Characterization of polymorphisms in *TNF-α*, *HLA-DRB1* and *HLA-DQB1* in patients with Hepatitis C and relevance to treatment response and outcome

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IN
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By

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2020

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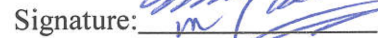
Student Name: **Mr. Muhammad Arshad** Signature: 

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List of Contents

Sr. No	Title	Page. No.
I	List of Contents	i
II	List of abbreviations	vi
III	List of Tables	ix
IV	List of Figures	xi
V	Acknowledgements	xii
VI	Summary	xiv
	1: Introduction and Literature Review	
1.1	Background	1
1.2	Natural history of HCV infection	2
1.2.1	Acute HCV infection	2
1.2.2	Chronic HCV infection	3
1.2.3	Fibrosis progression	3
1.3	HCV life cycle	3
1.3.1	HCV entry and uncoating	3
1.3.2	HCV RNA translation and replication	3
1.3.3	HCV assembly and release	4
1.4	Global epidemiology of HCV infection	5
1.4.1	Frequency rates	5
1.4.2	Prevalence rates in Pakistan	5
1.5	HCV genotype prevalence	6
1.6	Transmission and prevention	7
1.7	Diagnosis of HCV infection	7
1.7.1	HCV detection using Antibody production	8
1.7.1.1	Screening test: ELISA	8
1.7.1.2	Screening test: The quick, point-of-care test (POCTs)	8
1.7.1.3	Confirmatory tests	8
1.7.2	Detection of HCV RNA	9
1.7.2.1	Qualitative Diagnosis of viral RNA	9
1.7.2.2	Quantitative Detection of HCV RNA	9
1.8	Associated risk factors	9
1.9	Immune responses against HCV infection	10
1.9.1	Innate immune responses against HCV infection	10
1.9.2	Adaptive immune responses	11
1.9.3	HCV immune evasion strategies	12
1.10	HCV infection Treatment	13
1.11	Host Genetic factors associated with HCV infection	15
1.11.1	The HLA system	16
1.11.2	Role of polymorphism in Cytokine genes and HCV infection	20
1.11.2.1	TNF alpha and HCV infection	20

1.11.2.2	TNF alpha gene promoter	22
1.12	Concluding remarks	24
	2. Hypothesis, Aims and Objectives	
2.1	Hypothesis	25
2.2	Aims and Objectives	25
2.2.1	Aims	25
2.2.2	Objectives	25
	3: Materials and Methods	
3	Study design	27
3.1	Ethical approval for the study	27
3.2	Informed consent from patients	27
3.3	Sampling	27
3.4	Sample tagging and storage	27
3.5	Data entry and Patient Categories	28
3.6	Quantitative PCR and Genotyping	28
3.7	DNA extraction	29
3.8	Gel electrophoresis	31
3.9	DNA Quantitation	31
3.10	Candidate Gene selection	31
3.11	Primer designing	32
3.11.1	*TNF-α* primers	32
3.11.2	*HLA-DRB1* primers	32
3.11.3	*HLA-DQB1* primers	32
3.12	Primer optimization and troubleshooting	33
3.13	Polymerase chain reaction	33
3.14	Agarose gel electrophoresis	34
3.15	Gel purification	34
3.16	DNA Sequencing	36
3.17	Sequence analysis	36
3.18	Nucleotide Peak analysis	36
3.19	Reference SNP number and position on the chromosome identification	36
3.20	Generation of maps	37
3.21	Allele frequencies	37
3.22	Genotype determination	37
3.23	Haplotype determination	37
3.24	Multivariate logistic regression analysis	38
3.25	Data tabulation and comparative analysis	38
3.26	Linkage disequilibrium analysis	38
3.27	Dominancy models generation	38
3.28	Statistical Analysis	38

4: Results		
4.1 Results: Clinical and demographic analysis		
4.1	Clinical and demographic analysis of the studied patients	40
4.1.1	Gender and literacy based differentiation of the studied patients	40
4.1.2	Age intervals of the analyzed patients	41
4.1.3	Demography of included patients	43
4.1.4	Prevalence of symptoms in studied patients	43
4.1.5	Risk factors associated with HCV infection in analyzed patients	45
4.1.6	Occupational information of the studied participants	46
4.1.7	Literacy rates in studied patients	48
4.1.8	Prevalence of viral RNA in in anti-HCV positive patients	48
4.1.8.1	Prevalence of HCV RNA in anti-HCV positive male and female patients	49
4.1.9	HCV genotype prevalence in anti-HCV positive patients	50
4.1.10	Gender wise prevalence of viral load distribution in anti-HCV positive patients	51
4.1.11	Patient Categories	52
4.2 Results: Mapping Analysis		
4.2	Post-clinical and demographic analyses	54
4.2.1	DNA extraction	54
4.2.2	PCR amplification for selected candidate genes (*TNF-α*, HLA-*DRB1, DQB1*)	54
4.2.2.1	Gel image of the *TNF-α* -238/308 position amplification	54
4.2.2.2	Gel image of the *TNF-α* -863 position amplification	55
4.2.2.3	Gel image of the *HLA-DRB1* gene amplification	55
4.2.2.4	Gel image of the *HLA-DQB1* gene amplification	56
4.2.3	Gene sequencing	56
4.2.4	Mapping of the identified SNPs	56
4.2.4.1	Mapping of *TNF-α* gene SNPs	57
4.2.4.2	Mapping of *HLA-DRB1* gene SNPs	58
4.2.4.3	Mapping of *HLA-DQB1* gene SNPs	59
4.3 Results: Genotype and Allele Determination		
4.3	Patient genotype determination	62
4.3.1	Genotype distribution in responsive (R) and non-responsive (NR) patients	62
4.3.1.1	*TNF-α* -238, -308, -863 genotype distributions	62
4.3.1.2	*HLA-DRB1* genotype distribution in responsive and non-responsive patients	62
4.3.1.2.1	*HLA-DRB1*6151, 6167 and 6192 positions genotype distribution in responsive and non-responsive patients	62
4.3.1.2.2	*HLA-DRB1* 6228,6231, 6236, 6251genotype distribution in responsive and non-responsive patients	63

4.3.1.3	*HLA-DQB1* genotype distribution in responsive and non-responsive patients	64
4.3.1.3.1	*HLA-DQB1* -8288, -8298 and -8307 genotypes distribution in R and NR patients	64
4.3.1.3.2	*HLA-DQB1* -8331, -8344 and -8362 genotypes distribution in R and NR patients	65
4.3.1.3.3	Genotype distribution at *HLA-DQB1* -8442, -8447, -8465, -8471 in R and NR patients.	66
4.3.2	Genotype distribution in Chronically infected (CI) and Spontaneously recovered (SR) patients	68
4.3.2.1	*TNF-α* genotype distributions at -238, -308 and -863 position in SR and CI patients	68
4.3.2.2	*HLA-DRB1* genotype distribution in SR and CI patients	68
4.3.2.2.1	Genotype distribution in *HLA-DRB1* 6151, 6167 and 6192 positions in SR and CI patients	68
4.3.2.2.2	Genotype distribution in *HLA-DRB1* 6228, 6231, 6236 and 6251 positions in SR and CI patients	69
4.3.2.3	*HLA-DQB1* genotype distribution in SR and CI patients	70
4.3.2.3.1	*HLA-DQB1* -8288, -8298 and -8307 genotype distribution in SR and CI patients	70
4.3.2.3.2	*HLA-DQB1* -8331, -8344 and -8362 genotype distribution in SR and CI patients	71
4.3.2.3.3	*HLA-DQB1* -8442, -8447, -8465 and -8471 genotype distribution in SR and CI patients	72
4.3.3	Gender based genotypes distribution in studied patients.	74
4.3.3.1	*TNF-α* genotypes distribution in gender	74
4.3.3.1.1	*TNF-α* -238, -308 and -863 genotype distributions in male and females	74
4.3.3.2	*HLA-DRB1* genotypes distribution in males and females	74
4.3.3.2.1	*HLA-DRB1* 6151, 6167 and 6192 genotype distribution in males and females	74
4.3.3.2.2	*HLA-DRB1* 6228, 6231, 6236 and 6251 genotype distribution in males and females	75
4.3.3.3	Gender based *HLA-DQB1* genotypes distribution	76
4.3.3.3.1	*HLA-DQB1* -8288, -8298 and -8307 positions genotypes distribution in males and females	76
4.3.3.3.2	*HLA-DQB1* -8331, -8344 and -8362 positions genotypes distribution in males and females	77
4.3.3.3.3	*HLA-DQB1* -8442, -8447, -8465 and -8471 positions genotypes distribution in males and females	78
4.3.4	Allelic distribution in different groups of patients	79
4.3.4.1	Allelic distribution in the therapy group	80

4.3.4.1.1	Allelic distribution of *TNF-α* in responsive (R) and non-responsive (NR) patients	80
4.3.4.1.2	Allelic distribution of *HLA-DRB1 gene* in responsive (R) and non-responsive (NR) patients	80
4.3.4.1.3	Allelic distribution of *HLA-DQB1 gene* in responsive (R) and non-responsive (NR) patients	82
4.3.4.2	Allelic distribution in spontaneously recovered (SR) and chronically infected (CI) patients	84
4.3.4.2.1	Allelic distribution of *TNF-α* in spontaneously recovered (SR) and chronically infected (CI) patients	84
4.3.4.2.2	Allelic distribution of *HLA-DRB1* in spontaneously recovered (SR) and chronically infected (CI) patients	85
4.3.4.2.3	Allelic distribution of *HLA-DQB1* in spontaneously recovered (SR) and chronically infected (CI) patients	86
4.3.5	Gender based allelic distribution in studied patients.	88
4.3.5.1	*TNF- α* genetic polymorphisms in male and female patients	88
4.3.5.2	Gender based *HLA-DRB1* allelic variations in studied patients	88
4.3.5.3	Allelic variation in *HLA-DQB1* gene in male and female patients	89
4.3.6	Haplotypes observed in the studied patients	91
4.3.7	Linkage disequilibrium analysis	93
4.3.7.1	Linkage disequilibrium analysis for *TNF-α* gene	93
4.3.7.2	Linkage disequilibrium analysis for *HLA-DRB1* gene	95
4.3.7.3	Linkage disequilibrium analysis for *HLA-DQB1* gene	98
4.3.8	Dominancy models for genotypes in analyzed genes	102
4.3.8.1	*TNF-α* (-238) genotype dominancy model	102
4.3.8.2	*HLA-DRB1* (6151) genotype dominancy model	103
4.3.8.3	Dominancy model for *HLA-DQB1* 8288 genotypes	104
	5: Discussion	
5	Discussion	105
5.1	Conclusion	122
5.2	Future prospects	124
6	**6: References**	125
7	**7: Appendix**	172

List of Abbreviations

AASLD	American association for the study of liver disease
CD4T	Complementarity determining 4 T cells
CD8T	Complementarity determining 8 T cells
CDC	Center for disease control
cDNA	Complementary DNA
CH25H	Cholesterol-25-hydroxylase
CHC	Chronic hepatitis C
CI	Chronically infected
CLDN1	Claudin-1
CLRs	C-type lectin receptors
CTL	Cytotoxic T lymphocytes
DAA	Direct Acting Antivirals
DGAT1	Diacylglycerol acyltransferase-1
EASL	European association for the study of liver
EDTA	Ethylene diamine tetra acetate
EIA	Enzyme immune assay
ELISA.	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESCRT	Endosomal-sorting complex required for transport
ESLD	End stage liver disease
ExAc	Exome aggregation consortium
FDA	Food and Drug regulation authority
GWAS	Genome wide association studies
HAART	Highly active anti-retroviral therapy
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virrus
HCV G	Hepatitis C virus Genotype
HIV	Human Immune deficiency Virus
HuH	Human hepatoma cells
ICER	incremental cost effectiveness ration
IFN/RBV	Interferon/ribavin
IFN-ϒ	Interferon gamma
IL-2	Interlukin-2
IRES	Internal ribosomal entry site
IRF-3	interferon regulatory factor 3
ISLD	Islamabad
JAK	Janus kinases
KPK	Khyber Pakhtunkhwa
LD	Lethal dose
LDLR	Low density lipoprotein receptor
MAVS	Mitochondrial antiviral signaling protein

MDA5	melanoma differentiation-associated protein 5
MSM	Men sex with men
NANBH	Non-A, Non B hepatitis
NCBI	National center for Biotechnology Information
NF-κB	Nuclear factor kappa b
NK	Natural killer cells
NKT	Natural killer T cells
NLR	(NOD)-like receptors
NNPIs	Non-nucleotide polymerase inhibitors
NOD	nucleotide-binding oligomerization domain
NORI	Nuclear Medicine Oncology and Radiotherapy Institute
NR	Non- responsive
NS	Non-structural
NTR	Non-translated region
OAS- 1	2-5-oligoadenylate synthetase
OCLN	Occludin
ORF	Open reading frame
P7	Porin-7
PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1
pDCs	Plasmocytoid dendritic cells
Peg-IFN	Pegylated-interferon
PI	Protease inhibitors
PKR	Protein Kinase receptor
PLADs	pre-ligand assembly domains
POCT	Point of care test
PRR	Pattern recognition receptor
R	Responsive
RdRp	RNA dependent RNA polymerases
RIBA	Radio immune blot assay
RIG-I	Retinoic acid-inducible gene I
RLRs	(RIG)-I-like receptors
RTEs	Reverse transcriptase enzymes
RT-PCR	Reverse transcriptase-polymerase chain reaction
RWP	Rawalpindi
SD	Standard deviation
SIM	Simeprevir
SNP	Single nucleotide polymorphism
SNVs	Single nucleotide variations
SODD	Silence of death domain
SOF	Sofosbuvir
SR	Spontaneously recovered

ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
SVR	Sustained virological response
TACE	TNF alpha converting enzyme
TBE	Tris/borate/EDTA
TFA	Tubule forming agent
TGF	Transforming growth factor
TLR	Toll like receptors
TMA	transcription-mediated amplification
TRADD	Tumor necrosis factor receptor associated death domain
TRIF	TIR-domain-containing adapter-inducing interferon beta
UCSC	University of California Santa Cruz
UTR	Un-translated region
VLDL	Very low density lipoprotein
WHO	World health organization

List of Tables

Sr. No	Title	Page No.
3.1	Solutions used in purification of DNA	30
3.2	Set of Primers used for the amplification of the desired genes.	33
4.1	Gender and literacy based distribution of studied patients.	41
4.2	Viral load distributions in males and female patients	51
4.3	Mapped SNPs analyzed in this study	57
4.4	*TNF- α* (-238) genotype distribution in responsive and non-responsive patients	62
4.5	*HLA-DRB1*6151, 6167 and 6192 genotype prevalence in responsive and non-responsive patients	63
4.6	*HLA-DRB1* 6228, 6231, 6236, 6251 position genotype prevalence in R and NR patients	64
4.7	Distribution of genotypes in *HLA-DQB1* gene at-8288 , -8298 and -8307 position in R and NR patients	65
4.8	Genotype prevalence at 8331, -8344 and -8362 positions in R and NR patients	66
4.9	Genotype prevalence at -8442, -8447, -8465, -8471 positions in R and NR patients	67
4.10	Genotype distribution at -238, -308 and -863 position in SR and CI patients in TNF gene	68
4.11	Genotype prevalence at 6151, 6167 and 6192 positions in SR and CI patients	69
4.12	Genotype prevalence at 6228, 6231,6236 and 6251 positions in SR and CI patients	70
4.13	Genotype prevalence at -8288, -8298 and -8307 positions in SR and CI patients	71
4.14	Genotype prevalence at -8331, -8344 and -8362 positions in SR and CI patients	72
4.15	Genotype prevalence at -8442, -8447, -8465 and -8471 positions in SR and CI patients	73
4.16	*TNF-α* -238, -308 and -863 genotype distribution in male and female	74
4.17	*HLA-DRB1* 6151, 6167 and 6192 genotype distribution in male and female	75
4.18	*HLA-DRB1* 6228, 6231, 6236 and 6251 genotype distribution in males and females	76
4.19	Prevalence of genotypes at -8288, -8298 and -8307 positions in male and female patients	77
4.20	Prevalence of genotypes at -8331, -8344 and -8362 positions in male and female patients	78

4.21	Genotype distribution at -8442, -8447, -8465 and -8471 positions and their role in infection in male and female patients	79
4.22	Allelic distributions of *TNF-α* gene in the responsive and non-responsive patients	80
4.23	Therapy based distribution of alleles in observed SNPs in *HLA-DRB1* gene	81
4.24	Therapy based distribution of alleles in observed SNPs in *HLA-DQB1* gene	83
4.25	*TNF- α* genetic polymorphisms in SR and CI patients	84
4.26	Allelic distribution *HLA-DRB1* in spontaneously recovered (SR) and chronically infected patients	85
4.27	Allelic distribution *HLA-DQB1* in spontaneously recovered (SR) and chronically infected patients	87
4.28	*TNF- α* allelic polymorphisms in male and female patients	88
4.29	Gender based *HLA-DRB1* allelic distribution in studied patients**.**	89
4.30	Gender based *HLA-DQB1* allelic distribution in studied patients.	90
4.31	Most prevalent haplotypes detected in three of the studied genes.	92
4.32	Linkage disequilibrium analysis for SNPs observed in *TNF-α* gene	94
4.33	Linkage disequilibrium analysis for all SNPs in *HLA-DRB1* gene	97
4.34	Linkage disequilibrium analysis for all SNPs in *HLA-DQB1* gene.	99
4.35	Dominancy model for *TNF-α* (-238) genotype in gender response	102
4.36	Dominancy models for *HLA-DRB1* 6151 SNP in gender response	103
4.37	Genotype models for *HLA-DQB1*-8288 position.	104

List of Figures

Sr. No	Title	Page. No
4.1	Chart representing male and female distributions in studied patients	40
4.2	Chart representing the age intervals of the studied patients	42
4.3	Age based distribution of male and female participants	42
4.4	Demography of analyzed patients	43
4.5	Symptoms analysis in studied patients	44
4.6	Analyzed symptoms in males and females	44
4.7	Observed risk factors among studied patients	45
4.8	Risk factors prevalence in male and female	46
4.9	Chart representing occupation of studied participants	47
4.10	Gender based occupational differences among studied participants	47
4.11	Literacy levels in studied patients	48
4.12	Prevalence of HCV RNA in studied patients	49
4.13	Gender wise viral RNA prevalence in studied patients	49
4.14	HCV genotype prevalence in anti-HCV positive patients	50
4.15	HCV genotype prevalence in anti-HCV positive males and females patients	51
4.16	Categories of analyzed patients	53
4.17	Categorization of the patients on the basis of gender	53
4.18	Gel images of the extracted DNA from the blood samples	54
4.19	Gel image of the amplified product for *TNF-α* -238/308 locus	55
4.20	Gel image of the amplified product for *TNF-α* -863 locus	55
4.21	Gel image of the amplified product for *HLA-DRB1* gene	55
4.22	Gel image of the amplified product for *HLA-DQB1* gene	56
4.23	Figure showing SNPs in the promoter region of *TNF-α* gene.	58
4.24	Figure showing SNPs in Exon 2 of *HLA-DRB1* gene.	59
4.25	Figure showing SNPs in promoter region of *HLA-DQB1* gene	60
4.26	Combined map of all genes showing observed SNPs in the analyzed genes in this study	61
4.27	Linkage disequilibrium heat map for SNPs observed in *HLA-DRB1* gene	97

