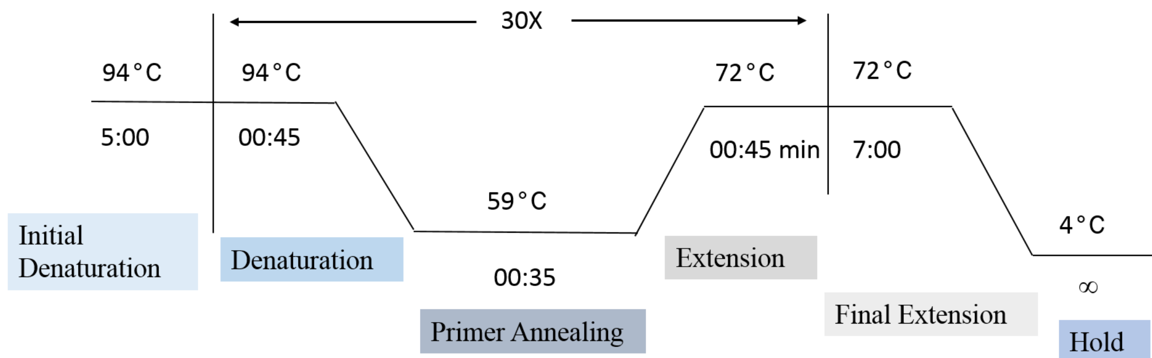

Figure 3.2 Diagrammatic presentation of PCR profile for amplification of KIR2DS2

The amplified PCR products were analyzed in 2.5% TAE-agarose gel for 30 minutes at 100 volts. As a reference 50 bp DNA ladder (Thermoscientific) was used.

3.3.4 PCR Amplification for Detection of HLA-C

Allele specific primers were designed for detection and calculation of frequency of HLA C1/C2 among the HCV patients in KPK.

Table 3.5 Primer information for HLA-C

Primer	Sequence	Product Size
C1/C2 Forward Primer	GAGGTGCCCGCCCGGCGA	332
C1 Reverse Primer	CGCGCAGGTTCCGCAGGC	332
C2 Reverse primer	CGCGCAGTTTCCGCAGGT	332

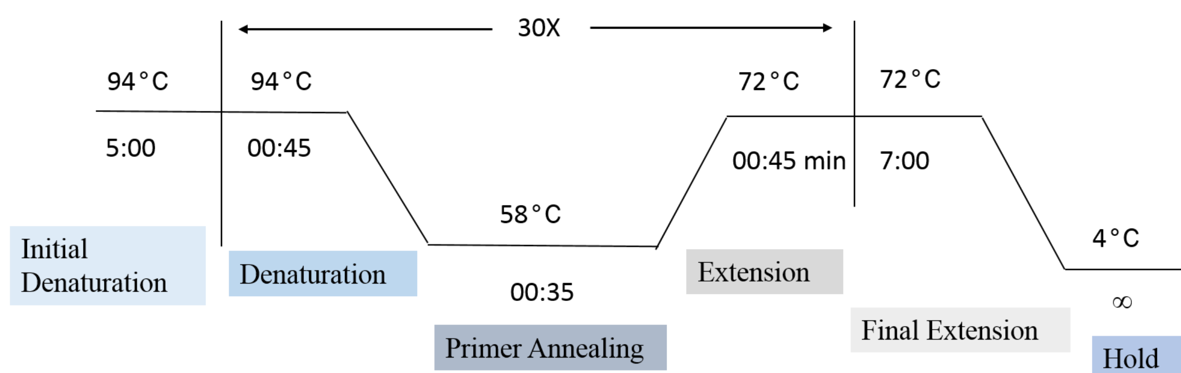

Figure 3.3 Diagrammatic presentation of PCR profile for amplification of HLA-C

The amplified PCR products were analyzed in 2.5% TAE-agarose gel for 30 minutes at 100 volts. As a reference 50 bp DNA ladder (Thermoscientific) was used.

PCR reaction was carried out at following conditions,

10X Taq Buffer---1.25 µl

2.5 mM $MgCl_2$---1.5 µl

2 mM dNTPs---1.25 µl

Forward primer--1 µl

Reverse Primer--1 µl

Nuclease Free Water----------------------------------4.3 µl

Taq Polymerase--0.2 µl

DNA Template---2 µl

Total Volume--12.5 µl

3.3.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was done to examine the result of PCR product. 2.5% gel was prepared in 50ml of TAE buffer. To stain the DNA, 5µl of ethidium bromide was added to gel. PCR products were loaded into wells along with bromophenol dye, electrophoresis gel was run at 100 volts for 30 minutes in 1X TAE buffer. Results of electrophoresis were analyzed in UV Tranilluminator.

3.4 Statistical Analysis

Graph Pad Prism for windows (Version 5.01) was used for statistical analysis. Chi-square test was applied for association analysis, Odd ratio and Relative risk ratio was calculated to determine the strength of association.

Chapter 4

RESULTS

4.1 DNA Extraction and PCR Amplification

Blood samples were collected from 102 HCV diagnosed patients from Lady Reading Hospital (LRH) and Khyber Teaching Hospital (KTH), Peshawar. Then genomics DNA was extracted using phenol-chloroform method. Genomic DNA was analyzed on 1% agarose gel. Two primer sets were used for one gene variant and PCR products were analyzed on 2.5% gel.

4.2 Gender Distribution

102 HCV diagnosed patients from KPK, participated in this study, out of these 102 patients 43 were females and 59 were males.

