Association of IL28B Genetic Variations with Spontaneous Clearance of Hepatitis C Virus, Treatment Response

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By

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"Verily! In the creation of the heavens and the earth, and in the alternation of night and day, there are indeed signs for people of understanding." (Al - Qur'an 3:190)

CERTIFICATE

This thesis, submitted by ***Salma Ghulam Nabi*** is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, **Quaid-i-Azam University, Islamabad, Pakistan** as satisfying the thesis requirement for the degree of Doctor of Philosophy in Microbiology.

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Dedicated To:

To my parents; especially to my late mother, Saeeda Begum.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

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LIST OF ABBREVIATIONS

AASLD	American association for the study of liver diseases
ALT	Alanine aminotransferase
AS	Antisense
ASO	Allele specific oligo-nucleotide
APOE	Apolipoprotein E
ASH	Allelic Specific Hybridization
AST	Aspartate aminotransferase
C	Core
CCR	Chemokine receptors
CXCR	C-X-C chemokine receptor
CXCL	C-X-C motif chemokine ligand
CCL	C-C motif chemokine
CD	Cluster of differentiation
cDNA	Complementary DNA
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
CTL	Cytotoxic T lymphocytes
DAA	Direct-acting antiviral
DAAT	Directly acting antiviral treatment
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
E1	Envelope 1
E2	Envelope 2
ENV	Envelope
EPG	Electropherogram
EVR	Early virologic response rates
FasL	Fas ligand
FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
GWAS	Genome wide association study
HAART	Highly active antiretroviral therapy
HALT-C	Hepatitis C antiviral long term treatment against cirrhosis
HBV	Hepatitis B virus
HCC	Hepatocellular HCV carcinoma
HCVcc	HCV cell culture
HCVcc	HCV core
HCV	Hepatitis C virus

HIV	Human immunodeficiency virus
HVR	Hyper variable Region
HLA	Human leukocyte antigen
IDUs	Injecting drug users
IFN	Interferon
IL	Interleukin
IL-28Rα	Interleukin-28 receptor α chain
IL28B	Interleukin 28B
INR	International Normalized Ratios
ISG	IFN-stimulated gene
Jak	Janus kinase
MELD	Model for End-Stage Liver Disease
NK	Natural killer
ORF	Open Reading Frame
PEG-IFN	Pegylated interferon
Pm	Picomole
PMN	Polymorphonuclear leucocytes
RBS	Binding solution
RBV	Ribavirin
RFLP	Restriction fragment length polymorphism
RL	Lysis solution
RNA	Ribonucleic acid
RVR	Rapid virological response
S	Sense
SC	Spontaneous Clearance
SNP	Single nucleotide polymorphism
STAT	Signal Transducers and Activators of Transcription
SVR	Sustained virological response
TGFβ1	Transforming growth factor beta 1
Th1	T helper 1
TNF	Tumor necrosis factor
TVR	Telepavir
VD	Variable durations

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Salma Ghulam Nabi

ABSTRACT

Among the health problems faced globally Hepatitis C virus (HCV) signify an important entity. The virus is found around the globe with varying occurrence in different countries. HCV has been found as chief factor causing chronic infection in liver leading to fibrosis, cirrhosis which can lead to hepatocellular carcinoma. Various studies have revealed that environmental, viral and host factors contribute to the differences in the disease expression and treatment response.

In order to initiate a thorough understanding of the various factors which could affect our set of population the present study was completed in two stages. In the first phase the focus was on analysis of viral factors; the prevalent genotypes in the region with associated viral loads. The investigation revealed the occurrence of genotypes 1 and 3 with additional subtypes 1a, 1b, 3a, 3b and mixed genotypes 1b + 3a, 1b + 3b and 3a + 3b. Quantification of Viral load was done in 151 patients who were found to be HCV positive. Genotype 3a was detected in 124 (82.12%) HCV positive patients, genotype 3b was recognized in 21 (13.91%), however other HCV genotypes were fewer than 2 %. Viral load was associated among various genotypes. Nevertheless, the rigorousness of disease was greater in genotype 1 as shown by comparatively higher viral load associated with this genotype.

The Second phase of the study was designed to determine the association of IL28B genetic polymorphisms as host factors playing a role in treatment response in patients having HCV genotype 3a infection. These genotypes are widely associated in different genome wide association studies (GWAS) with spontaneous as well as treatment induced HCV clearance.

DNA obtained from 169 HCV patients taking Interferon and Ribavarin base antiviral therapy was analyzed for the polymorphisms of IL28B gene by means of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Bidirectional sequencing was performed on a subset of the studied patients for confirmation of results obtained from PCR-RFLP technique. Information comprising on factors like age, alanine aminotransferase (ALT) levels and Hemoglobin (Hb) was tested. It was noted that ALT association was not significantly associated with Rapid virological response (RVR) but in one set of our study patients (group II) ALT levels showed significant association with Sustained virological response (SVR) (p=0. 010) and in these patients mean ALT levels of patients was 40 U/L.

Two IL28B genotypes were analyzed for their linked with RVR and SVR. In one group of study (group I) the occurrence of CC/CT/TT genotypes in rs 12979860 genotypes in patients who achieved SVR were found to be 79.7 %, 15.6 % and 4.7 %, respectively. For rs8099917 genotype, the TT/GT/GG distribution was 81.3 %, 10.9 % and 7.8 %, respectively. CC genotype at rs12979860 and TT genotype at rs8099917 were significantly higher in responders (p = 0.046 and 0.000, respectively). In second group of

study the frequency distribution of TT/GT/GG genotype at rs8099917 were 57.3 %, 20.7 % and 1.2 % respectively (p = 0.01). Lower baseline ALT and rapid viral response were also found to be associated with SVR.

In this study we have found that among patients infected with HCV genotype 3a there is significant association of successful treatment response in genotype CC in rs12979860 locus (p=0.04) and TT genotype at rs8099917 locus (p=0.00). Favorable C allele at IL28B rs12979860 locus was found in higher ratio (85.6%) than T allele (14.4%). Higher ratio of C allele in SVR achievers and T allele in SVR non-achievers was noted. At rs8099917 T allele was higher in both sets of patients (81.1% & 81.7%) whereas G allele frequency was low. Allele association with RVR and SVR point towards the part of genetic factors in clearance of infection. caused by HCV RVR was noted as a significant predictor of Sustained Virological response in both groups of our study (p =0.029, p =0.000),

Considering the analysis in view of early viral kinetics RVR was achieved in 75.6 % of the patients. Further analysis of rs12979860 polymorphism showed (66.5 %) was CC allele carriers while (19.5%) were CT allele carriers. Among, TT allele carriers RVR achievers were (3.4%). In analysis of rs8099917 those with TT allele RVR achievers were (63.2 %) those having TG allele RVR achievers were (20.7 %) and those having GG allele RVR achievers were (4.6 %). Similarly rs8099917 genotype TT was significantly associated with RVR in the group II of the study.

The observations highlight the importance of working towards personalized approach for patients where the funds are limited and the chance of success is the duty of specialists to bear in mind right patient for right therapeutic regimen.

It is recommended that a broad based strategy regarding parameters such as age, ALT levels, IL28B genotypes rs12979860 polymorphisms (CC, CT &TT) and rs8099917 polymorphisms (TT, GT & TT) and single allele (C, T & G) should be devised. High risks for HCV infection include, intravenous drug users, patients receiving multiple transfusions (Thalassemia and Leukemia patients), patients undergoing Hemodialysis and patients with HBV & HIV co-infections. The studies should be conducted to get a thorough insight into factors playing role in spontaneous and treatment induced clearance of HCV. The results of the detailed analysis might be used to guide treatment for chronic hepatitis C in Pakistani patients in the future.

REVIEW OF LITERATURE

2.1 VIRAL HEPATITIS AND TYPES

2.1.1 Hepatitis C Virus (HCV)

In 1989 HCV was discovered (Ryan & Ray, 2004) in the plasma of infected chimpanzees found to be negative for Hepatitis A or B viruses. Molecular cloning techniques were employed that led to its detection and it is categorized as an enveloped virus size ranging from 55-65 nm having single stranded +ve sense RNA genome (Kato, 2000). HCV is the only representative, in the family Flaviviridae of the genus named Hepacivirus (Safi et al., 2010). The virus is known to be hepatotropic having lymphotropic affinity (Akhund, et al., 2004), found to be infecting humans and only other host is chimpanzee (Ploss & Rice, 2009).

In order to visualize structure of HCV virions in vitro by electron microscopy, growth of viruses in cell cultures is central. Constricted host range and the association of HCV virus in serum with low density lipoprotein, scientist face difficulty in employing these cell cultures techniques (Kupfer, 2012). To deal with these difficulties different surrogate models consisting of the recombinant glycoproteins of HCV envelop have been developed (Wellnitz et al., 2002; Lambot et al., 2002). HCV-like particles (HCV-LPs) or HCV pseudo particles (HCVpp) and models like recombinant infectious HCV cell culture (HCVcc) have been developed for same purpose. (Popescu, et al., 2014; Da Costa et al., 2012).

2.1.1.1 Genome of Hepatitis C Virus

Study of complete genome of HCV was carried out in 1991 (Choo, et al., 1991; Clarke, 1997). HCV is a positive stranded RNA virus of ~9.6 kb genome. It has a single continuous open reading frame (ORF) that decodes a precursor protein comprising of three thousand amino acids. Cleavage by both the viral and host enzymes of this precursor protein gives rise to about ten proteins (Clarke, 1997). Three genes of ORF encode for structural protein; C (core), the two envelope proteins E1and E2 and (membrane protein) P7 with its role in forming a channel for ions (Griffin et al., 2003). A

significant rate of mutations is seen with envelope proteins E1 and E2 in their hyper variable regions designated 1 and 2 respectively. Four genes, non-structural type 4; NS4A, NS4B and non-structural type 5; NS5A and NS5B code for non-structural proteins (Pavlović et al., 2003; Figure 2.1). About 1.44×10^{-3} and 1.92×10^{-3} is the per year rate of genomic nucleotide substitution in HCV genome (Attaullah et al., 2011).

In the United States HCV infection is the most commomn chronic blood-borne infection about 3.2 million people suffer from chronic infection. Patients infected with HCV with proven cirrhosis commonly develop HCC in contrast to Chronic Hepatitis B Virus (HBV), infective patients (Hoshida et al., 2008).

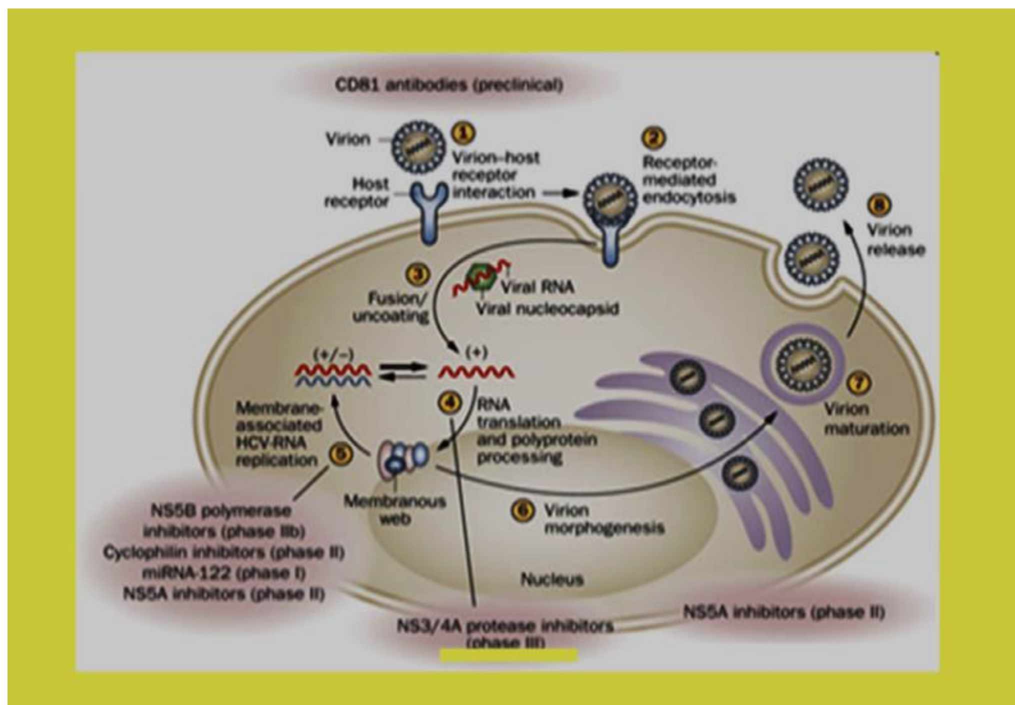


Figure 2.1 Structure of Hepatitis C virus, adopted from Nature Reviews Gastroenterology & Hepatology 8, 69-71(2011).

2.2 Prevalence and Geographic Distribution

Chronic HCV infection is affecting population around the globe, it shows variability in prevalence in different geographical regions and is found to be affecting about 2.8% of world population (Hanafiah et al., 2013). There are different incidence rates shown in

various subset of population , about 185 million people are found to be affected with the HCV virus .Studies have pointed that about 130-170 million people are suffering from chronic disease and 350,000 deaths are recorded each year due to various complication caused by the virus including advanced fibrosis, cirrhosis and hepatocellular carcinoma. HCV is found to be continually present in community and its epidemic surfaces effecting about 3 to 4 million people each year (Madhava, et al., 2002).

Different regions of the globe show variation in HCV infection. It has been noted that higher frequency of infected patients is found where the health sector gives less preference towards the curtailment of the disease. Higher proportions of infected persons are present in the South East Asian countries which have thickly populated regions. In this region the virus is found to effect more than 50 million people, however in North African and Middle Eastern region about 15 million people are found to be suffering from the infection. In developed countries a lesser ratio is noted equalling to about 10 million and in western region of Europe and in the region of North America about 4.4 million people are infected with the virus (Hanafiah et al., 2013).

It has been shown in different publications that threat to exposure of the population to HCV determines the number of infected patients within a country. The studies have shown people of United States of America who were born in the years from 1945 to 1965, had black ancestry and history of injectable drug usage had higher percentage (1.6 %) of infection. Genetic factor of transmission of infection through I/V route were overall factors assumed to be responsible for acquiring infection (Murphy et al., 2000). HCV has frequency of 10-30 % per year in the Injecting drug users (IDUs) of urban regions of USA and Europe, incidence rates show comparable ratio in cosmopolitan areas of Australia (Alonso et al., 2015).

Studies including (IDUs) have shown that in China 2.2 % prevalence rate was observed Detailed analysis show variations in different regions, the Henan province showing the higher 9.6 % and Fujian province having lower 2.1% prevalence in these sets of

population. (Madhava et al., 2002). It has been further found that in less than 30 % of infected IDUs clearance of HCV infection occur spontaneously (Cox et al.,2005).

Similarly patients receiving transfusions of blood and undergoing surgery are more susceptible to HCV infection. In Iran 0.87 % of people are found to be affected and in Afghanistan 1.1% have HCV among healthy blood donors. In Pakistan the fraction of infected persons amount to about 4.7 %, difference in range being from 4 to 33.7 % accordingly in different subsets of population (Umar *et al.*, 2010), the neighbouring regions like India showed 0.66 % rate of occurrence in its general population, while other neighbouring regions like Nepal show the percentage of 1.0 %, and Myanmar showing 2.5 % of prevalence rate (Umar et al., 2010) (Figure 2.2).

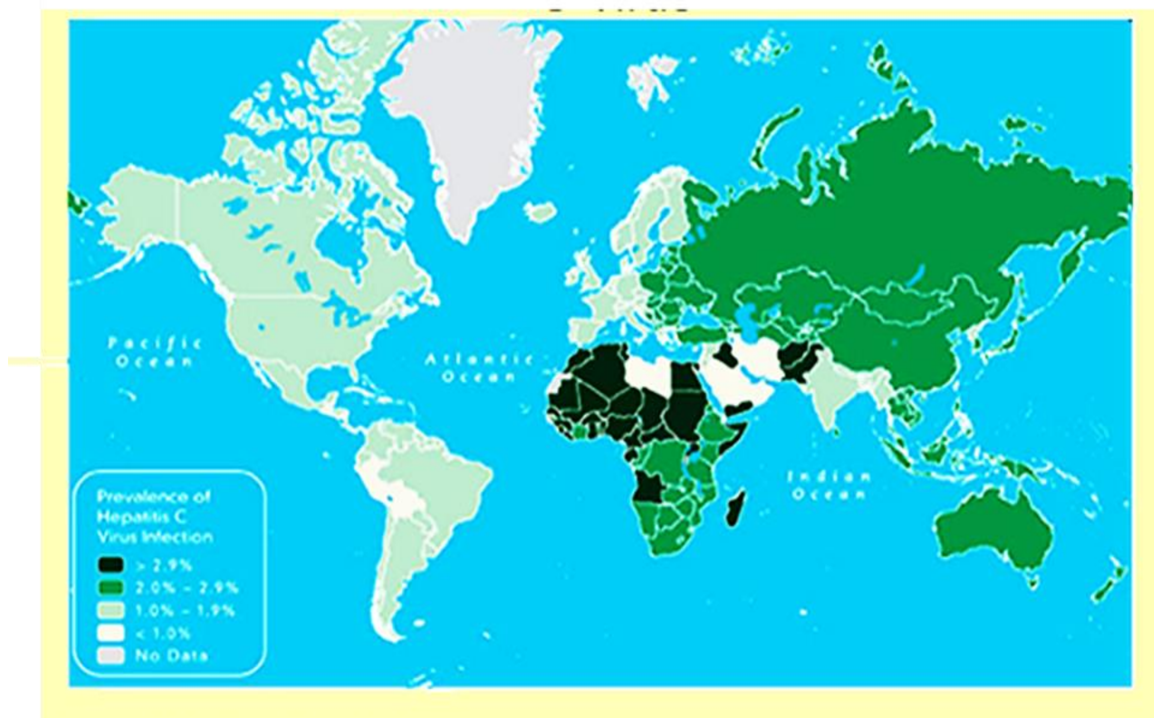


Fig. 2.2 Prevalence of hepatitis C virus around the globe (source CDC Yellow book, 2012)

2.3 Transmission

HCV infection can be categorized almost wholly as a blood-borne infection. Transmission of HCV occurs through various routes the most central of which is known to be the percutaneous comprising of intravenous drug use; blood transfusions; transplant taken from HCV positive donor; various procedures being categorized as therapeutic (use of equipment which are contaminated with the HCV , practice of infectious injection use) and professional procedures (needle stick). The infection can be transmitted through mucosal route including vertical and sexual routes (Alter, 2007).

The individuals acknowledged at greater danger of attaining infection are: present or previous injection drug users, even if they had injected only one time many years ago; Those persons who were given concentrates made up of clotting factors earlier than year 1987, are at greater risk of acquiring infection; The patients who received blood transfusions or patients who underwent surgeries for solid organ earlier than month of July 1992 are of greater risk because by then improved testing of blood donors were not accessible for patients. Similarly patients who are categorized as those undertaking chronic hemodialysis , HIV infected patients and children of mothers who are HCV-positive are at greater risk of acquiring infection. (Mast et al., 2005).

The most significant of these routes is through transfusion of blood and the injudicious use of intravenous drugs (Van Herck et al., 2008). The contamination of the products of blood and certain body fluids with HCV are also found to be responsible (Alavian et al., 2005; Murphy et al., 2000). The practice of routine screening of blood for HCV has constrained the spread of infection through transfusion (Donahue et al., 1992). Illegal usage of drugs through injectable route is now a days considered to be the main source through which HCV spread. The HCV infection through this route is forming a major proportion 40 % or above of its transmission through this route mainly in developing countries and in transitional economies (Figure 2.3). In the United states and western European countries this route is now considered as the main reason of spread (Wasley & Alter, 2000). It has been noted that the infection with hepatitis C is considerably higher in IDUs as compared to infection like HBV and HIV. (Garfein, et al., 1996).

In Pakistan the primary source of spread of HCV has been found to be nosocomial including a major subset of cases marked as sporadic, or the sources whose origin could not be traced (Ali et al., 2008; Idrees et al., 2008). The practice of reuse of syringes and needles without adequate sterilization is thought to contribute to approximately 61.45 % of HCV infections in Pakistan. Similarly improper sterilization of instruments during various surgical procedures and unsterilized dental treatments account for about 10.62 % of the overall transmission in this country. The improvement in screening for HCV has contributed towards a decline in transmission of infection through transfusion of blood and the various products of blood contributing 4.26 % share in overall transmission of HCV in Pakistan. Similarly the traditional ritual of circumcision and barber razor use for shaving at public places in the male populace, and other procedures like use of piercing tools contribute about 3.90 % towards the load of infection spread in Pakistan (Younus & Akhtar, 2009). Transmission through sexual route, the needle stick injury, and infants infected through mothers contribute about 1%. Sporadic factors with unrecognized routes account for approximately about 20.35 % in people infected with virus in Pakistan (Idrees et al., 2008).

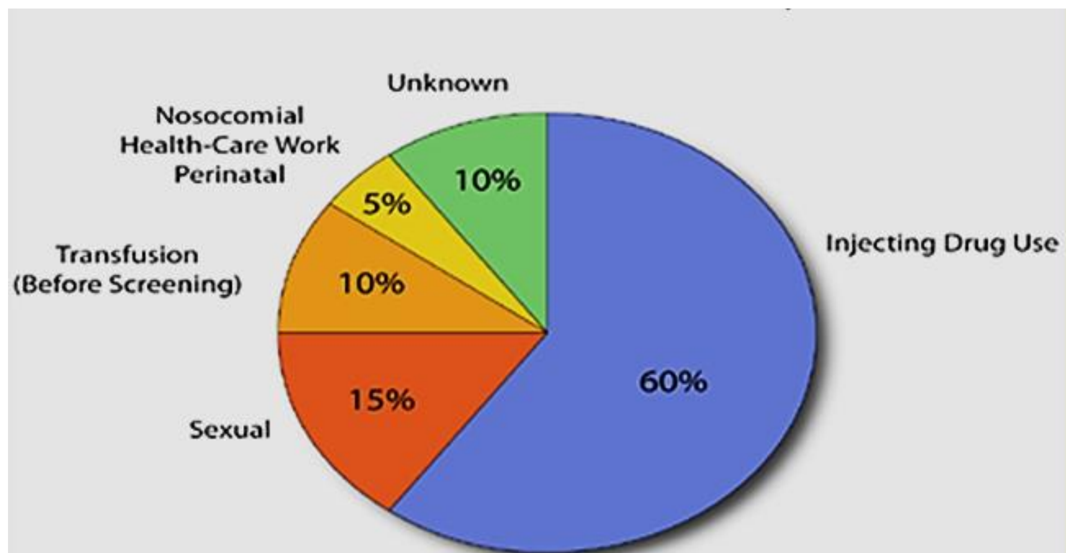


Fig. 2.3 Sources of infection for persons with hepatitis C virus (source, CDC DVH http:www.cdc.gov /ncidod/diseases/hepatitis/slideset .2000.

Infections transmitted through blood transfusions are considered as a challenge for the developing countries, especially Pakistan because of lack of policy, funding, malpractice and low literacy rate. Screening of the blood donors provides an indicator to get an idea to some degree of the prevalence in general population. Blood transfusion is an integral part of the health care system nevertheless it also serves as an important route for transmission of infections. Recent developments in science and expertise has upgraded donor screening and concurrently reduced the risk of viral transmission through transfusion (Vermeulen et al., 2009 ;Alaei et al., 2016; Reynolds et al., 2018).

Table 2.1 Frequency of HCV from different areas of Pakistan and its neighboring countries.