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Muhammad Arshad

Summary

Hepatitis C virus (HCV) is a major cause of chronic liver infection and is an important public health challenge in Pakistan. Host immunological factors are accountable for the differential outcome of HCV infection and response to anti-HCV therapy. However, not much is known about the spontaneous recovery and response to anti-HCV therapy for the patients under treatment. Moreover, prior to this report, none of the studies in Pakistan focused on the role of genetic polymorphism in key immunological factors *TNF-α, HLA-DRB1* and *DQB1* genes. This study was carried out to determine the relationship of polymorphisms in *TNF-α, HLA-DRB1, DQB1* genes and their possible association with response to anti-HCV therapy. Sampling for this study was done by collecting blood samples from the patients who were visiting Nuclear Medicine Oncology and Radiotherapy Institute (NORI) Islamabad. Patients under scrutiny were positive for anti-HCV and information regarding each patient's demography, signs and symptoms and risk factors were obtained using a designed proforma. Consent was obtained from all the patients whereas, ethical approval for this study was granted by Institutional Review Board of Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad. Samples were subjected to viral RNA quantification, along with genotyping of HCV. After viral RNA analysis, human DNA extraction was done by using phenol chloroform method. The extracted DNA was transported to the Laboratory of Virology and Infectious Disease, The Rockefeller University, New York USA. Three genes (*TNF-α, HLA-DRB1, DQB1*) were targeted for single nucleotide variation analysis. Primers were designed for *HLA-DRB1, DQB1*genes, whereas already reported primers were used for the amplification of *TNF-α* gene. PCR conditions were optimized for each gene and amplicons were confirmed on agarose gel by comparing the desired product size with a DNA marker. After PCR amplicons were purified using gel purification kit. DNA sequencing was done by Macrogen New York. After sequencing peak analysis of the sequences were carried out followed by patient's genotype and allele distributions and mapping of the identified SNPs. Haplotype determination was carried out for the studied SNPs and statistical analysis was performed. Gender and demographic data analysis for the studied patients revealed that the distribution of male and female patients in this study was almost equal, 49% vs 51%. The most common signs and symptoms distributed in studied group of patients were jaundice,

fatigue, abdominal discomfort, anorexia and malaise and revealed a significant association with HCV infection in studied patients (p<0.05). Demographic analysis showed that most of the patients analyzed in this study belonged to Rawalpindi and Islamabad region, followed by Punjab and Khyber Pakhtunkhwa. Similarly analysis for age groups revealed that most of the patients fell into age group, 31-40 years. Distribution of the risk factors in examined individuals revealed that the most prevalent risk factors were injections, intravenous infusions, barbers visits and skin piercing respectively (p<0.05). HCV genotype 3a was established to be the most prevalent genotype in the studied group of patients. Similarly literacy rates analysis depicted that the 86% of the patients were literate whereas, 14% of the patients were illiterate with matriculation being the most common education level followed by primary and middle education. Occupational information for the studied patients showed that the most of the male patients had Government jobs, laborer and shopkeeper respectively, whereas, most of the female patients were housewives followed by teachers. Viral RNA detection analysis depicted that viral RNA was detected in total of 51% of the studied patients, whereas the peak load in males was found to be 51,809,739 IU/ml, similarly the maximum HCV RNA load in female patients was reported to be 31,874,791 IU/ml respectively. Average viral load in both male and female patients was reported to be 5,315,791 IU/ml and 3,180,912 IU/ml, while the cut off value for viral RNA detection were 12 IU/ml. All the included patients in the study were categorized into two group that included therapy group and non-therapy group. Therapy group was further divided into responsive to interferon/ribavirin group (R) and non-responsive to interferon/ribavirin therapy group (NR). The non-therapy group was divided into chronically infected (CI) group and spontaneously recovered groups (SR). After sequencing analysis the obtained traces were analyzed for the presence of SNPs using different softwares, a total of twenty SNPs were examined in the analyzed set of patients. Three SNPs in Tumor necrosis factor alpha (*TNF-α*) were studied, whereas seven SNPs were analyzed in *HLA-DRB1* gene. In total ten SNPs were studied in *HLA-DQB1* gene and it was found that five of the total ten SNPs were novel. Mapping of the SNPs was carried out and we showed that SNPs in *TNF-α* gene mapped to the promoter region, similarly SNPs in *HLA-DRB1*gene mapped to the exon 2 of the gene. *HLA-DQB1* SNPs mapped to the promoter region of *HLA-DQB1* gene. After mapping genotyping of the studied SNPs

were carried out followed by allelic and haplotype determination. Genotype analysis showed that genotypes at *TNF-α* were not significantly associated with therapy or HCV disease outcome. However, differences in genotype distributions at -8362 position in the *HLA-DQB1* gene were statistically significant among spontaneously recovered and chronically infected patients. Similarly genotypes, TT and CC at position 6151 (rs1064663) in the *HLA-DRB1* gene were significantly associated in both male and female patients whereas, genotype distribution at 6231 (rs3167799) position in *HLA-DRB1* gene was found to be significantly associated with HCV infection in both male and female patients. Allelic distributions at the analyzed position showed that that allele T at 6228 (rs230382) position in *HLA-DRB1* gene was significant predictor of response to anti-HCV therapy (IFN/RBV combinatorial therapy). Similarly a novel allele in the *HLA-DQB1* gene at -8447 position was also responsible for response to interferon/ribavirin therapy in studied patients. Allele C/T distribution in both male and female patients in *HLA-DRB1* gene at 6151 (rs1064663) position was found to be statistically significant in male and female patients. Similarly Allele T/C variation at 6228 (rs230382) position in the *HLA-DRB1* gene was found to have a statistically significant association with infection outcomes in male and female patients, whereas allelic distribution at 6231 (T/C/G) (rs3167799) position revealed that allele G and T were significantly associated with defining the fate of HCV infection in male and female patients. Multivariate logistic regression analysis for *HLA-DRB1* (rs2308802) and *HLA-DQB1 (-8471)* showed that these variations were responsible for IFN/RBV therapy response. Haplotype analysis showed that none of the haplotypes were associated with therapy response or outcome of HCV infection. Linkage disequilibrium analysis for the genotypes revealed that there was genetic linkage among various genotypes at identified SNPs in *HLA-DRB1* and *HLA-DQB1*gene.

1. Introduction and literature review

1.1. Background

Hepatitis C Virus (HCV) is a non-cytopathic hepato-trophic virus that can lead to a wide range of clinical outcomes like, chronic HCV infection to cirrhosis, hepatocellular carcinoma (HCC) and liver failure (McPhee *et al*., 2019). According to the recent estimates, over the last 15 years, the sero-prevalence of HCV has reached up to 2.8% surpassing 185 million HCV infections globally (Lanini *et al*., 2019). Hepatitis C virus is an enclosed, positive sense ssRNA virus having genome of 9.6 kb and diameter of 55-65 nm. HCV is a member of *Flaviviridae,* family and genus *Hepacivirus* (Ferri *et al*., 2015; Drexler *et al*., 2013).

HCV genotypes are divided into seven recognized types; genotype seven being the recently reported in 2015. Each genotype is further divided into subtypes. HCV genotypes vary from one another, maximum up to 30% of the genomic sequence variations, while the subtypes differ by 15-23%. HCV genotypes have broad range of distributions, particularly in genotype 5 (Tsukiyama *et al*., 2018; Webster *et al*., 2015; Murphy *et al*., 2015; Smith *et al*., 2014). Additionally, HCV also unravel a great level of molecular diversity and till now HCV genotypes are characterized into 67 provisional subtypes (Yang *et al*., 2018). The large open reading frame (ORF) of HCV consists of 3000 amino acids, which are responsible for the production of ten viral proteins. The encoded viral proteins are divided into structural and non-structural proteins. Core HCV proteins along with E1 and E2 are structural proteins and P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B are non-structural proteins (Scheel and Rice, 2013). Molecular weight of structural protein is approximately 21kDa and is comprised of total 191 amino acids. The core protein is involved in modulating cell proliferation, gene transcription and metabolism interference resulting in oxidative stress, which may further progress towards hepatocellular carcinoma. The HCV proteins: E1 and E2 facilitate viral entry into the cell, whereas P7 proteins are responsible for the formation of ion channels and assembly of the virus (Sohail *et al*., 2018). HCV NS3 protein is a serine protease, involved in the cleavage of viral polyprotein at various locations, while NS5B plays a significant part in the replication of virus (Scheel and Rice,

2013). Life cycle of HCV starts with entrance, into host cell, using a complex series of events which are initiated at hepatocyte entry and fusion. Initial attachment of the HCV E2, to its corresponding receptor is enabled by heparin sulfate proteoglycans, which are expressed on the surface of hepatocytes (Dubinsson *et al*., 2014; Molina *et al*., 2007).

1.2. Natural history of HCV infection

There exists a substantial evidence regarding expansion of HCV infection, as number of patients advancing to chronic infection that leads to cirrhosis and HCC (Tengiz *et al*., 2016). Onset of acute HCV infection is silent and hence it is very difficult to decide time course of this infection. Therefore, transition of acute HCV infection to chronic may remain a sub-clinical process. HCV chronic infection is mostly associated with end stage liver disease (ESLD), HCC and death. Natural chronicle of this viral infection cannot be elaborated completely; however it is characterized by slowly progressing disease preceded by liver inflammation, consequently resulting in cirrhosis in 10-20% of the patients, remaining infected over 20-30 years of time period. After the progression to cirrhosis, the chance for progressing to HCC is 1-5%, while the odds for developing liver decompensation rise to 3-6% (Rachel *et al*., 2014). After hepatic decomposition risk of mortality increases up to 15-30%. The main hurdles in controlling HCV infection remains higher burden of chronically infected patients, along with the absence of vaccine. The treatment of HCV infection relays on anti- viral therapies. Although an anti-viral therapy significantly reduces risk of HCC development, prevention of HCV infection still remains a challenge (Sohail *et al*., 2018).

1.2.1. Acute HCV infection

This type of infection is minor and usually remains under-recognized. Acute HCV infection is diagnosed poorly. Initial features of acute infection are flu like symptoms, which also occurs in case of other viral infections. Other symptoms related to the acute HCV infection may include abdominal discomfort, dark urine, anorexia, jaundice and loss of appetite. Chronic HCV infection develops from acute infection which is considered a key shift in the course of infection though mute progression into chronic infection remain difficult to characterize (Lanini *et al*., 2019; Grebely *et al*., 2014). Progression from an acute HCV infection to a chronic may depend on immunogenetic factors, gender, mode of acquisition, weak immune response and HIV co-infection. A strong interferon gamma and NK cellular

response are considered important for recovery from this stage of infection (Kokordelis *et al.*,2014). Recovery from acute infection may result into formation of histological lesions. However, the significance of these lesions in acute viral infection still remains less understood (Morzov *et al.*, 2018).

1.2.2. Chronic HCV infection

There is a key relationship between chronic infection, HCC and ESLD that results into death in various populations across the globe. In the time span of 20-30 years approximately 10-20% of the patients develop cirrhosis which is mediated by slow disease conversion followed by permanent liver cell damage. Studies regarding the reports suggest that the progression rates to cirrhosis can vary from 2% to 51% (Pol *et al.*, 2018). Lacks of proper and timely diagnosis remain a challenge in preventing disease progress.

1.2.3. Fibrosis progression

In the total time span of approximately 20 years 16% of the patients, who are chronically infected may develop cirrhosis. Likewise, fibrosis progression rates may accelerate with aging (Khatun and Ray, 2019). Patients infected with HCV for 30 years may have cirrhosis rates 36 to 41%. Factors like immune therapy, HIV co-infection, diabetes and alcohol consumption have significant effect on the fibrosis progression. Similarly age and male gender are also one of the main factors responsible for the fibrosis progression (Khatun and Ray, 2019).

1.3. HCV Life cycle

1.3.1. HCV entry and uncoating

Entry of HCV into hepatocytes is the major defining step, which defines HCV tropism for hepatic cells. After transportation of virion by blood into the liver, HCV is in direct interaction with baso-lateral surface of the hepatocytes that result in interaction of HCV with surface receptors. HCV receptors such as heparan sulfate proteoglycan syndecan-1or 4 are responsible for the attachment of the virus to hepatic cells (Lefevre *et al.*, 2014). The initial school of thought was that HCV glycoproteins binds to heparan sulfate proteoglycans or scavenger receptor B1 (SRB1), however, studies showed that ApoE, entangles contact of virus to the cell (Dao *et al.*, 2012). The LDL receptor (LDLR) is also known to have a role in HCV entry; however, it can lead to potential degradation of HCV particles (Albecka *et al.*, 2012). After HCV attachment, four cellular factors are necessary

for HCV entry, including tight junction proteins (CLDN1) (Evans *et al*., 2007), occludin (OCLN) (Ploss *et al*., 2009), SRB1 (Scarselli *et al*., 2002), and tetraspanin CD81 (Pileri *et al*., 1998). SRB1 can contribute in viral attachment using virus-associated lipoproteins; SRB1 has lipid transfer activity that modifies the lipoprotein of the virus leading to exposure to CD81 binding site on E2 glycoprotein. The exact mechanism is not known, however, after binding to SRB1 HCV virion is primed for CD81 binding which is a main contestant in HCV life cycle (Feneant *et al*., 2014). After attachment, the virus is internalized inside the cell using pH dependent and clathrin mediated endocytosis (Ploss *et al*., 2009). HCV is endocytosed using clathrin mediated processing and it is also reported that CD81- CLDN1 complex is adopted using clathrin-dynamin based approach (Farquhar *et al*., 2012). After virus is internalized it is conveyed to Rab5a along with stress fibers made of actin for the fusion to take place, subsequently viral RNA is unrestricted at the cytosol for the synthesis of viral protein and genome replication.

1.3.2. HCV RNA translation and replication

Translation and replication of HCV is carried out by various cellular and viral factors, as the HCV genome is comprised of large ORF enclosed by 5’ and 3’ UTRs. These UTRs are crucial for viral genome replication (Lohmann, 2013). Both host and HCV proteases are responsible for the processing of polyprotein into ten individual proteins such as core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, while NS5B is responsible in conversion of positive ssRNA into negative ssRNA (Ganta *et al*., 2019). Newly generated genomes are translated and can also serve as basis for RNA multiplication followed by virion assembly. A hepatocyte RNA named as miR-122 employs Argonaute 2 to the 5’ end of the HCV RNA that helps in stabilizing the RNA along with protection from exonucleases (Li *et al*., 2013).

Higher degree of reorganizations takes place in membrane to form a microenvironment known as membranous web. These are vesicles those have a diameter of 150 nm and are present when viral RNA is replicating (Romero *et al*., 2012). The NS5A protein can only induce (double membrane vesicles) DMVs. None of these mentioned proteins can alone induce membranous web structure. There are many factors/parameters that can affect viral replication and translation.

1.3.3. HCV assembly and release

The viral assembly starts with the release of the genome and contact with core viral protein forms virion capsid. The core protein is located on the outside of ER membrane. Assembly is initiated in cytosol, before maturation and the viral particle release occurs by transporting newly produced particles through crossways of ER periphery into hepatic cells (Vieyres *et al*., 2013; Merz *et al*., 2011). The HCV assembly is accomplished in two phases: the initiation phase in ER cytosol and the maturation phase in the luminal periphery of ER. Different studies have reported cytosolic storage organelles known as LDs and the VLDL assembly pathway, which take place in the lumen of ER and are one of the major contributors in the HCV virion assembly. The newly produced viral particle buds into the secretary pathway and are released as lipoprotein coated virus at the cell surface and is ready to infect new cells (Vieyres *et al*., 2013; Merz *et al*., 2011; Jones *et al*., 2010).

1.4. Global epidemiology of HCV infection

1.4.1. Frequency rates

Due to the poor management and surveillance system around the globe, it is hard to estimate HCV burden globally. HCV infection is prevalent in Central and East Asia along with Middle East and North Africa, as HCV accounts for more than 3.5% infections in these populations. European countries, Switzerland, Germany, Sweden, France, United Kingdom and Denmark have frequency rates of less than 1% (Lanin *et al*., 2019).

Seroprevalence rates in north and Latin America were found to be less than 1.5%. Studies suggest that people who migrated from Asia and Africa make up considerable proportion of HCV infections in Europe and US (Umer and Iqbal, 2016). Prevalence rates in Middle East and North Africa were accounted to be 15 million followed by Southeast Asia, 11 million, and 10 million in Western Europe. About 20-40% of these infected patients recover spontaneously (Thrift *et al*., 2016). HCV prevalence rates in Egypt are higher than 14%, which makes it a country with the highest prevalence rates (Guerra *et al*., 2012; Mohamoud *et al*., 2013). In Asia the frequency of HCV is higher than 2% and the rates may differ among different countries. In the current scenario, Mongolia has the maximum rates of prevalence, which is more than 10%, followed by Uzbekistan with prevalence rate around 6% of the total population (Lavanchy, 2011).

1.4.2. Prevalence rates in Pakistan

A number of important studies have advanced our understanding of the epidemiology of HCV in Pakistan over the last decade. In 2007- 2008, an inclusive nation-wide survey was carried out to estimate HBV/HCV prevalence. (Arshad and Ashfaq, 2017; Qureshi *et al.,* 2010; CDC, 2011). Five sentinel sites were established, one in each of the four provincial capitals and in Islamabad. Data from these sentimental sites may not be sufficient due to limited access to rural areas, having higher incidence rates for HCV infection. Moreover, this national surveillance was only limited to screening of healthy patients. Previous data regarding HCV prevalence in Pakistan has been reviewed thoroughly (Khaliq *et al.,* 2018; Waheed *et al*., 2009; Bostan *et al*., 2010; Umar *et al*., 2009). Numbers of studies were conducted regarding the status of HCV in Pakistani population. These reports have added the data related to remote areas such as Azad Kashmir (Abbas *et al*., 2009) and Baluchistan (Ahmad *et al*., 2012; Khan *et al*., 2013). These studies also show that there is much work to be done regarding analysis and integration of newer studies in comprehensive manner, particularly to manage and design important strategies to prevent further infections in Pakistani population.

1.5. HCV genotype prevalence

HCV genotype distribution across the globe shows that prevalence of genotype 1 was 46% that makes it the most common genotype across the world, whereas the prevalence of other HCV genotypes, such as G3, G2, G4, G6 and G5 were found to be 22%, 13%, 13%, 2%, 1% respectively. Untypable or mixed genotypes are reported to be present in 3% of the total prevalent genotypes (Yang *et al*., 2018). Among these, genotypes, G 1b was the most prevalent genotype accounting for 22% of the total infections. Significant differences among the prevalence of subtypes were observed in Pakistan. In Europe, Latin and North America, the prevalent genotype were G1 (62-71%) with G1b responsible for 50%, 39% and 26% of all the HCV infections respectively (Guerra *et al*., 2012; Gower *et al*., 2014). Due to higher prevalence rates of G4 in Egypt, the total prevalence of G4 in Middle East and North Africa was 71%. The dominant genotype in Asia is G3 39%, preceded by genotype 1, up to 36%, which is mainly determined by HCV distribution in Indian and Pakistani populations. G1b was also found responsible for the 25% of total infected

population. Australia was dominated by G1 53% proceeded by G3 39% (Gower *et al*., 2014).

1.6. Transmission and prevention

Transmission of HCV with blood transfusion or other associated utilities were responsible for HCV propagation in patients before HCV discovery and identification. HCV diagnosis using anti-HCV antibody detection had diminished the risk of HCV spread via transfusion in developed countries like USA and Europe. Transmission of HCV via blood transfusion is a major route that allows HCV to transmit from one person to other and still remains a problem in developing countries (Coppola *et al.,* 2019). Intravenous drug injections, reuse of injecting needles are main source of HCV transmission (Cornberg *et al*., 2011). Parental transmission from mother to a child occurs in about 2-8% of the mono-infected mothers, the transmission may also be more prevalent in HIV co-infected mothers (Prasad *et al*., 2013). HCV sexual transmission in monogamous heterosexual couples is very unlikely (terrault *et al*., 2013). HCV vertical transmission is another important challenge in terms of viral infection (Pybus *et al*., 2009). Human Immune deficiency Virus (HIV) and HCV both share a common route of transmission and consequently, co-infection with these viruses can occur (Prasad *et al*., 2013). This condition results in 20–30% of the total 34 million HIV infected patients, co-infected with HCV. Co-infection of HCV with HIV has become one of the major reasons for higher mortalities among infected patients (Martin *et al*., 2013; Operskalski, *et al*., 2011). Chronic infection with HCV is prevalent in patients co-infected with HIV (Operskalski, *et al*., 2011).

1.7. Diagnosis of HCV infection

Defining a proper HCV treatment and consultation for HCV infected patients, it is necessary to properly diagnose this infection. Most of the HCV affected patients do not show any symptoms, yet may have HCV viraemia albeit normal serum ALT levels. Therefore, molecular methods of diagnosis should be preferred over clinical (Galli *et al*., 2018). Viral separation and propagation of antigens and RNA are direct methods for detection whereas, the indirect methods include detection of antibodies like IgM for acute and IgG for chronic HCV infection. It is also known that anti-HCV IgM is not detected in 50%-93% of acutely infected patients and 50% to 70% of chronically infected patients (Sagnelli *et al*., 2003). However, currently HCV isolation and culturing is not well

developed and anti-HCV IgM is not dependable indicator for HCV detection. In routine diagnosis of HCV, viral RNA, core antigens and anti-HCV antibody are reliable parameters (Tillmann, 2014).

1.7.1. HCV detection using anti-HCV

Serological analysis includes detection antibodies and confirmatory tests.

1.7.1.1. Screening test: ELISA

Third generation ELISA kits are used in diagnosis of HCV infection, which is based on the detection of antigens. Detection rates for 3rd generation EIA are 98.9% with specificity of 100% in patients with chronic infection (Li and Lo, 2015). ELISA based methods are easy to use, relatively cheaper and can be done by automation to process higher numbers of samples and are usually recommended for HCV testing. However, are not recommended for infant testing, as they might show reactivity because of maternal antibodies (Mack *et al.*, 2012). Food and Drug Administration (FDA) USA recommended several antibody-based assays for the diagnosis of HCV infections. Fourth generation EIA is currently available and can be used for the detection of anti-HCV antibodies (Mack *et al.*, 2012).

1.7.1.2. Screening tests: Rapid point-of-care tests (POCTs)

The POCTs are rapid direct tests and are carried outside laboratory. They are used to diagnose anti-HCV antibodies by great specificity and sensitivity (Scalioni *et al.*, 2014). Such tests can detect antibodies in specimen like finger stick, serum, plasma, whole blood, oral fluid and venipuncture. These methods can be used in settings which lack facilities. These tests are inexpensive, easy and quick to produce results (Lee *et al.*, 2010).

1.7.1.3. Confirmatory tests

Recombinant immuno-blot assays (RIBA); RIBA are employed to approve anti-HCV presence in patients, who are already positive by EIA (Martin *et al.*, 1998). This assay can use recombinant proteins and peptides from NS4A, NS4B, NS5A, NS3 helicase and E2 hyper variable region. Due to higher specificity and sensitivity of EIAs, RIBA are no longer needed and are carried out in specified diagnostic setups (Alborino *et al.*, 2011). These serological tests can detect anti-HCV antibodies; however they are not able to differentiate between past and present HCV infections. Nucleic Acid testing can be used to detect viral genomic RNA and serves as a better confirmatory tool (Alborino *et al.*, 2011).