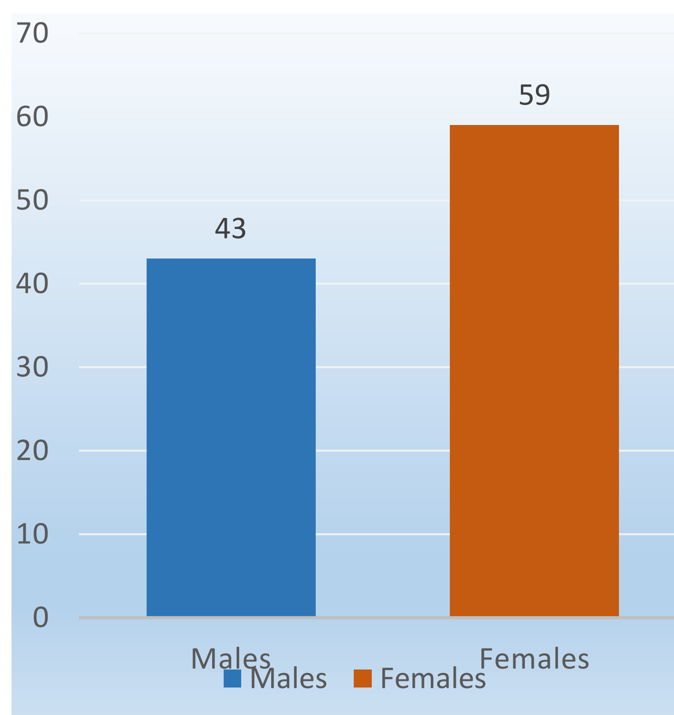

Figure 4.1 Gender distribution of HCV cases

4.3 Inhibitory Receptor KIR2DL1

F1R1 and F2R2 are the two alleles of KIR2DL1 gene , we genotyped these alleles in 102 KPK patients. Out of 102 patients 55 were the carriers of F1R1, 48 had F2R2 while 36 showed neither of the gene. 19 patients had homozygous F1R1-F1R1 alleles, homozygous F2R2-F2R2 allele was present in 12 patients and heterozygous F1R1-F2R2 was observed in 35 patients (Table 4.1).

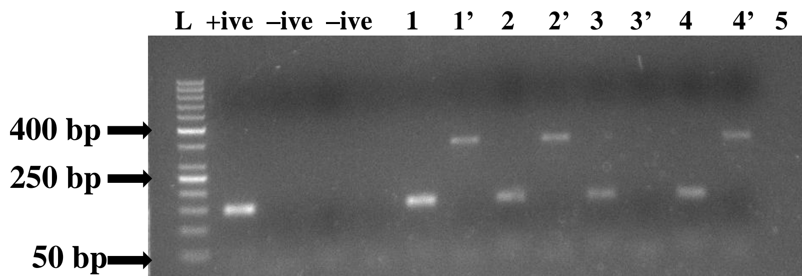

Figure 4.2 Representative gel of F1R1 and F2R2, gene variants of KIR2DL1 (1 indicates F1R1 allele and 1' F2R2 allele). Amplicon size for F1R1 allele is 146bp and F2R2 allele is 330bp. L denotes 50bp ladder. Lane 1 represents DNA ladder, lane 2 shows positive control, 3 & 4 represents the negative controls. Lanes labelled as 1,2,3,4 & 5 presents the F1R1 allele of KIR2DL1 while lane 1', 2', 3', and 4' represents F2R2 allele of KIR2DL1.

Table 4.1 Frequency of F1R1 and F2R2 alleles of KIR2DL1 in KPK patients

Gene Variant	Cases (102)	Control (30)
F1R1	55 (53.9%)	9 (30%)
F2R2	48 (47.0%)	7 (23.3%)
F1R1-F1R1	19 (18.6%)	7 (23.3%)
F2R2-F2R2	12 (11.7%)	5(16.6%)
F1R1-F2R2	35 (34.3%)	2 (6.66%)
Un-typed	36 (35.2%)	16 (53.3%)

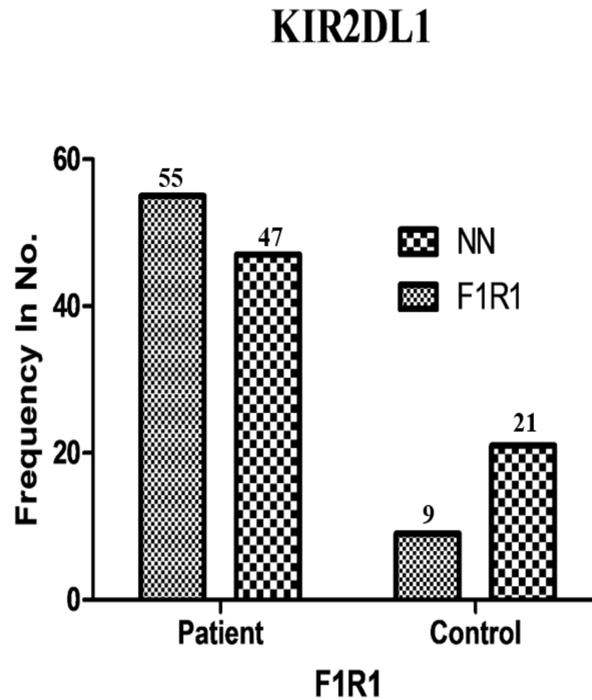

Figure 4.3 Control vs. F1R1 allele of KIR2DL1

This figure shows that 55 patients carried F1R1 allele out of 102 HCV diagnosed patients and 47 patients did not carry this allele, denoted by NN. In case of healthy individual (controls) 9 out of 30 were the carriers of F1R1 allele. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 5.311 (df, 1), the p-value was 0.0212 (P-value < 0.05). The odd ratio (OR) was 2.730 (CI. at 95% was 1.141 to 6.535) & relative risk (RR) was 1.797 (CI. at 95% 1.011 to 3.196). The values of OR & RR predicted that F1R1 allele of inhibiting receptor is associated with disease, so from these values it can be concluded that the carriers of this allele has a risk of disease progression.