Years of Study	**Area of study** **Country (city)**	**Total sample of study**	**HBsAg** **% positive**	**Anti-HCV** **%**	**Reference**
1996-2005	Pakistan (Lahore)	41 498	2.21	3.68	Sultan et al., (2007)
2008-2011	Pakistan (Peshawar)	1,27,828	2.68	2.46	Khan et al., (2011).
2009-2010	Pakistan (Karachi)	1600	3.12	1.44	Khan et al., (2011).
2007-2011	Pakistan (Rawalpindi)	2,46,611	1.63	2.92	Present Study & Nabi et al., 2013
2008-2010	Yemen	1483	2.40	0.79	Saghir et al., (2013)
2004-2008	India	39,060	1.27	0.23	Pallavi et al., (2011)

The bulk of the studies in India on blood donors revealed prevalence of HCV ranging from 0.3-1.85 % (Gupta et al., 2014). The trends showing a decrease in the incidence of HCV in blood donors is a positive feature and should be further sustained.

Blood transfusion is an integral part of health care on the other hand it also serves as an important route for transmission of blood borne infections. Infections transmitted through blood transfusions can be viral, bacterial or parasitic in nature, which are duly considered a challenge for the developing countries. The high rate of incidence reflects lack of policy, funding, malpractice and low literacy rate (Pallavi et al., 2011).

Indigenous studies on prevalence of HCV in different groups of population have contributed widely towards getting a holistic picture of HCV prevalence in respective regions. Various meta-analysis based on these studies have helped in solving the multifaceted issues arising with HCV infection. Study plans used to observe the prevalence of HCV infection in the population can contribute towards giving a holistic view of the problem facing dire need of a solution. (Younossi, Z. M., Birerdinc, A., & Henry, L. 2016)

The largest blood donor study with a sample size of 103,858 was published in 2002 and showed an overall prevalence of 4%. Higher rates were observed among rural donors and lower rates were seen among college students (Shah & Shabbeir, 2002). The frequency of HCV, from different areas of Pakistan and its neighboring countries is shown(Table 4. 2)

Compared to other countries it was noticed that in blood donors 0.13% was HCV prevalence in Iran (Alaei, et al., 2016) and 0.79 % in Yemen (Saghir et al., 2012). The similar blood borne HBV frequency was also high in Pakistani blood donors i.e. 1.63 % as compared to 1.27 %, 0.56 % in India and Iran respectively (Nabi et al., 2014). The alarming figures shown in another such studies highlights the increased proportions of HCV and HBV infection in the population reflected in blood donors demand for strict control measures (Ahmed et al., 2013).

The decreasing trend was also found in other areas of Pakistan as shown by Sultan in 2007 (Sultan et al., 2007). The bulk of the studies in India on blood donors HCV prevalence revealed range from 0.3-1.85 % (Gupta et al., 2014). In a study conducted in a university hospital in India it was found that for voluntary blood donors the ratio of anti-HCV patients has shown decreasing trend ranging from 0.19% in the year 2004 to a level of 0.05% in the year 2008, while in replacement donors, an increasing trend in

positivity rate from that of 0.07 % in year 2004 to amounting to 0.31 % was noted in year 2008 (Pallavi et al., 2011).

A recent study on various aspects of HCV infection in Pakistan showed that among risk factors the key risk factor was blood transfusions. The overall prevalence in our country is quite high. In province of the Punjab frequency of HCV was 6.7 %, It was 5 % in Sindh province, 1.5 % in Baluchistan, 1.5 % in Khyber Pakhtunkhwa (Formerly known as NWFP) 1.1 % (Mahmood & Raja, 2017). A recent study in China pointed out that 0.43 % people aged 1-59 years had anti HCV detected in their blood (Tan et al., 2018).

HBV and HCV are blood borne viral infections which are part of screening program for blood donors in Pakistan, It was found that the incidence of HCV was higher than HBV (Reynolds et al., 2017). In our study that took place in Rawalpindi in 2013 showing HBV prevalence in the same set of population (Nabi et al., 2013) and a study in Lahore (Pakistan) (Batool, et al., 2017) showed HBV prevalence to be higher as compared with HCV while in the present study HCV was showing higher frequency. HBV had highest frequency followed by HCV in a study in Peshawar (Pakistan) (Craxi et al., 2011; Shakeel et al., 2017). A study in India indicated the highest frequency for HBV followed by HCV (Khan et al., 2003).

In Pakistan HCV has a prevalence of about 6 % pointed by a recent studies (Ahmad, et al., 2018; Umer & Iqbal, 2016) as compared to its neighboring countries like Iran and India where the prevalence of HCV infection was around 1–1.9 % (Sievert et al., 2011). In Pakistan a study conducted in 2012 showed that in adult section HCV prevalence to be about 4.95%, in young people it was 3.64 % whereas the pediatric set of populace the prevalence was found to be about 1.72 %. It was further noted that HCV frequency was 48.67 % in the injection drug users and in patients receiving multiple blood transfusions (Mustafa et al., 2012). In Iran a study showed HCV sero-conversion of 4 % in patients receiving blood transfusion or its associated procedures (Sibley et al., 2015).

In Pakistan adult anti-HCV prevalence was found to be 6.7% (1.6%-10.0%), the Viraemic rate was found to be 87.4 %. Adult viraemic prevalence was 5.8 % (1.4-8.7%), Adult anti-HCV population (000) was detected as 8054 (1977-12,041), similarly adult

viraemic population (000) was found to be 7039(1728-10,524). In neighboring India adult anti-HCV prevalence was found to be 0.8% (0.4%-1.0%). The viraemic rate was found to be 80.8%. Adult viraemic prevalence was 0.7 % (0.4%-0.8.%), adult anti-HCV population in thousands (000) was detected as 74580 (3907-8879), similarly adult viraemic population was found to be 6026 range being (3157-7174). In Bangladesh adult anti-HCV prevalence was found to be 1.3% range being (0.2-2.2%) the viraemic rate was found to be 77.8%. Adult viraemic prevalence was 1.0 %, (0.2%-1.7%) Adult anti-HCV population was detected as 1384(000) ranging from (219-2444), similarly adult viraemic population was found to be 1077 (000) (171-1902). In Afghanistan adult anti-HCV prevalence was found to be 1.1% (0.6 %-1.9 %) the viraemic rate was found to be 58.1%. Adult viraemic prevalence was 0.6 % range (0.4%-1.1%). Adult anti-HCV population was detected as 179(000) (103-310), similarly adult viraemic population was found to be 104(000), range being (60-180) (Gower et al., 2014).

Chronic HCV infection leads to cirrhosis and HCC in patients. An action plan for inhibiting and regulating the infection can encompass non-communicable diseases such as liver cancer also , as adopted by china (Chen et al., 2013) and collection of related data from other countries also (IAfRoC, 2014), thus a curtailment of the cause such as HCV infection become possible.

Results of screening of blood donors for viral infections such as HCV unquestionably provide an indicator of its prevalence in general population. The prevalence rate in blood donors is higher in Pakistan as compared to its neighbors, the situation calls for strict practices regarding the containment of HCV infection .Similar studies on different sets of populations such as thalassemia patients, patients enrolled for dialysis, Surgical patients receiving emergency blood transfusions, Patients receiving blood products and similar should be screened on regular intervals, thus a country wise HCV alert should be formulated ,

HCV is developing as an immense problem in our region and actions should be taken to regulate its spread. The trends showing a decrease in the incidence of HCV in blood donors is a positive feature and should be further sustained.

2.4 VARIOUS FACTORS AFFECTING HCV INFECTION

2.4.1 HCV-specific cellular immune response

HCV infection induce cellular immune response that affect severity of liver damage, It has been noted that HCV perseverance is related with overpowering of HCV-linked T cells and not because phenotype of cytokine linked HCV clearence. The virus exerts its action by down regulating the stimulatory NK cells. Its infection has been seen as a cause of increase in the inhibitory receptors on natural killer cells and also CD8+ killer cells resulting in production of TGF-beta, leading to blockage of T cell activation and thus a decrease in IFN-gamma production (Jonjić et al., 2008). The increased occurrence of CD4+ CD25+ T cells considered as T cells categorized as regulatory type and their ex-vivo destruction of HCV-specific CD8 T cells proposes new part for regulatory T cells in the persistence of this infection (Sugimoto et al., 2003).

2.4.2 Alcohol intake

Alcohol consumption causes rapid progression to liver fibrosis. Even modest quantities of alcohol raises the danger of fibrosis. The habit of alcohol intake was linked to a high prevalence of anti-HCV antibodies in various studies (Gitto et al., 2009). It is recommended that alcohol should not be consumed by chronic HCV patients, and no level alcohol consumption are found to be safe for such patients (Hezode et al., 2003).

2.4.3 Daily use of marijuana

Patients having chronic hepatitis C infection should avoid use of marijuana (cannabis) daily. It was reported that HCV patients using cannabis daily were at significantly higher risk of moderate to severe liver fibrosis, or tissue scarring (American Gastroenterological Association. Science Daily, 29 January 2008). Hepatic receptors for cannabinoid are endogenous. These receptors are stimulated resulting in rapid progression of liver fibrosis (Hezode et al., 2003).

2.4.4 Specific host factors

Different stages of fibrosis are associated with certain genetic polymorphisms like B1 phenotype of tumour growth factor (TGF B1) and PNPLA3 (adiponutrin). Certain genetic factors are also linked to factors linked with steatosis (Jonsson et al., 2008).

2.4.5 Viral co-infections

When co-infected with HIV it has been noted that course of Hepatitis C infection is accelerated. Once chronic HCV infection occurs with acute Hepatitis B Virus (HBV) infection the course of chronic hepatitis shows increased severity. When compared with mono infected patients those co-infected with HBV show decreased replication of the Hep C virus whereas the predominant infection is still HCV. Likewise course of disease leading to damage to liver is more severe in these co-infected patients (Soriano et al., 2007).

2.4.6 Geography and environmental factors in development of Hepatocellular carcinoma (HCC)

There are some noticeable geographic differences linked with HCV and development of HCC (El–Serag & Rudolph, 2007). For example, the frequency of HCC is detected in Japan more commonly when compared with the United States. The cause being not very clear. (Altekruse et al., 2009).

2.4.7 Use of steroids

Intake of steroids raises HCV viral load, whereas the influence on liver enzymes such as aminotransferases is variable. Reduction in intake of corticosteroids brings HCV viral load to baseline. Nonetheless, the clinical impacts of corticosteroid use are unidentified. The short-term intake of corticosteroids is not linked with substantial changes in the prognosis of disease. Membranous nephropathy related with hepatitis C virus infection showed improvement when therapy with corticosteroids and usage of direct antiviral agent Sofosbuvir (Weng et al., 2017).

2.4.8 Host factors

Model for End Stage Liver Disease (MELD) score specifically evaluate comparative severity of the disease and expected recovery of patients who are planning transplant of liver as treatment strate. Similarly another score termed the Child score is also used in some settings (Tripodi et al., 2007).

As chronic hepatitis due to HCV follows a long course predictive models constantly seem to come to surface in order to build a consensus to the plan to successfully eradicate the HCV infection, these developments can be useful for judging the prognosis. Both clinical and laboratory parameters have been added in the models. Serum bilirubin levels and International Normalized Ratios (INR) being the diagnostic parameters and hepatic decompensation being acknowledged as clinical parameter but these models are not followed routinely in medical practice (El–Serag & Rudolph, 2007).

2.4.9 Host Genetic Factor (IL28B Polymorphism)

The Human body has the capacity to produce its own cytokines with ability to protect against different viral diseases. Cytokines induce responses leading to tissue injuries. Cytokines production genes form the basis that in different individuals cytokines are produced at different rates. Differences are due to single nucleotide polymorphism (SNP) present within coding region of cytokines production gene. Sometimes the chronicity of the disease or resistance to the interferon result due to the secretion of inappropriate amount of cytokines (Thio, 2008). Gene IL28B is linked with spontaneous HCV clearance and has been shown to code for IFN-λ3, which is a cytokine found to be vaguely related to family of interferon known as type 1 as well as to family of cytokine (Murphy et al., 2002; Fried et al., 2002).

Chromosome 19 (19q13) possess a cluster of three genes of cytokines INF type III family comprising IL29, IL28A and IL28B genes. These genes encode INF-λ1, INF-λ2 and INF-λ3 respectively. These INF-λ genes are co-expressed with INF type-I (α and β) genes and are found in variety of different cells like epithelial cell and peripheral blood mononuclear cell (Österlund, et al., 2007). The high levels of expression of these genes was observed in hepatocytes. After expression of genes the cells develop antiviral properties when verified *in-vivo* and also *in-vitro*. Janus Kinase signal transducer is known to cause inhibition of replication of HCV as an aftermath of expression of IL28B genes in the cells. It has been found that Single Nucleotide Polymorphism (SNP) near IL28B gene on chromosome 19, encoding IFN-λ3 are strongly effective against Hepatitis C virus and associated with treatment induced and spontaneous clearance of HCV from infected patients (Rauch et al., 2010b). SNPs rs8099917 and rs12979860 are related

with clearance of HCV spontaneously as well as greater (SVR) rates in infected patients (Suppiah et al., 2009). Many of these SNPs are identified and studied like rs8105790, rs12979860, rs11881222, rs8099917, rs8103142, rs28416813, rs4803219, and rs7248668 (Lange & Zeuzem, 2011).

2.5 HEPATITIS C VIRUS GENOTYPES

HCV demonstrates three different levels of variability in its genome being identified as types, subtypes, and quasi species. It is accepted that 65.7-68.9% similarity in the nucleotides sequences is categorized as Genotypes Types (GT), The subtype category showed 76.9-80.1% resemblance and under same criteria in quasi species 90.8-99% similarity was noted (Idrees et al., 2008).

The diversity in genotypes is found around the globe (Simmonds, 2004; Messina et al., 2015). HCV genotypes namely (1–7) have been recognized in different publications. Similarly the established genotypes 1 to 6 are further divided into subtypes (1a, 1b, 1c, 2a 2b, 2c). About 67 subtypes have been confirmed nearly 20 of these have been reported at various times in different researches (Philipsen et al., 2001; Smith et al., 2014). The unique feature of diversity of HCV genome is that it exerts its effect on course and progress of the disease thus indirectly affecting antiviral therapy. In development of vaccine against HCV the genotype diversity is a well-recognised issue. Special concern is seen on the effect of these genotypes and subtypes on advancement of infection. The diverse epidemiology of infection has lead to analysis of patients prior to treatment for successful results, likewise during replication the HCV virus characteristically shows high mutation rates, the result is high degree of intra host genetic diversity (Lavanchy, 2011).

Genotype 1 is noted as most widely dispersed around the globe, around 46 % of all cases are found to be infected by this genotype and about 30 % around the globe have HCV genotype 3 as the offending type, thus emerging as second more prevalent in the overall population, the rest of proportion is filled by GT 2, GT 4 and GT 6 bearing HCV virus accounting for approximately 22 % of the total of HCV cases GT 5 have revealed to have less than 1%, representation (Messina et al., 2015). In United States the most prominent genotype is GT1 with subtypes 1a and 1b and other genotype found were GT2 and GT3

(Zein et al., 1996). Europe has genotype 1 with subtypes 1a and 1b its infected population. In west Africa the genotype showing highest prevalence is GT 2 and where as in North Africa GT 4, is major genotype effecting people and in the middle east Genotype 5 whereas GT6 has been reported in south Africa and Hong Kong, whereas genotype 3 is found to be prevailing in South Asia (Attaullah et al., 2011; Smith et al., 2014)

It has been reported that under various selection pressures the molecular pliability of HCV allows rapid rearrangement of its genomic organization (Iles et al., 2014) resulting in a genetic variability which has made the development of a successful vaccine a difficult goal to achieve in near future. Specific genotypes have attained the limelight as strong predictors for the outcome of antiviral therapy against hepatitis C (Cruz-Rivera et al., 2013; Honegger et al., 2013). With a variety of studies focussing on epidemiological and various clinical features it has been noted that countries who were categorized as low income group had Genotype 4 and Genotype 5 in most of populace and genotype 1, 2 and 3 were found in upper income group (Messina et al., 2015).

2.5.1 Effects of Genotypes and Subtypes

In Pakistan and neighbouring India the most prevalent genotype is genotype-3 (GT-3) followed by genotype-1(GT-1). Genotype 3a is the subtype found most parts of Asia and the same subtype is most common in Pakistan (Husain et al., 2009). More than one genotype can infect the same person ; the term defining this situation is mixed genotype infection (Xia et al., 2008). It has been noted that different regions in Pakistan show diverse prevalence rate of mixed genotype infections (Xia et al., 2008) and similarly when different assay methods were analysed for detection of mixed genotypes (Idrees & Riazuddin, 2008).

Different studies have pointed towards HCV genotypes which are catogarized as untypable or mixed. To get a clearer view of the genotype picture inquiry has been carried on 1300 complete coding sequences to authenticate the prevailing genotypes and subtypes of HCV (Idrees & Riazuddin, 2008). It has been noted that with studies concentrated on whole genome sequences the analysis of reported differences in genotypes resulted in discovery of even greater number genotypes, and the further inter-

genotype which are recombinants and also the inter-subtype recombinants. The occurrence of recombinant forms are found to be rare (Preciado et al., 2014). Studies were also carried out for gaining awareness of the part of HCV genotype in progress of ailment, the association was found to be significant and varying among various genotypes. Hepatic steatosis and increased evolution to cirrhosis is found to be linked with genotype 3 (Bugianesi, et al., 2012; Hézode et al., 2013). Various subtypes are found within each genotype to be significant in disease progression. Genotypes have also been shown to exert effect on treatment response. Overall it has been shown that subtype 1b is more easily curable than genotype 1a. When compared with other genotypes observation has been made that genotype 1b infected individuals had higher susceptibility to development of cirrhosis. Compared to genotype 1, genotype 3 was linked with an 80% higher risk of HCC in a large cohort of patients from the USA Veterans Affairs medical system.(Kanwal et al., 2014)

Liver fibrosis leads to the development of portal hypertension, it has been established in some studies that genotype 3 was linked to increased rates of progress of the disease to such complications (Castera et al., 2004). Likewise in other studies cirrhotic patients are associated with HCC it has been found that genotype 3 can be independently linked with HCC. Similarly, the study on development of disease in patients suffering from hepatitis C have shown that with the infection of genotype 3 HCV patients a more frequent pattern of hepatic steatosis with relatively rapid progression to cirrhosis is noted (Nkontchou et al., 2011). When all genotypes of HCV were compared regarding the rate of progression to HCC genotype it was found that HCV genotype 1b showed the highest rate of severity of liver fibrosis leading to cirrohsis. Moreover, genotype 1 patients have shown highest turnover to HCC (Bruno et al., 1997). It was established in various studies that genotype 3 was the genotype most responsive to therapy (Muhammad et al., 2011).

2.5.2 Genotype distribution in Pakistan

Different studies have pointed that genotype 3 is the most prevalent in Pakistan (Idrees & Riazuddin, 2008; Bosanet al., 2010) A large set of population was analysed for the prevalence of various genotypes of Pakistan. Analysis revealed that regarding (GT-1) the percentage of population effected was 11.5% the two subtypes were subtype 1a having

about 8.3 % and subtype 1b having 3.0 % distribution. Genotype 2 (GT-2) was present in 8.4% of the studied population and two subtypes of (GT-2) being 2a having representative percentage of 7.5% and 2b having percentage of 0.8 % was notified in the Pakistani population. Similarly when genotype 3 was studied 67.5% of our population was infected with it, with two subtypes prevailing showing subtype 3a to be present in 49.1% and genotype 3b in 17.7 % of the population (Idrees & Riazuddin, 2008). Researches were also conducted with a small population size showing genotype 3 to be present as most prevalent being present in 81.0-86.7% of the infected population it was seen that genotype 1 was second most prevalent genotype in Pakistan (Bosan et al., 2010). The reason of the difference in the affected population infected might be size of sample of the population studied.

2.6 OUTCOME OF HCV

In approximately 30 % of persons exposed to HCV, clearance occur spontaneously, In remaining 70 % of patients, chronic disease follows eventually leading to hepatic cirrhosis continuing to hepatocellular carcinoma . In circumstances such as advanced cirrhosis and risk of developing HCC with consequential liver failure propositions of liver transplantation are given to patients (Martin et al., 2013).

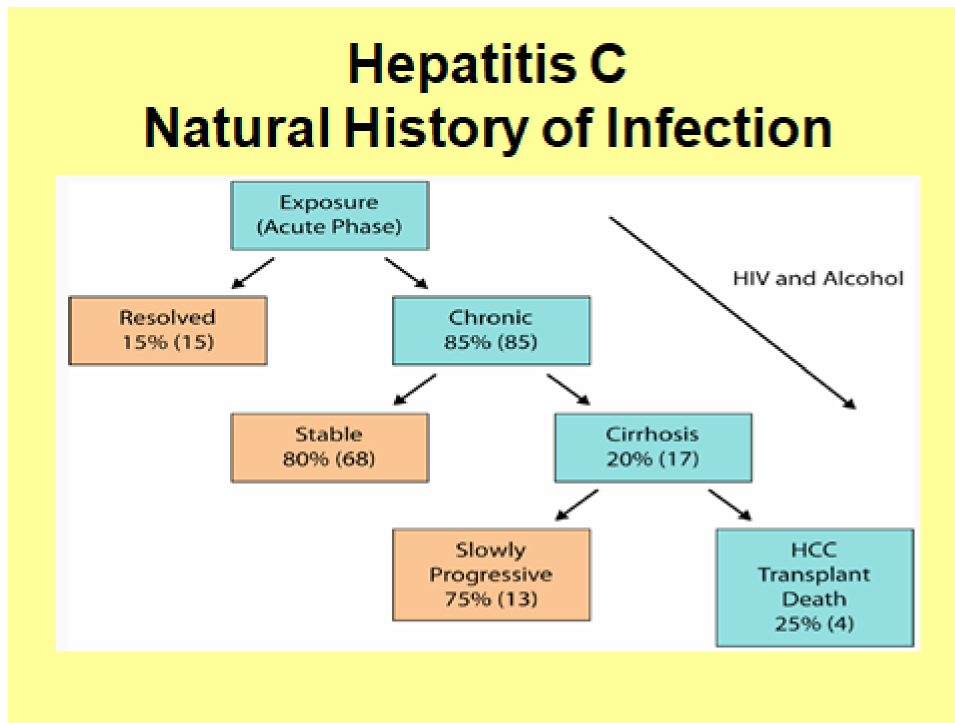


Fig. 2.4 Natural history of HCV infection (Hourigan et al., 1999)

2.6.1 Acute Hepatitis C Infections

Infection with HCV present for up to six months after inoculation of virus is considered acute hepatitis C (AHC) (Cox et al., 2005). The period of first two to twelve weeks comprise vague symptoms like nausea, malaise and occasionally accompanied pain in the upper right section of abdomen; these symptoms are somewhat similar to those found in illness associated with infection by other acute hepatitis causing viruses. In the forthcoming weeks a decline in symptoms accompanied with the fall of aminotransferase enzyme levels are noted in 40% of hepatitis C patients (Kim et al., 2008). In a period of about six to twelve weeks after exposure, raised levels of aminotransferases follow, this time period can range from one week to twenty six weeks, differing substantially among individuals. It has been noted that resolution of the HCV is not linked with normalization of aminotransferase levels (Kim et al., 2008).

Aminotransferase levels can rise to about 800 U/l equalling ten to thirty times the upper range of normal. Clinically the majority of patients of acute hepatitis present

asymptomatically, so the diagnosis of infection at this stage is quite hard. Viral and host factors have been related with natural and treatment-related HCV clearance.