1.7.2. Detection of HCV RNA

Nucleic acid amplification can be of different types, such as signal amplification, target amplification and probe amplification. Presence of HCV RNA in the sera is one of the most reliable markers for HCV infection. RT-PCR is the most widely used method (Arshad et al., 2019; Mack *et al*., 2012; Saludes *et al*., 2014). WHO standardized the titer of HCV RNA in terms of International Units (IU) (Pawlotsky, 2002).

1.7.2.1. Qualitative Diagnosis of viral RNA

These assays are based on target amplification by reverse transcription (RT-PCR) or TMA. Reverse transcription of the viral RNA is carried out using the reverse transcriptase enzyme that results in the formation of complementary DNA (cDNA). These products are visualized using probes by hybridization method. HCV 5' UTR is employed in the qualitative detection HCV infection (Saludes *et al*., 2014).

1.7.2.2. Quantitative detection

FDA already approved many assays for the quantitative detection (Albertoni *et al*., 2014). The most suitable method for quantitative detection is RT-PCR. However, not all genotypes of HCV can be detected using RT-PCR (Chevaliez *et al*., 2013). Antibodies against HCV are developed after several weeks of infection; therefore a positive anti-body test can be used after RT-PCR diagnosis. It is important to note that level of HCV RNA does not reflect severity of liver damage neither does it confirm cirrhosis and HCC (Pawlotsky, 2010).

1.8. Associated risk factors

Different risk factors can be related to HCV infection which may increase chance of HCV infection. Use of syringes, intravenous drug use, blood transfusions, hospitalizations, barber visits, surgery and dentist visits are the major risk factors for HCV disease (Manns *et al*., 2017). Transmission of HCV via sexual activity is unlikely but still one of the main risk factors for the homosexual men (MSM). Studies have reported that there is a co-relation of HCV infection and poverty, drug use and having a higher number of sex partners (Zibbell *et al*., 2015).

1.9. Immune responses against HCV infection

As a hepatotrophic virus, the host range of HCV is restricted as it can only cause infection in human and chimpanzee Protection against viral RNA can be conferred by factors like

RIG and MDA5 (Hiet *et al*., 2015). Activated pattern recognition receptors (PRRs) can lead to interferon secretion, which are involved in the anti-viral host response that could lead to the HCV suppression (Shi *et al*., 2017). Productions of type I and type II interferon can cause stimulation of natural Killer cells (NK) that can kill infected hepatocytes. The hepatic APC consists of DCs and kupffer cells and are responsible for HCV destruction, and to deliver HCV peptides to CD4 T and CD8 T cells (Shata *et al*., 2006; Shi *et al*., 2017). Studies show HCV directed CD8T cells responses are key factors in determining the outcome of HCV infection. It has been known that vigorous CD8T cell response lead to self-limiting HCV infection and in contrast weak CD8T cells responses can result in chronic HCV infection (Lechner *et al*., 2000). The HLA-A3, B-27 and B-27 are known to be involved in clearance of virus that confirms CD8T cells have a significant role in defining the follow-up of acute HCV infection (Shin *et al*., 2016).

1.9.1. Innate immune responses

These responses are activated by the recognition of PAMPs by PRRs. A Pathway responsible for PRR-mediated signaling was reported recently (Brubaker *et al*., 2015). In addition, HCV can be recognized by NLRs, TLRs or RLRs depending upon type of cells encountered by HCV. In plasma-cytoid, dendritic cells (pDCs) TLR7 acts as PRR for HCV PAMP, responsible for the production of interferon in pDC, co-cultured with infected hepatocytes (Xu *et al*., 2016). Cell to cell contact is required between pDCs and hepatocytes in order to produce IFNs exosomes (Dreux *et al*., 2012).

After phagocytosis of HCV particles, in phagocytic cells, HCV genome is detected by TLR7 that results in stimulation of pro- IL- 1b and pro-IL-18 transcription (Negash *et al*., 2013). Along with phagocytic uptake of HCV particles are delivered to macrophages by exosomes. The common receptors for HCV are RLR like RIG-I and MDA. Despite their similarities in sequences and signaling adaptor MAVS, both act differently in recognizing PAMP (Kato *et al*., 2008). In contrast, RIG-I can detect 5' triphosphate present at the end of dsRNA along with the ssRNA hairpins. The C terminal domain of both MDA5 and RIG-I is involved in ligand selectivity (Wu *et al*., 2013).

The PRR was initially identified for binding HCV in the liver cells. When a 3'UTR or full length HCV genome gets transfected into hepatocytes, it was noted that they were bounded by RIG-I and induce production of interferon (Uzri and Gehrke, 2009). The mechanism of

RIG- I to induce IFN signaling was difficult to elaborate as the HCV encoded NS3- 4A serine protease was responsible for the cleavage of MAVS thus halting activation of IFN signaling (Xu *et al*., 2016). In order to solve this query Huh7 cell lines were generated which expressed the mutant MAVS. The mutated MAVS was NS3-4A cleavage resistant and allowed interferon production after HCV re-infection. Study revealed that MDA5 has a vital function rather than RIG-I (Cao *et al*., 2015). Studies have also shown that both type II interferon and type I can inhibit HCV replication with cure rate of 80% of the total 49 chronically infected patients receiving therapy for HCV. The precise mechanism by which IFN inhibits viral replication is still unknown (Xiang *et al*., 2015). A number of interferon stimulating genes (ISGs) are induced during HCV infection and have been known to show their inhibitory effects on HCV, apart from these, some additional innate immunity factors are also responsible for controlling HCV infection (Chen *et al*., 2014).

Inhibition of HCV replication is mediated by a vital component known as cholesterol-25-hydroxylase (CH25H) (Anggakusuma *et al*., 2015). The 25HC is involved in immune regulation and is critical in lipid biosynthesis; in addition, it can also perform a vital role in inflammatory signaling, immune cell migration and antibody production (Simon, 2014). HCV infection can lead to the expression of CH25H in hepatocytes (Chen *et al*., 2014; Anggakusuma *et al*., 2015). CH25H can have a direct anti-viral activity against many enveloped and non-enveloped viruses. The exact mechanism involving CH25H and virus interactions is not completely understood, although CH25H halts virus replication by delaying the membrane fusion (Liu *et al*., 2013).

1.9.2. Adaptive immune responses

Disease outcome in HCV infection is directed by genetic factors encoding immune response against HCV. Chimpanzee models have shown that CD4 and CD8T cell interactions are necessary for protection against infections. The level of both CD4 and CD8T cells seem to be the driving force in progress towards spontaneous recovery against viral infection (Prieto *et al*., 2017). Progression towards chronic infection is mediated by the sequestering of CD8T cells into liver and non-availability in peripheral circulation. During acute HCV infection, CD4T cells can be detected but when there is a progression towards chronic infection, they tend to diminish (Prieto *et al*., 2017). In the mean while, in chronic HCV infection, HCV specific CD4T and CD8T cells can be elaborated by

manifestation of some indicators which include T cell immunoglobulin and mucin domain (Tim-) 3 and cytotoxic T lymphocyte-associated antigen-(CTLA) 4. The production of key cytokines like interferon (IFN)-γ, degranulation marker CD107a and tumor necrosis factor alpha, can further prevent differentiation of CD127-positive memory T cells (McKinney *et al*., 2016). Studies have shown that this phenotype cannot be regressed using different checkpoints inhibitors specifically directed against PD-1 (Bengsch *et al*., 2016). Continuous disclosure to higher loads of virus can result in diminished coding of PD-1. In addition, CD25, CD4 and Treg cells are known to suppress immune responses in HCV infection (Franzese *et al*., 2005).

The Treg cells are able to suppress CD8T cell responses even after spontaneous resolution of infection (Kondo *et al*., 2006). Treg cells are also known to suppress the IFNγ secretion by HCV specific CD8T cells, which indicates the function of Treg cells in defining the clinical outcome of HCV infection. This property is known to be conserved in those cells which exist in hepatocytes of chronically infected patients (Sugimoto *et al*., 2003), additionally these cells have higher expression levels of CD69 in absence or presence of αEβ7 integrin (CD103) respectively. This cell population consists of mixed numbers of γδ T cells and mucosal-associated invariant T (MAIT) cells. In HBV infected patients number of Trm CD69 and CD103 is amplified presenting 20% of the total memory CD8T cells. This population of cells can express higher PD-1 levels (Pallett *et al*., 2016; Prieto *et al*., 2017).

1.9.3. HCV immune evasion strategies

Recognition of HCV RNA by RIG-I or MDA5 presents a continuous danger to HCV (Du *et al*., 2016). In order to counteract interferon regulatory factor (IRF3); activation initiated by RIG-I/MDA5, HCV protease NS3/4a performs a dual role. Along with cleavage of viral proteins, these proteins are responsible for the cleavage of adapter proteins known as MAVS, which is a key player in RIG-I or MDA5 mediated signal transduction (Li *et al*., 2005). Viral proteases NS3/4a can also cleave TRIF which is mediator of TLR3 and is responsible for identification of dsRNA in endosomal compartments and hence prevents type I and III IFN production (Okamoto *et al*., 2014). NS3 is also known to associate with bind tank binding kinase (TBK) 1 and thus stopping IFN induction. To antagonize the paracrine IFN, the core protein of HCV is able to circumvent JAK/STAT signaling cascade

by openly binding to STAT1 and pNS4b preventing its phosphorylation and activation (Yao *et al*., 2005). The NS4b HCV protein can also inhibit type I interferon induction by targeting the TBK1 activation (Ding *et al*., 2013). Moreover, hepatitis C virus owns an elaborated structure for counteracting any host responses by sensing and avoiding detection. The whole HCV replication complex is enclosed in a membranous mesh and helps as a shield for the HCV particle against host defenses. This double membranous web can prevent the entry of PRR and blocks signaling (Neufeldt *et al*., 2016).

Other viral proteins like NS5a can reduce ISG expression by associating with 2, 5 OAS. The viral protein NS5a can also bind MyD88 and can block TLR mediated immune response along with PKR (Han *et al*., 2002; Tsutsumi et al., 2017). NS5b viral protein has polymerase activity but lacks the proofreading ability and can result in introduction of mutation in viral genome, that consequently are responsible for the development of viral quasispecies, a replication competent virus (Echeverria *et al*., 2015). Similarly changes in major histocompatibility complex I and II epitopes also result in loss of immune system ability to react (Sagnelli *et al*., 2004). HCV can also avoid the humoral immune response by using distinct suppressing cascades. HCV virion can also egress using cross-tight junction and infecting the nearby cells. This eliminates the risk of antibody encounters and neutralization. The exact mechanisms are unknown; however cellular lipids and glycosylation patterns shifting were suggested to be involved in this whole process (Gondar *et al*., 2015; Pantua *et al*., 2013).

1.10. HCV infection Treatment

The main goal of HCV treatment is to cure HCV infection, preventing disease progression and attaining sustained virological response (SVR) in the infected patients. SVR is defined as HCV RNA levels < 15I U/mL in the patient serum after completion of 12-24 weeks of antiviral therapy (Meer *et al*., 2012). Classical therapy for HCV included interferon and ribavirin for 48 weeks. For almost a decade pegylated interferon in combination with ribavirin was the basis for treating HCV infections (Poenisch *et al*., 2010). It has been reported that IFN-α therapy is known to rescue poly-functional CTL which are HCV targeted and can persist to one year even after discontinuation of anti-HCV therapy (Badr *et al*., 2008). Due to lower SVR, associated side effects and longer therapy time, alternative therapeutic possibilities were searched (Poenisch *et al*., 2010). Investigations regarding life

cycle of HCV has helped in understanding and progress in drug development such as protease inhibitors (PI) a drug that targets NS3/4A protease, and NS5B RNA-dependent RNA polymerase (Poenisch *et al*., 2010; Scheel and Rice, 2013). In 2011, the first DAAs tested were boeceprevir and telaprevir against HCV genotype 1 that resulted in the increased SVR. The viral protease inhibitors such as simeprevir (SIM) recommended for genotype 1 infected patients showed high effectiveness. Regimens for HCV genotypes 2 and 3 are also used and are comprised of RBV and SOF (McHutchison *et al*., 2009; Powdrill *et al*., 2010). HCV has high replication rate of 10^{12} copies per day. The viral replication protein known as NS5B, is a RNA-dependent RNA polymerase (RdRp) that lacks the proof reading activity with higher error rates and resulting in viral quasispecies production.

These viral quasispecies have less ability to replicate in comparison to wild type virus; however, when there is selection pressure due to anti-HCV therapy, number of wild type viruses' decreases and these viral quasispecies gain replication fitness. Viral genome mutations lead to the modifications of structures and sites on which DAAs act, hence resulting in the resistance to DAAs. There is no available anti-HCV vaccine that prevents HCV infection. HCV vaccine development is hindered by many factors including HCV immunobiology and host immunogenetics. Anti-HCV therapy might also need a multidrug approach to treat HCV infections (Sentandreu *et al*.,2008; Jazwinski *et al*., 2011). *In vitro* cell culture system development for HCV had greatly contributed to escalation in desiging anti-HCV agents and some of the newly discovered agents are already in clinical trials (Bartenschlager *et al*., 2013).

1.11. Host Genetic factors associated with HCV infection

There are various agents involved in the establishment of viral infection, disease establishment and progression towards further HCV related disease outcomes. The most important factors in this regard are HCV genotype along with the host genetic background (Ellwanger *et al*., 2017). The immunogenetic profile of the host decides how an infected patient deals with HCV infection. In addition to that some environmental factors are also involved which reflect the outcome of the HCV infection (Ellwanger *et al*., 2017). Studies regarding the immunogenetic clearance of HCV in infected patients might be influenced by type I interferons. Single nucleotide polymorphisms in these genes along with those of

innate and acquired immune system can define the molecular signatures for understanding HCV immunology (Buchanan *et al*., 2015). Genetic polymorphism in the genes of immune system is very important in context of HCV disease outcomes. Here in we discussed the different immune system genes and possible part in defining HCV fate related outcomes. Epidemiological factors can also play an important role in HCV infection outcome; factors like human immunodeficiency virus (HIV) co-infection, gender, age and ethnicity can determine the HCV related disease outcomes (Matsuura and Tanaka, 2015).

There is clear difference in the individual ability to define the viral infection outcome and it has been noted that it is not the virus alone responsible for HCV infection or therapy, as the immune system has key role to play, even if the population is epidemiologically homogeneous (Sun *et al*., 2015). Similarly, once chronic HCV infection is established the immune system of the host is responsible for the liver damage as HCV is non-cytopathic for the hepatocytes. One of the major hurdles in understanding HCV and immune responses interplay is the lack of animal models or culture systems except humans and chimpanzee. HCV infection can lead to activation of interferon genes which play key role in responses to viral infection (Heim *et al*., 2014; Matsuura and Tanaka, 2015). Similarly polymorphism in the interferon-induced genes, like: RNA dependent protein kinase (PKR) 2-5-oligoadenylate synthetase (OAS- 1) and myxovirus resistance genes can also play a key role in HCV infection outcome (Knapp *et al*., 2003).

TGF-beta 1 gene polymorphism also have been reported to influence HCV infection outcomes and HCV fibrogenesis (Westbrook and Dusheiko, 2014). Genome wide association studies (GWAS) have led to the identification of polymorphism in the interferon-I3, I4, also known as interleukin -28B (IL-28B). IL-28B is present on 19q13.13 and was found to be one of the main determinants of response to anti-viral therapy in shape of pegylated interferon (PEG-IFN) along with ribavirin (Prokunina *et al*., 2013).

1.11.1. The *HLA* system

The *HLA* (Human Leukocyte Antigen) cluster located on chromosome 6p21 comprises many genes involved in immunity like *HLA* and tumor necrosis factor alpha (Crux *et al*., 2017). After its discovery in 1958 (Thorsby, 2009), Human leukocyte antigen (*HLA*) is known to be one of the most important host genetic factors associated with infectious disease susceptibility. *HLA* plays an important role in fine-tuning immune pathways. Host

genetic factors are capable of differentiation among "self" and "non-self" antigens and are distributed into three classes class I, II and III (Hudson *et al*., 2016). *HLA*s are one of the most polymorphic genes studied in human genome, which results in the coding of large number of functional peptides (Robinson *et al*., 2015).

The increased level of polymorphism in the MHC region can be one of the tools to combat a large number of microorganisms; however it may also lead to certain complications like surging the possibility of autoimmune diseases (Trowsdale *et al*., 2013). Investigation of *HLA* allelic polymorphism is crucial to understand potential relationship between HCV infection and *HLA* association, like *HLA- B27* that plays its role on the generation of CD8T cell responses, which are specific against HCV and directed against a single viral epitope (Shin *et al*., 2016). Immune system cells CD94+T cells are triggered by attachment of T cell receptors (TCR) to the peptides, which are presented by Human Leukocyte Antigen (*HLA*s) molecules like *HLA-DQ, DR* and *DP*. Alpha and beta chains mold *HLA* class II peptide groove. The differences in peptide binding loci are due to different type of alleles in each individual (Yue *et al.,* 2015). *HLA* class protein is divided into 3 types known as *HLA*-class I, II and III. Proteins of class I are explicated on all nucleated cells in varying degrees and is composed of one heavy chain and has trans-membrane and three extracellular domain.

The domains are α1-3 and beta-2-microglubin, which is comprised of light chain. *HLA* class I functions by the presentation of antigens and peptides from inside the cell to TCR, which are present on CD8T cells which lead to immune system mechanisms and destruction of infected cells. This *HLA* interaction with KIR present on NK cell surface leads to inhibition of NK activity. (Dyer *et al.,* 2013; Li and Raghavan, 2010).The expression *HLA* class II proteins is only constrained to antigen presenting cells, their expression varies with type of cells and inflammatory condition or infection. Class II *HLA* molecules are comprised of α and β chains, which are expressed by the MHC cluster. Each chain has two extracellular domains and the distal domain act as a binding groove for peptides that are self or non-self peptides.

After binding of the *HLA* class II proteins with the presented peptides, proteins interact with CD4T helper cells for the presentation to TCR (Klein and Stato, 2014). In human genome, MHC genes are considered to be one of the highly polymorphic genes. According

to immuno polymorphic database (IPD), the total number of alleles in class A is 6,192 and 7,431 in class class B and 6,067 in class C. Similarly the number of alleles in DRB1, DQB1 and DPB1 are reported to be 3,391, 1,857 and 1584 respectively (IPD-IMGT/HLA, 2017). This extreme allelic polymorphism can be an evolved mechanism by the human immune system in order to cope with large number of different antigens and peptides (Nicole and Elahi, 2017). A slight difference in amino acid sequence results in change of *HLA* molecules binding cleft for peptides. It is known that the α1 and α2 domains of *HLA*-class I and α1, β1 domains of *HLA*-class II are responsible for the higher grade of polymorphism. In addition, the membrane domain α3 which is proximal domain in class I and α 2 domain in the class II along with cytoplasmic domains and transmembrane domains have limited polymorphism (Wagner *et al.,* 2012).

HLA association with HBV and HCV infections has been a hot topic regarding disease severity, vaccine response and interferon therapy and has been intensively investigated across global population (Singh *et al.,* 2007). Studies have indicated that *HLA-DQB1*-0301 and *HLA-DRB1* are responsible for the clearance of HCV infection (Hong *et al.,* 2005). *HLA-DRB1* member of class II beta chain paralogs family and is constituted of α and β chain both anchored in the membrane and play role in peptides presentation to immune cells.

HLA-DRB1, β chain encoded by 6 exons are coded on macrophages, dendritic cells and B lymphocytes having size approximately of 26-28 kDa. Exon 1 encode leader peptide, extracellular domains are coded by exon 2, 3, transmembrane domain by exon 4 and cytoplasmic tail by exon 5. Specifications to bind to diverse peptides are result of all the polymorphisms restrained by *DRB1* complex β chain. Linking and typing of Hundreds of *DRB1* alleles SNP is usually carried out for kidney transplantation and bone marrow analysis (NCBI, 2008). The *HLA* class II β chain paralogs family also included *HLA-DQB1*, having a heterodimer containing α and β chain both anchored in membrane. *HLA-DQB1* plays vital role in presenting antigens to the immune cells. Both, alpha and beta chains of *HLA-DQB1* molecule possess the polymorphism required to increase antigen-binding specificities (NCBI, 2011). Many studies identified the association of class II alleles with HCV disease outcome. In many populations across the globe, the *HLA-DRB1**1101 and *DQB1**0301 were reported to be associated with clearance of HCV

infection (Scotio *et al.,* 2003; Harris *et al.,* 2008). In Irish and USA cohorts *DRB1**01 and *DQB1**0501 alleles were also found to be associated with HCV clearance (McKiernan *et al.,* 2004; Wang *et al.,* 2009).