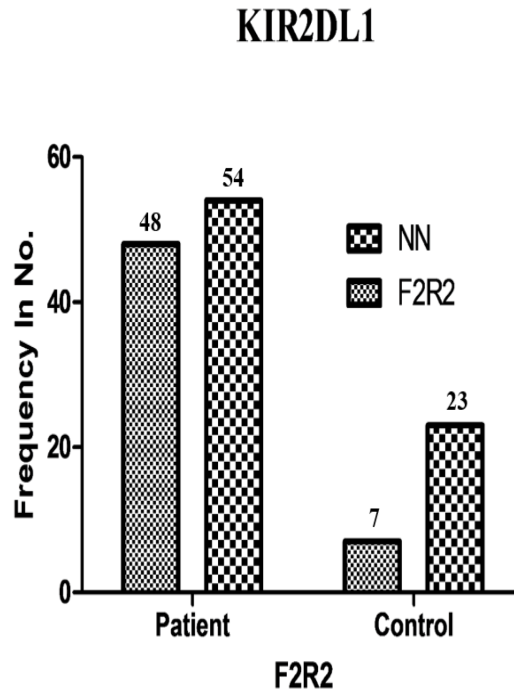

Figure 4.4 Control vs. F2R2 allele of KIR2DL1

This graph shows that 48 patients carried F2R2 allele out of 102 HCV diagnosed patients and 54 patients did not carry this allele, denoted by NN. In case of healthy individual (controls) 7 out of 30 were the carriers of F2R2 allele. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 5.369, (df, 1), the p-value was 0.0205 (P-value < 0.05). The odd ratio (OR) was 2.921 (CI. at 95% was 1.151 to 7.412) & relative risk (RR) was 2.017 (CI. at 95% 1.021 to 3.984). The values of OR & RR predicted that F2R2 allele of inhibiting receptor is associated with disease, so from these values it can be concluded that the carriers of this allele has a risk of disease progression.

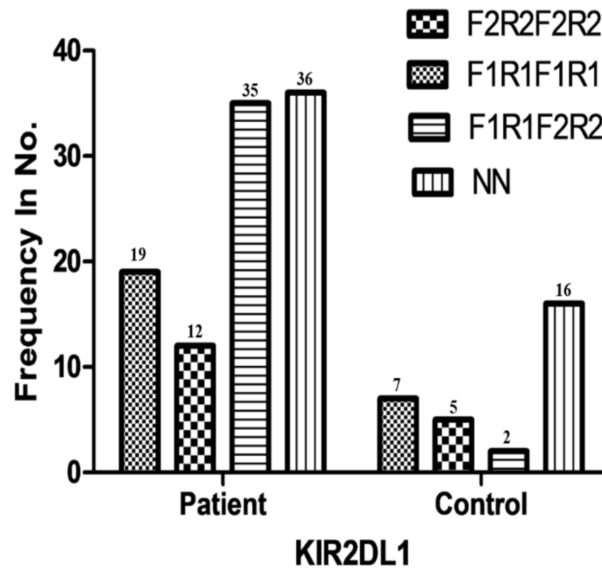

Figure 4.5 Frequency distribution of F1R1-F1R1, F1R1-F2R2 and F2R2-F2R2 alleles of KIR2DL1

This graph represnts that 19 patients carried F2R2-F2R2 allele and 12 patients had F1R1-F1R1 allele, 35 carried heterozygous F1R1-F2R2 genotype and 36 did not carry any of the combination, denoted by NN. In case of healthy individual (controls) out of 30, 7 were the carrier of F1R1-F1R1 genotype, 5 individuals carried F2R2-F2R2 and 2 had heterozygous F1R1-F2R2 genotype while 16 did not carry this gene variant. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 8.930, (df, 3), the p-value was 0.0302 (P-value < 0.05).The chi-square value predicted that KIR2DL1, inhibiting receptor is associated with disease, so it can be concluded that the carriers of KIR2DL1 has a risk of disease progression and the p-value suggested that these findings are significant.

4.4 ACTIVATING RECEPTOR KIR2DS2

F1R1 and F2R2 are the two gene variants of KIR2DS2, we genotyped this gene in 102 KPK patients. Out of 102 patients 40 were the carriers of F1R1 gene variant, 30 had F2R2 while 32 showed neither of the gene variant. 21 patients had homozygous F1R1-F1R1 alleles, homozygous F2R2-F2R2 allele was present in 27 patients and heterozygous F1R1-F2R2 was observed in 22 patients (Table 4.2).

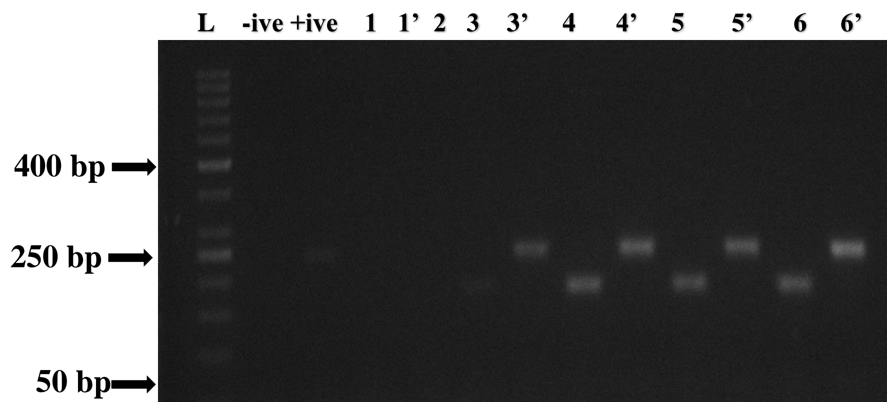

Figure 4.6 Representative gel of F1R1 and F2R2 two alleles of KIR2DS2 (1 indicates F1R1 allele and 1' F2R2 allele).). Amplicon size for FIR1 allele is 157 bp and F2R2 is 240 bp. L denotes 50bp DNA ladder. Lane 1 presents DNA ladder, lane 2 & 3 shows negitive & positve controls, respectively. The lanes denoted as 1,2,3,4,5 and 6 represent F1R1 allele and 1',2',3',4',5' and 6' represent F2R2 allele of KIR2DS2.