Incubation period of HCV infection varies considerably ranging from a few days to about two months in patients infected with HCV (Hoofnagle, 1997).

HCV antibodies in patient serum can be detected using ELISA technique. These antibodies are present in the serum from eight weeks to months after first inoculation of virus in the body (Thimme et al., 2001). Presence of HCV RNA in blood and liver in acute HCV is done by PCR technique (Pawlotsky, 2002). In about 20 % of patients cure from the disease occurs specified by undetectable HCV RNA in the blood.

In acute infection jaundice become apparent in 5 % or less of patients. It has been pointed out that rest of patients comprising a large proportion adopt a course with unapparent symptoms posing a dilemma while diagnosing acute hepatitis C infection (Vogel & Nelson, 2009). This has led to adaptation of new strategies involving the practice of periodic screening of intravenous drug users similarly in a section of HIV patients recognised as MSM are those men having sex with men. (Vogel & Nelson, 2009).

HCV genotype was noticed as one additional competent to foresee the aftermath of infection with hepatitis C virus (Grebely et al., 2014). HCV genotype 1 was associated with elimination of HCV infection when compared to other HCV genotypes. Another factor which is associated with outcome of HCV is the route of HCV transmission. Intravenous drug users have been significantly related with development of chronic hepatitis C (Lavanchy, 2011).

The patients presenting symptoms of acute HCV infection are about 20-30% , rates equalling to 16-42 % yearly of acute HCV infection have been reported in developed country like US (Hagan et al., 2001). Chronic HCV infection can also remain asymptomatic for a quite long period which can last for decades .(Kamal et al., 2006).

Table 2.2 Host factors responsible for treatment response and non-response

Gender	Age& Disease Progression	Race	HCV Infection & Age Associated With SVR	IL28B
Not meaningful differences occur between men and women when the whole sample is examined without stratification by age *(*Belci, P *et al)*	The disease progression of chronic HCV infection often accelerates after20 years of infection, with lifestyle factors key drivers of hepatic fibrosis (Hajarizadeh, B.,*et al)*	The importance of race as an internal (genetic, metabolic) and external (social, economic,cultural) factors (Wilder,J., Saraswathula,2016)	Overall, patients aged < 40 years experienced higher rates of SVR than did those Aged > 40 years (84.2% vs 30.2%). Antonucci G, et al 2007)	Genetic Variants can exert impact on IL28 B production & response to chronic hepatitis C interferon-α and ribavirin therapy (Tanka et al 2009)

2.6.2 Hepatitis C Virus Chronic Infection

Chronic HCV is defined as presence of viral RNA in the blood stream for minimum 6 months (Chen et al., 2018). HCV infection can cause chronic liver disease (Lavanchy, 2011). Different clinical settings of chronic infection with HCV include : Minimal changes in the liver histology, widespread fibrosis and liver cirrhosis with hepatocellular carcinoma (HCC) or cirrhosis not associated with HCC. No symptoms are observed during the initial few years of chronic infection but subsequently symptoms develop. Liver cirrhosis is characterized by different features like abdominal fluid accumulation, bleeding of upper gastrointestinal tract, portal hypertension, jaundice, enlarged veins, and cognitive syndrome known as hepatic encephalopathy becoming the common reasons of liver transplant (Chen et al., 2018). The severity and clearance of HCV infection is greatly affected by many features, comprising age, gender, ethnicity, time of occurrence of infection, development of jaundice during acute hepatitis, viral load, and host genetic factors (Lavanchy, 2011).

HCV has an assessed worldwide frequency of about 3%, nearly 180 million are estimated carriers and approximately 350,000 infected persons or more expire each year as a result of infection from hepatitis C related disease of liver (Lavanchy 2011). Patients with liver cirrhosis have also been shown to develop hepatocellular carcinoma and undertake liver transplant for controlling of End-stage liver disease being presented as a consequence of long course of HCV infection. (Missiha et al., 2008).

2.7 TREATMENT OF HCV INFECTION

Many drugs have been approved by FDA for the treatment of HCV infection (Table 2.2). The regimens for treatment of chronic HCV patients is Ribavirin combined with Interferon (Booth, et al., 2001; Wang,et al., 2003). It is accepted as gold standard therapy for Chronic Hepatitis C (CHC) but evidence has shown the drugs are not tolerated very well, studies have shown that long course of the disease might have an effect towards the success of regimen. Such researches have led to development of two types of pegylated interferon which have better chemical composition and improved pharmacokinetics than non pegylated preparation of interferons (Lindsay et al., 2001; Manns et al., 2001).

Among these preparations better achievement of SVR is seen with Peginterferon alfa-2b which has a linear polyethylene glycol moiety of a 12-kD and a combination of Ribavirin when compared with Interferon alfa-2b and Ribavarin therapy. Similarly, it is noted that a 40 kD Peginterferon alfa-2a which is a polyethylene glycol recognized as branched moiety possess a prolonged half-life possessing a continuous viral suppression extending for about seven days, this led to administration of once a week dose and improved clinical efficacy (Dore, 2012; Caraglia et al., 2005).

A varied success rate of the regimen against the infection has led the scientist to work on predicting factors promising the success of regimen (Idrees et al. 2011). Polymorphism upstream of IL28B Gene, female gender, the accompanied jaundice and early age are prognostic factors of treatment, it is worth noting that none of these factors precisely predicts spontaneous clearance when seen at individual level (Asselah, 2010). Cytokines are among one the most important host defences against viral infections. Human body have the capacity to produce its own cytokines which protects against different viral diseases but these cytokines also induces inflammatory responses which often leads to tissue injuries (Fallahi et al., 2012). Sometimes the chronicity of the disease or resistance to the interferon is often resulted due to the secretion of inappropriate amount of cytokines (Kiser, et al., 2013). Cytokines production is based on genetic component which explains why different individuals produce cytokines at different rates studies have revealed that cytokine production gene single nucleotide polymorphism exert effects seen as varied response to treatment (Steinke & Borish, 2006). It has been noted that various features can be designated as predictors of success to interferon & Ribavirin combined treatment.

Among many host factors studies have pointed towards gender and race of the patient, basal metabolic index and the stage of liver fibrosis and favourable IL28B genotype. Similarly, among viral factors viral load and its genotypes have shown to be affecting the course of disease. Patients with genotype 3 HCV with advanced fibrosis do not benefit from extended therapy with pegylated interferon and ribavirin (Shoeb et al., 2014). It was noted that despite the fact that Ribavarin has a mechanism of action which is still

speculative, it is being used in the therapy (Fukuchi, et al., 2010). In HCV patients who are considered non responders develop liver cirrhosis. These patients mostly are those infected with more resistant HCV genotypes, which do not respond to these therapeutic regimens. As with other drugs adverse reactions such as symptoms resembling influenza-like ailments, hematologic idiosyncrasy, and certain indications being psychiatric in nature, can led to untimely cessation of treatment. A HCV standard treatment comprise of one direct acting antiviral (DAA), a protease inhibitor ,combined with Peg-IFN & ribavirin has almost doubled the likelihood of favourable response to therapy. (Maylin et al.2008, European Association for The Study of The Liver. 2018).

With the improved therapy with Direct Acting Antivirals the coming decade will be a critical period in the progress towards attaining improved response against infection with the HCV virus. With addition of a protease inhibitor with combined pegylated interferon, ribavirin in early phase will be linked with augmented toxicity and difficulty of management of therapy but, strategies could be developed towards the regimens with improved tolerability and simple dosing programmes (Zampino et al., 2018).

Table 2.3 FDA approved list drugs and Treatments for Hepatitis C (https://www.fda.gov/forpatients/illness/hepatitisbc/ucm408658.htm)

Brand Name	Generic Names	Manufacturer Name	Indication
CoPegus	Ribavirin	Roche	Use in combination with Pegasys or with Roferon of adults with chronic hepatitis C virus infection.
Daklinza	Daclatasvir	Bristol-Myers Squibb Company	An NS5A replication complex inhibitor, used with sofosbuvir for the treatment of patients with chronic HCV genotype 3 infections.
Epclusa	sofosbuvir, velpatasvir	Gilead	Afixed-dose combination of sofosbuvir, and velpatasvir, and is indicated for the treatment of adult patients with chronic HCV genotype 1, 2, 3, 4, 5, or 6 infection -without cirrhosis.
Harvoni	ledipasvir/ sofosbuvir	Gilead	A fixed-dose combination of ledipasvir, and sofosbuvir, and is indicated for the treatment of chronic hepatitis C (CHC) genotype 1 infection in adults.
Incivek	Telaprevir	Vertex Pharmaceuticals	Uses in combination with peginterferon alfa and ribavirin, for the treatment of genotype 1 chronic hepatitis C with compensated liver disease.
Infergen	interferon aphacon-1	Three Rivers Pharma	Treatment of chronic hepatitis C in adult patients with compensated liver disease who have anti-HCV serum antibodies and/or HCV RNA
Intron A	interferon alpha-2b	Schering	Treatment of chronic hepatitis C in adult with compensated liver disease.
Mavyret	glecaprevir and pibrentasvir	AbbieVie	Indicated for the treatment of adult patients with chronic HCV genotype 1, 2, 3, 4, 5 6 infection without cirrhosis.
Olysio	Simeprevir	Janssen Pharmaceuticals	Treatment of chronic hepatitis C genotype 1 infection

Pegasys	pegylated interferon	Roche	Treatment of adults with chronic hepatitis C virus infection with compensated liver disease and have not been previously treated with interferon alpha
Pegintron	pegylated interferon alpha-2b	Schering	in combination with rebetol, is indicated for the treatment of chronic hepatitis C in patients 3 years of age and older with compensated liver disease.
Rebetol	Ribavirin	Schering	use in combination with Pegintron for treatment of chronic hepatitis C in patients with compensated liver disease.
Roferon	interferon alpha-2a	Roche	treatment of chronic hepatitis C in patients 18 years of age or older
Sovaldi	Sofosbuvir	Gilead Sciences	for the treatment of chronic hepatitis C as a component of a combination antiviral treatment regimen
Technivie	Ombitasvir, paritaprevir and ritonavir	AbbVie Inc.	The product is indicated in combination with ribavirin for the treatment of patients with genotype 4 chronic hepatitis C virus.
Victrelis	Boceprevir	Merck & Co.	treatment of chronic hepatitis C (CHC) genotype 1 infection, in combination with peginterferon alfa and ribavirin,
Viekira Pak	Ombitasvir, paritaprevir and ritonavir tablets	AbbVie Inc.	use with or without ribavirin for the treatment of patients with genotype 1 chronic hepatitis C virus
Vosevi			for patients who have been previously treated with the direct-acting antiviral drug sofosbuvir, or other drugs for HCV that inhibit a protein called NS5A.
Zepatier	elbasvir, grazoprevir	Merck Sharp Dohme	a fixed-dose combination product containing elbasvir, and grazoprevir, an HCV NS3/4A protease inhibitor, and is indicated with or without ribavirin for treatment of chronic HCV genotypes 1.

2.8 SINGLE NEUCLEOTIDE POLYMORPHISMS IN IL28B AND HCV INFECTION

Treatment of HCV infection is regarded as a challenge yet to be resolved by the medical community (Barth, 2015). Independent genome wide association studies (GWAS) are found to be beneficial for planning better individualized treatment regimens for HCV infection (Venegas et al., 2012). According to these GWAS genetic variants found in proximity of IL28B gene are sturdily linked with response to interferon (IFN)-based therapy (Ge et al., 2009). Interest arose initially when several GWAS studies highlighted that favourable single nucleotide polymorphism rs12979860 found 3kb upstream IFNλ3 promoter gene (IL28B) is linked to better treatment response of chronic HCV. This favourable detection has led to a series of studies on the polymorphisms exact location (Alavian et al., 2014). New searches designate two strong bi allelic Linkage disequilibrium among IL28B gene single nucleotide polymorphisms: namely rs12979860 and rs8099917 it was also noted that there were specific divergences amid these two sets of genotypes suggesting that rs12979860 to be a better pointer for IL28B phenotype when compared with rs8099917. (De Castellarnau et al., 2012; Kobayashi, et al., 2012).

Similar favourable response is shown by the favourable alleles of the two above mentioned genotypes are shown in patients infected with HCV genotype 4 (Antaki,et al., 2013), favourable pattern was also shown in children and adults showing spontaneous clearance of virus (Grebely et al., 2014). The positive response by these favourable alleles was shown in HCV and HIV coinfection (Rallón et al., 2010).

The noticeable variation in incidence of favourable IL28B genotype in different areas of the world have resulted in diverse SVR rates. Higher frequency is found in in East Asia amounting to about 70 % of population and nearly about 30 % in North American and Western European people (Thio et al., 2009). Sustained virological response is achieved more frequently with rs12979860 CC (vs CT or TT) giving CC the recognition of favourable allele. In case of rs8099917 TT is favourable (vs GT or GG) is linked to higher SVR rate when they were given treatment with PEG-IFN and ribavirin to patients of HCV genotype 1, irrespective of the race of the HCV patient.

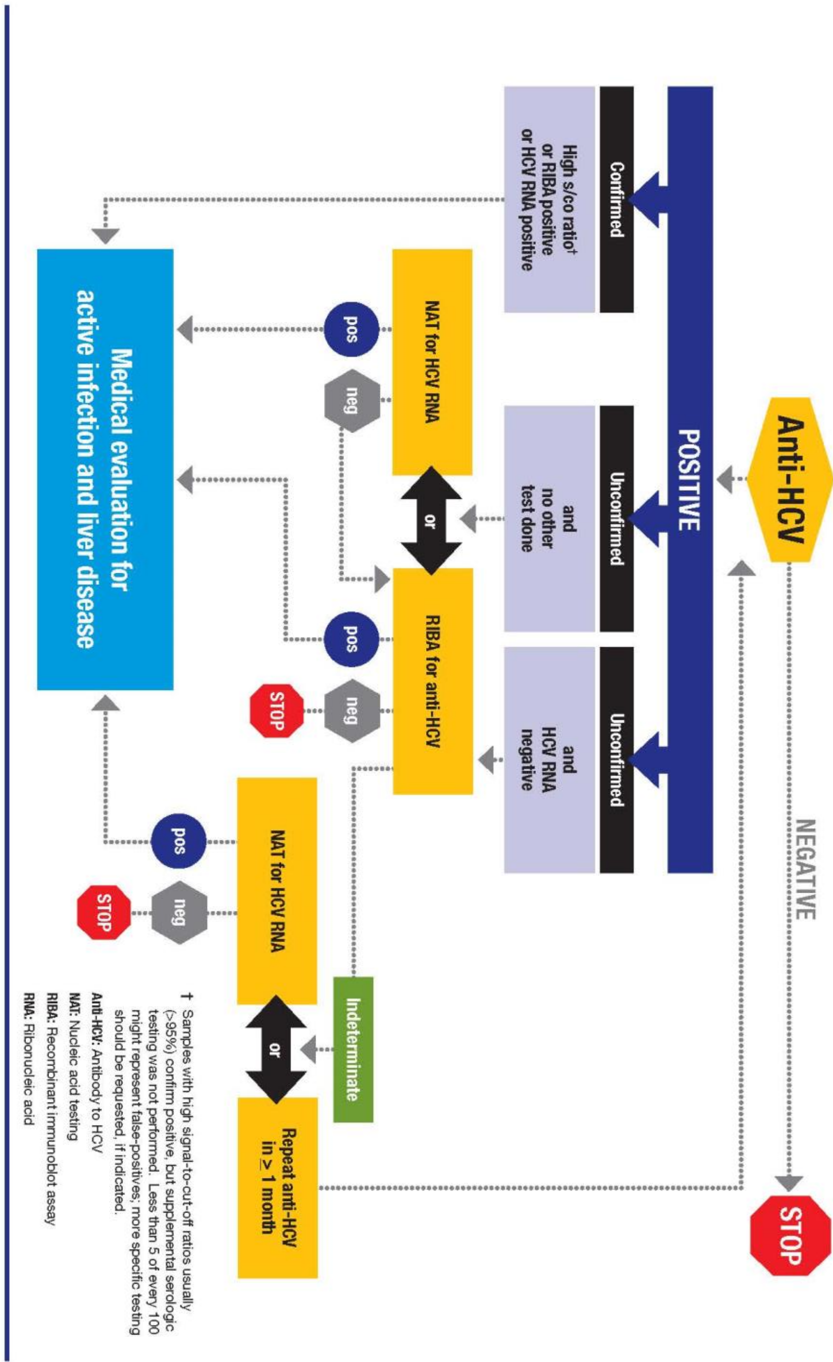
The effect of IL28B on the response to therapy in patients infected with viral genotypes 2 and 3 have been the aim of several studies, showing a link between IL28B polymorphisms with SVR, though some studies have not shown such association (De Castellarnau et al., 2012).

The subsequent studies pointed that in patients having favourable allele rs 12979860-CC there is no need to additionally evaluate 8099917 genotype. When included in a multivariate analysis with base line features which are linked with SVR such as viral genotypes1b, previous responders to treatment or the patients who partially responded to therapy, the stage of advance fibrosis of liver, female sex, and finally the viral load measure found to be a lesser amount than 800,000 IU/mL, it was found despite analysis of these parameters 12979860 genotyping has significant status of prediction of response in patients who are treatment naïve and relapsers andfor the set of and also for patients who displayed the ability to successfully resolve the infection when treated with PEG-IFN/RBV (Calisti et al., 2015). In contrast when assessed in treatment-failure patients given therapy comprising of telaprevir forming the bases of therapy (REALIZE study), IL28B was not found to be meaningfully affectiing SVR (Pol et al., 2013).

In African population the frequency of C allele was fewer than in Caucasians (Thio et al. 2009). It was noticed that the largest fraction of allele recognized as favourable was found amongst Asians and smallest in Africans, which explains the increased rate of good response to interferon (IFN) therapy in population belonging to Asian origin in comparison with the population such as of African origin.(Fellay et al. 2009). It was deduced from different studies that rs12979860 C allele of chronic HCV infection shows positive response during antiviral treatment (Thompson et al., 2010; Ruiz et al., 2011). In Indian populations is still not understood that what is the effect of IL28B associations in interferon responsiveness (Gondeau et al., 2015).

2.9 DIAGNOSIS OF HCV:

Diagnostic tests for hepatitis C comprise serologic assays for measuring antibodies produced in response to HCV infection and molecular virologic assays to directly identify HCV RNA (Fg 2.5)


Hepatitis C Virus (HCV) Infection Testing for Diagnosis
Anti-HCV
NEGATIVE
STOP
POSITIVE
Confirmed
High s/co ratio†
or RIBA positive
or HCV RNA positive
Unconfirmed
and
no other
test done
Unconfirmed
and
HCV RNA
negative
NAT for HCV RNA
or
RIBA for anti-HCV
pos
neg
pos
neg
STOP
Indeterminate
NAT for HCV RNA
or
Repeat anti-HCV
in ≥ 1 month
pos
neg
STOP
Medical evaluation for
active infection and liver disease
† Samples with high signal-to-cut-off ratios usually (>95%) confirm positive, but supplemental serologic testing was not performed. Less than 5 of every 100 might represent false-positives; more specific testing should be requested, if indicated.
Anti-HCV: Antibody to HCV
NAT: Nucleic acid testing
RIBA: Recombinant immunoblot assay
RNA: Ribonucleic acid
DEPARTMENT OF HEALTH & HUMAN SERVICES
Centers for Disease Control and Prevention
Division of Viral Hepatitis
www.cdc.gov/hepatitis

2.9.1 SNP Genotyping Methods

When a single base mutation occurs in different individual it is known as Single nucleotide polymorphism (SNP). These SNPs are likely associated to phenotypic differences of various diseases in different population. SNPs play a chief share in drug development, response and clinical trials. Due to the impacts of SNPs on health care different SNP high through put methods are developed by biotechnological and pharmaceutical industry, different SNP high through put methods are developed. All the SNP genotyping methods are based on two major types, Allelic Discrimination and Signal Detection Methods (Twyman, 2005).

2.9.2 Allelic Specific Hybridization (ASH) Methods

The simplest method of allele discrimination is ASH in which allele specific oligo-nucleotide probes (ASO) are used (Hoshida et al., 2008). Molecular beacons and taqman both rely on ASH. For each specific allele one probe is needed. ASH is modified with allele specific-PCR in which alleles are discriminated by using allele specific primers (Pawlotsky, 2002). Another modification of ASH is allelic specific ligation. In this method of ligation, two oligonucleotides anneal adjacent to each other and ligases are used to join them together (Lizardi et al., 1998).

2.9.3 Single Base Primer Extension (Mini Sequencing) Method

In mini sequencing method primers are design that anneals one upstream of the variable site. This technique is more reliable than ASH method because of using greater diversity of labeling for example four different labeling tags can be used for 4 different nucleotides given different results of SNPs at the same time (Syvanen, 1999).

2.9.4 Enzymatic cleavage Method (RFLP)

A technique based on principle of enzymatic cleavage of allelic specific site of DNA is known as it is known to be one of earliest techniques which is widely used (Beckmann & Soller, 1986). In this process restriction endonuclease enzymes are used that recognize

specific sequence on DNA and causes cleavage at that site. RFLP technique is mostly adopted in PCR-free genotyping technique known as Invaders (DE burns, 2002).

2.9.5 Fluorescence Resonance Energy Transfer (FRET)

Fluorescence Resonance Energy Transfer (Lambot et al., 2002) technique uses allele specific oligonucleotide cassettes generating signals in a single homogenous reaction vessel (Syvanen 1999; (Koch, 2004). In this method energy transfer takes place between donor fluorophore and accepter fluorophore and emission of light intensity is measured. Two assays use the FRET principle, TaqMan assay, in which taq polymerases produce fluorescent signal from ASO probe (Sobrino & Carracedo, 2005); Livak et al., 1995) and Molecular Beacon assay based on probes labeled with donor and acceptor fluorophore having self complementary ends (Tyagi & Kramer, 1996).

2.9.6 Amplification Refractory Mutation System (ARMS)

ARMS-PCR is easy, rapid, reliable and economical SNP genotyping assay (Li et al., 2014). In this assay primers are designed that can amplify the variant site of DNA. Base mismatch is inserted at the 3’-end of the primer so that it will attach to the mutant strand of DNA and amplify that region while the other matches and amplify the wild type (Punia et al., 2009).

2.9.7 Pyrosequencing

Pyrosequensing assay is based on pyrophosphate detection during DNA synthesis. Pyrophosphate is generated during DNA synthesis which is processed by ATP sulfurylase to produce ATP. This ATP activates luciferin by luciferase enzyme emitting light signals (Ronaghi et al., 1998).

INTRODUCTION

The World Health Organization (WHO) recognizes HCV infection presence worldwide (Thomson et al., 2008; Basnayake & Easterbrook, 2016). Studies have reported that among different geographic areas around the globe there is variability in the percentage of the patients suffering from this infection. It is estimated that almost 10 million population of Pakistan is suffering from HCV (Raja & Janjua, 2008; Umar et al., 2010). In India nearly 12.5 million of population is found to be infected with HCV (Firdaus et al., 2014; Deterding et al., 2017). Pakistan has HCV sero positivity more than its neighbours (Tahir et al., 2016). The main routes by which the virus spread is via injudicious intravenous usage of drugs, inappropriate blood transfusions practices and unsterilized equipment used in various therapeutic techniques (Van Herck et al., 2008). Over the past two decades the growing number of HCV infected individuals associated liver disease has resulted in increased demands on health care systems. The need for cure of this complex infection has contributed to advancement in diagnostic techniques and a considerable progresses in treatment and restraint of the infection (Northrop, 2017).