In mixed European, Polish, Irish and Thai populations *HLA- DRB1**0701 was found to be associated with viral persistence (Wawrzynowicz*et al.,* 2000) while opposite relationship of *HLA-DRB1**0301 had already been reported in European and Asian populations (Yoon *et al.,* 2005). These results show that the allelic distribution and its association with HCV disease outcome are dependent on the ethnicity of the analyzed population. A genome wide scan analysis conducted in European and African patients by Duggal et al 2013 reported that the *HLA-DQB1* allele rs4273729 and *IFNL4* allele rs12979860 were independently associated with spontaneous clearance (Duggal *et al*., 2013). Similarly another GWAS conducted in Japanese cohort consisting of 481 CHC patients and 2,963 healthy patients. They reported that the intronic region SNP rs9275572 was related to chronic viral disease. They also performed genotyping of *HLA-DQA1* and *HLA-DQB1* alleles and they found that these alleles were associated with protection against chronic viral infection (Miki *et al*., 2013).

Matusura and Tanaka performed genotyping of *HLA- DRB1* and *HLA-DQB1* in a GWAS that reported that rs8099917 non-TT allelic homozygosity was related to stubborn viral infection. Similarly they also reported *HLA-DQB1* 03:03 were allied to spontaneous clearance in patients with HCV (Matsuura *et al*., 2016). Studies have reported that during chronic HCV infection the serum levels of the classical Ia molecules are elevated as compared to the controls. These levels are expected to further increase during IFN treatment and return to normal levels after the cessation or end of therapy (Murdaca *et al*., 2017; Puppo *et al*., 2000).

Determination of levels of these soluble markers during IFN treatment could be a useful predictor for the HCV infection as a study reported that the levels of s*HLA*-G were higher in the serum of those patients who were positive for chronic HCV disease (Weng*et al*., 2011). Polymorphism in these classical and non-classical genes can really determine the fate of HCV disease outcomes in infected patients. Due to its major role in immune stabilization single nucleotide polymorphisms in these gene can serve as predictor for anti-viral therapy responses (Murdaca*et al*., 2017). Of all these classical and non-

classical molecules *HLA*-B is the most widely investigated in the context of HCV infection. Alleles of *HLA* class I specifically *HLA*-B are known to have protective effects in Irish women cohort who were believed to be infected by HCV genotype 1 (McKiernan *et al*., 2004).

In contrast, these associations were not found to be linked with HCV disease in German cohort consisted of genotype 1b infected women contaminated via anti-rhesus D immunoglobulin (Ziegler *et al*., 2013). Study conducted by Neumann-Haefelin et al. showed that HCV genotype 3a peptides are not recognized CD8T cells in developed cell lines and hence the consequences of *HLA* B*27 are restricted to genotype 1 only (Haefelin*et al*., 2010). These associations were also confirmed in US population, however they were not found in Easter German patients (Haefelin*et al*., 2010).

Genotype specific variations in the *HLA*-B*57 restricted epitopes against both HCV genotype 1 and 2 can result in increased recognition of the viral epitopes along with the subsequent clearance of the virus among the patients who are carrying *HLA*-B*57 genotype (Kim *et al*., 2011). Proteosomal cleavage is responsible for the generation of viral epitopes which are then transported to endoplasmic reticulum and are loaded to *HLA* class I. Along with *HLA*- class I genes, a non-synonymous mutation in Tapasin gene was also associated with protection against HCV infection. This non-synonymous substitution was present in the exon 4 of the Tapasin gene. This protection was found to be present in European but not in US populations (Ashraf *et al*., 2013). A study reported strong association of *HLA* class I allele A*03 with the viral clearance however, this effect is not replicated in independent patients with HCV. It is possible that the *HLA*-A *03 NS3 1080–1088epitope is immunodominant in Irish cohort and hence associated with protective effect (Fitzmaurice *et al*., 2015; Nitschke*et al*., 2016). *HLA* class C alleles are also known to be related to HCV clearance and perseverance. These *HLA-C* *01 and *HLA-C* *04, which are constrained HCV definite *CD8T* cell ligands and were described very recently. The part of *HLA*-C single nucleotide variations may interrelate to NK cells receptors, they might have an important role in HCV related outcomes by communicating with NK cells rather than halting of HCV directed *CD8+ T*-cell signaling (Nitschke*et al*., 2016).

1.11.2. Role of polymorphism in Cytokine genes and HCV infection

Although cellular interactions are responsible for immune responses, it's the cytokines microenvironment can play a very important role in immune system interactions (Buchanan *et al*., 2015). The Th1 related cytokines which includes IFN γ, tissue necrosis factor (*TNF*)-α and IL-12 can encourage robust cytotoxic T-cell cascade, while (TGF)-β, IL- 10, IL-13 provides the adjusting environment (Buchanan *et al*., 2015). Polymorphism in the cytokines genes can affect consequence of viral infection.

1.11.2.1. Tumor necrosis factor alpha and HCV infection

Tumor necrosis factor alpha (*TNF-α*) is a pleiotropic cytokine and was identified in 1975. It is an endotoxin-tempted glycoprotein that can lead to the hemorrhagic necrosis of sarcomas transplanted into mice (Carswell *et al.,* 1975). Human *TNF*α cloning was carried out in 1985 for the first time and it was shown that cloned *TNF* molecules are able to cause hemorrhagic necrosis of sarcomas in mice models. *TNF-α* is known to have important role in wide range of inflammatory, malignant and infectious conditions (Pennica*et al.,* 1985).

TNF is produced by T lymphocytes and active macrophages as a 26kDa protein, which is encoded on plasma membrane. The expressed protein is cleaved by matrix metalloproteinase which releases 17 kDa soluble type of *TNF*. The biological activities of both soluble form and membrane bound are distinct and these molecules are in trimeric forms. The *TNF*-alpha converting enzyme known as TACE mediates the release of *TNF* molecules from the cell periphery (Black *et al.,* 1997). TACE can also result in release of proteins which are associated with the cell membrane such as receptors for *TNF* that can neutralize the effects of *TNF* (Wang *et al.,* 2003). TACE can act as pro-inflammatory or anti-inflammatory depending upon the effectors like endothelial cells or macrophage. Normally *TNF* is not detected in healthy people but in case of inflammatory and infectious conditions elevated levels can be detected. Serum levels of *TNF* can be related to severity of infections (Rego*et al.,* 2014).

The main sources of *TNF* production are monocytes and macrophages; however a number of different cells can result in production of *TNF* such as NK cells, mast cells, B and T lymphocytes and cardiac muscles cells (Bradley, 2008). Signal transduction pathways for *TNF* are not known, but the transcriptional regulation of NF-κB is the most important factor for the signal transduction. Studies have shown 221 molecular associations which results

in the *NF-κB* pathway modulation (Bouwmeester, *et al.,* 2004). Responses by *TNF* are initiated by binding of *TNF* to its receptors *TNFR*1 also known as *TNFRSF1A*, *CD120*a, p55 and *TNFR*2. These receptors are regulated on different cell types depending upon the condition of cell (Wang *et al.,* 2013).

The *TNF* receptors are diverse in function as they can bind both identical and unrelated molecules. Mice model studies involving knockout mice have shown that *TNFR*1 is responsible for the function like cell death and pro-inflammatory pathways which are responsible for tissue injury. *TNFR*2 signaling is not well characterized however, it may play role in tissue repair and angiogenesis (Jones *et al.,* 1999). The *TRADD* is responsible for the downstream signaling of *TNF* along with other factors like Silencer of Death domain (SODD), death domains (DD), and pre-ligand assembly domains (PLADs) (Chan *et al.,* 2000; Jiang *et al.,* 1999).

Subsequent signaling includes activation of Mitogen activated protein kinase (*MAPK3K*) along with other factors which subsequently lead to interaction with *NF-κB* and enters nucleus and results in the initiation of gene transcription (Devin *et al.,* 2001). Single nucleotide polymorphism in the *TNF*-alpha promoter region can play an important role in deciding the outcomes of HCV related disease. HCV infections are investigated across the global population, as discussed below. The gene for *TNF*-alpha protein is located on chromosome 6 on p21.33 position. It is located between the alpha and beta lymphotoxin loci closer to *HLA*-B. It is proposed that *TNF* can play an important part in HCV disease response (Dogra *et al.,* 2011). *TNF-α* is one of the key mediator of inflammatory responses and critical for host defense against various pathogens. *TNF*- alpha regulate both innate immunity and inflammatory responses and regulation of this gene in pathological infections poses a challenge.

1.11.2.1.1. *TNF- α* gene promoter

The regulation of the gene is mainly controlled by promoter of this gene (Grandi *et al.,* 2014). Polymorphism in gene regulating region of *TNF* alpha can affect the overall expression and secretion of cytokine. Studies have shown that polymorphism in *TNF-α* genes affects the process of inflammation, autoimmune response, infectious diseases and allograft rejection (Yen *et al.,* 2008, Schneider *et al.,* 2004). SNP present in the respective genes that are responsible for the both pro and anti- inflammatory effects may help in the

antiviral therapy responses. *TNF-α* and *IL-10* performs a major role in regulation of cellular and immune responses to HCV infection (Dogra *et al.,* 2011).

TNF- α is an important and potent pro-inflammatory cytokine and also acts as antagonist to *IL-10*. Well-characterized SNPs at different positions like -863, -308 and -238 have been shown to influence the expression of *TNF*- α expression (Cheong *et al.,* 2006). These SNPs have been already reported to be associated with the pathogenicity of HCV acute and chronic infection along with viral persistence and response to IFN-α therapy (Amini*et al.,* 2012; Thio, 2008).

TNF-α gene polymorphism has shown to contain many binding sites for the factors, which are involved in the gene transcription, the presence of these single nucleotide variations in the regulatory region of *TNF*-alpha gene might influence the regulation of transcription of *TNF* gene. Several studies have shown that *TNF*A-238G/A, *TNF*A-308G/A and*TNF*A-863C/A may be associated with hepatocellular carcinoma in Asian patients (Yang *et al.,* 2012). Similarly another study reported that there is no significant association was found between tumor necrosis factor alpha polymorphism -238 G/A and -308 G/A with the anti-HCV treatment response in Brazilian patients (Grandi *et al.,* 2014).

There are a large number of studies that reported different outcomes of HCV infection and *TNF* SNPs, like *TNF*-308 SNP was related with hepatic fibrosis in HCV infected Taiwanese population (Jeng*et al*., 2007). Barret et al (2003), reported no association of *TNF* alpha with the outcomes of HCV infection. SNP at -238 and -308 were studied by Hohler et al and they reported that -238 was associated with HCV outcomes while no such relations were found with -308 (Hohler *et al*., 1998). A study conducted in patients who were receiving anti-HCV therapy was carried out in order to determine the connotation of *TNF-α* -238 (G/A) promoter variations versus antiviral therapy outcomes. They found that there was a higher trend of allele of A allele in HCV infected patients, however, they did not find a significant association of *TNF*α -238 (G/A) polymorphism in both controls and patients (Hugo *et al*., 2003).

Similarly, Yee et al found that the *TNF* alpha gene promoter SNPs at -238 A and -308 A were related to increased chances for advanced liver cirrhosis respectively (Yee*et al*., 2000). Analysis performed in Mexican patients revealed that there was no association between *TNF* alpha promoter gene polymorphisms at regions -238(G/A), -308(G/A) and

chronic HCV infection in 48 patients who were chronically infected with HCV (Murillo *et al*., 2010). A single cohort analysis of patients comprised of co-infected patients with HCV and HIV for the -238 (G/A) and -308 (G/A) were analyzed. It was reported that *TNF* alpha -238(G/A) variations a major part to play in cirrhosis progression along with previously defined factors like age, alcohol consumption gender and immunodepression (Corchado*et al*., 2013).

Another study reported that G/A transition at the promoter region of *TNF* alpha gene might be able to predict the Interferon/ribavirin therapy response in patients who received anti-HCV therapy (Dai et al., 2006). Similarly another analysis reported that *TNF* alpha promoter gene polymorphism at -308 region is associated with fibrosis severity and quantitative load in infected patients (Dai *et al*., 2006). A meta-analysis performed by Zhou et al in Chinese patients to determine the relationship of *TNF*-238 single nucleotide polymorphism with cancer. It was revealed no noteworthy relationship of *TNF*-238G/A variation and risk for cancer development (Zhou *et al*., 2011).

1.12. Concluding remarks

Globally, many studies have focused on the association of host immune-genetic factors and their outcomes in HCV infection and anti-HCV therapeutics. Such studies can provide insights for the future endeavors for developing anti-HCV vaccines and therapies. Pakistan has a multi ethnic population comprised of Pashtuns, Punjabis, Sindhi, Balochi and Baltistani. Seroprevalence rates of HCV in Pakistan is around 6.8% (Umer *et al*.,2016).

HCV genotype 3a is common in Pakistani population. With such high prevalence rates of HCV in Pakistan, it is necessary to analyze, the host genetic backgrounds of Pakistani population and their association with different outcomes of HCV related disease and anti-HCV therapy outcomes such as peg-interferon/ribavirin. This analysis was aimed to investigate association of host immunogenic factors and their association with the outcomes of HCV infection in group of analyzed patients. The selected patient's clinical and demographic information were obtained and compared with the patient genotype for the analyzed genes. The candidate genes selected for this investigation were: *TNF-α*, *HLA-DRB1* and *HLA-DQB1*. Tumor necrosis factor alpha gene was analyzed for three SNPs in the regulatory region of *TNF-α* gene and HCV infection outcome was determined in the studied group of patients. Similarly seven SNPs were analyzed in the *HLA-DRB1* region for the studied groups of patients along with ten SNPs in the *HLA-DQB1* region. SNPs, patient's genotypes along with haplotypes on the corresponding chromosomes were analyzed for the studied patients.

2.1. Hypothesis

Host immune response is vital for defining the outcome of anti-HCV therapy. Single nucleotide polymorphism in the immune system genes like *TNF-α*, *HLA-DRB1* and *HLA-DQB1* can influence response to anti-HCV therapy and ultimately outcome of HCV infection.

2.2. Aims and Objectives

2.2.1. Aim of this study

To decipher role of genetic variation in *TNF-α, HLA-DRB1, DQB1* genes in spontaneous recovery and response to interferon/ribavirin therapy.

2.2.2. Objectives

1. To determine demographic information of the patients along with therapy and disease history.

2. To scrutinize signs and symptoms and associated risk factors for HCV infection.

3. To determine the viral load of infected patients and disease outcomes.

4. To determine the rate of responsiveness to interferon therapy in HCV infected patients.

5. To study association of gender based allelic and genotype variations and their role in HCV infection along anti-HCV therapy outcomes.

6. To generate genotypes and haplotypes of population under study and to compare these for anti-HCV therapy response and disease outcome.

7. To establish linkage disequilibrium analysis for the above set of genes for the group of patients under study.

8. To analyze the association of *TNF-α* gene polymorphisms and its association with HCV disease outcome and therapy outcome in Pakistani population.

9. To relate the association of *HLA-DRB1* and *DQB1* single nucleotide polymorphisms with HCV infection and therapy outcomes in Pakistani population.

3. Study design

3.1. Ethical approval for the study

Ethical approval for this study was granted by the Institutional Review Board (IRB) of the Department of Microbiology, Quaid-i-Azam University, Islamabad (#Micro/2016/1027). Necessary guidelines were followed in the light of Helsinki Declaration.

3.2. Informed Consent from Patients

Consent from all patients/guardian/parents included in this study was obtained. A proforma containing patient's information e.g. name, gender, age, symptoms, risk factors and information regarding anti-HCV therapy was collected by direct interview of the patients. Additional details were also obtained regarding the status of infection where possible.

3.3. Sampling

For the current study a total of 245 blood samples were collected from the patients visiting Nuclear Medicine Oncology and Radiotherapy Institute (NORI) hospital Islamabad from May to September 2015. The sample size was calculated according to the following formula. $S = Z^2 * p * (1-p) / M^2$. NORI is a public sector cancer hospital located in sector G-8 Islamabad, Pakistan. Samples of all the patients were collected in EDTA tubes and stored at -20˚C. A trained phlebotomist collected two separate blood samples from each patient. One of the two blood samples was used for the viral RNA detection and analysis, whereas, the other sample was used for the isolation of human DNA and analysis of host genetic factors. Standard precautionary measures were followed during collection of blood sample from infected patients. Student himself processed all the experiments and DNA extractions from human samples which were used for DNA sequencing by automated DNA sequencer at the facility of Macrogen, New York.

3.4. Sample tagging and storage

All the collected blood samples were properly tagged according to the patient's ID number allotted to each patient by the sample collection center. The collected blood samples were then tagged immediately and transferred to -20˚C to avoid any damage and deterioration to the samples.

3.5. Data entry and Patient Categories

After sampling and PCR analysis, data of the patients was entered in excel work sheet. Patients analyzed in this study were categorized mainly into two groups, patients that were receiving interferon/ribavirin for the past 24 weeks and patients that were not receiving any therapy. Therapy group comprised of patients receiving antiviral combination therapy with Pegylated IFN (180lg) once a week and ribavirin (800–1200 mg) according to the patient's body mass index. The standard recommended therapy for HCV treatment is comprised of a subcutaneous intake of 3 MU/ml of Interferon replicating three times in a week plus oral intake of 10mg/day/kg of the body weight of ribavirin continued for period of 24 weeks (Shiffman *et al*., 2011; Tsertsvadze *et al*., 2016). Therapy group was further divided into two groups; responsive (R) to interferon/ribavirin and non-responsive (NR) to interferon/ribavirin therapy group. These groups were defined on the basis of presence or absence of viral RNA. Patients that had detectable HCV RNA after completing IFN/RBV therapy were categorized as non-responsive (NR), whereas those patients that did not have any detectable viral RNA after completion of IFN/RBV therapy were categorized into responsive (R) group. The non-therapy group was further divided into two types that included, chronically infected (CI) and spontaneously recovered (SR) group. Chronically Infected group was defined by the presence of viral RNA for at least six months with presence or absence of clinical symptoms (Westbrook and Dusheiko, 2016). Spontaneously recovered patients were defined by the fact that these patients were positive for anti-HCV antibody, however they were negative for HCV RNA via real time PCR and studies have suggested that approximately 25% of the patients usually clear the virus, whereas the remaining 75% progress towards chronic infection (Gunal *et al*., 2017; Danilau *et al*., 2017).

3.6. Quantitative PCR and Genotyping

HCV RNA quantification and analysis was carried out by Smart Cycler II Real-time PCR (Cepheid, Sunnyvale, Calif. USA), using HCV RNA quantification kit (Sacace Biotechnologies, Milano, Italy). The bottom line detection limit was 12 IU/mL. Genotype analysis of HCV was carried out to determine the exact genotype linked to the infection. Briefly 10 µl (50ng) of HCV RNA was reverse-transcribed to cDNA using 100 U of M-MLV RTEs (Thermoscientific, Massachusetts, USA) at 37°C for 50 minutes. About 2µl of

the synthesized cDNA was then used for the amplification of 470bp of HCV NCR region located at the 5´ end of the viral RNA. Reaction was further processed for the two second rounds of nested PCR amplifications with mixI and mixII set of primers (total reaction volume of 10µl). Mix-I had specific primer set for 1a, 1b, 1c, 3a, 3c and 4 genotypes while mix-II contained specific primer set for 2a, 2c, 3b, 5a and 6a (Ali *et al*., 2011).Viral load and genotypes were determined for the patients under scrutiny and data was tabulated.

3.7. DNA extraction

DNA from the collected sample was extracted using DNA purification kit (Qiagen, Hilden, Germany) and by phenol chloroform method. Each DNA Purification kit was used for the purification of 50 blood samples according to manufacturer's instructions. A total of 200 µl of blood was mixed with 400 µl of lysis buffer that was incubated at 65°C for 5 min. The frozen samples were thawed before adding lysis buffer. The samples were incubated at 65°C for 10min with occasional inverting and shaking of Eppendorf tube. About 600µl of chilled chloroform was added that was inverted 3-5 times for emulsification that followed centrifugation step at 13,000rpm (17,900g) for 2 min. The precipitation solution was prepared by mixing 720 µl of deionized water with 80µl of 10X precipitation solution. The aqueous phase was transferred to a fresh tube and 800 µl of precipitation solution was added and was centrifuged at 13,000 rpm (17,900g) for 2 min. Supernatant was completely removed DNA pellet was dissolved in 100 µl of 3M NaCl solution, tube was vortexed to dissolve DNA completely. DNA was precipitated by adding 300 µl of cold ethanol and placing at -20°C for 10min. Samples were centrifuged at 13,000 rpm (17,900g) for 3-4 min. Ethanol was removed and pellet was washed with 70% chilled ethanol and centrifuged twice at 13,000 rpm (17,900g), for 3-4 min. After washing, ethanol was discarded and pellet was allowed to dry at room temperature for 10min. After drying the DNA pellet, 100 µl of TE buffer was added to suspend it by gentle vortex and stored at -20°C till further processing. Conventional, phenol chloroform method was used for the purification of human DNA for 195 patients.

First step of the protocol used for the purification was to take 750 µl of blood sample in a 2ml. Eppendorf tube and 750 µl of solution A was added (Table 3.1). The sample and the solution were mixed by inverting the tube 4-6 times and that was kept at room temperature for 5-10min. Eppendorf tube was centrifuged at 13000rpm (17,900g) for 1 min. After

centrifugation, the supernatant was discarded and nuclear pellet was resuspended in 400 µl of solution A. The pellet was dissolved in the solution A by tapping several times until the pellet was not seen attached to the bottom of the tube. Samples were then centrifuged at 13000rpm (17,900g) for 1min, supernatant was discarded and the nuclear pellet was resuspended in 400 µl of the solution B along with 12 µl of 20% SDS and 5 µl of Proteinase K (Thermoscientific, Massachusetts, USA), mixed and incubated at 65˚C for 3 hours or at 37˚C overnight.