Table 4.2 Frequency of F1R1 and F2R2 alleles of KIR2DS2 in 102 HCV patients from KPK

Gene Variant	Cases (102)	Control (30)
F1R1	40 (39.2%)	18 (06%)
F2R2	30 (29.4%)	15 (50%)
F1R1-F1R1	21 (20.5%)	4 (13%)
F1R1-F2R2	22 (21.5%)	10 (33.33%)
F2R2-F2R2	27 (26.5%)	1 (0.1%)
Un-typed	32 (31.3%)	13 (43.3%)

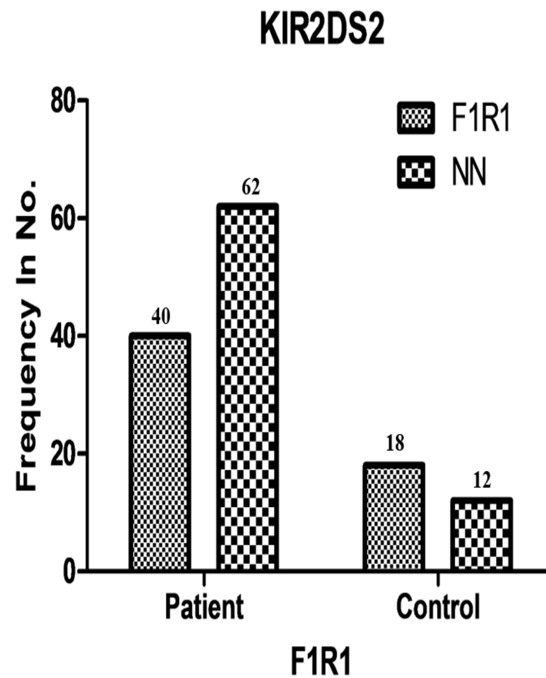

Figure 4.7 Control vs. F1R1 allele of KIR2DS2

This graph shows that 40 patients carried F1R1 allele out of 102 HCV diagnosed patients and 62 patients did not carry this allele, denoted by NN. In case of healthy individual (controls) 18 out of 30 were the carriers of F1R1 allele. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 4.065 (df, 1), the p-value was 0.0438 (P-value < 0.05). The odd ratio (OR) was 20.4301 (CI. at 95% was 0.1872 to 0.9881) & relative risk (RR) was 0.6536 (CI. at 95% 0.4473 to 0.9550). The values of OR & RR predicted that F1R1 allele of activating receptor is not associated with disease, so from these values it can be concluded that the carriers of this allele has the ability to clear the virus. KIR2DS2 help in enhancing the immune response against virus and helps in viral clearance.

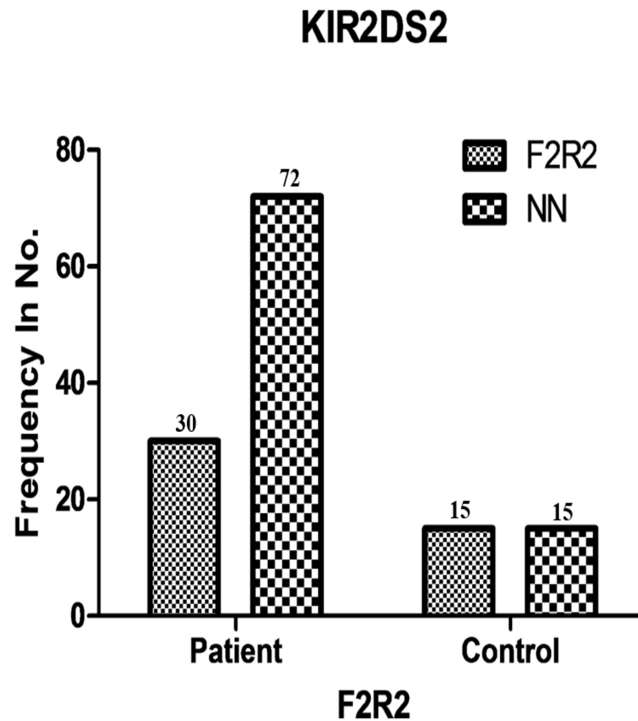

Figure 4.8 Control vs. F2R2 allele of KIR2DS2

This graph shows that 30 patients carried F2R12 allele out of 102 HCV diagnosed patients and 72 patients did not carry this allele, denoted by NN. In case of healthy individual (controls) 15 out of 30 were the carriers of F2R2 allele. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 4.373 (df, 1), the p-value was 0.0365 (P-value < 0.05). The odd ratio (OR) was 0.4167 (CI. at 95% was 0.1811 to 0.9584) & relative risk (RR) was 0.5882 (CI. at 95% 0.3686 to 0.9388). The values of OR & RR predicted that F2R2 allele of activating receptor is not associated with disease, so from these values it can be concluded that the carriers of this allele has the ability to clear the virus. KIR2DS2 help in enhancing the immune response against virus and helps in viral clearance.