Hepatitis C Virus

For perceiving a better understanding of HCV kinetics in a consensual international classification a study of complete HCV genome was carried out and it was affirmed that HCV is a positive strand RNA virus which has ~9.6 kb genome with a single continuous open reading frame (ORF) that translates three thousand amino acids containing precursor protein (Choo et al., 1991; Houshmand & Bergqvist, 2003).

An HCV infection lead to liver disease, and its incidence is even more raised in at risk populations. Most of the spread seems to be caused by healthcare procedures. Keeping these features in fore front national priority should be given towards curtailment of the disease (Al Kanaani, et al., 2018). Most of the patients infected with HCV are asymptomatic for decades and in the course of infection may lead to cirrhosis of liver and its progression to hepatocellular carcinoma, curtailing to end stage liver disease (Westbrook & Dusheiko, 2014). In 15 to 25% of patients the HCV-RNA become untraceable and ALT becomes normal while around 75 to 85% develop chronic hepatitis

i.e HCV RNA sustained its presence in the blood stream for at least 6 months. (Chen & Morgan, 2006).

Treatment of chronic HCV comprise Interferon-based therapy consisting of Pegylated interferon (PegIFN) in combination with ribavirin (RBV). Recently the addition of direct-acting antivirals to the regimen have materialized as an option to accomplish virus eradication from the body .The aim of therapy is to curtail the development of liver fibrosis which may lead to cirrhosis & hepatocellular carcinoma (HCC) (Maylin et al., 2008).

Viral factors are known which can envisage response towards resistance to interferons, among them HCV genotype and associated viral load are the strongest ones. In Pakistan and neighboring India, the most prevalent viral genotype is GT-3 followed by genotype GT-1. Genotype 3a is the subtype found most parts of Asia and the same subtype is the most common in Pakistan. It is of great interest for the researchers that more than one genotype can infect the same person referred to as mixed genotype infection (Ali et al., 2011). However, when different assay methods were evaluated for detection of mixed genotypes in same set of people revealed different results with different assays (Idrees & Riazuddin, 2008).

The treatment schedule consisting of weekly pegylated interferon-α with oral ribavirin daily for 24 to 48 weeks is applied against the HCV infections, but a varied success rate of the regimen has led researchers to work on predicting factors towards the success of regimen (Akram et al., 2011; Yarlott, et al., 2017). HCV genotype 3 has been associated with higher rates of treatment success nearing about 70% in those patients without prior interferon therapy and up to 55-60 % with prior treatment with standard regimen of (PegIFN)-α & ribavirin (Ghany et al., 2011). Patients with chronic HCV infection are currently treated with direct acting antivirals (DAA) in conjunction with interferon-based therapy. Comparative insensitivity of genotype 3 to available protease inhibitors has been noticed which points to the fact that universal remedy against the infection is still not attained (Zeuzem et al., 2013; Deterding et al., 2017).

IL28B Polymorphism

Different factors have been found to play role in treatment response to standard regimens (Ahmed & Felmlee, 2015). Polymorphism upstream of IL28B Gene, female gender, young age group are factors associated with clearance of virus spontaneously, it is worth noting that none of these factors precisely predicts spontaneous clearance when seen at individual level. It is vital to find new yet undiscovered determinants of treatment response (Asselah, 2010). Development of jaundice during acute hepatitis and role of some cytokine producing genes are also recognized as host factors (Lavanchy, 2009).

Cytokines production gene affects the response to treatment through single nucleotide polymorphism. Sometimes the chronicity of the disease or resistance to the interferon often results due to the secretion of inappropriate amount of cytokines (Kiser & Flexner, 2013). In the present-day period, new HCV standard treatment comprise of one direct acting antiviral (DAA), a protease inhibitor combined with Peg-IFN & ribavirin. The usage of the therapy has almost doubled the likelihood of favorable response but the patient has to bear the cost of increased toxicity (Maylin et al. 2008; Rauch et al., 2010).

In order to initiate a thorough understanding of the HCV patients, the present study was completed in two phases; in the first phase the focus was on analysis of viral factors such as prevalent genotypes in the region with associated viral loads in the host. In the second phase association of polymorphism of IL28B gene at rs12979860 & rs8099917 loci with spontaneous and treatment induced clearance of HCV was analyzed. With the aim that the results of the detailed analysis might be used to guide treatment for chronic HCV infection in Pakistani patients in the future.

HYPOTHESIS

Genotypes of IL28B have association with Hepatitis C Virus spontaneous clearance, treatment response in genotype 3 patients in Pakistan.

Aim and Objectives

The main aim of the study was the investigation of various factors associated with HCV in our sets of population and their role in treatment response. To fulfill the aim following objectives were laid down.

PHASE 1

1. Determination of common Hepatitis C virus genotypes in study set of population and their demographic pattern for age and gender.
2. Determination of mean viral load specific to each HCV genotype.

PHASE 1I

3. Determination of Frequency of patients achieving rapid virological response (RVR) and SVR and determination of Association of SVR with RVR, age, ALT levels and Hemoglobin.
4. Determination of Frequency of rs8099917 and rs12979860 genotype polymorphism of IL28B in HCV infected patients.
5. Determination of IL28B genotype 8099917 & rs12979860 allelic frequency.
6. Analyses of effects of IL28B polymorphism on HCV spontaneous clearance and treatment response.

MATERIALS AND METHODS

3.1 PREVELANCE OF HCV GENOTYPES (Study Phase 1)

3.1.1 Patient Selection

Cross-sectional Study was conducted after ethical approval of the project was taken from Microbiology dept. QAU, Islamabad and NORI, Islamabad, written informed consent was taken from the patients .

The basic aim of first phase of study was to find out the association of genotypes with host age, gender and viral load. This study included 320 patients with chronic hepatitis C virus (HCV) infection who were referred to the hospital between November 2011 and July 2012. Out of these 151 who were found to be positive when tested for HCV RNA and were treatment naive. All those patients were excluded from the study found co-infected with HBV, HIV or patients with the alcoholic liver disease.

For second phase of study for Association of IL28 B polymorphism analysis two groups were included in the study .

Inclusion Criteria; Only those patients were selected who were currently on Peg-IFN/RBV treatment or received their 24 weeks of combine therapy of Peg-IFN/RBV and agreed to provide blood sample.

Exclusion Criteria: All those patients were excluded from the study that were co-infected with HBV, HIV or patients with the alcoholic liver disease.

In first group105 patients were enrolled in the study for IL28B SNP genotyping out of which 18 patients failed to meet inclusion criteria or refused to participate. For IL 28 B Genotyping patients were selected who were currently on Peg-IFN/RBV treatment or received their 24 weeks of combine therapy of Peg-IFN/RBV and agreed to provide blood sample. In second group 82 patients were included with the same inclusion and exclusion criteria for IL28B genotyping.

The samples (5-7 mL) blood was collected from the patients in EDTA tube and stored at −80°C in super cool temperature. All HCV RNA positive samples were subjected to

genotype determination using restriction fragment length polymorphism and type specific PCR followed by direct sequencing. The analysis revealed the presence of genotypes 1 and 3 with further subtypes 1a, 1b, 3a, 3b and mixed genotypes 1b + 3a, 1b + 3b and 3a + 3b. Genotyping of Hepatitis C was done by amplification targeting the core region of Hepatitis C Virus isolates by using specific primers described by Ohno et al., (1997). In Table 3.1 the Primer sequences used for the genotypes are mentioned.

3.1.2 HCV Genotypes determination

3.1.2.1 Plasma Separation

Centrifugation at 14,000 rpm for 4 minutes of blood samples resulted in the separation of plasma using clean 1.5 ml eppendorf tubes. RNA was extracted from the tubes containing plasma at room temperature or was stored at -20°C for the purpose to be used afterward.

3.1.2.2 Extraction of Viral RNA

Aj Roboscreen, instant virus RNA Kit was used for extraction of viral RNA from the blood plasma following the protocols set by the manufacturers. Extraction of RNA from the samples was carried out in the controlled environment of Biosafety level II cabinet (BSL II), to avoid contamination of the samples, and to minimize the exposure of laboratory personals and the laboratory environment to viral RNA.

Lysis solution RL (450 µl) was added to the extraction tubes, and then 150 µl of sample was added and mixed thoroughly by pulsed vortexing for 10 seconds. Extraction tubes were incubated for 15 minutes. During the incubation period the samples were vortexed 3-4 times to increase the lysis efficiency of the solution. After the incubation the tubes were centrifuged to remove the condensate from the lid of the tubes. After the lysis, 600 µl of Binding solution RBS was added to the extraction tubes and mixed vigorously by pipetting up and down several times to get a homogenous solution. In the next step 650 µl of the sample was applied to the 2.0 ml Receiver Tube having Spin-Filter and centrifuged at 12,000 rpm for 1 minute. These Receiver Tubes having Filters were discarded and the residual samples were loaded onto the Spin-Filter after placing it into another 2.0 ml Receiver Tube. Then the Receiver Tubes were centrifuged at 12,000 rpm for 60 seconds. Receiver Tubes having filters were discarded again and a new Spin-Filters was placed

into another 2.0 ml Receiver Tube. Next the 500 µl of Washing Solution WS was poured and then centrifuged at 12,000 rpm for 1 minute. The Receiver Tubes with the filters were discarded and the Spin-Filters were placed into the new 2.0 ml Receiver Tubes. Again the previous step was repeated with the addition of 650 µl of Washing Solution LS. The mixture was then centrifuged at maximum speed for 2 minutes to remove all the traces of ethanol and discarded the Receiver Tubes. In the final step the Spin-Filters were placed into a 1.5 ml Elution Tube and 30 µl of RNase-free water was added to the tube. Then the Tubes were incubated at room temperature for 2 minutes and centrifuged at 8,000 rpm for 1 minute. Additional RNase-free water of 30 µl was added to the Spin-Filter and again centrifuged at 8,000 rpm for 1 minute. The Spin Filters were discarded and the Elusion Tube containing the combine elutes was vortex and centrifuged shortly. Since the RNA is highly susceptible to degradation, the elution tube was placed on ice immediately after extraction. This RNA was processed by the PCR amplification within 20 minutes or stored at -30°C.

3.1.2.3 cDNA Synthesis

Antisense primer (A-c2) for the core region was used to synthesize cDNA from RNA (Table 3.2). 10 µl of master mix was prepared containing 2 µl of dNTPs mix (10 mM each), 4 µl of 5X reaction buffer, 25 pM antisense external primer (A-c2), 40 U of RNase inhibitor (Fermintas, USA), and 200 U of M-MLV RT (Fermentas, USA). Then the master mix was added to 10 µl of RNA and reaction was run. The synthesis conditions are given below.

42°C→60 minutes

92°C→ 02 minutes

The cDNA was either utilized for the PCR or was kept for storage at -20°C.

The first round PCR was carried out with 20 µl of master mix containing 4 µl of template cDNA, 10 µl of Go-Taq Green (Promega), 1.25 pMol of each sense (S-c2) and antisense (A-c2) primers (Table 3.1). Following are the synthesis conditions for the first round PCR.

Cycles repeated 20 times 94°C for1 minute

45°C for1 minute

72°C for1 minute

20 cycles again for 94°C for1 minute

60°C for1 minute

72°C for1 minute

3.1.2.4 Use of the genotype Specific Primers (PCR Second Round)

Two distinct mixtures were made for the amplification of PCR during second round. For both mixtures same reagents but different primers were used. Mix-1 constitute 10 µl of Go-Taq Green master mix (Promega, USA), 1.25 pmol of S-2a, S-7, G-2a, G-1b, G-3b, G-2b primers, and added with 2 µl of first round PCR amplified product. While 1.25 pmol of S-7, G-4, G-3a, G-1a, G-6a, and G-5a primers (Table 3.1) were used for mixture-2. The optimized conditions for PCR amplification are given below.

95°C→04 minutes

29 cycles 94°C→30 seconds

62°C→30 seconds

72°C→60 seconds

72°C→7 minutes (Elongation)

3.1.2.5 Electrophoresis using Agarose Gel

In order to analyze the amplified product electrophoresis with 2% of agarose gel was used. The gel was prepared by adding 2 grams of agarose to 100 ml of 2X TBE buffer, it was mixed and heat was applied to completely dissolve all the agarose crystals. In order to visualize DNA on gel after cooling of the solution of gel 30 µl of Ethidium Bromide (EtBr) was mixed in the gel. The prepared solution was then transferred into the gel tray, which has the comb. A molecular marker of 100 bp (MBI Fermentas, Catalogue # SM1153) was run in parallel with the samples in order to compare sizes of fragment. In order to visualize the resulting PCR product UV light of “BIO-RAD Gel Doc XR” imaging system was used.

Table 3.1 Primer Sequences used in Genotyping of HCV

Primers	Sequence
S-c2	5'-GG(GA)GGTCTCGTAGACCGTGCACCATG-3'
A-c2	5'-GAGACGGGTATAGT(AC)CCCATGAGAGTCGGC-3'
S-7	5'-AGACCGTGCACCATGAG(CA)C-3'
S-2a	5'-AAC(AC)TAACCGTCGCCCACAA-3'
G-1b	5'-CC(TG)CCCTCGGGTTGGCTAAG-3'
G-2a	5'-CACG(TG)GCTGGGATCGCTCC-3'
G-2b	5'-GGCCCCAATTAGGACGAGAC-3'
G-3b	5'-CGCTCGGAAGTC(TT)ACGTAC-3'
G-1a	5'-GGATAGGCTGACGTCTAC(CT)-3'
G-3a	5'-GCCC(A)GGACCGGCCTTCGCT-3'
G-4	5'-CC(CG)GGAACTTAACGTCCAT-3'
G-5a	5'-GAACC(TC)GGGGGGA(GA)GCAA-3'
G-6a	5'-GG(TC)ATTGGGGCCCCAATGT-3'
A-5	5'-TACGCCGGGGGT(CA)TGTGA(GG)GCCCCA-3'

3.1.3 Demographic Analyses of Samples

3.1.3.1 Distribution Pattern of Hepatitis C Virus Genotypes According to Age

Patients were divided into 04 age groups consisted those patients from 18 to 30 years were categorized as group 01 less than 30 years, age group 02 from 31-40 , age group 03 from 41-50 years, age group 04 greater than 50 years patients were tested for presence of HCV single and mixed genotypes infection genotype 3a, genotype 3b and other genotypes like 1a and 1b similarly mixed infections of genotype 3 like 3a + 3b and genotype 1 i.e. 1a + 1b were also analyzed.

3.1.3.2 Distribution Pattern of Hepatitis C virus genotypes According to Gender

In total of 151 patients frequency of HCV genotypes were analyzed for their distribution in male and female population

3.1.3.3 Mean Viral Load of Patients Infected with Different HCV Genotypes

3.1.3.3.1 HCV Quantitative test

HCV RNA quantification was conducted by using Smart Cycler II Real-time PCR (Cepheid, Sunnyvale, Calif. USA) along with HCV RNA quantification kits (Sacace Biotechnologies,Italy). The Smart Cycler II is a (PCR system) by which amplification and diagnosis were accomplished at same time with TaqMan technology (Applied Biosystems,Foster City, Calif) fluorescent probes were used to inspect amplification after every replicating cycle. The lower and upper detection parameters of the used assay were 250 and 5.0×10^8 IU/mL, respectively. Specimens giving values above the upper limit were diluted 100-fold, retested and the attained values were multiplied by this dilution factor to determine the real HCV RNA concentration in international units per ml (Popescu et al 2011).

3.2 EFFECT OF IL28B POLYMORPHISM ON HCV SPONTANEOUS AND TREATMENT RESPONSE (STUDY PHASE 2)

In phase 2 of the study the effect of IL28B gene polymorphisms (rs8099917 & rs12979860) on spontaneous clearance, treatment response was determined. The cross-sectional study was conducted in two settings. The first group (group I) was of the same patients selected for phase 1 of study consisted of patients having HCV genotype 3a which was found to be the most prevalent in the set of patients included in phase 1 of the present study. All those patients were not incorporated in the present study that had HBV, HIV co-infection or were suffering from alcoholic liver disease. The patients were considered as those achieving Rapid Virological Response (RVR) and Sustained Virological Response (SVR). Association IL28B gene polymorphisms (rs8099917 and rs12979860) with RVR and SVR was determined.

Similarly HCV patients were registered from a private sector hospital in Islamabad having genotype 3a and same inclusion criteria as above. This group was categorized as group II in our study. Association IL28B gene polymorphism rs8099917 with RVR and SVR was determined.

The samples were collected from the patients attending Social Security Hospital Islamabad & from a Private Clinic in Islamabad; 5-7 ml of the patient blood was taken in EDTA tube and stored at -80°C in super cool temperature. After approval from board the blood samples were processed in the Department of Pathology, NORI Hospital, Islamabad for HCV genotyping, viral load and IL28B genotyping.

3.2.1 Application of Technique Restriction Fragment Length Polymorphism (RFLP)

Analysis using RFLP technique was done by amplifying rs8099917 and rs12979860 region of the gene IL28B by using specific primers described by Venegas et al, (2011). Primer sequences are given in Table 3.2. The restriction pattern of IL28B genotype rs8099917 and rs12979860 was analyzed on 2% agarose gel.

3.2.1.1 Genomic DNA extraction

Phenol/chloroform method was used for the whole blood Genomic DNA extraction. 3 different solutions A, B, and C were prepared in different molar concentrations. Solution A consists of 5 mM $MgCl_2$, 0.32 M sucrose, and 10 mM of Tris, and Solution B has the combination of 2 mM of EDTA, 10 mM Tris (pH 7.5) and 400 mM NaCl. Solution C consists of Iso-amyl alcohol (1 volume) and Chloroform (24 volumes) with the ratio of 24:1. Phenol buffered with either 10 mM Tris or Distilled water was used in order to remove its impurities. Steps involved in the genomic DNA extraction from the whole blood are given below.

Blood (700 µl) of was transferred into a clean 1.5 ml of Eppendorf tube and 500 µl of solution A was added and mixed it. The solutions were kept at room temperature for about 8-10 minutes. Centrifugation of mixture was carried out at 13000 rpm for 1-3 minutes and then the supernatant was separated and discarded, and the nuclear pellet was obtained which was re-suspended in 400 ml of solution A and mixed thoroughly by inverting several times. The mixture was again centrifuged, and the supernatant was separated and discarded and 400 µl of solution B was used to re-suspend the nuclear pellet and 12 µl of 20 % of SDS and 25 µl of proteinase-K was added to the mixture in order to degrade proteins and other impurities and incubated at 37°C overnight. After incubation, 0.5 ml of equal volume of solution-C and Phenol was added and inverted

numerous times and centrifuge at 14000 rpm for 8-10 minutes. The upper layer was transferred into a new Eppendorf tube and equal volume of solution C was added to the mixture and centrifuged at 14000 rpm for 8-10 minutes. The aqueous phase was collected in Eppendorf tube, and the DNA was precipitated with 55 µl of sodium acetate and 1000 µl of 100 % chilled ethanol by inverting the tube several times. Then the tube was centrifuged at 13000 rpm to settle DNA and the supernatant was removed and discarded. 0.5 ml of 70 %-ethanol was added to the DNA mixture and centrifuged the tube to settle DNA. The DNA pellet was obtained by discarding the supernatant and then the DNA pellet was air dried at room temperature by placing the tube inverted on the tissue paper. The precipitated DNA was dissolved in 60 µl of TE (10 mM Tris, 0.1mM EDTA) and stored at -20°C.

3.2.1.2 PCR Amplification for rs8099917

The rs8099917 of IL28B gene was amplified by using specific pair of primers, 8099S and 8099AS (Table 3.2). PCR amplification was carried out with 50 µl of master mix containing 20 µl of Go Taq, 15 µl of PCR water, 2.5 µl of each primer 8099A and 8099AS and 10 µl of DNA template was added. The synthesis conditions were as follow;

	94°C→10 minutes
40 cycles	94°C→1 minutes
	58°C→40 seconds
	72°C→1 minutes
	72°C→7 minutes (Elongation)

3.2.1.3 **Electrophoresis on Agarose Gel of Amplified product of Region of IL28B**

From the region of IL28B amplified product of 404 bp was separated by the process of electrophoresis using 2% agarose gel. For the comparison of the size of the fragments a molecular marker constituting 100 bp was used (MBI Fermentas, catalogue # SM1153).

3.2.1.4 **Enzymatic Digestion of Amplicon for rs8099917**

The PCR amplified product was digested with BseMI (BsrDI) #ER1261 restriction enzyme at incubation temperature of 37°C lasting for 16 hours. The agarose gel was used to analyze the digested product for separating the DNA fragments and as a result the pattern of bands was obtained. For comparison of the sizes of bands a 100 bp ladder was used.

3.2.1.5 PCR Amplification for rs 12979860

The region rs12979860 of IL28B gene was amplified by using specific pair of primers, Sense and anti sense (Table 3.2). PCR amplification was carried out with 50 µl of master mix containing 20 µl of Go Taq, 15µl of PCR water, 2.5µl of each primer S and AS and 10 µl of DNA template was added. The synthesis conditions were as follow

94°C→10 minutes

40 cycles 94°C→1 minutes

58°C→40 seconds

72°C→1 minute

72°C→7 minutes (Elongation)

Table 3.2 Sequences of Primers used in RFLP-PCR amplification

SNP	Primer	Sequence (5′-3′)
rs8099917	Sense	5′-TTC ACC ATC CTC CTC TCA TCC CTC AT-3′
	Anti-sense	5′-TCC TAA ATT GAC GGG CCA TCT GTT TC-3′
rs12979860	Sense	5′-AGCAGGACAGATTGGCAAAG-3
	Anti-sense	5′-CACAATTCCCAACCACGAGAC-3′.

3.2.1.6 Enzymatic Digestion of Amplicon for rs12979860

The amplified product was digested with Hpy166II restriction enzyme at incubation temperature of 37°C lasting for 16 hours. The agarose gel was used to analyze the digested product for separating the DNA fragments and as a result the pattern of bands was obtained. For comparison of the sizes of bands a 100 bp ladder was used.

3.2.1.7 Purification of Amplified Product from Gel

To extract the pure DNA product from the agarose gel the manufacturer protocols of Gene JETTM Gel Extraction Kit were followed. A 2 % agarose gel, was used to visualize the bands using the gel doc system UV light. In order to obtain DNA bands sharp blade was used to excise it from the gel. An Eppendorf tube was weighed and again its weight was determined after addition of gel slice and the difference was noted. Binding Buffer measuring 100 µl for agarose gel equal to 100 mg was added to the tube with the gel slice. Incubation at 60°C lasting for 10 minutes, during this period of incubation inversion of tube facilitated the melting of the gel. When the gel was fully dissolved, the ratio of 100 µl of 100 % iso-propanol for every 200 µl of mixture was added and then mixed well. The subsequent step was the transfer of sample to the purification column of GeneJET^{TM} and centrifugation was done for 1-2 minute at 13,000 rpm, the fluid mixture which was passed inside the column was removed and the column was positioned back. The process was repeated by mixing 700 µl of wash buffer in which

Ethanol was added to the GeneJET^{TM} column being used for purification. To totally eliminate the wash buffer for another one minute centrifugation was done on the empty column. The GeneJET^{TM} column was shifted inside a recovery tube and then at the center of purification column membrane, elution buffer 50 µl pre-thawed (60°C) was applied and then incubation was done for 3 minutes and centrifugation was done at 14,000 rpm. Then in this finally prepared purified sample sequencing was performed.