Equal volumes (500 µl) of mixed Solution C and Solution D were added to the incubated samples and centrifuged at 13000rpm (17,900g) for 10min. Upper layer (aqueous phase) was collected into a fresh 1.5 ml Eppendorf tube and equal quantity (500 µl) of Solution D was added to the newly collected aqueous layer and mixed. The mixture was then centrifuged at 13000rpm (17,900g) for 10min. Again the aqueous phase was transferred to a 1.5 ml Eppendorf tube and DNA was precipitated by adding 55 µl of sodium acetate (3M, pH 6), and equal volume of iso-propanol or 2X volumes of ethanol. Tube was inverted several times, to precipitate the DNA. After DNA precipitation the samples were centrifuged at 13000 rpm (17,900g) for 10min and the supernatant was discarded. To the DNA pellet 200 µl of 70% ethanol was added and centrifuged for 7min at 13000rpm (17,900g). After centrifugation, ethanol was discarded and the DNA pellet was dried at 37˚C for 20min. The dried DNA pellet was dissolved in 200 µl of 1X TE or deionized water. The purified DNA was stored at -20˚C for further use.

Table 3.1. Solutions used in purification of DNA

SOLUTION A	**SOLUTION B**	**SOLUTION C**	**SOLUTION D**
0.32M Sucrose 10mM Tris (pH 7.5) 5mM $MgCl_2$ Autoclaved, add Triton X-100 1% (v/v)	10mM Tris (pH 7.5) 400mM NaCl 2mM EDTA (pH 8.0)	Phenol	Chloroform-isoamyl alcohol 24:1

3.8. Gel Electrophoresis

Agarose Gel Electrophoresis was carried out to confirm the purified DNA. Agarose gel (1%) was prepared by dissolving 1g of Agarose 100ml of 1X TBE buffer by heating in oven for 1 min. The solution was allowed to cool down before pouring it into gel casting tray, containing comb for the formation of wells. The gel was left for 10 min to solidify and later the gel tray was placed in the BioRad Gel (BioRad, California, USA) tank containing 1X TBE buffer. The quantity of running buffer in the gel tank was adjusted to cover the surface of the gel. Samples were prepared for loading into the gel by mixing 5 µl of DNA sample with 1 µl of 6X loading dye (Fermentas, Massachusetts, USA). Thermoscientific 100bp DNA marker (Thermoscientific, Massachusetts, USA) was loaded as marker for determining the size of DNA. Samples were loaded into the wells, placed the lid on the gel tank and gel was run for 30 min at 90V. Afterwards, the gel was observed on UV illuminator for the presence of DNA bands. After confirmation these samples were transported to the Laboratory of virology and infectious disease, The Rockefeller University, New York for further analysis.

3.9. DNA Quantitation

Purified DNA was quantified using Thermoscientific Nanodrop (Nanodrop 1000 spectrophotometer, Thermoscientific, Massachusetts, USA). Quantity of the measured DNA was recorded in terms of ng/µl.

3.10. Candidate Gene selection

To analyze the Single Nucleotide Polymorphism (SNP) and their effect on outcome of HCV infection, three sets of genes in the immune system were targeted. The targeted gene belonged to the Human Leukocyte Antigen class II and III (HLA-Class II, III) region and consisted of three loci.

1. Promoter region of Tumor Necrosis Factor Alpha (*TNF-α*)
2. Second Exon of *HLA-DRB1* region
3. Promoter region of *HLA-DQB1* region

These regions were selected on the basis of their function in the immune system pathways as the *TNF-α* is a pro-inflammatory cytokine that plays its role in the regulation of cytokines and subsequent immune reaction (Tahan *et al.,* 2016). Promoter region of *TNF-*

α gene control the transcription and regulation of *TNF* gene and hence SNPs analysis in the context of HCV infection and therapy is crucial to understand in Pakistani population. Exon 2 of the *HLA-DRB1* gene codes for the extracellular domains of HLA proteins involved in antigen presentation. Analysis of SNPs in exon2 in the can be associated with response to anti-HCV therapy and infection (El-Bendrey *et al.,* 2019). Similarly SNPs analysis in the promoter region of *HLA-DQB1* gene can also play a vital role in defining the outcome of anti-HCV therapy and infection outcome IN Pakistani population (Arshad *et al.,* 2019). SNPs in these genes were determined using DNA amplification and sequencing analysis and analyzed for the corresponding outcome of HCV infection. *HLA* cluster is one of the recombination hotspot as reported by many studies, so it would be interesting to investigate the patterns of allelic polymorphism in these specific genes and their impact on the outcome of anti-HCV therapy and HCV disease outcome.

3.11. Primer Designing

3.11.1. *TNF-α* primers

A total of three positions were targeted in *TNF-α* gene and SNPs at specific regions were analyzed. The regions and variations selected were -238(G>A), -308(G>A) and -863(C>A). Primers for these regions were used as previously reported by Kim *et al*. (2003) with some modifications. A pair of primers was used to amplify both -238(G>A) and -308(G>A) regions, while a separate pair of primers was used to amplify -863(C>A) region. Primers used for the amplification of region of interest are given in Table 3.2.

3.11.2. *HLA-DRB1* Primers

Single Nucleotide Polymorphisms in Exon 2 of the *HLA-DRB1* region were targeted for the analysis of SNPs. Primers (Kaneshige *et al*., 1994) used for the amplification of region of interest are given in Table 3.2.

3.11.3. *HLA-DQB1* primers

Primers for the analysis of SNPs in *HLA-DQB1* region were designed by downloading the DNA sequence of *HLA-DQB1* region from ensemble browser. The downloaded DNA sequence was analyzed in MacVector version 6 and the promoter region of *HLA-DQB1* gene was targeted for the analysis of SNPs. A single pair of primers was used to amplify the desired gene. The designed primers were subjected to BLAST in the UCSC genome

browser for complementarities within the genome at other sites than the desired sequences. Sequences of the selected primer used for the amplification are given in Table 3.2.

Table.3.2. Set of primers used for the amplification of desired genes.

Position/Gene	**Primers**	**Product size (bp)**
-238/-308		
Forward (5'-3')	GGAGGCAATGTTTTGAGG	200
Reverse (3'-5')	GGTTTCTTCTCCATCGATGG	
-863		
Forward (5'-3')	GACCACACATGGGTATTCG	180
Reverse (3'-5')	GCTCTCACTTCTCAGGATGG	
HLA-DRB1		
Forward (5'-3')	GTGACGGTGTGTAGCACGTTCC	250
Reverse (3'-5')	CCGCTGCACTGTGAAGCTCT	
HLA-DQB1		
Forward (5'-3')	TTTGAAGACACAGTGCCAGGCACTG	350
Reverse (3'-5')	CGGCTCTGAGACAGCTGCCCTGCAC	

3.12. Primers optimization and troubleshooting

Along with above-mentioned primers, some other pairs of primers were also tried to amplify gene of interest as incase of *HLA-DQB1* and *HLA-DRB1*. Polymerase Chain Reaction was carried out for the concerned primers by using ExTaq polymerase enzyme along with different gradient temperatures for the primer annealing. Gradient PCR (LabOne thermocycler) was used for the optimization of each set of primers and various annealing temperatures were checked. After optimization of all the conditions such as annealing temperature, sample quantity and annealing time, PCR was finally carried out by using the optimized conditions. Each gene was optimized separately for PCR reaction.

3.13. Polymerase Chain Reaction

Polymerase Chain Reaction was carried out using LabOne and Biometra thermocyclers (Applied Biosystems, Massachusetts, USA). A total of 50 µl of the reaction mix was prepared for each sample. The premix consisted of 5ul of 10X PCR buffer, 1ul of 10mMol

dNTPs, 1ul of 50pm of both forward and reverse primers, 0.5ul (5U/ul) units of Taq polymerase 1ul of 50mM Mgcl2 and 5ul of 40ng/ul DNA template. The final volume was adjusted to 50ul reaction mixture by adding 35.5 ul PCR water

The PCR conditions set for the reaction were as follows:

1. Initial denaturation at 95˚C for 5min
2. Denaturation at 95˚C for 2 min.
3. Annealing at 58˚C for 30 sec.
4. Initial extension at 72˚C for 2min (repeat from step 2, 35 cycles)
5. Final extension at 72˚C for 10min.
6. Final hold at 10˚C for indefinite period of time.

Annealing temperature for each set of primer was different for the studied genes.

3.14. Agarose Gel Electrophoresis

After PCR, samples were checked for amplification on Agarose Gel. One percent agarose Gel was prepared by dissolving 1g Agarose in 100ml of 1XTBE buffer. After the preparation and solidification of the Gel PCR amplified sample was mixed with 1µl of 6X loading dye that was loaded in the wells of the Gel Matrix. DNA marker, 1kb (solisbiodyne, Tartu, Estonia) was loaded in order to compare the size of amplicon and correct size. Agarose Gel was visualized on the UV illuminator (Biometra, Applied Biosystems, Massachusetts, USA).

3.15. Gel Purification

After getting the desired amplicons by PCR, products were purified from the gel. Agarose gel (1X) was made and the remaining 47 µl of the amplified product was loaded into the Gel. Gel was run for 40min at 100V; products were purified using Qiagen Mini Gel purification Kit (Qiagen, Hilden Germany). The final purified product was stored at -20˚C. The protocol for gel purification is as follows.

1. The DNA band containing the desired amplified was excised from the gel using a clean, sharp scalpel. Removing the extra agarose minimized the size of excised Agarose gel.

2. The excised gel slice was weighed in a clean 1.5 ml Eppendorf tube and three volumes of buffer QG was added to 1 volume of gel slice. i.e. 100mg = 300µl.

3. The tube was then incubated at 50˚C for 10 min with occasional vortexing and tapping so that the gel was dissolved completely. Incubation time was increased incase of 2% agarose gels for complete dissolution.

4. After the gel was completely dissolved the color of the mixture was checked to be yellow, if the color was violet or orange then 10 µl of 3M sodium acetate was added to the tube containing the mixture.

5. To the mixture, 1 volume of iso-propanol was added, i.e. 100 µl =100mg of gel. This step was carried out for the product size of less than 500bp to increase the yield of DNA fragments. For DNA fragments larger than 500bp iso-propanol was not added.

6. A QIAquick spin column was placed into 2ml collection tube, and the whole mixture from the tube was transferred to QIAquick spin column.

7. The column was centrifuged for 1min at 13000 rpm (17,900g), the flow through was discarded and the column was placed back to the collection tube.

8. To the column 0.5ml of QG buffer was added again and centrifuged for 1 min at 13000 rpm (17,900g), flow through was discarded and again column was placed into the collection tube.

9. For washing, 750 µl of buffer PE was added. Ethanol 96-100% was already added to the buffer PE, the column was let to stand for 2-3min and then centrifuged at 13000 rpm (17,900g) for 1 min.

10. Flow through was discarded and column was again placed into the collection tube column was centrifuged again 13000 rpm (17,900g) for 1min.

11. The column was transferred into a new clean 1.5ml Eppendorf tube and 50 µl of Elution buffer was added into the column, centrifuged at 13000 rpm (17,900g) for 1min.

12. The flow through was again placed back into the column and centrifuged was done at 13000rpm (17,900g).
13. Column was discarded and the purified product was stored at -20˚C.

3.16. DNA Sequencing

Automated Sanger DNA sequencing for each purified sample was carried out by sending the samples to Macrogen, New York which was analyzed further. In order to assure the data quality, sequencing of both forward and reverse strands were carried out.

3.17. Sequence Analysis

The DNA sequences were analyzed for variations by comparing with the reference sequence downloaded from ensemble genome browser. Sequence for each sample was analyzed using the reference DNA sequence and the obtained DNA sequence for each sample was then compared by the BioEdit software. Nucleotide variations in each sample were noted and identified.

3.18. Nucleotide Peak Analysis

Nucleotide peak analysis was carried out to fine tune variations to compare with reference DNA sequence. Each sample was analyzed separately.

3.19. Reference SNP number and position on the chromosome identification

Reference SNP number (rs number) is the number assigned to each identified SNP present in different databases. The rs numbers and position of our nucleotide variants were identified by comparison. Mutation Taster (http://www.mutationtaster.org) and ExAc (http://exac.broadinstitute.org/) databases were used for the identification of rs numbers and exact position of the nucleotide on corresponding chromosome. Sequence variant along ten nucleotides upstream and downstream of the variant was pasted into the mutation taster along with the variation and reference nucleotide enclosed in brackets. The name of the gene along with information regarding transcript ID and exonic or intronic information were entered in mutation taster. After entering the information a new window was opened which showed all the reported info about the SNP of interest including rs number, position, allele frequency and other relevant information. The rs numbers were identified for all SNPs except for unreported. Positions for all SNPs were identified based on the available information in the ExAc database.

3.20. Generation of Maps

After the identification of SNPs, maps were generated. For this purpose, rs number of each SNP was pasted in the UCSC genome browser, in the hg19 genome assembly. Clicking the get DNA downloaded upstream and down stream sequence of the SNP, around 30 nucleotides both upstream and downstream were downloaded, the minimum number of nucleotides was 20. The reference for the corresponding gene was downloaded from the UCSC genome browser. After downloading both SNP sequence and the reference sequence for each corresponding gene, all the sequences were pasted and numbered accordingly in a separate word file. Once the sequence file was complete, the whole sequence files were subjected to BLAST in UCSC genome browser by selecting the Basic Local Alignment Search Tool (BLAST). A sequence identity window was generated after BLAST which showed the percent identity of our query sequence with the corresponding gene. The sequence file which has the maximum similarity index was selected; similarity index confirming identity of 99-100% was selected. Opening the link resulted in the generation of a genomic Map which showed the position of each SNP in the gene as shown in the *HLA-DQB1* map (https://genome.ucsc.edu/).

3.21. Allele frequencies

The Allelic frequencies for the studied SNPs were also determined from the UCSC genome database. Allelic frequencies for both reference nucleotides and the variant were obtained from the browser. Analysis of the allelic frequencies were carried out in each studied group of the patients and compared to the allelic frequencies available in the UCSC database (https://genome.ucsc.edu/).

3.22. Genotype determination

Genotypes for the identified SNPs in the studied patients were also determined. Both forward and reverse strands of the sequenced samples were analyzed and genotypes were determined. Resulted genotypes were then compared with the reference studies and the genotypes in the genome databases in order to have a clear view of the genotype distributions in different subset of patients.

3.23. Haplotype determination

Haplotypes were constructed for all sets of genes analyzed in this study. Haplotypes were generated using online freely available tool Haplo2ped

(http://bighapmap.big.ac.cn/software.html). A complete haplotype map along with the percentiles of prevalent important haplotypes was drawn.

3.24. Multivariate logistic regression analysis

Multivariate logistic regression analysis for the significant SNPs was carried out and association was generated using Graph Pad Prism and SPSS software.

3.25. Data tabulation and comparative analysis

The analyzed data was tabulated in Excel sheet and comparative analysis between patient demographic and clinical information was done by studying the allelic and genotype distribution among HCV infected patients. Allelic and genotype prevalence in different patient groups like spontaneously recovered (SR), chronically infected (CI), recovered (R) and non-recovered (NR) were categorized. Patient´s gender, literacy rates, signs and symptoms and risk factors were compared and analyzed. HCV genotype, viral load, anti-HCV therapy, allelic and genotype distribution of the studied SNPs of the analyzed group of patients was co-related.

3.26. Linkage disequilibrium analysis

Linkage disequilibrium (LD) analyses were carried out using online tool SNP stats. Heat maps and tables were generated for the analyses.

3.27. Dominancy models generation

Dominancy models for the analyzed genotypes were developed by using online tool SNP stats.

3.28. Statistical Analysis

Allelic and genotype frequencies for all the SNPs were compared between the responsive and non-responsive (R vs. NR), and between chronically infected and spontaneously recovered (CI vs. SR) groups of patients. Statistical analyses were carried out through GraphPad Prism and STATA (11.0) softwares. The significance of the differences from random distribution was estimated through Chi-square and T-test statistics. Yates' corrected Chi-square and Fisher's exact tests were also employed. The level of significance for p-value was <0.05. The magnitude of the effect was analyzed by calculating odds ratios (OR) at 95% confidence interval (95% CI). For multivariate analyses, logistic regression was performed for the both R/NR and CI/SR groups separately. Groups were coded as dichotomous variables and SNPs and demographic factors as independent variables. A

stepwise regression model was applied by including all variables and excluding the non-significant ones.

4. Results

4.1. Clinical and demographic analysis of the studied patients

A total of 245 HCV infected patients were included in this study. Patients were positive for anti-HCV antibody. The patients positive for HCV infection registering in hospital during defined period of this study were included.

4.1.1. Gender and literacy based differentiation of the studied patients

Gender based differentiation of the analyzed patients as follows; n=121 (49%) of the participants were male patients, whereas, n=124(51%) were females (Figure 1). Literacy based distribution showed that, n = 114(53.7%) of the male and n= 98(46.2%) of the female participants were literate (Table 1).

Figure 4.1. Chart representing male and female distribution in studied patients.

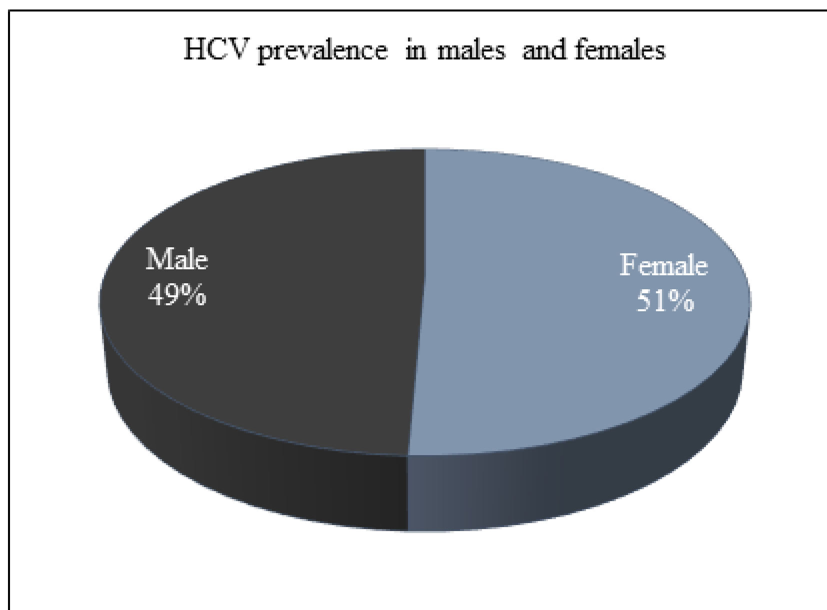


Table 4.1. Gender and literacy based distribution of studied patients

Gender	Literate: n (%)	Illiterate: n (%)	*p* value
Male	114(54)	7(21)	p=0.0005, S
Female	98(46)	26 (79)	
Total	**212**	**33**	

n=number of patients, S= statistically significant, *p* value was determined using chi square distribution

4.1.2. Age intervals of the analyzed patients

Age interval for the studied participants revealed that majority of the participants belonged to age group 31-40 years having prevalence rates n=76(31%) , similarly the age group 19-30 years showed second highest rate of prevalence n=57(23%), that was followed by age group 41-50 years, n=54(22%). Age groups 51-60 years and 61-70 years had relatively lower prevalence rates, n=43(18%) and n=15(6%) respectively (Figure 4.2). Gender based differentiation of age intervals in studied patients revealed that male gender n=10 (67%), n=30 (53%) of the patients belonged to two age groups, 61-70 years and 19-30 years respectively. The male gender in age group, 31-40 years, 41-50 years and 51-60 years were n=34 (45%) n=24 (44%) and n=23 (53%). Likewise, female gender in the studied age group was n=30 (56%) in age group 41-50 years, followed by n=42 (55%) in 31-40 years, n=27 (47%) in age group 19-30 years respectively. The prevalence in age groups 51-60 years and 61-70 years were n=20(47%), n=5(33%) (*p*=0.008, statistically significant) (Figure 4.3).