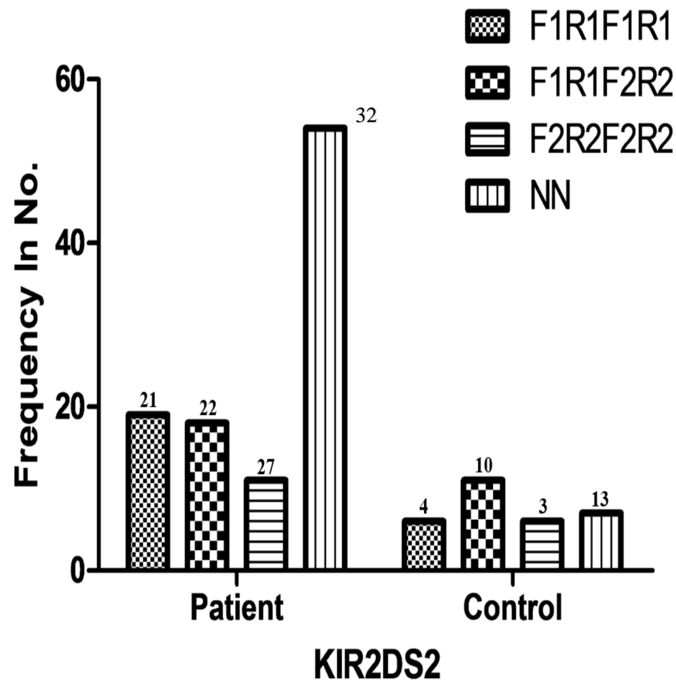

Figure 4.9 Frequency distribution of F1R1-F1R1, F1R1-F2R2 and F2R2-F2R2 allele

This graph shows that 21 patients carried homozygous F1R1-F1R1 allele and 22 patients had heterozygous F1R1-F2R2 allele, 27 carried homozygous F2R2-F2R2 genotype and 32 did not carry any of the combination, denoted by NN. In case of healthy individual (controls) out of 30, 4 were the carrier of F1R1-F1R1 genotype, 10 individuals carried F1R1-F2R2 and 3 had homozygous F2R2-F2R2 genotype while 13 did not carry this gene variant. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 9.766, (df, 3), the p-value was 0.0201 (P-value < 0.05).The chi-square value predicted that KIR2DS2, activating receptor is not associated with disease, so it can be concluded that the carriers of KIR2DS2 has the ability to clear the virus and the p-value suggested that these findings are significant.

4.5 Human Leukocyte Antigen-C (HLA-C)

C1 and C2 are the two gene variants of HLA-C, we genotyped this gene in 102 KPK patients.Patients who typed solely C1 was denoted as homozygous C1 allele (genotype C1C1), while types with C2 solely are designated as C2 allele and genotype C2C2, remaining were designated as heterozygous C1-C2 genotype. Out of 102 patients, 48 were the carriers of C1 gene variant, 44 had C2 while 28 showed neither of the gene variant. 22 patients had homozygous C1-C1 alleles, homozygous C2-C2 allele was present in 27 patients and heterozygous C1-C2 was observed in 25 patients (Table 4.3).

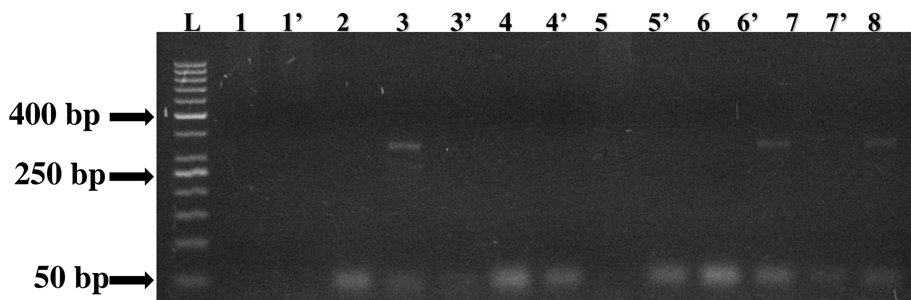

Figure 4.10 Representative gel of C1 and C2 gene variants of HLA-C (1 indicates C1 allele and 1’ C2 allele). Amplicon size for both alleles is 332bp. L denotes 50bp ladder.

Table 4.3 Frequency of C1 and C2, gene variants of HLA-C in 102 HCV infected patients from KPK 8

Gene Variant	Cases (102)	Control (30)
C1	48 (47.05%)	21 (70%)
C2	44 (43.13%)	22 (73.3%)
C1C1	22 (21.5%)	12 (40.0%)
C1C2	25 (24.5%)	2 (6.67%)
C2C2	27 (26.5%)	2 (6.67%)
Un-typed	28 (27.4%)	14 (46.6%)

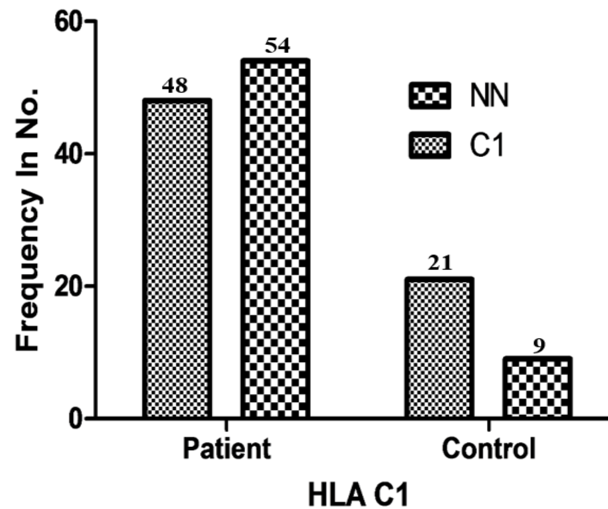

Figure 4.11 Control vs. HLA-C1 allele of HLA-C

This graph shows that 48 patients carried C1 allele out of 102 HCV diagnosed patients and 54 patients did not carry this allele, denoted by NN. In case of healthy individual (controls) 21 out of 30 were the carriers of C1 allele. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 4.890 (df, 1), the p-value was 0.0270 (P-value < 0.05). The odd ratio (OR) was 0.3810 (CI. at 95% was 0.1592 to 0.9116) & relative risk (RR) was 0.6723 (CI. at 95% 0.4921 to 0.9184). The values of OR & RR predicted that C1 allele of HLA-C is not associated with disease, so from these values it can be concluded that the carriers of this allele are able to clear the virus.