3.2.1.8 Sequencing

The sequencing step of the selected samples was done on ABI automated sequencer. Then to observe the nucleotide sequence chromas software version 2.0 was used. To remove the obscurities, Electropherogram outcomes of the, forward and reverse primers were equated with each other as and with the IL28B NCBI sequences.

3.2.1.9 BLAST Study

The BLAST was used to compare sequences through comparison with the database and query of the nucleotide sequences were submitted to and sequences were compared with the databases, to catch out the similarity with IL28B sequences. (www.ncbi.nlm.nih.gov/BLAST/).

3.2.2 Demography of Patients for IL28B Genotyping

Age and gender was analyzed in group I and group II. Mean-median of age was calculated. Patients were grouped as follows: group 1 < 20 years, group 2: 21- 30, group 3: 31-40, group 4: 41-50 years and group 5: >50 years.

3.2.3 Clinical Parameters of the Patients

Clinical parameters of hemoglobin and ALT were analyzed for each patient by the following method.

3.2.3.1Analysis of Hemoglobin:

Hemoglobin was analyzed using an automated hematology analyzer XP100.

3.2.3.2 Analysis of ALT:

ALT was analyzed using Roche Cobas 6000 in which coupled-enzyme technique with continuous UV monitoring of NADH disappearance was used.

3.2.4Association of SVR and age

SVR was analyzed with different age group of patients as mentioned above. Patients were divided in two groups whether they achieved SVR or not.

3.2.5Association of SVR and ALT

SVR was analyzed with different levels of ALT. The level of ALT was divided into two groups, below 30 IU and more than 30 IU compared with whether SVR was achieved or not.

3.2.6Association of SVR and HB :

The levels of haemoglobin was divided into three groups (group1:<10, group 2:10-12, group 3:>12) which were compared with SVR status.

3.2.7 Association between RVR and SVR

Patients RVR status was analysed with their SVR status.

3.2.8. Calculation IL28B genotype 8099917 Allelic Frequency T&G

F(T)=[(2 x TT)+TG] /2 x Total

F(G)=[(2 x GG)+TG] /2 x Total

3.2.9 Calculation IL28B genotype 12979860 Allelic Frequency C&T

F(C)=[(2 x CC)+ CT] /2 x Total

F(T)=[(2 x TT)+CT] /2 x Total

3.3 STATISTICAL ANALYSIS

Statistical analysis was performed by using SPSS 21 (Statistic Package for Social Sciences, Inc. Chicago, Illinois, USA). Nature of data was determined by applying Kolmogorov-Smirnov (K-S) test. Descriptive statistics were carried. Quantitative data was summarized as mean and standard deviation. For categorical variables, frequency and percentages were calculated. Multivariate logistic regression analysis was performed to identify IL28b genotypes for predicting SVR. p value $\leq$ 0.05 was recognized as statistically significant. Calculated sample size is 73using the following formula.

Z2 x P (1-p) /MOE 2 Z (Critical value of normal distribution)= 1.96

P (Estimated sample proportion)=95%

MOE (Margin of error)= 5%

RESULTS AND DISCUSSION

4.1 PREVALENCE OF HCV GENOTYPES (STUDY PHASE I)

For a person having HCV infection it is important to identify which genotype is infecting him. The patient treatment plan is specific for the infecting HCV genotype with regards to duration and medication ((Abbas et al., 2009; Strader et al., 2004; Hadziyannis et al., 2004).

Out of 320 HCV positive patients as diagnosed by the presence of antibody was tested for the presence of HCV RNA, 151 patients were HCV RNA positive. All HCV RNA positive samples were subjected to genotype determination using restriction fragment length polymorphism and type specific PCR followed by direct sequencing. The analysis revealed the presence of genotypes 1 and 3 with further subtypes 1a, 1b, 3a, 3b and mixed genotypes 1b + 3a, 1b + 3b and 3a + 3b (Figure 4.1& 4.2).

The amplified products of viral genotypes and subtypes showed that virus genotype 3a which is most prevalent in Pakistan consist of 232 bp, genotype 1b which is second in prevalence in Pakistan showed 234 bp size and 1a consist of 209 bp, The size of the amplified product associated with 3b consist of 176 bp (Table 4.1).

4.1.1 HCV Genotypes with corresponding Amplified Product Size:

Various HCV Genotypes were analyzed with Corresponding Amplified Product Size (table 4.1)

Table 4.1 HCV genotypes with corresponding amplified product size

HCV GENOTYPE	PRODUCT SIZE (bp)	RESULT
3b	176	Detected
2a	190	Not Detected
1b	234	Detected
2b	337	Not Detected
4	99	Not Detected
1a	209	Detected
3a	232	Detected
5a	320	Not Detected
6a	336	Not Detected

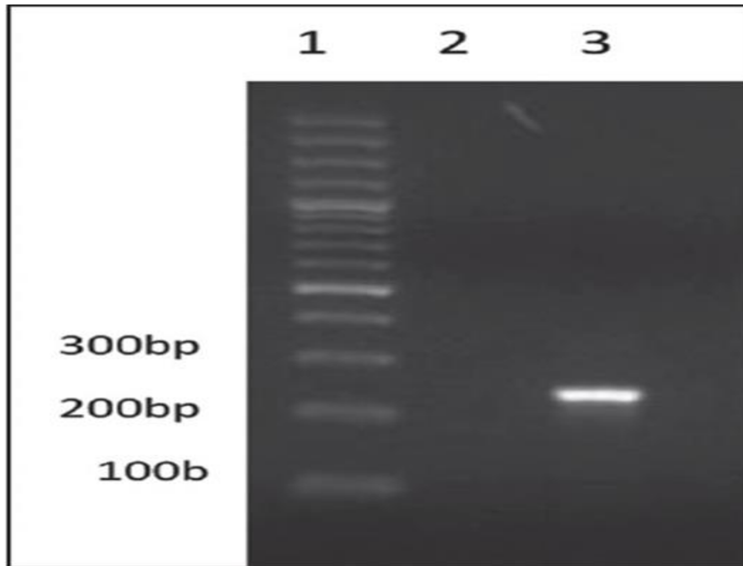


Figure 4.1 The electrophoresis pattern of genotype 1a : Lane 1 showing 100 bp deoxyribonucleic acid ladder, Lane 3: Polymerase chain reaction product of genotype 1a (209 bp).

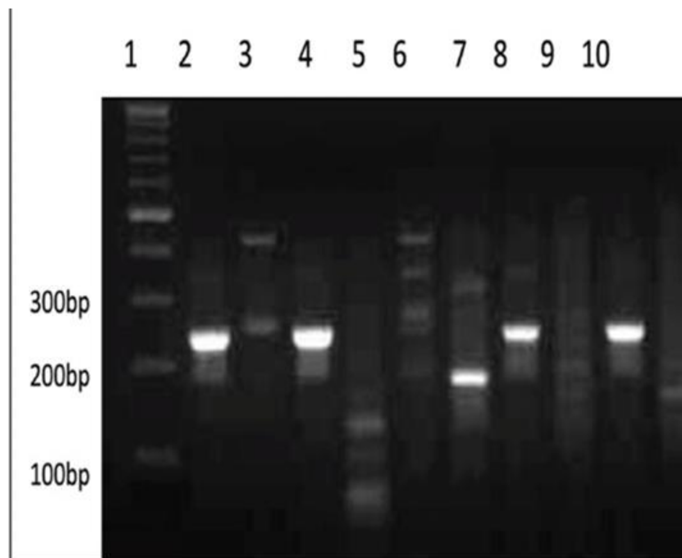


Figure 4.2 The Electrophoresis Pattern of HCV Genotypes. Lane 2-4, Polymerase chain reaction (PCR) product of HCV genotype 1b constituting (234 bp), Lane 5, negative control. Lane 7, PCR product of genotype 3b (176 bp) and lane 8 and lane 10 PCR product of genotype 3a (232 bp).

4.1.2 Distribution Pattern of Hepatitis C Virus Genotypes According to Age

Total 151 patients were divided into 4 age groups, in the group of less than 30 years, out of 46 patients 30 patients were found to be infected with genotype 3a. In the group from 31-40 years, out of 43 patients 37 patients were genotype 3a. In the group from 41-50 years, out of 50 patients 44 patients were having genotype 3a. In the group from >50 years, out of 12 patients 10 patients were genotype 3a. When distribution sub-genotype in different age group was studied it was found in the group of less than 30 years, out of 46 patients 13 patients were genotype 3b. In the group from 31-40 years, out of 43 patients 5 patients were having genotype 3b. In the group from 41-50 years, out of 50 patients 5 patients were having genotype 3b. In the group from >50 years, out of 12 patients 1 patient was having genotype 3b. In the group of less than 30 years, the group from 31-40 years, and in the age group from 41-50 years, none of the patients were having genotype 1b. In the group from >50 years, out of 10 patients 1 patients was found to be infected with genotype 1b. When occurrence of genotype 1a was studied in different age groups it was observed that 1 out of 46 in age group < 30 and 1 out of 50 was in age group 41-50. While in the age group from 31-40 years and >50 years none of the patients were infected with genotype 1a. For the mixed genotype 1 patient out of 46 and 1 out of 10 were found to be infected with genotype 3a+3b in the age group less than 30 and greater than 50 years respectively. In the group from 31-40 years and 41-50 years no genotype 3a+3b were recorded. The mixed genotype 1b + 3a was detected only in 1 patient in the age group of 31-40 and there was no occurrence of this genotype in other age groups (Table 4.2).

Table 4.2 Distribution Patterns of Hepatitis C Virus Genotypes According to Age

AGE groups	Genotype 3a	Genotype 3b	Genotype 1b	Genotype 1a	Genotype 3a+ 3b	Genotype 1b + 3b	Genotype 1b+3a	TOTAL
less than 30 years N=46	30 65.21%	13 28.26%	0	1 2.17%	1 2.17%	1 2.17%	0	46 100%
31-40 years N=43	37 86.04%	5 11.62%	0	0	0	0	1 2.32%	43 100%
41-50 years N=50	44 88%	5 10%	0	1 2%	0	0	0	50 100%
>50 years N=12	10 83.3%	1 8.3%	1 8.3%	0	0	0	0	12 100%

4.1.3 Distribution Pattern of Hepatitis C Virus Genotypes According to Gender

In total of 151 patients genotype 1a and 1b were found in none of the male patients while genotype 3a was found in 58 patients (82.8%), genotype 3b was found in 10 male patients (14.29 %), genotype 1b +3b was found in 1 patients (1.43 %) and genotype 3a +3b was found in none of the male (Table 4.3, Figure 4.4).

Table 4.3 Distribution Patterns of Hepatitis C Virus Genotypes According to Gender

Gender	Genotype 1a (%)	Genotype 1b (%)	Genotype 3a (%)	Genotype 3b (%)	Genotype 1b+3b (%)	Genotype 1b+3a(%)	Genotype 3a+3b (%)
Male	0 (0.00)	0(0.00)	58 (82.86)	10 (14.29)	1 (1.43)	1 (1.43)	0 (0.00)
Female	2 (2.47)	1(1.23)	66 (81.48)	11 (13.58)	0 (0.00)	0 (0.00)	1 (1.23)
Total	2 (1.32)	1(0.66)	124 (82.12)	21(13.91)	1 (0.66)	1 (0.66)	1 (0.66)

In total of 151 patients genotype 1a was found in 2 (2.4 %) of the female patients and 1b was found in none of the female patients. While, 66 (81.48%) and 11 (13.58 %) females were infected with genotype 3a and 3b respectively. Mixed genotype 1b +3b (1.43%) and 3a +3b (1.23 %) was found only in 1 of the female patient each (Figure 4.3).

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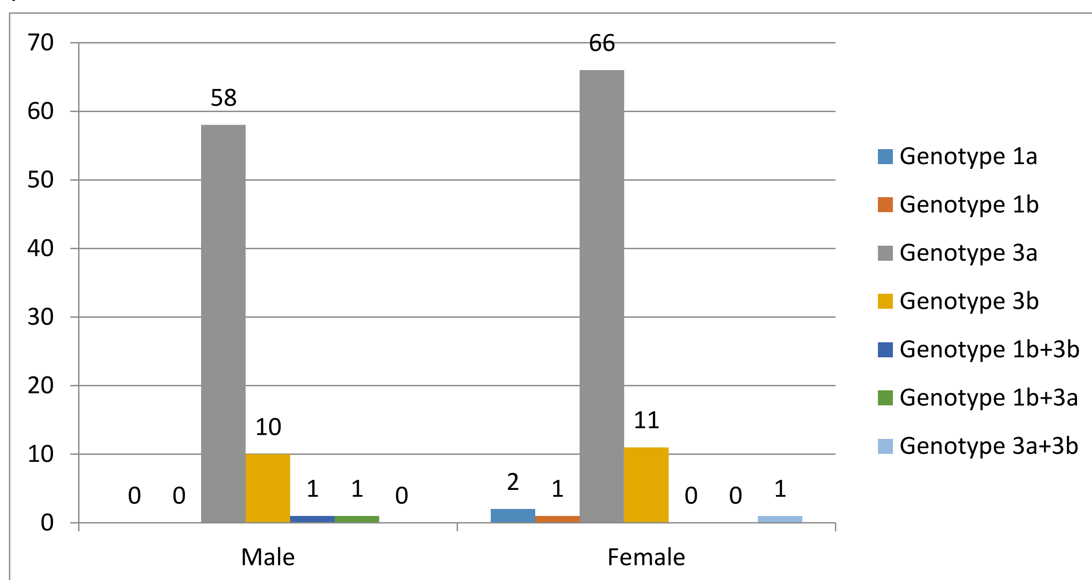

Fig. 4.3 Frequency of Genotypes in Male and Female Gender (N=151) in Pakistan

4.1.4 Mean Viral Load of Patients Infected with Different HCV Genotypes

Mean viral load calculated for HCV genotype 1a, 1b, 3a, and 3b were 2.75 x10^6 , 3.9 x 10^6, 2.65 x10^6 and 2.51 x10^6 respectively. HCV genotype 1b + 3a showed mean viral load equal to 3.41x10^6 and HCV genotype 1b+3b showed mean viral load of 2.71x10^6, while HCV genotype 3a+3b showed mean viral load equal to 3.51x10^6 (Table 4.4).

Table 4.4 Mean Viral Load of Patients Infected with Different HCV Genotypes

HCV GENOTYPE	MEAN VIRAL LOAD
1a	2.75 x10^6
1b	3.9 x 10^6
3a	2.65 x10^6
3b	2.51 x10^6
1b + 3a	3.41x10^6
1b +3b	2.71x10^6
3a+3b	3.51x10^6

4.1.5 Discussion

In the present study most common genotype of Hepatitis C was genotype 3 which was followed by genotype 1. The analysis revealed the presence of genotypes 1 and 3 with further subtypes 1a, 1b, 3a, 3b and mixed genotypes 1b + 3a, 1b + 3b and 3a + 3b .Studies conducted in the regions of Punjab showed that seventy percent prevalence was shown by genotype 3, approx. 14 % by genotype 1 and genotype 4 showing prevalence of 7 % (Ijaz et al., 2011). Likewise a study which was conducted in the University of Karachi genotype 3 showed 68 % prevalence followed by 14 % of genotype 1 (Simmonds, 2013). Another research showed prevalence of different genotypes as follows genotype 3 approximately 64% genotype 2 approx. 40 % genotype 2 about 6% prevalent (Smith et al., 2014).

A research done in France the prevalence of HCV genotypes was found to be in the following distribution pattern genotype 1b having approx. 46 %, 1a showing 16 %, 3a having 21% followed by 2a having 7.5%, type 4 with 4.5%. This study also revealed genotype 5 having 0.7 % prevalence (Simmonds, 2013).

In the present study patients were divided in different age groups those in the set of less than 30 years, It was found that 65.21 % were having infection of genotype 3a similarly genotype 3b was present in 28.26 % of patients, the other genotypes like 1a and were in very low percentages similarly mixed infections of genotype 3 like 3a + 3b and genotype 1 ie 1a + 1b were also rare. In second set of age group 31-40 years genotype 3 was seen with subtypes 3a in 86.04% of population and 3b in 11.62 % of population belonging to this group. An increasing trend was seen of genotype 3a in patients belonging to age group of 41-50 years where 88 % of the population was infected with genotype 3a and 10 % of the population from genotype 3b (Alaei et al., 2016; Safi et al., 2010).

In our study the patients in the age group which was less than 50 years genotype 1b was not traced while in more than 50 years it was traced to be having 8.33%. Similarly it was noted that genotype 1a was 1% in set of population of 41-50 years and 2.17 % in age groups 31-40 years.

A Chinese study which was done in the southwest region in year 2009 also has shown similar trends when compared among various genotypes revealing the increasing

trends shown by genotypes 3 and also of genotype 6 in young patients infected with HCV while decreasing trends were shown in HCV genotypes 1b and 2a in younger population (Rao et al., 2014; Smith et al., 2014). A European study also substantiated a relationship of younger population with genotype 3a while HCV genotype 1b was found to be associated with older group (Petruzziello et al., 2013).

Regarding gender no difference in spread was noted among various genotypes. There was equivalent distribution pattern of different genotypes of HCV when a comparison was made with gender.

HCV viremia is also acknowledged as plasma viral load and its measurement unit is copies per millilitre. In our study when mean viral load was specified by each viral genotype it was observed that in genotype 3b the mean viral load was 2.51×10^6 and that of genotype 3a was 2.65×10^6. Thus among genotype 3 the subtype 3b had somewhat higher viral load than subtype.

In subtype 1a the mean viral load was 2.75×10^6 and 1b mean viral load was 3.9×10^6 thus showing that viral loads show fluctuations with various HCV genotypes. Our results run parallel with a study conducted by Agha Khan University revealing significantly lower viral loads in patients infected with genotype 3 (37 meq/ml) as compared with genotype 1 (8.63 meq /ml) $P < 0.001$(Moatter et al., 2002). In this study the higher viral loads were observed as compared to the present study. Similar study in India also revealed that the mean viral load associated with genotype 3 was 1.9×10^5 and that associated with genotype 1 was 1.6×10^5 showing lesser values than our study in which mean viral load for genotype 1a was found to be 2.75×10^6, that of genotype 1b 3.9×10^6, while the most prevalent genotype 3a showed the viral load to be of 2.65×10^6, and that of genotype 3b was found to be 2.51×10^6. Furthermore it was noticed that mixed genotypes as subset of 1b+3a showed mean viral load of higher value 3.4×10^6, and subset consisting of genotype 1b+3b showing mean viral load of 2.7×10^6 and the subset having mixed genotype infection of 3a+3b the highest mean viral load of 3.5×10^6 was observed.

In our study the patients in the age group which was less than 50 years genotype 1 b was not traced while in more than 50 years it was traced to be having 8.33%.Similarly

it was noted that genotype 1a was 1% in set of population of 41-50 age and 2.17 percent in patients having age group belonging to 31-40 years.

The study discovered the presence of genotypes 1 and 3, also found were subtypes 1a, 1b, 3a, 3b and also mixed genotypes 1b + 3a, 1b + 3b and 3a + 3b. Lower percentages of genotype 3b infections were noted with increasing age groups. Genotype 1b was not found in patients less than 50 years while its prevalence was 8.33% in patients of age more than 50 years.

A Chinese study which was done in the southwest region in year 2009 also has shown similar trends when compared among various genotypes revealing the increasing bents shown by genotypes 3 and also of genotype 6 in young patients infected with HCV while decreasing trends are shown in HCV genotypes 1b and 2a in younger population (Zhou et al., 2009). A European study also substantiated a relationship of younger population with genotype 3a while HCV genotype 1b was found to be associated with younger older group (López-Labrador et al., 1997).

4.2 EFFECT OF IL28 POLYMORPHISMS RS8099917 & RS12979860 ON HCV SPONTANEOUS AND TREATMENT RESPONSE (PHASE 2)

Two sets of patients were included in the study N=87 patients were enrolled in the study from Social Security Hospital located in Islamabad a semi government organization while the other N= 82 patients of population was included from a private Hospital based in Islamabad the capital city of Pakistan. Both the sets of population (Group I &II) received interferon based therapy.

4.2.1 Demography of patients for IL28B Genotyping (Group I & II)

Total 169 patients were included in the study. The patients were divided into two groups in different hospital settings in Islamabad. In group 1 total 87 patients were included in the study 35 (40.2%) patients were male and 52 (59.8 %) were female. While using 95% estimated sample proportion with 5% margin of error the calculated sample size is 79 The patients were divided into five age groups. In the group < 20 years 2 were males and no female, in age group 21-30 years 4 patients were male and 5 were females. While in age group 31-40 years distribution of male and female were 13 and 33 respectively. The age group of 42-50 years 21 were males and 30 were females and there was only 1 male and 2 females in age group 51-60 years (Table 4.5, Figure 4.4).

In (group II) total 82 patients were included in the study 35 (42.7%) were male and 47 (57.3 %) were female. In the group < 20 years 01 was male and 02 were female, In age group 21-30 years 3 patients were male and 12 were females, while age group 31-40 years 12 males and 8 females were included. The distribution of male and female in age group 42-50 were 14 and 11 respectively and only one male and 2 females were included in age group >51-60 (Table 4.6, Figure 4.5).

Demographic characteristics of patients were analyzed statistically in both the study groups (I & II) it was observed that in group I, females were 59.8 % while in group II females were 57.3x%. In group I males were 40.2c% while in group II males were 42.7 %. The mean age of the patients was 40.3 in group I and 42.7 in group II(Table 4.7 and 4.8). The median of ages were 40 in group I and 41.5 in group II. Mean value of serum Alanine transferase was 58.6 Units/dl and that in group II was 48.8 Units/dl. The median of ALT values was 40 in group I and 41.5 in group II. Hemoglobin levels were 11.6 in group I and 11.9 in group II with the median of Hb levels of 12 in group I & II (Table 4.9).

Table 4.5 Demography of patients for IL28B Genotyping (Group1)

Valid	Frequency	Percent	Valid Percent	Cumulative Percent
Male	35	40.2	40.2	40.2
Female	52	59.8	59.8	59.8
Total	87	100.0	100.0	100.0

Table 4.6 Demography of patients IL28B Genotyping (Group II)

Valid	Frequency	Percent	Valid Percent	Cumulative Percent
Male	47	57.3	57.3	57.3
Female	35	42.7	42.7	100.0
Total	82	100.0	100.0	

Table 4.7 Demography of clinical characteristics of patients showing different variables

Variables	**Total population (n= 87)**	**Total population (n= 82)**
Gender		
Female (%)	52 (59.8%)	47 (57.3%)
Male (%)	35 (40.2 %)	35(42.7%)
Age		
Mean ± SD	40.3±11.6	41.5± 7.0
Median	40	41.5
ALT		
Mean ± SD	58.6 ± 32.4 Units/dl	48.8± 24.4 Units/dl
Median	54	51
Hb		
Mean ± SD	11.6 ±1.5	11.9 ±1.9
Median	12.0	12

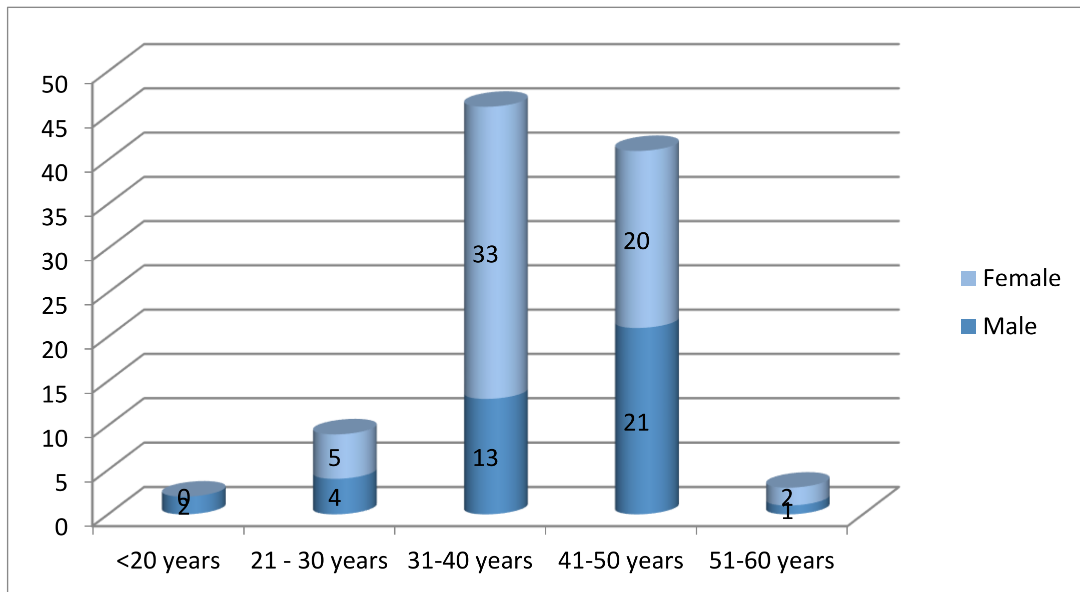

Fig. 4.4 Demographic distribution of patients included in the study for genetic polymorphism in IL28B from Social security Hospital (group 1) with respect to gender and age.