Figure 4.2. Chart representing the age intervals of the studied patients

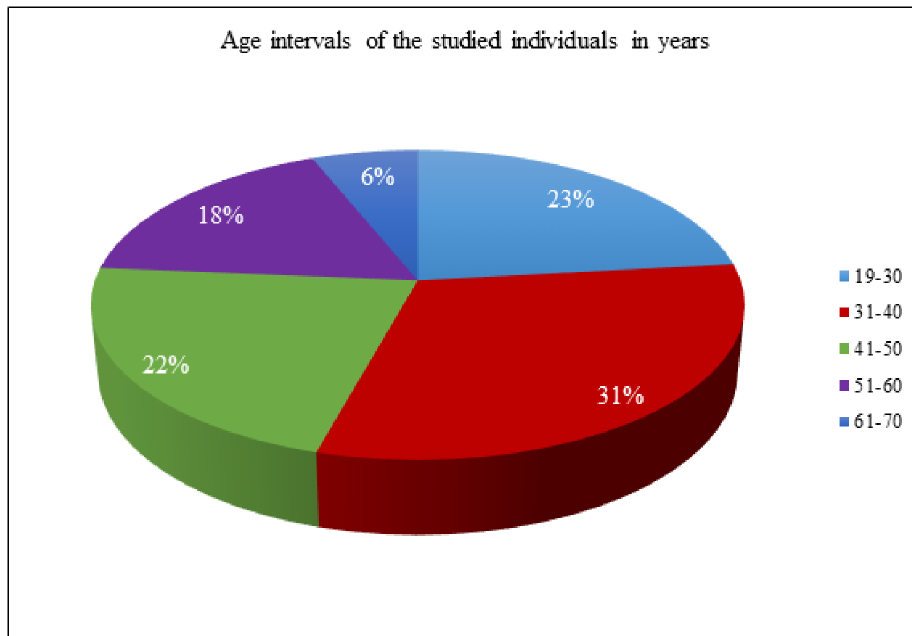
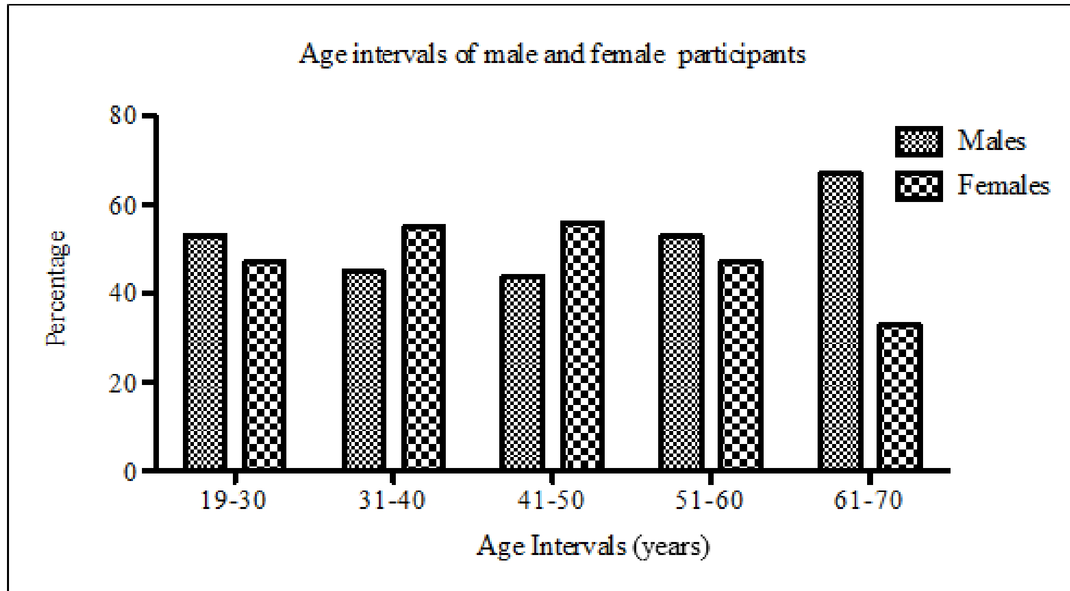


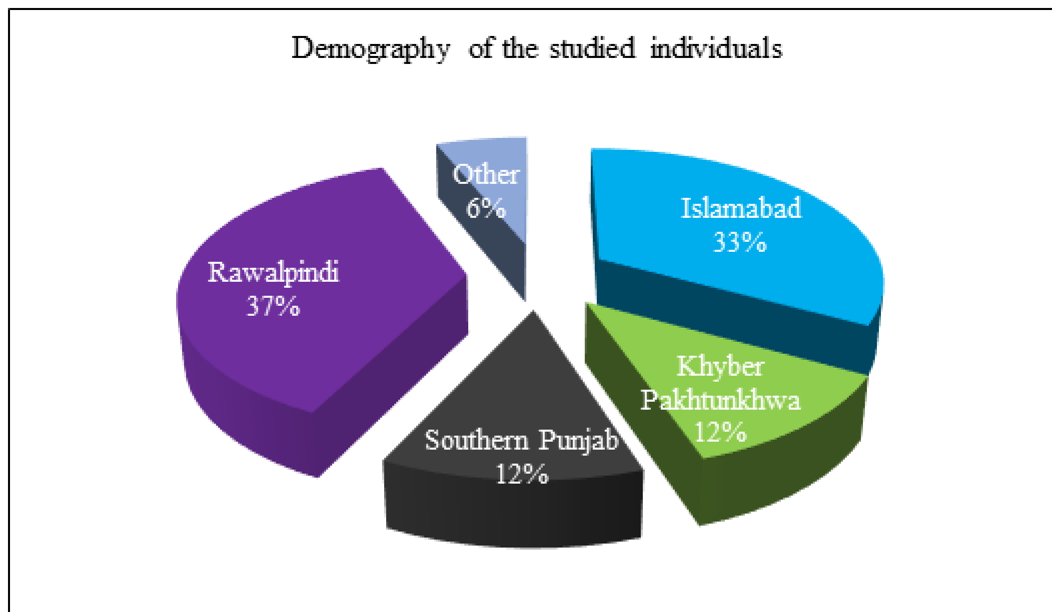
Figure 4.3. Age based distribution of male and female patients



4.1.3. Demography of included patients

Demographic analysis for all participants revealed that most of the patients belonged to District Rawalpindi of Punjab province, n=90(37%) followed by Islamabad the capital territory n=81(33%). Participants from Khyber Pakhtunkhwa and Southern Punjab were n=29 (12%) each. participants from the other region of the county were n=15(6%) (Figure 4.4).

Fig 4.4. Demography of analyzed patients



4.1.4. Prevalence of symptoms in studied HCV patients

Symptoms observed in the studied patients revealed that the most prevalent symptom was fatigue followed by Jaundice and fever, having the rates of n=136(19%), n=117(16%) and n=98(14%) respectively. Prevalence rates for the other symptoms observed in this study like abdominal discomfort, malaise and dark urine are shown in the Figure 4.5. Symptom differentiation in male and female participants revealed that jaundice was the most prevalent symptom in male patients in comparison to females, n=70(58%) vs. n=47(38%). The other major symptom was fatigue, as males had the prevalence rates of n=58(48%) compared to females n=78(63%) respectively. Symptoms like abdominal discomfort, anorexia were almost comparable among male and female patients (shown in Figure no. 4.6). Chi square distribution showed probability value of 0.172 and hence is statistically not significant.

Figure 4.5. Symptom analyses in studied HCV patients

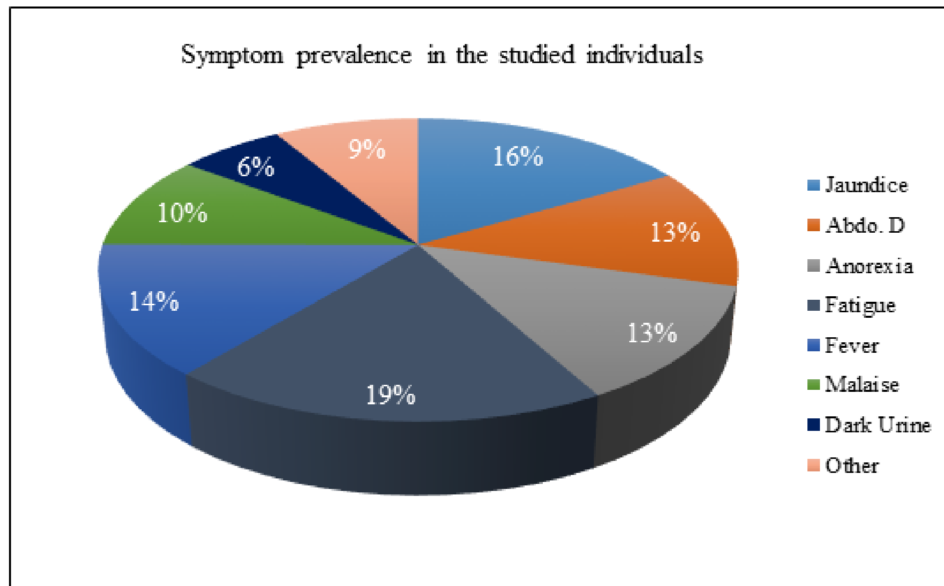
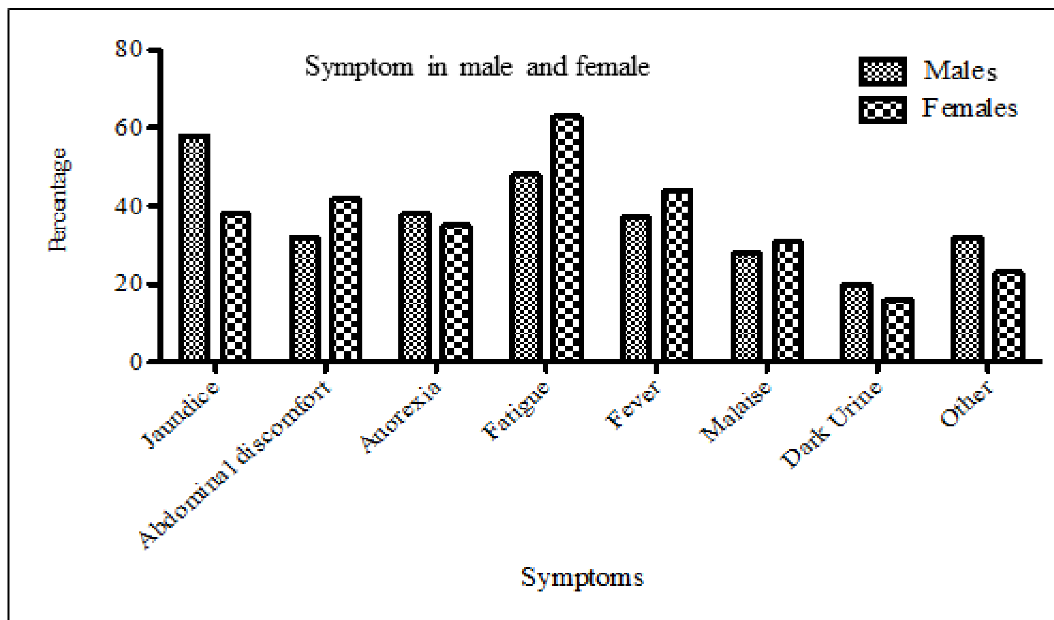


Figure 4.6. Analyzed symptoms in males and female patients



4.1.5. Risk factors associated with HCV infection in analyzed patients

Risk factors analysis showed that the most widely distributed risk factor was injection use n=243(23%), followed by intravenous infusions, n=145(14%). Skin piercing and barber visits were also the most commonly distributed risk factors in the studied patients having rates of n=124(12%) and n=116(11%) respectively. Distributions for other risk factors such as dentist visits, surgery, hospitalization etc. are shown in Figure 4.7.

Gender based differentiation of risk factors among participants revealed that most of the studied risk factors were equally distributed, however skin piercing in females was n=114(92%), whereas barber visits in males n=116(98%), were the most frequent risk factors studied patients. Distributions for the rest of the risk factors are shown in Figure 4.8. Chi square distribution showed probability value (*p*) of <0.001, and hence the association was found to be statistically significant.

Figure 4.7. Observed risk factors among studied patients

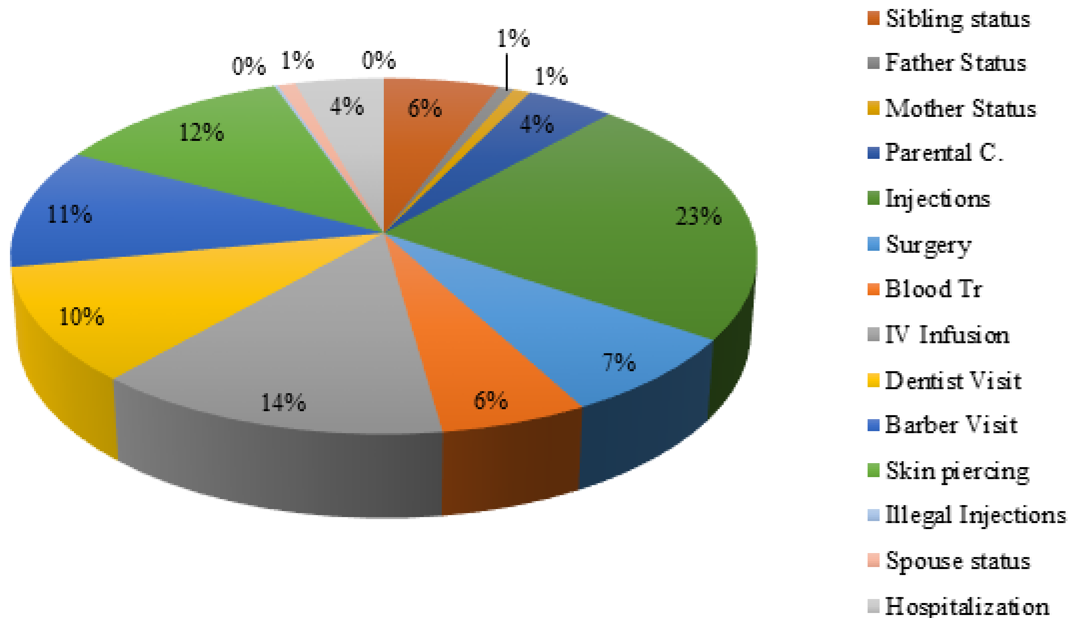
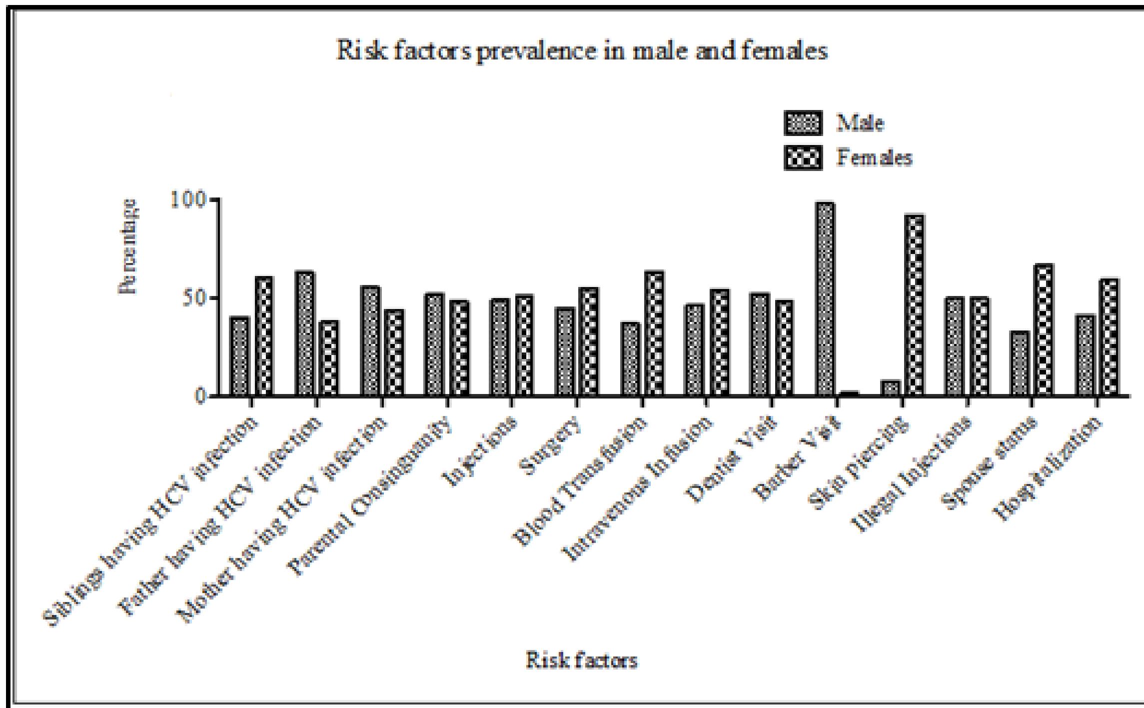


Figure 4.8. Risk factors prevalence in male and female patients



4.1.6. Occupational information of the studied participants

Data regarding the occupation of the participants was also obtained and it was found that the occupation was dominated by house wives, n=102(42%) belonging to females patients. Public servants and laborers were the second most prevalent occupation in analyzed patients having rates of n=43(18%) and n=25(10%) respectively. Data for the rest of the occupations is shown in Figure 4.9. Differences in the occupations of the studied patients on the basis of gender are shown in Figure 4.10. Similarly all laborer and driver participants were only males. Prevalence rates for other occupations like teachers, business, students and public servants are shown in Figure no. 4.10. Statistical analysis for association of occupation with infection revealed significant associations, i.e. $p<0.001$.

Figure 4.9. Chart representing occupation of studied patients

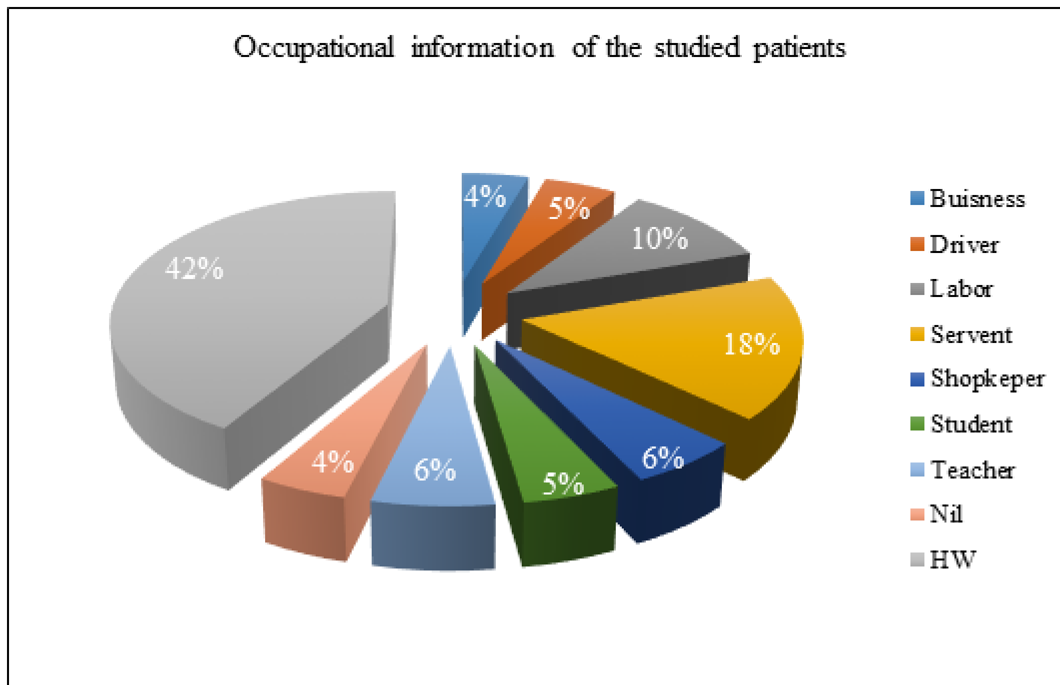
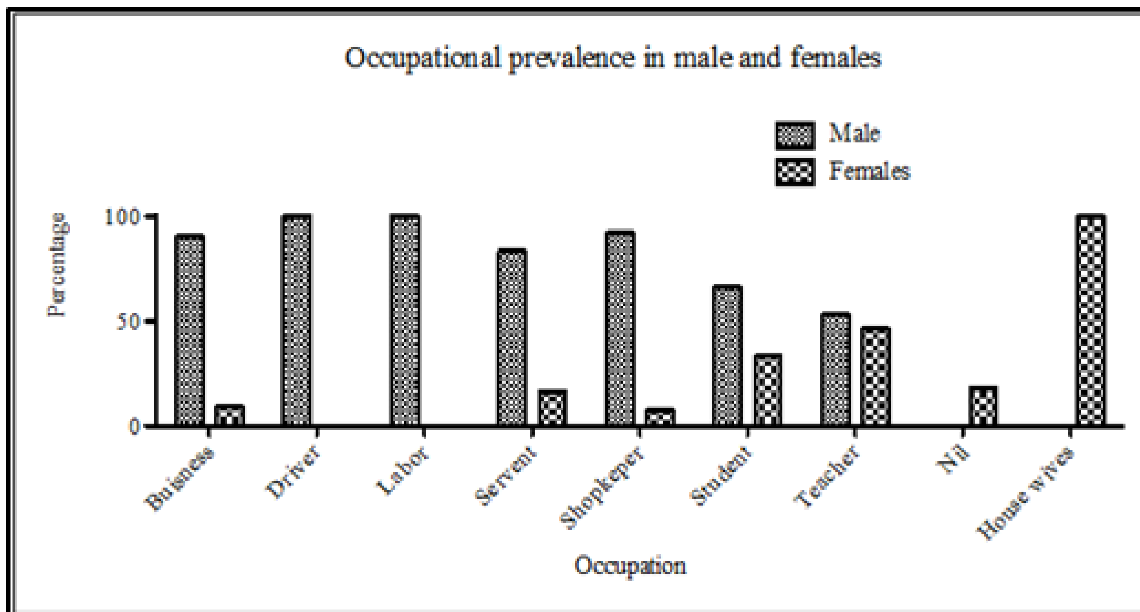


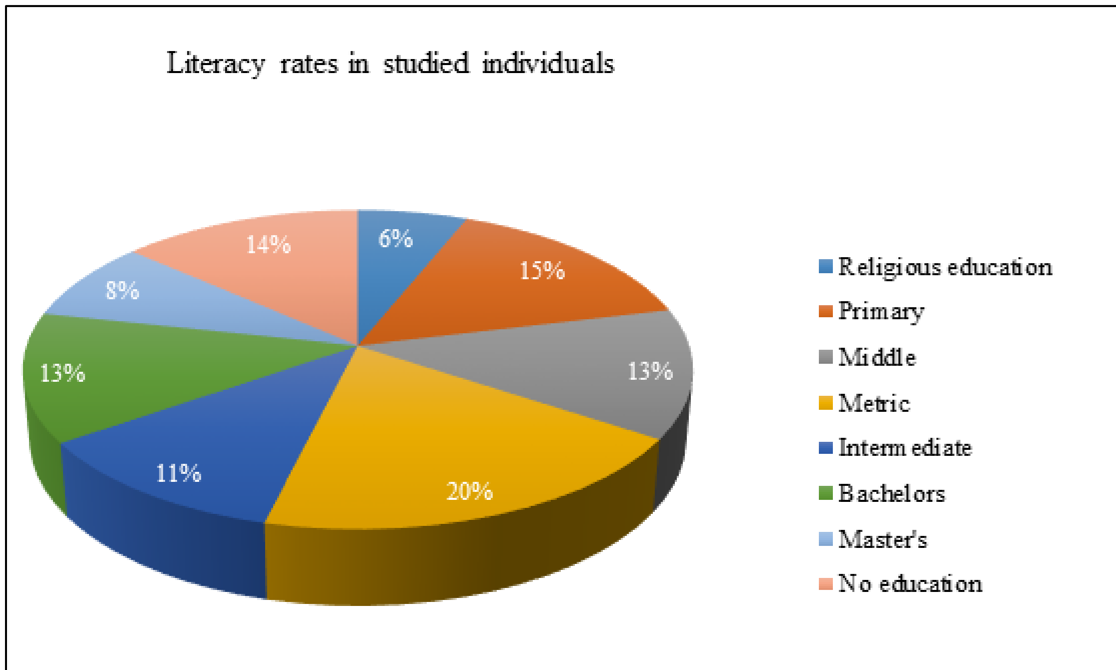
Figure 4.10. Gender based occupational differences among studied patients



4.1.7. Literacy rates of studied patients

Literacy rates in analyzed patients revealed that most of the participants had matriculation, n=48(20%) followed by primary and middle levels of education having rates of n=37(15%) and n=32(13%) respectively. Most of the patients were literate and their literacy along with literacy levels are shown in Figure no. 4.11. Chi square distributions for literacy rates in males and females revealed probability values of <0.003 and hence found to be statistically significant.