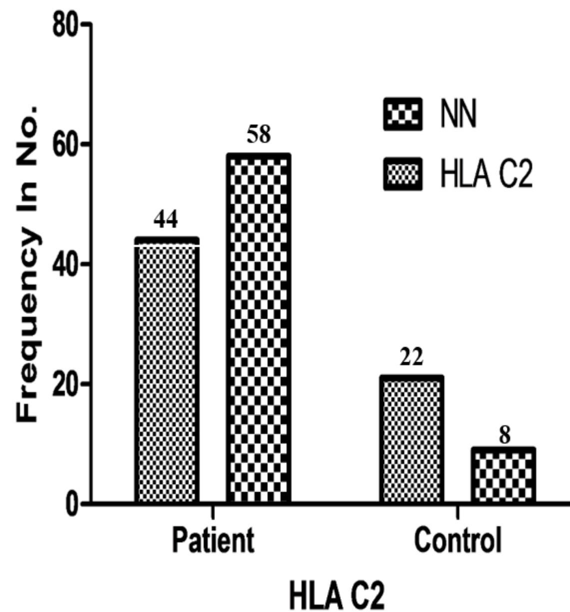

Figure 4.12 Control vs. HLA-C2 allele of HLA-C

This graph shows that 44 patients carried C2 allele out of 102 HCV diagnosed patients and 58 patients did not carry this allele, denoted by NN. In case of healthy individual (controls) 22 out of 30 were the carriers of C2 allele. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 8.455 (df, 1), the p-value was 0.0036 (P-value < 0.05). The odd ratio (OR) was 0.2759 (CI. at 95% was 0.1122 to 0.6780) & relative risk (RR) was 0.5882 (CI. at 95% 0.0.4313 to 0.8022). The values of OR & RR predicted that C2 allele of HLA-C is not associated with disease, so from these values it can be concluded that the carriers of this allele are able to clear the virus.

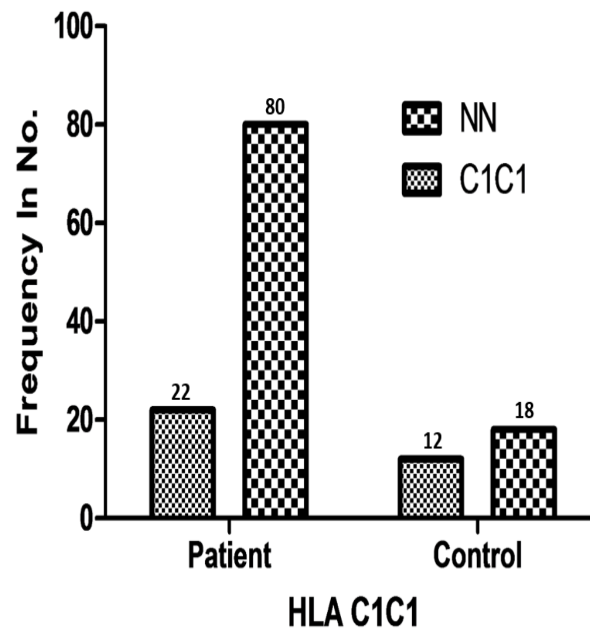

Figure 4.13 Control vs. HLA-C1C1 allele of HLA-C

This graph shows that 22 patients carried homozygous C1C1 genotype out of 102 HCV diagnosed patients and 80 patients did not carry this genotype, denoted by NN. In case of healthy individual (controls) 12 out of 30 were the carriers of C1C1 genotype. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 4.118 (df, 1), the p-value was 0.0424 (P-value < 0.05). The odd ratio (OR) was 0.4215 (CI. at 95% was 0.172 to 0.984) & relative risk (RR) was 0.5392 (CI. at 95% 0.0.303 to 0.957). The values of OR & RR predicted that C1C1 allele of HLA-C is not associated with disease, so from these values it can be concluded that the carriers of this allele are able to clear the virus.

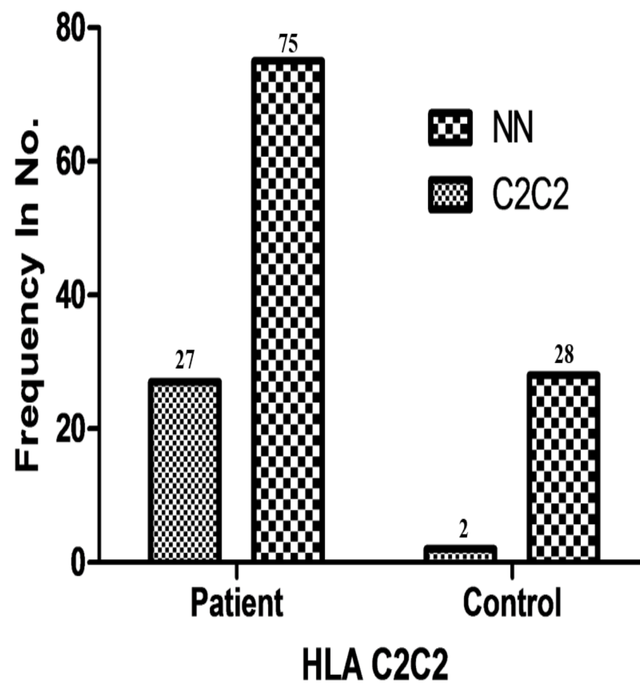

Figure 4.14 Control vs. HLA-C2C2 allele of HLA-C

This graph shows that 27 patients carried homozygous C2C2 genotype out of 102 HCV diagnosed patients and 75 patients did not carry this genotype, denoted by NN. In case of healthy individual (controls) 2 out of 30 were the carriers of C2C2 genotype. When these results were analyzed in Graph pad prism, this interpreted the chi-square value as 5.304 (df, 1), the p-value was 0.0213 (P-value < 0.05). The odd ratio (OR) was 5.040 (CI. at 95% was 1.124 to 22.61) & relative risk (RR) was 3.971 (CI. at 95% 1.001 to 15.75). The values of OR & RR predicted that C2C2 allele of HLA-C unlike C1C1 is associated with disease, so from these values it can be concluded that the carriers of this allele are more prone to disease progression.