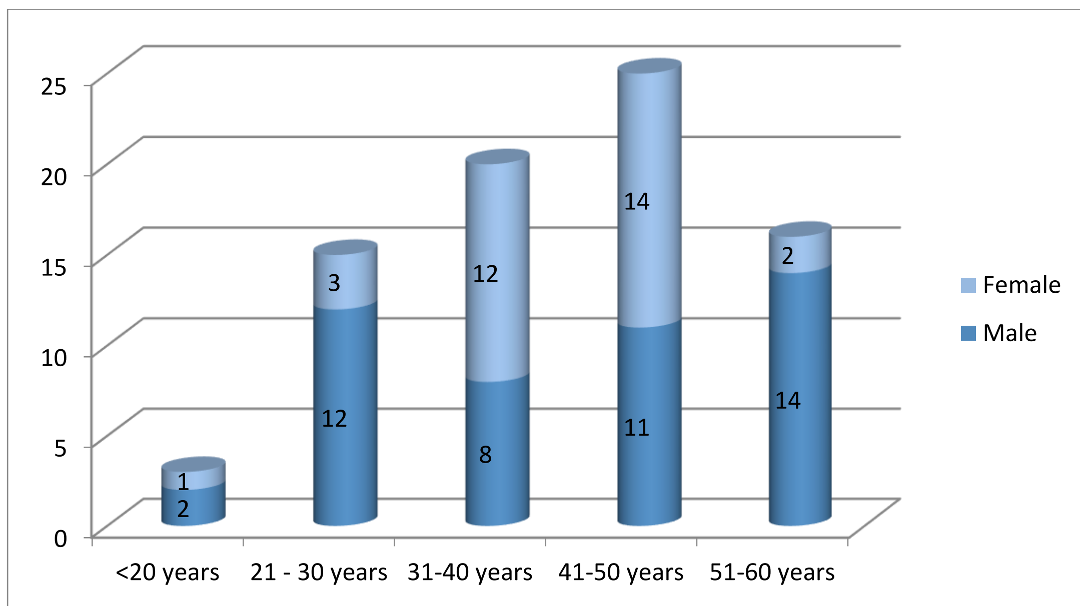

Fig. 4.5 Demographic distribution of patients included in the study for genetic polymorphism in IL28B from Private Hospital in Islamabad (group 1I) with respect to gender and age.

4.2.2 Association of SVR and age

For analysis of SVR with age the patients were divided into 5 age groups. In study group I only1 patient achieved SVR and none of the patient was without SVR in patients of age group <20 years. In group II of study in the same age group 3 patients were able to achieve SVR and none of the patient was without SVR. For the age group 21-30 years, 8 patients achieved SVR and none of the patient was without SVR (group I) while the second study group (group II) 13 patients were able to achieve SVR and 2 patients were without SVR in the age 21-30 years. The age group 31-40 years should maximum number of patients achieving SVR (33 and 17 in study group I and II respectively) while 7 and 3 patients were without SVR in these groups. In study group I and age group 41-50 years 19 patients achieved SVR and 16 patients were without SVR. While in the same age group 21 patients were able to achieve SVR and 4 were without SVR in study group II. The patients with older age (51-60 years) 3 patients achieved SVR and none was without SVR (study group I) and 11 were able to achieved SVR and 8 patients were without SVR in the same age group (study group II) (Table 4.8).

To observe the association of age group and SVR chi square test was applied. It is observed that age group and SVR in patients categorized as (group I) of our study has significant relationship with p value = 0.01316. In the same manner it was observed that age group and SVR in patients categorized as (group II) of our study has significant relationship with p value = 0.0121 (Table 4.10).

4.2.3 Association of SVR and ALT

Association of liver enzymes Alanine Aminotransferse (ALT) in patients with SVR was analysed in the present study. The study enrolled patients were separated in two groups. ALT group 1 was designated to those with ALT level below 30 U/L and group 2 ALT was above 30U/L. In ALT group 1, 14 patients were found to be achieving SVR while 9 patients did not achieve SVR (study group I) while 51patients achieved SVR while 8 did not achieve SVR. Total 65 were successful in achieving SVR while 17 did not attained SVR. To observe the association of ALT group and SVR chi square test was applied. It is observed that ALT group and SVR in patients categorized as (group I) of our study has insignificant relationship with p value =

0.551. In the same manner it was observed that ALT group and SVR in patients categorized as (group II) of our study has significant relationship with *p* value =0.010 (Table 4.9).

Table 4.8 Association of SVR and age

SVR	Group 1 Age10-20 years	Group 2 Age21-30 Years	Group 3 Age31-40 years	Group 4 Age41-50 Years	Group 5 Age51-60 Years	P value
GROUP I (n=87)						
SVR Achieved	**1**	**8**	**33**	**19**	**3**	
SVR Not achieved	**0**	**0**	**7**	**16**	**0**	**0.01316**
GROUP II (n=82)						
SVR Achieved	**3**	**13**	**17**	**21**	**11**	
SVR Not achieved	**0**	**2**	**3**	**4**	**8**	**0.0121**

Table 4.9 Association of SVR and ALT

SVR	Group 1 ALT ≤30 U/L	Group 2 ALT>30 U/L	*P* value
GROUP I (n=87)			
SVR Achieved	**18**	**46**	
			0.551
SVR Not Achieved	**5**	**18**	
GROUP II (n=82)			
SVR Achieved	**14**	**51**	
SVR Not Achieved			**0.010**
	9	**8**	

4.2.4 Association of SVR and HB

Association of Haemoglobin (Hb) in patients with SVR was analyzed in our study. Theenrolled patients were separated into three groups. Hb group 1 was designated to those with Hb level below 10 g/dl and those in group 2 the level of Hb was from 10.1-12 g/dl and group 3 above 12 g/dl. In Hb level below 10 g/dl, 15 patients achieved SVR while 2 patients did not achieve SVR in study group I while in study group II, 15 patients achieved SVR while 5 did not achieve SVR. In Hb level between 10.1-12 g/dl 26 achieved SVR while 17 patients were non achievers of SVR (study group I). While in study group II, 22 patients achieved SVR while 5 patients did not achieve SVR with Hb levels of 10.1-12 b/dL. In Hb levels of >12 g/dl 23 patients achieved SVR and 14 patients did not achieve SVR (group I) while in the study group II, 28 achieved SVR while 7 were non achievers of SVR in the same group of Hb levels. To observe the association of Hb group and SVR chi square test was applied. It is observed that Hb group and SVR in patients categorized as (group I) of our study has non-significant relationship with p value =0.23. In the same manner it was

observed that Hb groups and SVR in patients categorized as (group II) of our study has non-significant relationship with p value =0.855 (Table 4.10).

Table. 4.10 Association of SVR and Hb levels in the study groups I and II

Hb levels	≤10	10.1-12	>12	P value
GROUP I (n=87)				
SVR Achieved	**15**	**26**	**23**	**0 .23**
SVR Not achieved	**2**	**17**	**14**	
GROUP II (n=82)				
SVR Achieved	**15**	**22**	**28**	**0 .855**
SVR Not achieved	**5**	**5**	**7**	

4.2.5 Association of RVR with SVR

Frequency of patient achieving RVR was determined in group I and group II Out of 77 (88.5%) patients who achieved RVR patients 60 (69.0%) achieved SVR and 10 (11.5%) did not achieve SVR. Similarly out of 23 (26.4%) patients who did not achieve RVR 17(19.5%) achieved SVR while 6 (6.9%) did not achieve SVR (study group I) (Table 4.13).

In study group II out of 65 (79.3%) patients who achieved RVR, 59(72 %) achieved SVR and 3 (7.3%) did not achieve SVR. Similarly out of 17 (20.7%) patients who did not achieve RVR (3 .7%) patients achieve SVR while 14 (17.1%) did not achieve SVR (Table 4.13).To observe the association of RVR and SVR chi square test was applied. It is observed that RVR and SVR in patients categorized as (group I) of our study has significant relationship with p value =0.029. In the same manner it is observed that RVR and SVR in patients categorized as (group II) of our study has significant relationship with p value =.000 (Table 4.13).

Table 4.11 Frequency of patients achieving Rapid Virological Response (RVR) in study group I

RVR	Frequency	Percentage
Achieved	**77**	**88.5**
Not Achieved	**10**	**11.5**
Total	**87**	**100**

Table 4.12 Frequency of patients achieving Rapid Virological Response (RVR) in study group II

RVR	Frequency	Percentage
Achieved	**65**	**79.3**
Not Achieved	**17**	**20.7**
Total	**82**	**100**

Table 4.13 Association of RVR with SVR

		Group 1		Group 2		
RVR	SVR Achieved N= 77	SVR not Achieved N= 10	P value	SVR Achieved	SVR not Achieved	P value
Achieved	**60**	**4**		**59**	**3**	
Not Achieved	**17**	**6**	**0.029**	**6**	**14**	**0.00**

4.2.6 Polymorphism of IL28 B genotype rs8099917

In this section of study we compared polymorphism of rs8099917 in IL28B gene. Two sets of patients were included in the study 87 patients from Social security hospital located in Islamabad ,while 82 patients were included from a private clinic based in Islamabad. Both the sets of patients received interferon based therapy. Electrophoresis pattern of the amplified product and enzymatic digest is shown in (Figure 4.6 and 4.7).

Table 4.14 Restriction with BseMI (BsrDI) e rs 8099917

No of bands	Size of base pairs of bands	Genotype
01	401	TT
02	251,150	GG
03	401,251,150	TG

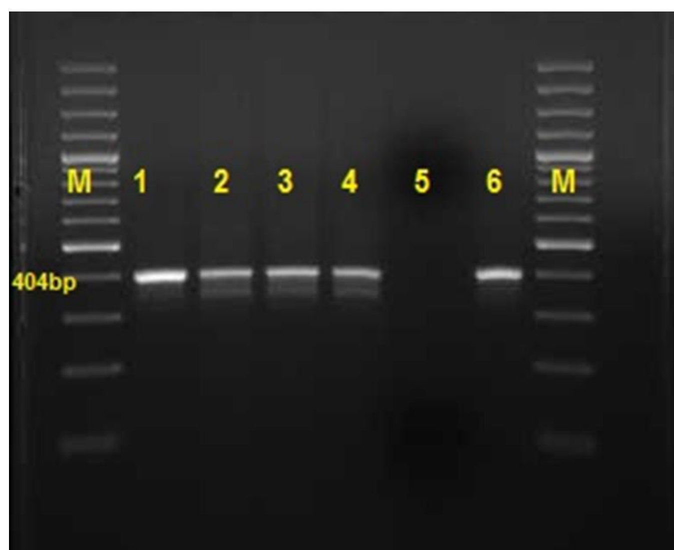

Fig. 4.6 The Electrophoresis Pattern of RFLP of IL28B amplified product of rs 8099917, Lane 1-4: Amplified product of rs8099917 with 404bp fragment size, Lane 5: -ve control, Lane 6: +ive control, Lane M: 100bp DNA ladder.

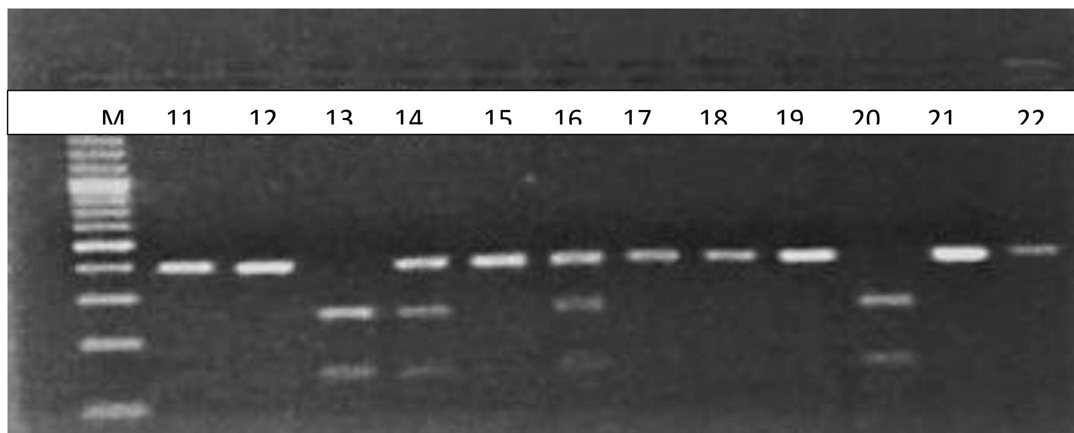

Fig 4.7 Agarose gel electrophoresis pattern of rs8099917 genotype , a 404-bp product was amplified .The amplified product was digested with and ana BseMI (BsrDI) elyzed on 2% agarose gel. Lane 11,12, 13,17, 18, 19, 21, 22 (TT) genotype ; (401 bp) Lane 13,20 (GG) genotype (251 bp,150 bp); Lane 14,16 (TG) genotype. (401bp, 251bp, 150bp).

4.2.7 Analysis of IL28B genotype SNP rs 8099917 (Group1)

Three different genotypes TT homozygous,TG heterozygous and GG homozygous were found in both group of patients characterized as SVR achievers (responders) and SVR non-achievers or (non-responders) in group I in which 87 patients were enrolled in Social security hospital. In group II in a total of 82 patients SVR achievers, non-achievers and relapsers were considered.

In group 1 out of 87 patients the most frequent genotype was homozygous TT which was found in 60 (69 %) patients followed by genotype TG in 21 (24%)patients whereas GG genotype was found in 6 (6.9 %) of the patients. (Fig 4.8)

In group 1 patients further analysis revealed the presence of genotype TT in 52 (81.3%) of (responders) and 8 (34.8 %) of non-responders. The hetrozygous TG genotype was found in 7 (10.9 %) of responders and 14 (16.9 %) of non-responders. The homzygous GG was found in 5 (7.8 %) of responders and 1 (4.3 %) of patients in non-responders to treatment (Table 4.14, Fig 4.9).

To observe the association of the three different genotypes TT homozygous, TG heterozygous and GG homozygous of rs8099917 and SVR, chi square test was applied. It is observed that rs8099917 polymorphism and SVR in patients categorized as (group I) of our study has significant relationship with p value =.000 (Table 4.16).

4.2.8 Analysis of IL28B genotype SNP rs 8099917 (Group II)

While analyzing IL28B polymorphism another set of patients was included in study. It was given name of group II patients and were categorized into three categories responders, relapsers and non-responders. Out of 82 patients the homozygous TT genotype was found in 56 (67.5%) of total HCV patients while TG frequency was lower than TT and detected in 22 (26.5%) of patients and GG genotype was found in 4 (4.8 %) of patients (Table 4.15, Figure 44.9).

In group II, out of total of 82 patients SVR was achieved by 65 patients. In TT genotype, total SVR was achieved by 47 patients (57.3%). In TG genotype, 17 (20.7%) patient achieved SVR In GG genotype, 1 (1.2%) patient achieved SVR,. Statistical analysis showed that rs8099917 polymorphism and SVR in patients categorized as (group II) of our study has significant relationship with p value =0.010 (Table 4.16).

Table 4.15: Frequency of rs8099917 genotype polymorphism in patients (n=87 Group I) and (n=82,Group II)

	Group I		**Group II**	
	Frequency	Percent	Frequency	Percent
TT: Homozygous	60	69.0	56	68.3
TG: Heterozygous	21	24.1	22	26.8
GG	6	6.9	4	4.9
Total	87	100.0	82	100

Table 4.16: Association of SVR with rs8099917 genotypes

il8099917		Group 1			Total	P value	Group2			Total	P value
		TT	TG	GG			TT	TG	GG		
SVR	Achieved	52 59.8%	7 8.0%	5 5.7%	64 73.6%	0.000	47 57.3%	17 20.2%	1 1.2%	65 79.3%	0.010
	Not Achieved	8 9.2%	14 16.1%	1 1.1%	23 26.4		9 11%	5 6.1%	3 3.7%	17 20.7%	
Total		60 69.0%	21 24.1%	6 6.9%	87 100%		56 68.3%	22 26.8%	4 4.9%	82 100%	

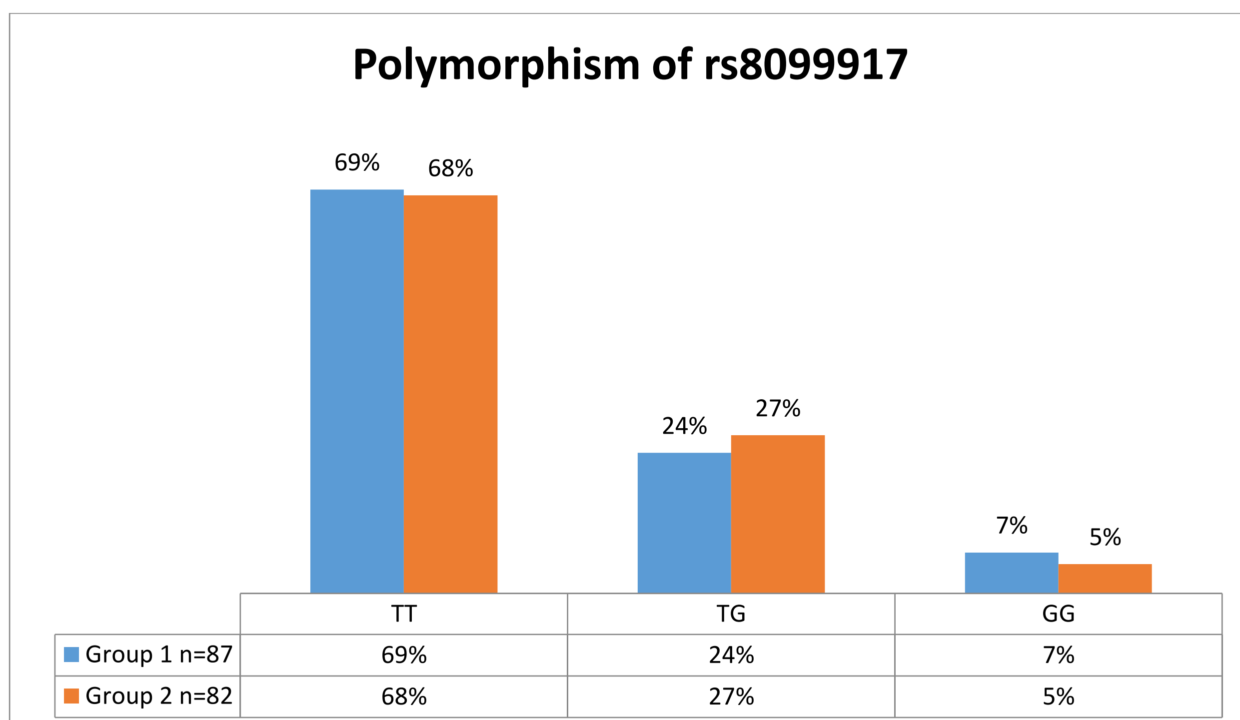

Fig. 4.8 RFLP analysis of 8099917 genotype polymorphism : TT genotype was found in 69% of patients, TG genotype was found in 24% of patients, GG genotype was found in 7% of patients.

TABLE 4.17 SVR 8099917 CROSS TABULATION (Group I &II), Association of SVR With Genotypes 8099917 Polymorphism

GROUP 1	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	23.068[a]	2	.000
Likelihood Ratio	21.237	2	.000
Linear-by-Linear Association	8.284	1	.004
N of Valid Cases	87		

GROUP 2	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.962[a]	1	.010
Likelihood Ratio	6.246	1	.024
No of valid cases	82		

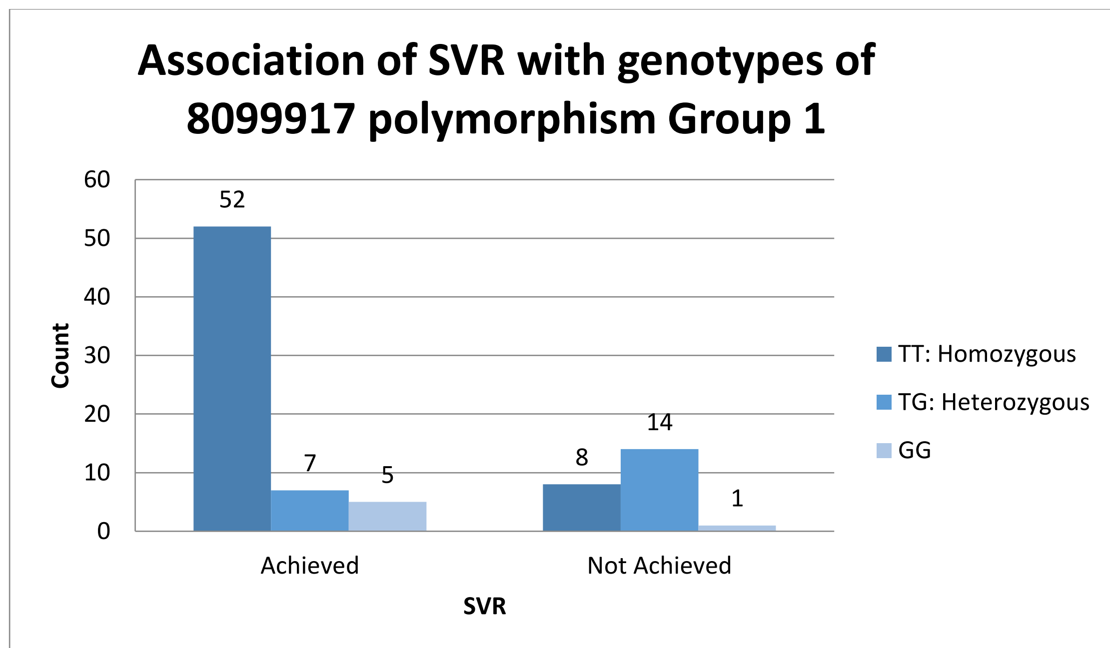

Fig 4.9 IL28B Gene Genetic Variation: % SVR to pegIFN+RBV in HCV-Mono infected Patients

4.2.9 IL28B genotype 8099917 single Allelic Frequency(Group I & II)

In order to further analyze polymorphism at genotype 8099917b single allele frequency of the two alleles T and G were calculated. The result revealed that in a total of 82 patients frequency of T allele was 81.1% and frequency of G allele was 18.9 in (Group I). Similarly single allele frequency of rs 8099917 polymorphism two alleles T and G revealed that out of total of 87 patients frequency of T allele was 81.7% and frequency of G allele was 18.3 (Group II, Table 4.18).