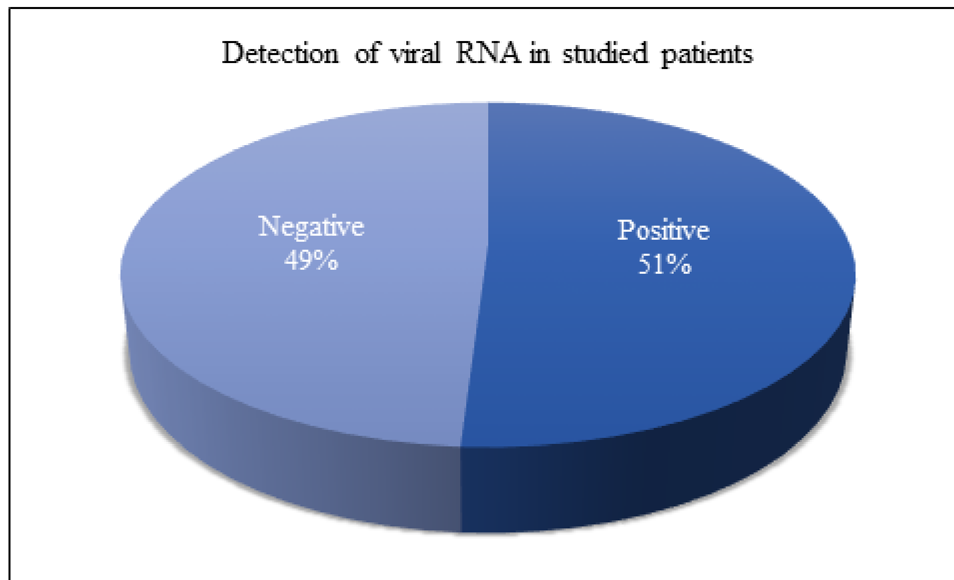
Figure 4.11. Literacy levels in studied patients



4.1.8. Prevalence of viral RNA in in anti-HCV positive patients

Viral RNA detection was carried out for all 245 patients, and it was found that viral RNA was detected in n=125 (51%) of the total patients, while 120 (49%) of the patients were negative for HCV RNA presence (Figure 4.14).

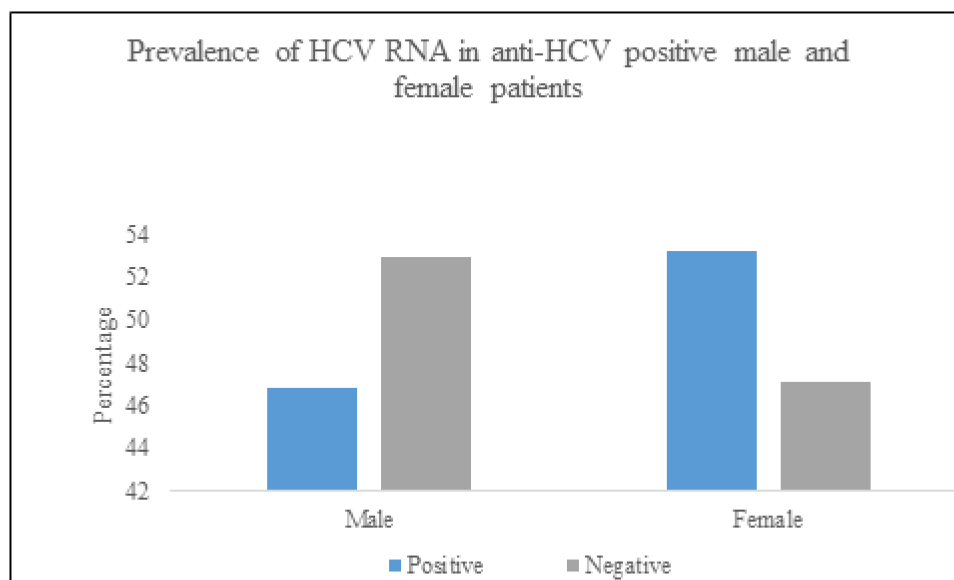
Figure 4.12. Prevalence of HCV RNA in studied patients



4.1.8.1. Prevalence of HCV RNA in anti-HCV positive male and female patients

Gender based viral RNA distribution revealed that viral RNA was present in n=66 (47%) of the studied males patients, while n=55 (53%) of the male patients were negative for viral RNA. Similarly viral RNA distribution in females was n=75 (53%), while n=49 (47%) of the females patients were negative for HCV RNA (Figure 4.15). (*p*=0.347, statistically not significant)

Figure 4.13. Gender wise viral RNA prevalence in studied patients



4.1.9. HCV genotype prevalence in anti-HCV positive patients

Due to limited availability of resources a total of n=100(40%) patients with HCV were analyzed for HCV genotype and it was found that out of total analyzed patient's genotype 3a was detected in 94% of the patients. Genotype 1a, 1b and un-typable were found to be present 2% each, Figure 4.12. Gender based prevalence of HCV genotype revealed almost equal distribution for genotype 3a, genotype 1a, 1b and untypable only prevalent in male patients, data shown in Figure 4.13. Chi square distribution showed *p* value of >0.553, and hence not statistically significant.

Figure 4.14. HCV genotype prevalence in anti-HCV positive patients

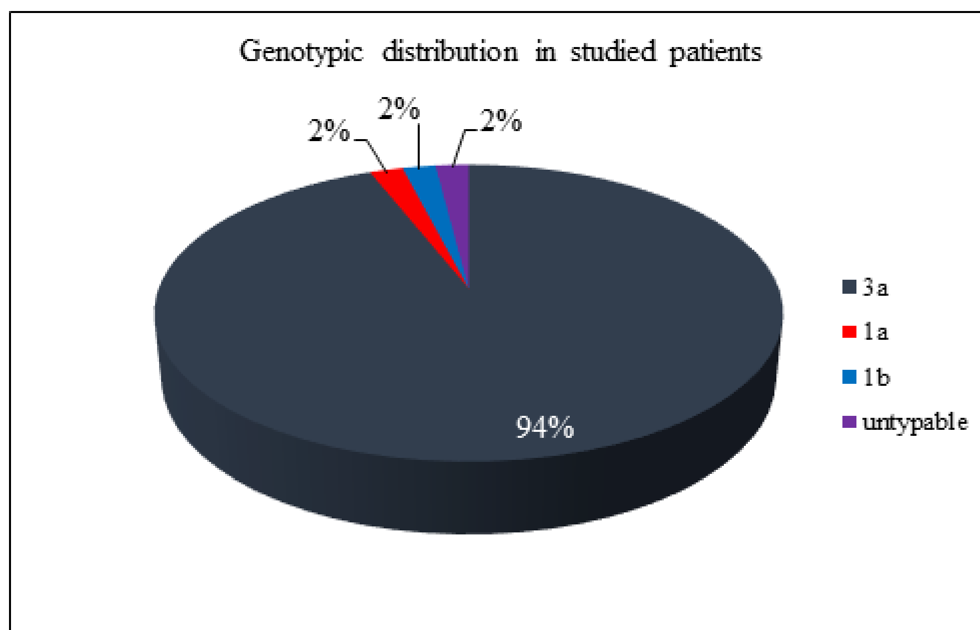
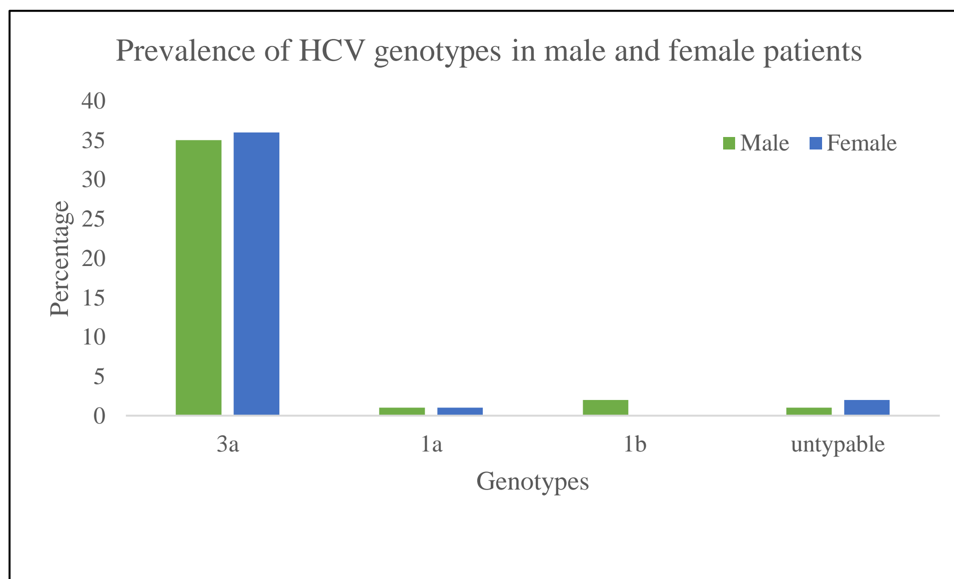


Figure 4.15. HCV genotype prevalence in anti-HCV positive males and females



4.1.10. Gender wise viral load distribution in anti-HCV positive patients
Viral load distribution revealed that the lowest viral load in male patients was 12 IU/ml, whereas, the lowest viral load in female patients was 15 IU/ml respectively. Similarly the highest viral load among the male infected patients showed that the viral load was 51,809,739 IU/ml, while the highest viral load among female patient was 31,874,791 IU/ml respectively. The average viral load in males was 5,315,791 IU/ml and 3,180,912 IU/ml in female patients (Table 4.2).

Table 4.2. Viral load distribution in male and female patients

Gender	Average Age (years)	Lowest (IU/ml)	Average (IU/ml)	Highest (IU/ml)
Male	41.1	12	5,315,791	51,809,739
Female	41.0	15	3,180,912	31,874,791

IU/ml= international units per ml. Average viral load was higher in male patients as compared to females.

4.1.11. Patient Categorization

All the patients included in this study were categorized into two main groups, patients receiving anti-HCV therapy, n=110 (45%) and patients not receiving any anti-HCV therapy (n=135 (55%). The therapy group comprised the patients who were receiving antiviral combination therapy with Pegylated IFN (180lg) once a week and ribavirin (800–1200 mg) according to the patient's body weight. These groups were further categorized into two groups each. The therapy group was divided into responsive (R) to therapy group and non-responsive to anti-HCV therapy group with the relative prevalence of n=68 (62%) and n=42 (38%) respectively. Responsive group comprised of the patients who had negative viral RNA levels after the completion of six months of therapy, whereas non-responsive patients did not clear the virus after completion of the therapy. Similarly the non-therapy group was further distributed to chronically infected, n=99 (73%) and spontaneously recovered group n=36 (27%) (Figure 4.16). In chronically infected group those patients were included who were treatment naïve and HCV RNA was detected in their serum. Spontaneously recovered group included those patients who recovered from HCV infection and had negative viral load levels with out taking any therapy.

Gender based categorization of the patients showed that n=33 (49%) of the responsive to therapy group were males, while n=35 (51%) of the responsive patients were females. The Non-responsive group comprised of n=23 (54%) of male patients and n=19 (46%) of female patients. Similarly the chronically infected group was comprised of n=43 (43%) of males and n=56 (57%) of female patients. In the spontaneously recovered group of patients n=22 (62%) of patients were males, while n=14 (38%) were females (Figure 4.17) (p=0.051, statistically not significant).

Figure 4.16. Categories of analyzed patients.

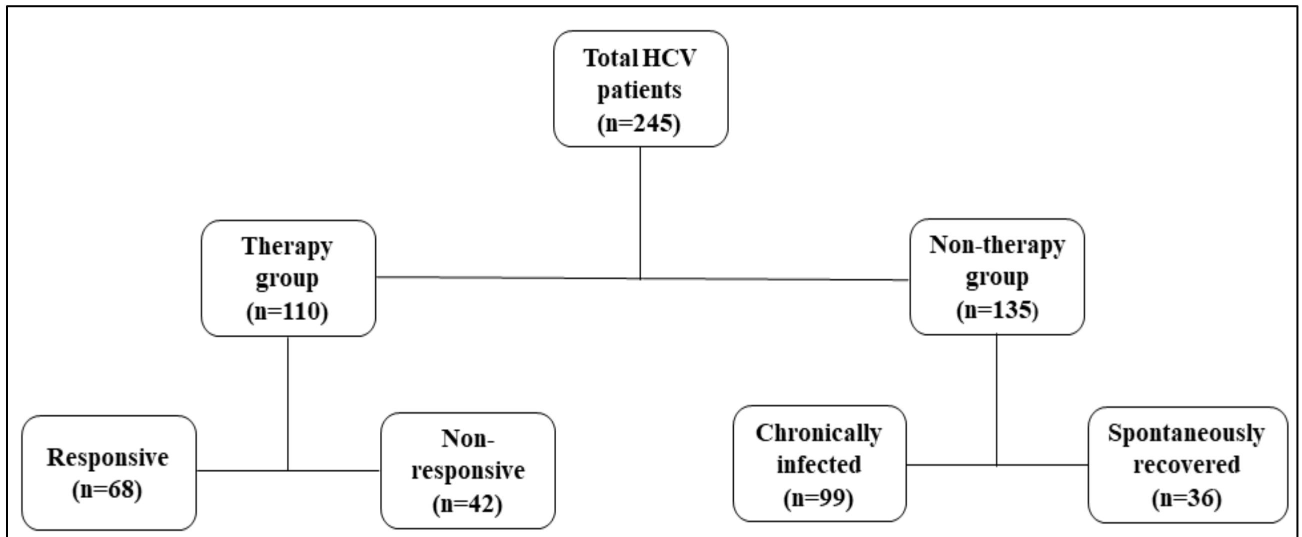
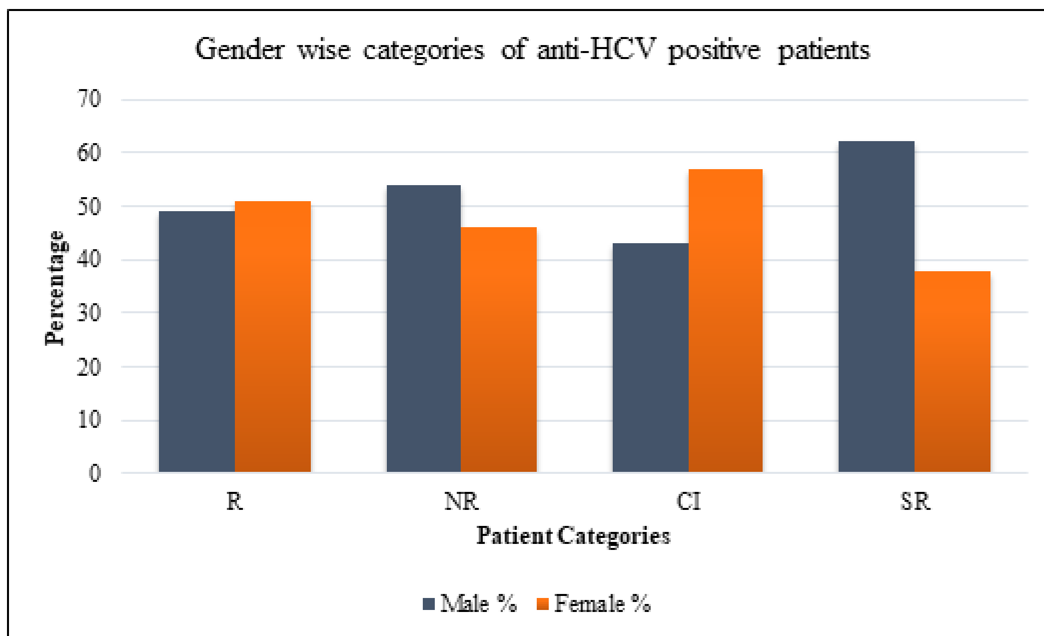


Figure 4.17. Categorization of the patients on the basis of gender



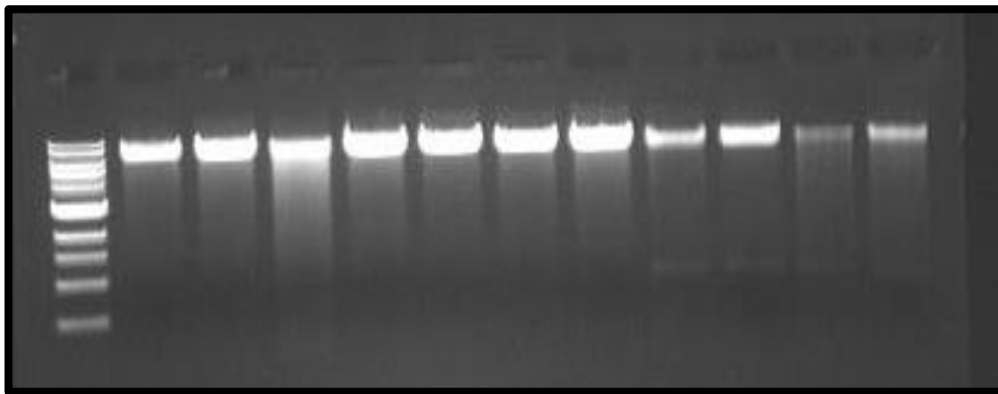
2. Post-clinical and demographic analyses

After the clinical and demographic analysis all the blood samples were subjected to DNA extraction, PCR and sequencing as described in the materials and methods section. Detailed analysis of the observed single nucleotides polymorphism in the proposed set of genes was carried out which included the following steps.

4.2.1. DNA extraction

As mentioned earlier DNA from the collected samples were extracted using phenol-chloroform method and using, Thermoscientific DNA extraction kit.

4.18. Gel image of the extracted DNA from the blood samples



M= marker, sample number 1-11.

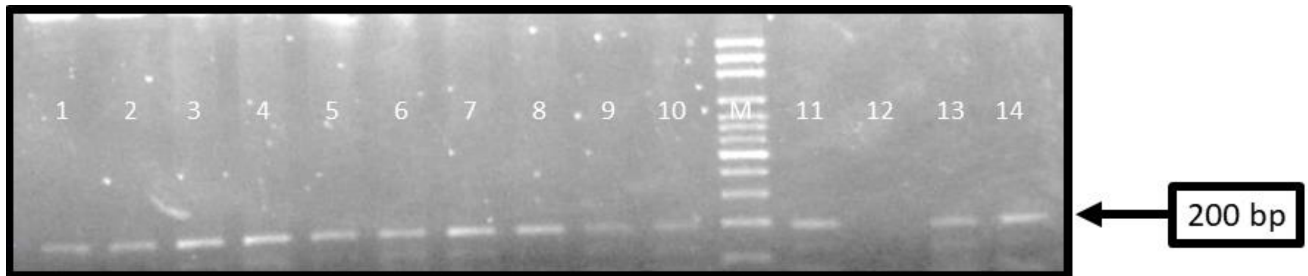
4.2.2. PCR amplification for selected candidate genes (*TNF-α*, HLA-*DRB1, DQB1*)

PCR amplifications were carried out for the above mentioned genes after primer desig and PCR optimization. PCR conditions and reagents concentrations are mentioned in the material and methods section. Gel images of amplified genes are shown below.