4.6 Treatment Response (Follow-Up Data)

In order to validate statistical analysis, I went for the follow-up data of the patients who had completed their IFN/RBV therapy against HCV infection.

Out of 102 patients, 33 were the carrier of HLA-C2+KIR2DL1 combination (Table 4.4). Out of these patients 9 achieved sustained virological response, 20 patients with this combination did not achieve sustained virological response (Table 4.5). These findings validate our statistical findings, which suggested that presence of inhibitory receptors (KIR2DL1) is associated with disease progression. Similar statistical findings for homozygous C2-C2 allele and KIR2DL1 were obtained.

Table 4.4 Carriers for the KIR2DL1 and HLA-C2 allele combination 9

	KIR2DL1		
	F1R1-F2R2	**F1R1-F1R1**	**F2R2-F2R2**
HLA-C2	18	9	6

Table 4.5 No. of patients achieved SVR or NSVR

SVR	NSVR	Unreachable
9 (27.2%)	**20 (60.6%)**	**4 (12.12%)**

Out of 102 patients, 49 were the carrier of HLA-C1+KIR2DS2 combination (Table 4.6). Out of these 49 patients 31 achieved sustained virological response, 12 patients with this combination did not achieve sustained virological response (Table 4.7). These findings validate our statistical findings, which suggested that presence of activating receptors (KIR2DS2) is helps in viral clearance. Statistical results for HLA-C1 and KIR2DS2 variants also suggested that these genes are associated with viral clearance and not associated with disease progression.

Table 4.6 Carriers for the KIR2DS2 and HLA-C1 allele combination

	KIR2DS2		
	F1R1-F2R2	F1R1-F1R1	F2R2-F2R2
HLA-C1	8	21	20

Table 4.7 No. of patients achieved SVR or NSVR

SVR	NSVR	Unreachable
31 (63.2%)	12 (24.4%)	6 (12.24%)

Chapter 5

DISCUSSION

Hepatitis C Virus is a severe burden worldwide on health system (Ashfaq, Javed, Rehman, Nawaz, & Riazuddin, 2011). According to WHO, Report 2017, 71 million people globally are infected with chronic HCV infection and approximately 399 000 patients die every year due to HCV, (WHO 2017). Annually, 3% of the world's population is affected by HCV (Liver, 2012). No vaccine against HCV is known till date, but present studies on HCV lifecycle has enabled to develop effective treatments including direct-acting antivirals (DAAs), however, resistance against DAAs have also been reported in HCV patients (Pawlotsky, 2016; D. L. Wyles, 2012). If treatment therapy is left incomplete, it results in adverse effects. Massive efforts have been put for development of vaccine against HCV, but due to certain limitations no vaccine has been developed yet (Q. Choo et al., 1991). In case of HCV, the treatment outcome depends mainly on viral-host association. In case of viral factors, core proteins, p7, NS2, NS3 and NS5, viral load and most important is viral genotype. Among host factors, age, gender, ethnicity, social life, environmental factors and SNPs in various genes are the vital influencing factors (Bengsch et al., 2009). In the current study, we focused on the host HLA and KIR genotype prevalence in Pakistani patients, specifically in KPK patients. This study included HLA C-1 and HLA-C2 genes, and KIR2DL1 (two alleles) and KIR2DS2 (two alleles). Total patients included were 102. They were all from KPK, Pakistan. The correlation between the type of receptor and HLA was studied, using Chi-square test. Allelic frequency was calculated. We selected these widely studied genes to test their association with HCV progression or clearance specifically in Pakistani patients. These genes have already been studied in Caucasian populations and needed to be verified in Pakistani patients due to the difference in ethnicity and genetic factors. This study was designed to predict the treatment outcome in case of HCV infection, based on host gene variants. The genotyping of certain host gene factors can be helpful in predicting that will patient response to treatment or not? In case of KIR2DL1, we selected two gene variants and denoted them as F1R1 and

F2R2, two alleles of KIR2DS2 were selected and denoted by F1R1 and F2R2. In case of HLA-C, C1 and C2 alleles were selected.

In case of KIR2DL1 frequency, F1R1 allele was present in 53.9% of patients and F2R2 in 46.1% of the population. The odd ratio and relative risk ratio values at 95% confidence interval of these variants were calculated as 2.730, 2.921 and 1.797, 2.017 respectively. These values suggested that the patients with these gene variants are more prone to disease progression. This suggests their association with absence of treatment-induced viral clearance. The chi-square value i.e 5.311 also suggest their association with disease progression. The similar results were cited in literature many times. The receptor-ligand bond of KI2DL1 with HLA-C2 is said to be the strongest one among inhibitory receptors (Moesta & Parham, 2012). The inhibitory receptor-ligand, weaker bonds are thought to be protective, because it is more easy for activating receptors to override the weaker associations as compare to the stronger bonds (Salim I. Khakoo et al., 2004). KIR2DL1-C2 strongest bonding is associated with disease progression or absence of viral clearance (Moesta & Parham, 2012). An interaction between KI2DL1 and HLA-C2 is said to be the cause of increased disease risks (Moesta & Parham, 2012). Homozygous C2C2 genotype has also been cited as one of the factor that fails the viral clearance when treatment is given to the patient. In our study, out of 102 patients, 27 were the carrier of this genotype. According to follow-up data of these patients, 33 were HLA-C2 and KIR2DL1 carrier, and out of these 33 patients, 20 patients did not achieve SVR. This response of patients also predict that C2C2 genotype is associated is one of the factors for failed treatment outcome. The odd ratio value at 95% CI was obtained as 5.040 (CI =1.124-22.61), this suggests that patients with this genotype have 5.040 times more chance of treatment failure. Similar results were obtained for relative risk at 95% CI (CI= 1.001-15.75) i.e 3.971, this value also suggests the higher strength of association with failure of viral clearance. Similar results were cited in a work done by Suppiah *et al*, in 2011 (Suppiah et al., 2011). They suggested that C2C2 genotype increases the risk of treatment failure.