Table 4.18 Frequency of T allele & G allele

	Frequency Group 1	**Frequency Group 1I**
T allele	81.1%	81,7%
G allele	18.9%	18.3%

4.2.10 Agrose gel electrophoresis of IL28B Genotype SNP rs 12979860

In order to visualize restricted products for rs12979860 the Restriction with enzyme Hpy166II enzymewas used. Electrophoresis pattern of genotype in rs 12979860 with enzyme showed that restricted PCR product forming a band of 529 bp,& 165 bp indicate the T/T genotype, 509 bp, &185 bp indicate the C/C genotype, and 139 bp + 509 bp, 185 bp, 165 bp C /T genotype. Table 4.19; Figure 4.10).

Table 4.19 Restriction with for Hpy166II enzymers12979860

No. of Bands	Size of bands (bp)	Genotype
02	509, 185	CC
02	529, 165	TT
03	509, 185, 165	CT

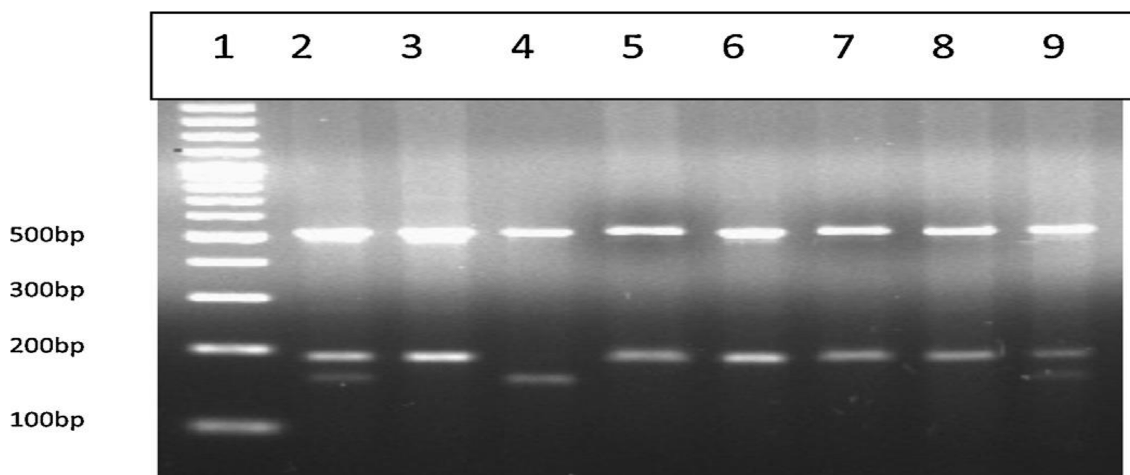

Fig 4.10 Agarose gel electrophoresis of rs 12979860 genotype, a 694-bp product was amplified.: lane 1 showing 100-bp DNA ladder; lanes 2 and 9 : genotype CT; lane 4: genotype TT; lanes 3 and 5–8: genotype CC.

4.2.11 IL28B Genotype rs 12979860

4.2.11.1 Frequency of IL28B genotype rs 12979860

Three different genotypes CC, CT and TT were found in patients. Homozygous CC genotype was found in 65 (74.7%) patients. Heterozygous CT genotype was detected in 23 (21.8) patients and Heterozygous TT was found in 3 (3.4%) patients (fig4.12) chromatogram displaying heterozygous G/T genotype. C/T polymorphism also confirmed the findings (fig4.13)

Out of total 87 patients 64 (73.6%) achieved SVR and 23 (26.4 %) did not achieve SVR. Three different genotypes CC, CT and TT were found in both groups of patients. Homozygous CC genotype was found in 51 (79.7%) of responders. Heterozygous CT genotype was detected in 10 (15.6%) of responders patients and Heterozygous TT was found in 3 (4.7%) of responders patients. Similarly CC genotype was found in 14 (60.9%) of non-responders, genotype CT was found in 9

(39.1%) of non-responders and genotype TT was found in 0% of non responders (Table 4.20).

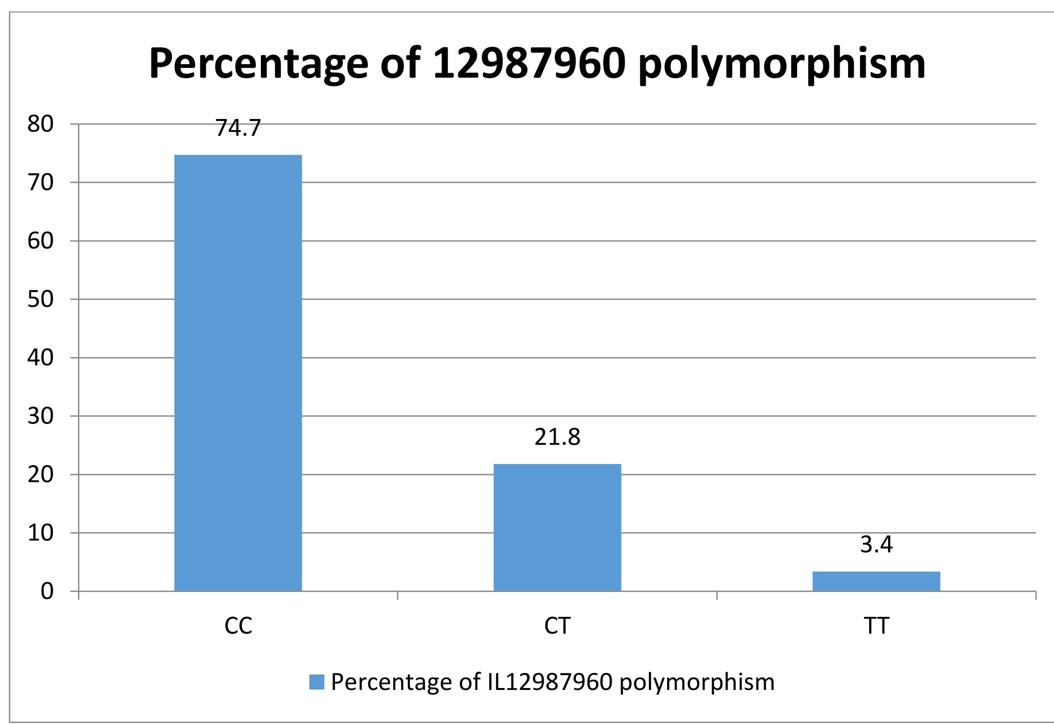

Fig 4.11 Genetic Variation in IL28B Gene showing Percentage of IL28 by rs12979860

Table 4.20: IL28B Frequency rs12979860 genotypes with Relation to SVR

SVR	Frequency	Percent
SVR Achieved	**64**	**73.6**
SVRNot Achieved	**23**	**26.4**
Total	**87**	**100.0**

Table 4.21: Association of SVR to rs12979860 genotypes

	12979860	Total

			CC	CT	TT	
SVR	Achieved	Count	51	10	3	64
		% of Total	58.6%	11.5%	3.4%	73.6%
	Not Achieved	Count	14	9	0	23
		% of Total	16.1%	10.3%	0.0%	26.4
Total		Count	65	19	3	87
		% of Total	74.7%	21.8%	3.4%	100%

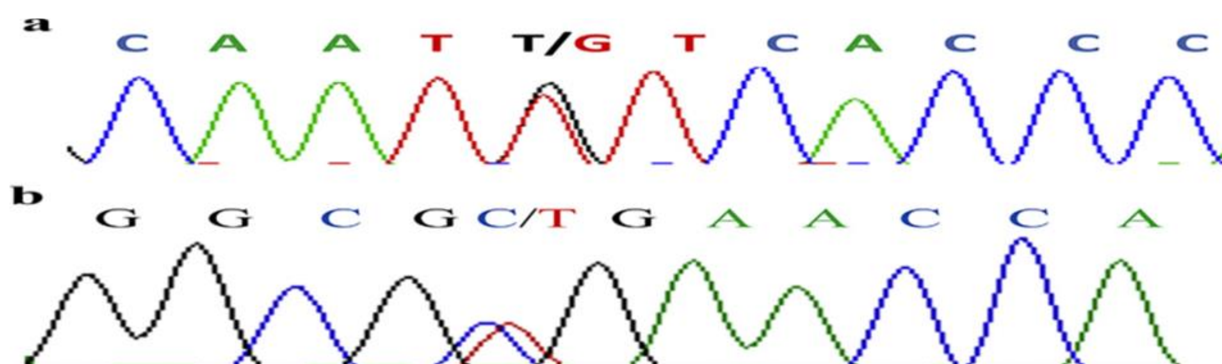

Fig 4.12: chromatogram displaying heterozygous G/T genotype. (b) chromatogram displaying C/T polymorphism

4.2.12 Allele Frequency of IL28B Genotypes SNP rs 12979860

IL28B genotype rs 12979860 single Allelic Frequency (Group I)

In order to further analyze polymorphism at genotype rs12979860B single allele frequency of two alleles C and T were calculated. The result revealed that in a total of 87 patients frequency of C allele was 85.6% and Frequency of C allele was 14.4 in (Group 1).

F (C)= [(2 x 65) +19] / 174 = 0.856 = 85.6%

F (T)= [(2 x 3) +19]/ 174 = 0.144 = 14.4 %

4.2.13 Multivariate Analysis of IL28 B Genotypes

Multivariate logistic regression analysis of IL28B genotypes was done as shown in Table 4.21. Genotypes TT, TG, GG of 8099917 (p value < 0. 000) and CC,CT ,TT of rs 12979860 (p value = 0.046) appear as significant predictor of SVR. Multivariate logistic regression analysis as done showing IL28 B genotypes as significant predictor of SVR (p value < 0.05) (Table 4.22).

Table 4.22 Frequency of IL28B genotypes and Multivariate analysis

Group I

Polymorphisms	SVR Achieved (R)(N= 64)	SVR not Achieved (NR) (N=23)	P value
rs 12979860			0.046
CC	51 (79.7 %)	14 (60.9 %)	
CT	10 (15.6 %)	9 (39.1%)	
TT	3 (4.7 %)	0 (0.0 %)	
rs 8099917	SVR Achieved (R)(N= 64)	SVR not Achieved (NR) (N=23)	0.000
TT	52 (81.3%)	8 (34.8 %)	
TG	7 (10.9 %)	14 (16.9 %)	
GG	5 (7.8 %)	1 (4.3 %)	

SVR Achieved (R)= Responder, SVR not Achieved (NR) = Non responder,

Group II

rs 8099917	SVR Achieved (R) (N= 65)	SVR not Achieved (NR) (N=17)	P value 0.019
TT	47 (57.3%)	9 (11.0 %)	

TG	17 (20.7 %)	5 (6.1 %)	
GG	1 (1.2 %)	3 (3.7 %)	

SVR Achieved (R)= Responder, SVR not Achieved (NR) = Non responder

Table 4.23: Frequency of IL28B genotypes and Multivariate analysis (Group I and II) Treatment Response; RVR

Polymorphisms	RVR Achieved ((N= 77)	RVR not Achieved (N=10)	P value
rs 12979860			0.799
CC	57 (66.5 %)	8 (9.2 %)	
CT	17 (19.5 %)	2 (2.3%)	
TT	3 (3.4 %)	0 (0.0 %)	
rs 8099917			0.168
TT	55 (63.2%)	5 (5.7 %)	
TG	18 (20.7 %)	3 (3.4 %)	
GG	4 (4.6 %)	2 (2.3 %)	
rs 8099917	.(Group II)(N= 65)	.(Group II)(N=17)	0.019
TT	47 (57.3%)	9 (11.0 %)	
TG	17 (20.7 %)	5 (3.1 %)	
GG	1 (1.2 %)	3 (3.7 %)	

4.3 **DISCUSSION**

Hepatitis C virus infection is regarded as a main reason of chronic liver disease resulting in liver fibrosis, cirrhosis & HCC. (Barth, 2015). Peg IFN-α-2a and PegIFN-α-2b are the preparations combined with Ribavarin a used in interferon based therapy to treat the infection (Ghany et al., 2009). There are different response rates observed among patients in different geographic areas receiving therapy (De Re et al., 2014). European origin patients have significantly better treatment response than those of African origin (Conjeevaram et al., 2006, Bochud et al., 2011). Association IL28B gene polymorphisms with natural and treatment induced curtailment of HCV infection has been pointed in various studies. (Rauch et al.,2010, Yang et al.,2013). Polymorphisms of IL28B genotypes are viewed as predictors of response to therapy thus considered important in assessing response to treatment. In patients infected with HCV (genotype 1) link between SVR and two SNPs near IL28B gene region rs12989760 and rs8099917 has been noticed (Lin et al., 2011). Interferon based treatment against chronic HCV infection is often not tolerated well due to side effects that avert some patients from finishing treatment. The personalized approach to treatment against chronic HCV built on IL28B genotypes, the viral genotype infecting the patient and the response to therapy is the outcome of discoveries towards solving the infection (Ghany et al., 2009).

In the present study the above mentioned SNPs rs12979860 and rs8099917, were analyzed in genotype 3a patients. Patients were analyzed for the effect of IL 28B genotypes in treatment response and spontaneous clearance. The study included Female (59.8%), Male (40.2 %) (Group I) and Male (57.3%) Female (42.7%) (Group II). In the present study age was significantly associated with SVR in both groups of patients (p =0.01316, p=0.0121).

The association of RVR with age was not significant. The fact that both groups in the patients showed significant relationship with SVR point towards the effect of age on treatment response (Crespo et al., 2013). The point is further highlighted in a study where association of HCV infection with age predicted that hepatitis C cirrhosis and its outcomes will exert their effect and in coming decade the complications associated with infection will be more evident in patients more than 60 years of age thus they will be needing newer therapies to deal with these complications. Current treatment patterns are seen as casting little effect on these complications (Davis et al.,

2010). The initiation of HCV infection can only be known through serology in patients with presence of antibodies but no viral RNA (Lechner et al., 2000).

Viral genotype and age at initial stage of therapy were recognized as predictors of response comparable to our study where age was associated with SVR in both groups of study (Thomson et al., 2008). A study further relate age with prevalence, it was pointed different region show different ranges of age showing peak prevalence, and is predicted to also depend on local epidemiology of HCV transmission (Hanafiah et al., 2013). The present study HCV genotype, IL28B polymorphism and age were factors which might predict viral clearance. The same grouping of the viral load, age and IL28B polymorphisms was used to yield a precise pre-treatment likelihood of SVR (Fattovich et al., 2011).

Several Genome Wise Association studies have depicted that in patients who are being treated for HCV infection certain variants in IL28B gene predicts response (Ge et al., 2009). SNPs (rs12979860 and rs8099917) are known for their association for forecasting treatment response is strengthened by accompanied clinical based observations (Bellanti et al., 2012). Nowadays these polymorphisms are regarded as the best in predicting outcome of interferon based treatment in patients having HCV genotype 1 infection. The polymorphisms at IL28B are also considered for their association in spontaneous clearance of virus; the suggestion was that mechanisms for natural and treatment-induced clearance of virus are shared (Rembeck et al., 2012). In the present study the favorable CC homozygous allele at rs12979860 and TT homozygous allele at rs8099917 were in higher ratio than unfavorable genotypes (CT, TT) at rs12979860 and (TG, GG) at rs8099917.

Limited data is available on the role of these polymorphism in spontaneous clearance, treatment response in genotype 3 subtype (3a) infected patients. Genetic factors are observed to be playing their role towards clearance of virus as various studies pointed towards African Americans having lower rate of favorable allele of these polymorphisms and found unable to clear the infection as compared to Caucasians in which GWAS have pointed for a larger ratio of favorable alleles (Mizokami, 2012 ; Bochud et al., 2011). Getting to the point of IL28B polymorphisms contribution in spontaneous clearance (SC) it was noted SC ratio was higher in those persons having

rs12979860 CC and rs8099917 TT genotypes (Bellanti et al., 2012). In Caucasian, Asians and Africans populations rs12979860 CC was related with SC, similarly when role of rs8099917 TT genotype for spontaneous clearance was analyzed it was seen to be associated with Caucasian ancestry (Yang et al., 2013).

Factors responsible for spontaneous clearance of HCV infection are unpredictable and a major percentage of persons infected result in persistence of virus in the body leading to chronic liver disease. An interesting strategy is to treat acute HCV and to put a check to the progression to chronic hepatitis (Barth, 2015). Numerous studies have demonstrated that treating acute hepatitis C infection in the acute phase is related with high SVR rates (Santantonio et al., 2008). There is agreement that interference in acute period of disease is linked to better viral clearance. Important clinical queries have stayed unanswered like optimizing treatment regimens and search of predictors of clearance of virus (Kamal & Nasser, 2008).

It has been found that rs 12979860 polymorphism having favorable CC allele had 2.5 % stronger association to SVR in genotype 1 patients as compared to unfavorable TC/ TT allele. In a multi-ethnic study, which was based on population the C allele was found in a much larger ratio leading the researchers to draw conclusion of C allele association with spontaneous clearance of virus (Ge et al., 2009).

4.3.1 IL28 B Polymorphism association of T and C alleles with viral kinetics (VKs)

Studies have pointed towards IL28B variants mainly influence the initial drop in levels of HCV RNA (Honda et al., 2010). The same type of observations was made depicting early virological response to get understanding of early viral kinetics regarding the role of IL28B polymorphisms (Scherzer et al., 2011). In a study, T at rs12979860, or G at rs8099917, in patients were found to be significantly linked with the raised baseline induction of ISGs and were regarded as risk alleles (Dill et al., 2011), the cause of their association was thought to be due to persistence of disease but their association with IFN-stimulated gene (Honda et al., 2010). In our study the C allele in rs12979860 were in higher ratio (85.6%) than T (14.4%) alleles. While analyzing patients in (group I) frequencies in rs 8099917 T allele was (81.1%) similarly in (group II) T allele frequency was (81.7%) and G allele frequency was

(18.3%) respectively. In rs12979860 genotype the higher ratio of C allele in SVR achievers and T allele in SVR non-achievers was noted, similarly in 8099917 higher ratio of T allele in SVR achievers and G allele in non-achievers was noted. The higher frequency of C may be considered as a controlling factor for better clearance of virus or link to spontaneous clearance. In a study on genotype 3 patients, T allele of rs12979860 was linked with a reduction in the first phase decline of viral load, the same study pointed that Polymorphisms in IL28B showed strong association with the initial clearance of HCV receiving peg interferon-α/ribavirin treatment, regardless of HCV genotype, these polymorphisms were found to be involved in early virological kinetics, but not with a second phase reduction in HCV levels (Bochud et al., 2011). In a study increased risk of HCV associated HCC were linked to T-allele of rs12979860 (Trinks et al., 2017). In a large Italian study on IL28B allelic distribution in overall data significant relationship was found in IL28B C allele and spontaneous clearance of HCV. Likewise it was observed that T allele showed a weak association with advancement to hepatocellular carcinoma. A hepatocellular carcinogenic model showed IL28B TT genotype, is found to play a role in causing persistent chronic hepatitis resulting into hepatocytes injury and chronic inflammation, over all effects contribute towards facilitation of HCC development (De Re et al., 2014). Another study concluded that the above mentioned associations were seen as link in decision-making for management of acute HCV infection in genotype 3 patients (Pearson & Manolio, 2008). Similarly like the present study the C allele was found in higher ratio in HCV-3- (0.692) infected patients (Falleti et al., 2011). Current management plan in case of acute HCV commends an observation period lasting for 3-month to allow time for spontaneous clearance (SC) (Craxi et al., 2011). This period is most suitable for favorable alleles possessing patients, showing spontaneous clearance ratios >50%, linked with better treatment response irrespective of whether treatment is in the acute or chronic setting (Lagging et al., 2011). In patients with unfavorable IL28B genotypes, particularly if anicteric, the rates of spontaneous clearance in such patients is low and instant start of treatment might maximize the response to treatment in such situations (this has not yet been proven). It seems a realistic clinical approach to keep the track of IL28B polymorphism rs12979860 and rs8099917 in HCV patients was the inference drawn in another such study (Lagging et al., 2011). Similarly it was of

interest for the conductors of another study that IL28B genotype was found to be involved in early viral kinetics (VKs) in HCV patients (Feeney & Chung, 2014).

4.3.2 ALT level Association with HCV infection

ALT analysis is effective in identifying patients with severe liver disease thus enhancing the significance of ALT as an important screening tool for diagnosing chronic liver disease In group I of our study we found an insignificant relationship of ALT levels with patients achieving RVR (p = 0.493) while in group II significant relationship of ALT levels was found with RVR (p = 0.010). Similarly in group I of our study we found an insignificant relationship of ALT levels with patients achieving SVR (p =0.551) while in group II significant relationship was found with SVR (p = 0.010). The normal range of the ALT kit was 0-40U/L. Another study carried out in Punjab University detected that raised ALT levels with negative antibodies against HCV and negative HCV RNA in blood samples are associated with occult infection in liver (Idrees et al., 2011). The mean ALT levels in our study group I was (58.6 U/L). In group II mean of ALT of patients was 48.8 U/L. Comparable to this study a research conducted in NIBGE, Pakistan showed mean (79.2U/L) ALT activity in HCV patients (Ahmad et al., 2007). Similarly, patients having HCV genotype 3 infection CC carriers at rs12979860 showed upper level normalized alanine aminotransferase (ALT) levels when compared to TT allele carriers showing a advanced degree of inflammation and fibrosis (Rembeck et al., 2012). In the present study also T allele in rs12979860 is associated with patients characterized as non-responders. Comparable to a study in Italy where elevated ALT levels were seen even in HCV RNA negative patients while receiving PEG-IFN and ribavirin treatment is fairly frequent. It was further noted that patients in relapsers there is a tendency of achieving higher ALT levels (Basso etal,. 2008).

4.3.3 Haemoglobin (Hb) Association with Peg interferon & Ribavarin Treatment Response

It is vital to study parameters, which can predict the outcome of long treatment regimens like Peg interferon Ribavarin. In the present study hemoglobin levels were analyzed in group I & II. The mean Hb levels were found to be 11.6mg/dl (group I) and 11.9 mg/dl (group II). This is in comparison to a study in which it was seen that

the likelihood of anemia rises relative to dose of ribavirin. It was observed that low-dose 800 mg/day of ribavirin is adequate for achieving SVR in patients with HCV genotypes 3 and is linked to a lesser possibility to anemia (Snoeck et al., 2006). It has been noted that there is association of low levels of hemoglobin with triple therapy ie with addition of Telepavir (TVR) with Peg interferon & Ribavarin (Ogawa et al., 2013). The demarcation of severe anemia in antiviral treatment is (Hb) <85 g/L and it was concluded that anemia is recognized as threat for every patients receiving TVR-based triple therapy (Jacobson et al., 2011). In our study in patients receiving interferon based therapy no association was found with HB levels and SVR.