Second, host gene variant we included in our study was KIR2DS2, an activating receptor, which binds to HLA-C1 ligand. These activating receptors help the immune system in viral clearance by enhancing the activity and production of natural killer cells. It has been reported that KIR2DS2 is the receptor that is expressed on both

acquired as well as innate arms of immune system (S. H. Wang et al., 2008), this suggests that it might help in clearing the virus by enhancing the cellular and innate immune response (Sung et al., 2011). Literature provides evidence that KIR2DS2 carriage effects the treatment outcome in HCV infection. We selected two alleles i.e F1R1 and F2R2. Out of 102 HCV diagnosed patients 40 patients carried F1R1 allele and 30 were the carrier of F2R2 allele while remaining 32 patients carried neither of the two alleles. The odd ratio value for F1R1 allele is calculated as 0.4301 at 95% CI (CI= 0.1872-0.9881), this indicates that KIR2DS2 is not associated with disease progression, rather helps the immune response to clear the virus on therapy. The relative risk value obtained at 95% CI was 0.6536 (CI= 0.4473-0.9550), also suggest that patients with this variant are able to clear the virus. A similar trend was observed in case of F2R2 allele, the odd ratio at 95% CI was 0.4167 (CI= 0.1811-0.9584). A study conducted on the Korean population presented the very same results that KI2DS2 helps in viral clearance (Portela et al., 2017). Recently, it has been reported that KIR2DS2 recognizes a specific sequence from HCV helicase and leads to activation of NK production. They reported that KIR2DS2 as sufficient to fight against HCV infection by inhibiting the replication of virus (Mohammed M Naiyer et al., 2017). Another study conducted by Medhat *et al,* also verified the obtained results that KIR2DS2 helps in clearing the Hepatitis virus when therapy is given to the patient. They reported that the absence of KIR2DS2 is associated with poor response to treatment. According to Medhat *et al,* the patients who did not achieve SVR was due to the factor that they were not the carrier of KIR2DS2 gene. Presence of KIR2DS2 is linked with achieving SVR (Askar et al., 2009). In our study, when we went for the follow-up data of patients who completed their RBV/IFN therapy, we found almost similar results. From 102 patients 49 had KIR2DS2+HLA-C2, out of these 49 patients 31 achieved SVR, which verify our findings from statistical analysis and are also according to the literature. So these results suggest that KIR2DS2 gene is one of the critical host gene factors that helps in achieving the SVR and the patients who carry this gene might respond better to the IFN/RBV therapy and has more chance to achieve SVR.

In case of Human Leukocyte Antigen-C, we had two alleles C1 and C2 in our study. They are ligands to KIRs. We found that 48 HCV diagnosed patients carried C1, 44 had C2 and remaining 22 showed neither of them. The odd ratio of 0.3810 at 95% CI shows that this gene is not associated with infection progression. The relative risk of 0.6723 at

95% CI (CI= 0.4921-0.9184) indicates that patients who carry C1 gene variants has 0.6723 times more chance to clear the HCV infection. A similar trend is observed in case of homozygous C1C1 allele. Studies suggest that C1C1 homozygous allele helps in clearing the virus (Salim I Khakoo et al., 2004). Another study reported that the patients who achieved SVR have greater frequency of homozygous C1C1 allele, which suggests that it exhibits protective effect against viral clearance when given RBV/IFN therapy (Vidal-Castiñeira et al., 2010). Suppiah *et al,* also reported that C1 is associated with viral clearance (Suppiah et al., 2011). But in case of homozygous C2C2, the trend was totally opposite, homozygous C2C2 genotype has been cited as one of the major host factors that fails the viral clearance when treatment is given to the patient. The odd ratio value at 95% CI was obtained as 5.040 (CI =1.124-22.61), this suggests that patients with this genotype have 5.040 times more chance of treatment failure. A similar results were obtained for relative risk at 95% CI (CI= 1.001-15.75) i.e 3.971, this value also suggests the higher strength of association with failure of viral clearance. Similar results were cited in a work done by Suppiah *et al*, in 2011 (Suppiah et al., 2011). They suggested that C2C2 genotype increases the risk of treatment failure.

Chapter 6

Conclusion & Future Prospects

Genetic make-up majorly affects the treatment outcome in case of Hepatitis C Virus, different host genetic factors respond differently to treatment. Our results indicated that specific KIR and HLA cells play significant role in treatment outcome by regulating the natural killer cell production. We found a statistically significant association between KIR and HLA gene Variant and HCV treatment. Finally, it would not be wrong to conclude that carriage of particular HLA-C in combination with specific KIRs alters the treatment outcome.

This study needs to be performed with a large sample size so that the significance would be more evident and needs to be conducted on patients from different regions and different ethnic groups and more parameters should be included. An *in-silico* study can be designed to investigate the changes at the translational level then these results would be confirmed through wet lab tests.

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