4.3.4 IL28 B Polymorphism association with Genotype 3 HCV infection

The patients included in the present study were having HCV genotype 3 infection, in these patients analysis IL28B polymorphism revealed rs12979860 genotype homozygous CC genotype in (74.7%) patients. Heterozygous CT genotype was in 21.8 % patients and heterozygous TT was found in (3.4%) of patients. In contrast to a study conducted in USA on HCV genotype 3, IL28B gene rs 12979860 polymorphism analysis showed (37.5%) of patients had CC, (54.2%) of patients had T/C, and (6.9 %) patients had T/T genotype (Scherzer et al., 2011). Our results having higher frequency of CC genotypes of rs12979860 in genotype 3a are comparable with a study in which the most frequent genotype CC of rs 12979860 was found in increased proportion in genotype 3 infected patients while in genotype 1 patients the ratio of CC genotype was lower (Sarrazin et al., 2011). Similarly in HCV genotype 3–infected patients IL28B polymorphisms are linked with achievement of SVR to PEG-IFN therapy; and analysis of rs12979860 and rs8099917 genotypes help to recognize patients who are likely to relapse after SVR for extended treatment regimens or in case of adjunct therapy (De Re et al., 2014; Moghaddam et al., 2011). In our study of rs12979860 polymorphism homozygous CC genotype, and rs 8099917 homozygous TT genotype are strongly linked with SVR ($p = 0.046$, $p = 0.00$ respectively). In another study rs12979860 CC genotype was significantly

associated with SVR in patients having HCV genotype 3 infection (p = 0.01) (Sarrazin et al., 2011). The findings are in comparison with a study done in Europe where genotype 3 showed strong association with favorable CC and TT genotypes of IL28B genotypes (McCarthy et al., 2010). Likewise IL28B genetic polymorphisms have been identified as important prognostic factors for progression of steatosis usually seen as an accompaniment of HCV genotype 3 infection beside playing a role in treatment response in CHC (Tillmann et al., 2011). In contrast to our study it was seen that the race of patient and HCV genotypes place threat of forthcoming liver events and death. It was found that HCV genotype 3 had a greater danger for treatment failure than HCV genotype 2 (regarded as at lowest risk) or HCV genotype 1 patients (Tucker, 2013). Irrespective of the previous treatment history or HIV co-infection with HCV no association was shown by the above polymorphisms near IL28B in response to treatment in genotype 2 and 3 in a Swedish study (Jia et al., 2012).

4.3.5 IL28 B Polymorphism association with Rapid Virological Response (RVR)

Rapid Virological Response (RVR) is achieved when HCV RNA is (<15 IU/mL) at day 29 of Peg interferon Ribavarin treatment. Or when HCV RNA becomes negative after 4 weeks of antiviral treatment. RVR is an important predictor for achievement of Sustained Virological Response (SVR) and a valuable guide in treatment of HCV patients. Interferon based treatments regimens can be categorized as those lasting for 24 weeks called the standard duration or can be identified as variable durations of therapy (VD). Those receiving variable durations (VD) the patients achieving Rapid Virologic Response (RVR) can be given therapy for 12 weeks (VD12) while those not achieving RVR are given treatment for 24 weeks (VD24) (Mangia et al., 2010). In the present study when association of RVR with rs12979860 genotype was analyzed it was found that in 75.6% of patients achieved RVR in group I and 66.5 % of CC allele carriers were able to achieve RVR & SVR. It was seen that in 19.5 % of CT allele carriers who achieved RVR were successful in achieving SVR. In a similar study in RVR & SVR achievers favorable CC allele carriers were 77%, while in instances of patients who were relapsers heterozygous CT alleles of rs 12979860 and heterozygous TG alleles of rs 8099917 seemed to predominate (Firdaus et al., 2014).

While further analyzing results of rs 8099917 polymorphism in our study (group I) 88.5% achieved RVR while 11.5 % of patients were unable to achieve RVR. Similarly in group II RVR was achieved in 75.6 % and not achieved in 24.4 %. When comparison was made between patients with RVR achieving SVR was It was noted that in patients rs8099917 among having TT allele RVR achievers were (67.8%) and non-achievers were (16.07%), those having TG allele RVR achievers were (67.8%) and non-achievers were (16.07%). RVR was established as important predictor of SVR in both groups of our study (p=0.029, p =0.000; Table 4.23). To highlight the importance of association of RVR and IL28B genotype it is in practically recommended that in case of patients unable to achieve RVR but having favorable genotype HCV treatment plan cannot be shortened (Mangia et al., 2010). There are some studies suggesting that in non RVR patients the therapy should be intensified. In a study conducted in USA it was seen that a total of 43 out of 67 patients had RVR and the frequency of alleles in the patients was CC: allele frequency was 77.8%, and TC or TT allele frequency was 50.0% (Scherzer et al., 2011). In contrast in another study it was found that patients who were not able to achieve RVR, the CC genotype of rs12979860 was seen as sole predictor the SVR (Eslam et al., 2014).

In another research done by Lin et al., the patients achieving RVR ,79.0% of the cases also achieved SVR, this ratio was higher in comparison to those without RVR (SVR: 44.83%, p <0.001) (Lin et al., 2011). In another study IL28B polymorphism was associated with achievement of RVR but no relationship was found with SVR (Moghaddam et al., 2011).

4.3.6 Association SVR with IL 28B rs12979860 Polymorphism

The aim of therapy in chronic HCV infection is to attain sustained elimination of HCV (SVR), (Undetectable HCV RNA in serum 12 weeks after the antiviral treatment is completed) and to stop progress to liver cirrhosis, hepatocellular carcinoma (HCC), and finally progression to decompensated liver disease necessitating liver transplantation (Vinod, et al., 2019; Panel et al., 2015). In the present study rs12979860 genotype CC was found in 74.7% of patients; similarly CT, had 21.8 %; and genotype TT showed 3.4 % occurrence. It was found that 79.7 % of carriers of CC genotype attained SVR similarly in CT allele carriers 15.6 % of HCV infected patients were able to achieve SVR whereas SVR achievers were 4.7 %

having TT genotypes ($p = 0.046$). According to present study SVR rates in genotype rs1279860 are ominously higher in CC allele carriers when compared with CT & TT allele carriers. Similar results were shown by a study done in 2014 where CC alleles at rs12979860 were linked with SVR even in patients attaining high viral load (Firdaus et al., 2014).

In patients with genetic variations at rs12979860 it was shown that 94.6 % of patients having CC genotype attained SVR while 32.3 % of patients having CT & TT achieved SVR $p \leq .001$ (Ahlenstiel et al., 2012).

Another study showed the existence of different alleles at rs12979860 locus CC homozygous allele frequency was 37 %, CT allele frequency was 48 %; and similarly TT allele showed frequency of 15 % where by in the same study 82 % of HCV patients carrying CC allele achieved (SVR), similarly 75 % off patients having CT allele and 58 % having TT allele were able to achieve SVR ($p = 0.0046$) (Mangia et al., 2010).

In another research on HCV genotype 3 patients the genotype incidences of controls and chronic HCV patients showed frequency of CC allele as 47.0 % in controls and 32.6 %, in HCV patients, while unfavorable CT was 41.8 % in controls and frequency was 52.8 % in HCV infected patients similarly frequency of TT was 11.2 % in controls and 14.6 % in HCV patients ($p < 0.0001$) (Falleti et al., 2011).

An Indian research revealed the at rs12979860 locus presence of favorable allele CC was 59.09% (Sivaprasad et al., 2012). Several studies at different countries have shown association of rs12979860 to SVR in genotype 4 patients as achieving higher ratio with CC allele compared with CT/TT genotype were (88% versus 38%) results shown by patients infected with HCV genotype 1 revealed the association of CC genotype with higher SVR ratio and similarly an established predictor of response in Asian, Caucasian and also in Afro-American, patients (Basso et al., 2008; Bochud et al., 2011; Ghany et al., 2009; Yang et al., 2013). Similarly in a study carried out, SVR proportions were higher in those having CC genotype than the patients who were carriers of CT &TT genotypes ($p = 0.001$) (De Nicola et al., 2012).

4.3.7 Association of SVR with IL 28B rs8099917 Polymorphism

Variants near IL28B gene have their impact on IL28B production. Genotype rs8099917 is located 8kb upstream of IL28 B gene on chromosome 19 (Motolla et al., 2015). Genetic variations near IL 28 B gene at rs8099917 and 12979860 was studied in genotype 1b, analysis in the study showed SVR to be attained by 83.8 % of patients having TT genotype while 29.6 % of patients having TG allele and 0 % with GG allele were able to achieve SVR (Akuta, et al., 2010). Results of the present study rs8099917 genotype showed 81.3 % of patients among TT genotype achieved SVR while 10.9% of patients of overall TG genotype and 7.8% amid GG genotype achieved SVR (group I) and 76.8% in TT genotype patients achieved SVR, while 21.4% of patients amid TG genotype and 1.8 % of patients amongst GG genotype achieved SVR (group II). It is cited that both the groups were on interferon based therapy. In another study rs 8099917 favorable TT genotype revealed frequency (69%), TG genotype (24.1%) and GG genotype (6.9%). Similarly, SVR rates seen was 73.6 % of patients included in the study. In patients having TT genotype SVR was achieved in 59.8%, where as 8 % TG allele, followed by 5.7% GG allele carriers

S.No	Number of Patients	SVR (%)	Region	Reference

achieved SVR (Kobayashi et al., 2012). In a study conducted in Japan almost 90% of patients who had IL28B rs 8099917 homozygous TT genotype attained SVR while low expectancy of SVR was noted in patients found to be having advanced liver fibrosis, the patients who were showing prior partial response to therapy or were showing null response to therapy, this predisposition was earlier found in patients belonging to Caucasian race (Tanaka et al., 2009). The TT allele of rs8099917 in HCV Korean patients presented a considerably greater frequency as paralleled with other origins; highlighting role of ethnicities in pharmacokinetics of this infection (Lyoo et al., 2011). Similarly in Japan a study noted different ethnicities showing various frequency of the polymorphism near IL28 B gene (Kobayashi et al., 2012). In a study conducted in the same region in 2004 SVR rates were claimed to be (78.06 %) (Mahsud et al., 2008). In another study 88 % of patients achieved SVR (Abbas, et al.,

1	82	65%	Larkana	(Shaikh,et al ., 2003)
2	183	82%	Peshawar	(Farooqi et al., 2002)
3	65	F=81% M=86%	Peshawar	(Farooqi ,et al., 2005)
4	350	78.5%	Buner	(Muhammad, et al., 2004)
5	403	74.7%	Islamabad	(Aziz et al., 2011)
6	44	75%	Rawalpindi	(Shafi et al., 2011)
7	27	74.7%	Kohat	(Ali et al., 2011)
8	236	82.2%	Islamabad	(Aziz et al., 2013)
9	220	84.92%	Islamabad	(Qureshi et al.,, 2014)
10	170	73.5%	Abbotabad	(Jadoon & Nazar, 2014)
11	105	68.6%	Islamabad	(Aziz ,et al., 2015)

2002) (Table 4.24).

Table 4.24 A review of SVR achieved in different areas of Pakistan

4.3.8 Highlights of IL28B Rs8099917 & Rs12979860 Polymorphism Clinical Importance

IL28B genotype evaluation is helpful to initiate therapy for homozygous CC rs12979860 carriers. Moreover the conclusion that IL28B variability had no influence on liver histopathology or viral load amongst genotype 2 infected patients suggests that IL28B may differentially control the progression of genotype 2 and 3 infection (Rembeck, 2015).

Many clinical observations have suggested that donor liver grafts carrying the clinically favorable IL-28B allele may exert a valuable effect in improving sustained viral responses and decreasing the 5-year mortality rate of recipients with HCV infection (Chiu et al., 2018). It is observed from the comparisons given above that both the favorable genotypes TT and CC achieved SVR in greater proportions thus attaining the status of favorable alleles in our settings also. Looking at this phenomenon through different prospective a recent study has established through pyro

sequencing of hepatocytes isolated through Laser capture micro dilution (LCM). Ensuing living donor liver transplantation (LDLT) the TT-to-GT change prevailed in IL28B genotype rs8099917, and the CC-to-CT variation predominated in rs12979860 (Chiu et al., 2018).

In a study conducted in Japan SNP rs8099917 nearby IL28B gene was depicted as having most substantial association with SVR (Tanaka et al., 2009). In several studies conducted on patients receiving interferon therapy with direct acting antivirals DAA (Telepavir) SVR was strongly associated with rs rs8099917 TT genotype and hence it was agreed upon as one of the predictive factors responsible for achieving SVR (Jacobson et al., 2011; Ogawa et al., 2015; Petta et al., 2013). Similarly patient having un favorable TG/GG had higher ratio in non-responders (Ogawa, 2015).

In another study in Ukraine it was pointed that the polymorphism variants CC (rs12979860 and TT (rs8099917) of the gene IL28B are considered favorable (less severe of fibrosis in the disease advancement in chronic HCV infection in children). Variant TT (rs12979860) of the gene IL-28B is linked to the progression of hepatitis quicker advancement ion of liver fibrosis (Berezenko, Tsaryova, & Dyba, 2016). IL28B region rs12979860-CC genotype emerged as the most valued predictor of SVR and it was recommended that a clinical prediction model be made founded on IL28B genotype and clinical variables, can give beneficial personalized predictions of the possibility of treatment success that could be helpful in attaining better SVR rates and reduce the rate of unsuccessful treatment amongst patients with CHC (O' Brient et al., 2011). It was found in another study that IL28B genotype cannot be regarded as independent predictor of liver fibrosis (Lundbo et al., 2014). Favorable genotypes rs12979860 (C/C) and rs8099917 (T/T) were linked with HCV clearance in patients receiving interferon based therapy. It was observed from the comparisons in the study that both the genotype TT and CC achieved SVR in greater proportions thus attaining the status of favorable alleles. In our setting also detailed analysis showed rs12979860-CC and rs 8099917-TT genotypes as significant predictor of SVR. In our settings funds are inadequate and success of therapy is the duty of authorities considering right patient for right therapeutic regimen. The observations highlighting the studies achieving personalized approach should be encouraged for choosing patients in our settings.

Favorable C allele in IL28B at rs12979860 locus was found in higher ratio (85.6%) than unfavorable T allele (14.4%). At rs8099917 favorable T allele was higher (81.7%) and unfavorable G allele frequency was lower in frequency (18.3%). When analyzing rs12979860 the higher ratio of C allele in SVR achievers and T allele in SVR non-achievers was noted. Association favorable alleles with RVR and SVR point towards the role of genetic factors playing role in clearance of HCV infection. It is observed that favorable alleles possessing IL28B patients, with spontaneous clearance rates are >50%, and thus treatment response proportions will be high, irrespective of whether treatment is in the acute or chronic settings. The observation can be authenticated by population based study on the allele frequency helpful to further analyze the effects on IL 28B & other cytokines productions.

Detailed evaluation revealed that in group having mean ALT levels of 40 U/L significant associations was shown with SVR. This again point to trend of ALT level association with achieving SVR, the pathology of the disease can be interpreted by this parameter, a detailed analysis of the patient ALT level and further models constructed for association of ALT with RVR &SVR can provide a cheap forum for the reflection of the response to treatment.

The genotypes of IL28B leading to better response CC, TT of rs 1297860 &8099917 are found in considerably higher frequency in our patients achieving RVR also, establishing the ethnic link with clearance of virus in early viral kinetics. In the present study RVR was found in 75.6% of the patients 66.5 % of CC allele carriers achieved RVR while those having CT allele RVR achievers were (19.5%) and non-achievers were (2.3%). those having TT allele RVR achievers were (3.4%) and non-achievers were (0%). .in patients rs8099917 patients among having TT allele RVR achievers were (63.2%) and non-achievers were (5.7%), those having TG allele RVR achievers were (20.7%) and non-achievers were (3.4%). those having GG allele RVR achievers were (4.6%) and non-achievers were (2.3%). RVR was found to be a significant predictor of SVR in both groups of our study (p=.029,p =0.000), While 8099917 genotype TT was significantly associated with RVR in group II of the study.(p= .019)

In patients enrolled in group II at rs8099917 among patients having TT allele RVR achievers were (57.3%) and non-achievers were (11.00%), those having TG allele

RVR achievers were (20.7%) and non-achievers were (6.1%). those having GG allele RVR achievers were (1.2%) and non-achievers were (3.7%). Significant relation rs 8099917 genotype alleles were noted with RVR in group II patients of our study. The above mentioned findings guide towards a personalized approach to antiviral therapy thus a base adopted in HCV treatment can lead to the similar pattern to be observed for other diseases requiring prolonged therapy such as cancers of various origins exerting a considerable burden to health care system.

HCV management and prevention should now be a national urgency in Pakistan. Major development of infection control in clinics and hospitals, as well as implementation of the WHO guidelines for the usage of safety-engineered syringes should be adopted.

It is vital to identify the impact of HCV on progress of liver disease and work towards new healing strategies. Despite the fact that frequency of new HCV infections have shown a decline, because of chronicity and insidious nature of disease there are concerns that patients with complications due to advance stages of will come to the forefront as progression of fibrosis can be enhanced by features such as older age, the time period HCV infection has lasted and similarly resistance of insulin can be a factor for development of accompanied hepatic steatosis, progression of fibrosis and inflammation. If more effective remedies are not adopted for HCV, there is a fear that large number of patients could progress to cirrhosis, hepatic decomposition, or HCC affecting the health care system severely.

Keeping in view of the findings of the present study it is recommended that a broad based strategy regarding categorizing patients with base line characteristics such as Age , ALT levels, IL 28 B genotypes rs12979860 (CC, CT &TT) and rs 8099917(TT,GT & TT) alleles along with single allele frequency of C , T & G alleles should be employed in high risk patients such as intravenous drug users, patients receiving multiple transfusions (Thalassemia and Leukemia patients), patients undergoing Hemodialysis ,and patients with HBV,HIV co infections .Monitoring sero conversion similarly periodic screening and HCV RNA detection, should be employed to get a thorough insight into various factors pointed to as contributing factors in spontaneous and treatment induced clearance of virus.

In spite of the growing number and diversity of HCV genome sequences being discovered, the system of organization of variants into genotypes and subtypes has proven unexpectedly robust. The seven established genotypes have strong support and the division of these genotypes into subtypes that vary over a whole coding region sequence by >15% exposes a natural interruption in the dissemination of sequence distances. Areas of doubt still remain with respect to the areas of endemicity of genotype 5, indicated by a single subtype isolated in Europe, Brazil, North Africa, and South Africa, and genotype 7, isolated from a migrant from the Congo. We might also anticipate the more discoveries of other HCV-like viruses in the genus and variants closer genetically to it. A more robust analysis keeping these points should be carried out in future.

Studies pointing towards causal variants exerting their effect by increasing IFN-λ3 expression or by the expression of IFN-λ4. In case of treatment response, rs368234815 seems to be the causal variant, correspondingly case-control studies can be designed to analyze SNP rs117648444 to explore role of IFN-λ4 in treatment response

One potential role of IL28B genotype analysis could be to identify patients who, although not presently eligible for antiviral treatment i.e. the patients with advanced fibrosis, can benefit by personalized approach, directed differentiation of induced pluripotent stem cells produces patient-specific liver tissue accommodating to HCV and responds to infection with a strong innate immune response "personalizing" the treatment of infection.

In the present study HCV genotype, IL28B gene polymorphisms at (rs8099917, rs1297986) and age were the factors, which influence viral clearance. Further research regarding wide geographic regions such as all the provinces sections comprising primary hospitals on the first step and later all the tertiary care hospitals be scrutinized to establish a more wider aspect of these three parameters found to be significantly associated with treatment response.

4.3.9 Association of DAA with IL 28B rs8099917 Polymorphism

Direct-acting antivirals (DAAs) have been approved to be used for treatment in 2011, in HCV genotype 1b, 2a and 2b infected patients, significant improvement in SVR

rates is noted with IL28B rs 8099917 TT allele carriers. Despite other factors like prior therapy (interferon–Ribavarin),in patients having IL28B TG/GG genotype, only 12.5% patients achieved SVR with their use. (Ogawa et al., 2014). Of specific note is that if the patients had unfavorable TG or GG allele with severe fibrosis the previous partial and null responders were unable to achieve SVR, in total contrast SVR was achieved in patients with mild or no fibrosis with heterozygous TG and homozygous GG genotype, with DAA usage. Treatments with new Direct Acting Antivirals have shown an immense progression. The data on previous non-responders has shown 20-30% improvement in SVR with addition of Bocepavir (BOC). It has been noted that with Telepevir (TPV) the non-responders to standard therapy showed up to 37% improvement and up to 55 % improvement was shown in patients showing partial response to therapy. In the cases of failure of therapies in remaining 70% of population in which treatments are repeated over a time mutations of virus may evolve. The choice of therapy in these cases requires an insight into a new IL28B polymorphism (Smith et al., 2011). It has also been advocated to wait for availability of newer therapeutic antivirals in the patients with unfavorable IL28B genotype. The patients in our study also showed the association of TG/GG genotype with fewer patients achieving SVR. In Japan, some patients infected with HCV have the IL28B major genotype, which may indicate a favorable response to interferon-including regimens; however, certain patients within this group are also interferon-intolerant or ineligible. In Japan, interferon-free 24-week regimens of Sunaprevir and Daclatasvir are now available for HCV genotype 1b-infected patients who are interferon-intolerant or ineligible or previous treatment null-responders. The treatment response to interferon-free regimens appears better, regardless of IL28B genotype. May be other interferon-free regimens will widely be available soon. In conclusion, although some HCV-infected individuals have IL28B favorable alleles, importance of IL28B will be reduced with availability of oral interferon free regimen (Kanda et al, 2015).

CONCLUSIONS

1. Genotype 3a is the major genotype in this geographical region trailed by genotype 1 nevertheless, the rigorousness of disease was more in genotype 1 as judged by higher viral load.
2. Genotype 3a individuals carrying protective rs12979860 CC genotype and rs8099917 TT genotype have significantly higher SVR than unfavorable rs1297980 CT or TT alleles (p=0.046) and rs8099917 TG and GG (p=0.00)
3. Significant relation of rs 8099917 genotype alleles were noted with RVR in (group II) patients of our study. Association favorable alleles with RVR and SVR point towards the role of genetic factors playing role in clearance of HCV infection
4. The genotypes of IL28B CC, TT of rs 1297860 & 8099917 respectively are found in considerably higher frequency in our patients achieving RVR also, establishing the link with early viral kinetics.
5. When analyzing rs12979860 the higher ratio of C allele in SVR achievers and T allele in SVR non-achievers was noted.
6. Favorable C allele in IL28B at rs12979860 locus was found in higher ratio (85.6%) than unfavorable T allele (14.4%). At rs8099917 favorable T allele was higher (81.7%) and unfavorable G allele frequency was lower in frequency (18.3%). When analyzing rs12979860 the higher ratio of C allele in SVR achievers and T allele in SVR non-achievers was noted. It is observed that favorable alleles possessing IL28B patients, with spontaneous clearance rates are >50%, and thus treatment response proportions will be high, irrespective of whether treatment is in the acute or chronic settings. The observation can be authenticated by population based study on the allele frequency helpful to further analyze the effects on IL 28B & other cytokines productions.
7. Detailed evaluation revealed that in group having mean ALT levels of 40 U/L significant associations was shown with SVR. Further models constructed for association of ALT with RVR &SVR can provide a cheap forum for the reflection of the response to treatment.

8. The results suggest that IL28B variants have an impact on the treatment outcome of patients thus should be incorporated as a routine test before start of treatment. In our settings funds are insufficient the observations in the study stress the importance of achieving personalized approach should be encouraged while choosing patients with greater success rate.

FUTURE RECOMMENDATIONS

I. Emergence of new HCV genotypes with improved diagnostic techniques are noteworthy and their viral load association with currently used DAA is the need of the day.

II. HCVgenotypes,IL28B gene polymorphisms at (rs8099917, rs1297986) and age were the factors which influence viral clearance. Further study on these parameters is recommended in larger scale.

III. Personalized approach to antiviral therapy can form emphatic bases to be observed for other diseases demanding prolonged therapy such as cancers of various origins.

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