4.2.2.1. Gel image of the *TNF-α*-238/308 position amplification

TNF-α-238/308 position was amplified using above mentioned conditions by PCR. The amplified product for these sets of primers was 200bp. Fermentas 100bp gene ruler was used as a marker in order to determine the correct size of the amplified product. Gel image of the amplified product is shown below.

Figure 4.19. Gel image of the amplified product for *TNF-α* -238/308 locus

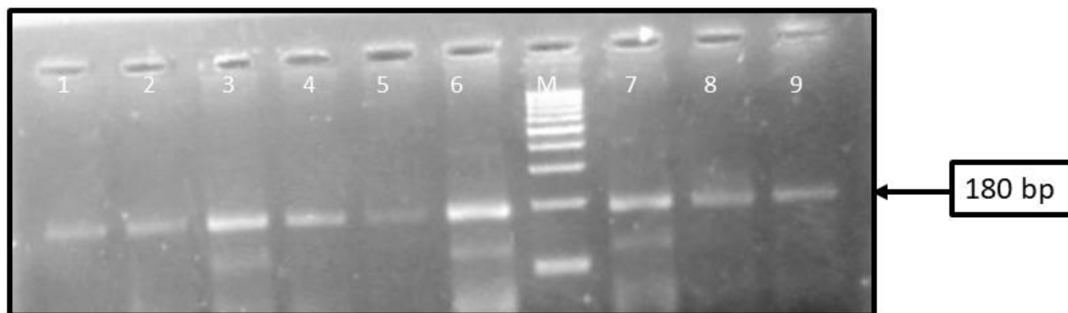


Numbers 1-14 represents amplified product, marker is represented by M.

4.2.2.2. Gel image of the *TNF-α*-863 position amplification

TNF-α-863 position was also amplified using the PCR conditions which are described above. PCR product of 180bp was observed on the gel for this particular variant. Fermentas 100bp gene ruler was used as a marker in order to determine the correct size of the amplified product. Gel image of the amplified product is shown below.

Figure 4.20. Gel image of the amplified product for *TNF-α* -863 locus, samples

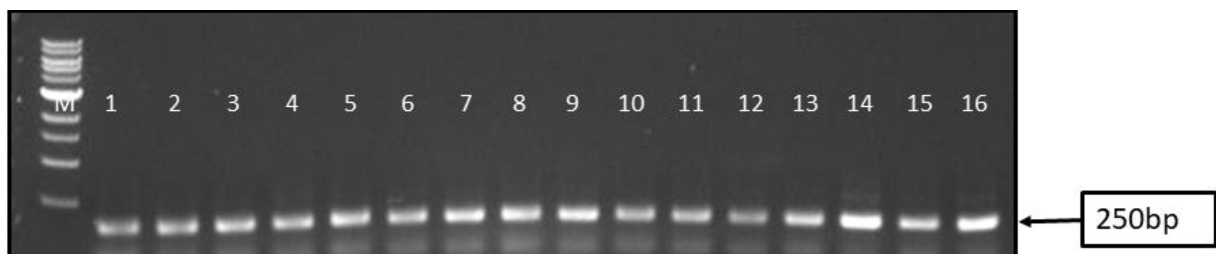


Numbers 1-14 represents amplified product, marker is represented by M.

4.2.2.3. Gel image of the *HLA-DRB1*gene amplification

HLA-DRB1 gene was amplified. PCR product of 250bp was observed on the gel for this particular variant. Fermentas 1kbp gene ruler was used as a marker in order to determine the correct size of the amplified product. Gel image of the amplified product is shown below.

Figure 4.21. Gel image of the amplified product for *HLA-DRB1*gene

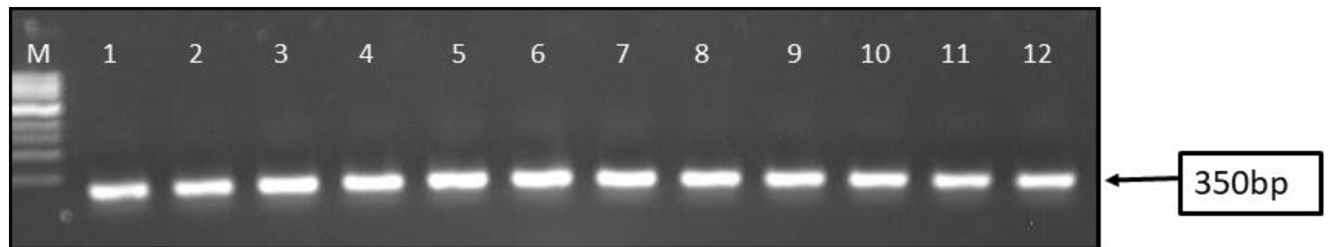


Samples are represented by numbers 1-16, while marker is represented by M. The smallest band in the marker is 500bp.

4.2.2.4. Gel image of the *HLA-DQB1*gene amplification

HLA-DQB1 gene was amplified. PCR product of 350bp was observed on the gel for this particular variant. Fermentas 1kbp gene ruler was used as a marker in order to determine the correct size of the amplified product. Gel image of the amplified product is shown here below.

Figure 4.22. Gel image of the amplified product for *HLA-DQB1*gene



Samples are represented by numbers 1-12, while marker is represented by M. The smallest band in the marker is 500bp

4.2.3. Gene sequencing

As mentioned in the chapter 3, all the amplified samples were subjected to the Sanger sequencing by Macrogen, New York. After sequencing the analyzed sequences were obtained and the sequences were screened for the presence of single nucleotide variations (SNVs).

4.2.4. Mapping of the identified SNPs

All the analyzed SNPs in the corresponding genes mapped into chromosome 6p21.3. Identified SNPs were analyzed using online browsers and databases such as NCBI databases, mutation taster, Exome Aggregation Consortium (ExAC), University of California Santa Cruz genome browser (UCSC browser) and ensemble genome browser. Table 4.3 represents the total SNPs their positions on chromosome and reference SNP numbers analyzed in this study.

Table 4.3. Mapped SNPs analyzed in this study

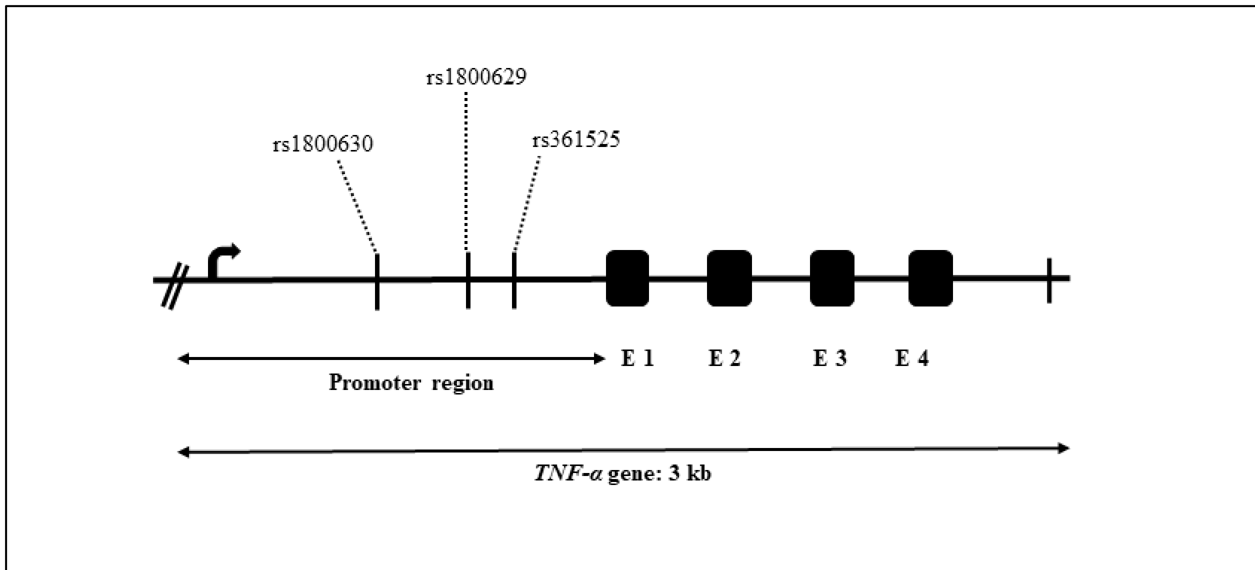
Gene/position	Nucleotide Variation	rs number	Position on chromosome Hg-19
TNF-α			
-238	G/A	rs361525	Chr6:31543101
-308	G/A	rs1800629	Chr6:31543031
-863	C/A	rs1800630	Chr6:31542476
HLA-DRB1			
6151	T/C	rs1064664	Chr6:32552075
6167	(A/C/T)	rs707957	Chr6:32552059
6192	(C/T)	rs776046212	Chr6:32552034
6228	(C/T)	rs2308802	Chr6:32551998
6231	(T/C/G)	rs3167799	Chr6:32551995
6236	(A/C)	rs1059586	Chr6:32551990
6251	(A/T)	rs397844204	Chr6:32551975
HLA-DQB1			
-8288	(C/G)	rs9273598	Chr6:32629093
-8298	(C/G)	rs9273592	Chr6:32629083
-8307	(A/C/G)	rs9273588	Chr6:32629074
-8331	(T/G)	Not reported	Chr6:32629051
-8344	(T/C)	Not reported	Chr6:32629038
-8362	(A/C/G)	Not reported	Chr6:32629020
-8442	(G/C)	rs34644981	Chr6:32628939
-8447	(C/A/T)	Not reported	Chr6:32628934
-8465	(G/C)	rs9273552	Chr6:32628916
-8471	(A/G/C)	Not reported	Chr6:32628910

Table shows analyzed SNPs in the studied patients along with their reference number and position.

4.2.4.1. Mapping of *TNF-α* gene SNPs

A total of three polymorphisms were analyzed in *TNF-α* gene. These polymorphisms mapped into the promoter region of *TNF-α* gene. The SNPs position, rs numbers and nucleotide variations are shown in Table number 4.2. Map representing *TNF- α* gene and the respective SNPs is shown below.

Figure 4.23. Figure showing SNPs in the promoter region of *TNF-α* gene

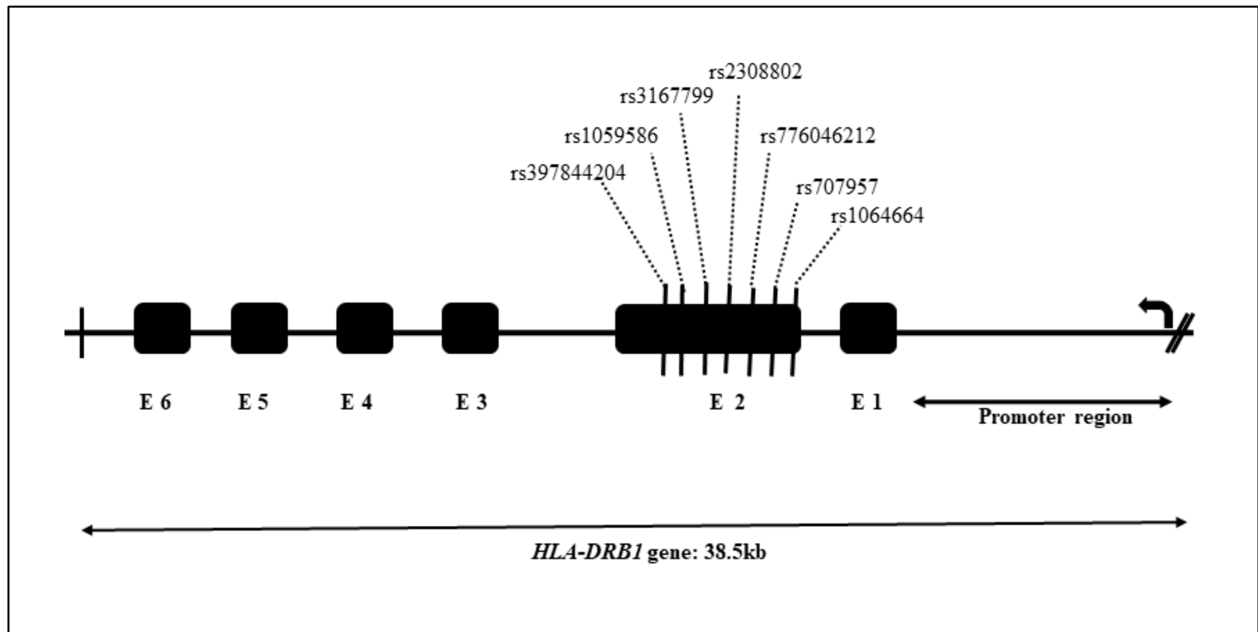


This map shows three SNPs in the promoter region of *TNF-α* gene. Double oblique cross shows start of the gene while the bend arrow shows the promoter and direction of the gene. Dotted line represents the identified SNPs along with their rs numbers, while black blocks represents the Exons of the gene denoted as E1,E4. Gap between each exon shows the introns while a single cross at the last shows that ending of the gene. The *TNF-α* gene is 3kb long (ensemble data base).

4.2.4.2. Mapping of *HLA-DRB1* gene SNPs

In the *HLA-DRB1* gene, seven SNPs were observed in this study. All of these SNPs mapped into the exon 2 of the *HLA-DRB1* gene. Information regarding the position of SNPs on chromosome 6 and reference SNP number are available in the Table. no. 2. Generated map for *HLA-DRB1* gene is shown in Figure no. 4.24.

Figure 4.24. Figure showing SNPs in Exon 2 of *HLA-DRB1* gene.

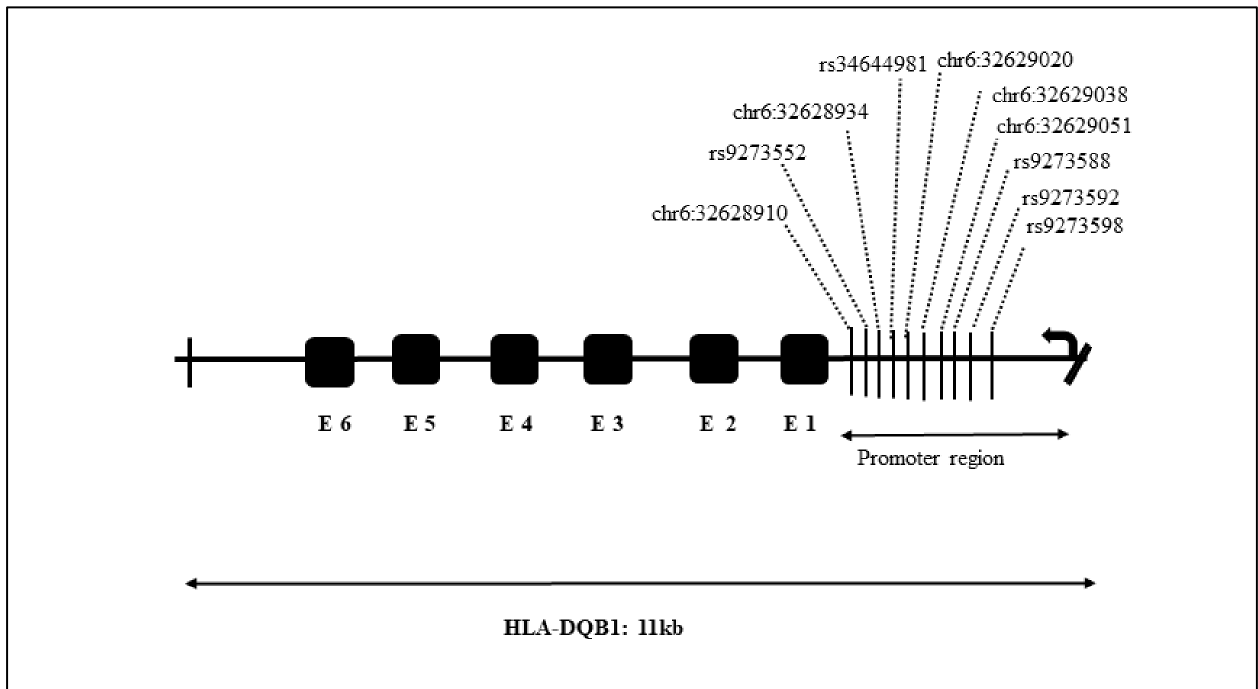


This map shows three SNPs in exon 2 of *HLA-DRB1* gene. Double oblique lines shows start of the gene while the bend arrow shows the promoter and direction of the gene. Dotted line represents the identified SNPs along with their rs numbers, while black blocks represents the Exons of the gene denoted as E1…E6. Gap between each exon shows the introns while a single cross at the last shows that ending of the gene. The *HLA-DRB1* gene is 38kb long (ensemble data base).

4.2.4.3 Mapping of *HLA-DQB1* gene SNPs

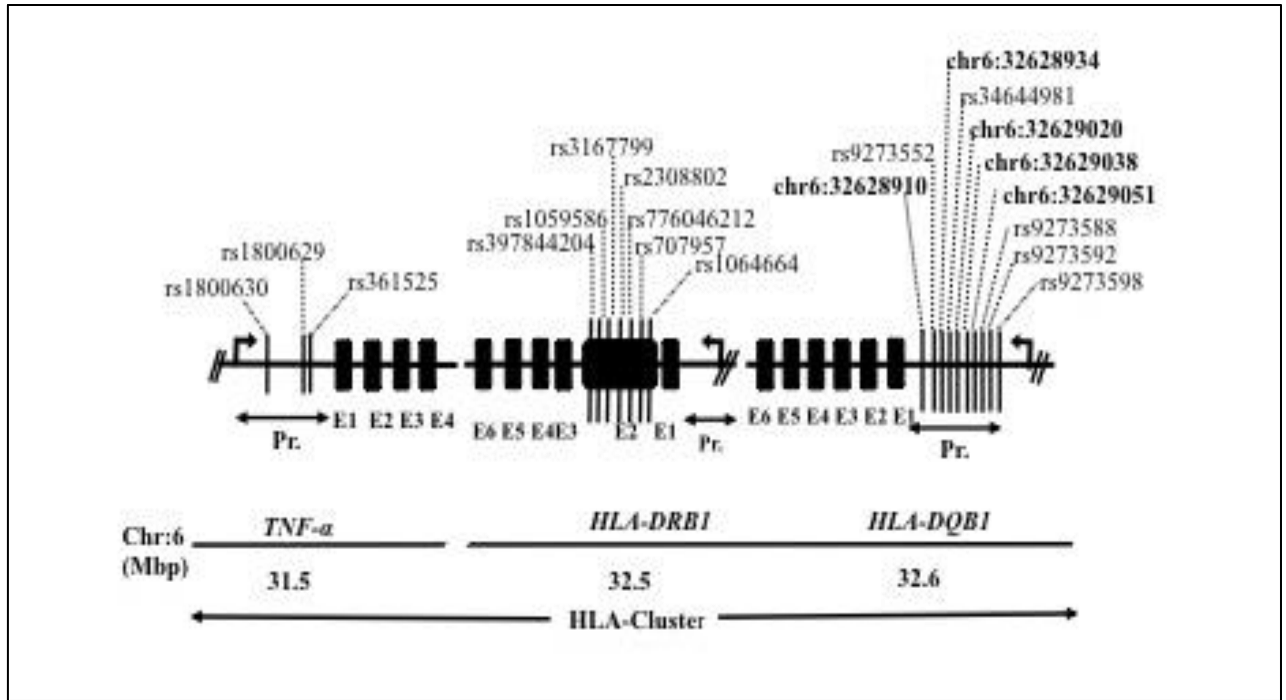
In the *HLA-DQB1* gene 10 SNPs were observed in this study. All of these SNPs mapped to the promoter region of *HLA-DQB1* gene. Information regarding the position of SNPs on chromosome 6 and reference SNP number are available in the Table. no. 2. Generated map for *HLA-DQB1* gene is shown below in Figure no. 4.25.

Figure 4.25. Figure showing SNPs in promoter region of *HLA-DQB1* gene



This map shows three SNPs in the promoter region of *HLA-DQB1* gene. Double oblique cross shows start of the gene while the bend arrow shows the promoter and direction of the gene. Dotted line represents the identified SNPs along with their rs numbers, while black blocks represents the Exons of the gene denoted as E1…E6. Gap between each exon shows the introns while a single cross at the last shows that ending of the gene. The *HLA-DQB1* gene is 11 kb long (ensemble data base).

Figure 4.26. Combined map of all genes showing observed SNPs in the analyzed genes in this study



Arrows indicate the direction of reading frame of gene. Novel SNPs detected at *DQB1*are shown in boldface. E= Exon; Pr.= promoter.

4.3. Patient genotype determination

Genotypes of the analyzed patients were determined by analyzing the nucleotides on the both sequenced DNA strands. Each group of patients were analyzed and compared for any association with HCV disease outcome.

4.3.1. Genotype distribution in responsive (R) and non-responsive (NR) patients

4.3.1.1. *TNF-α*-238, -308, -863 genotype distributions

Prevalence of genotypes at -238, -308 and -863 positions in *TNF-α* gene revealed no significant association with anti-HCV therapy outcome in studied patients. Data regarding probability values, odd ratios and confidence interval is shown in Table number 4.4.

Table 4.4. *TNF- α* genotypes distribution in responsive and non-responsive patients

Locus	**Genotype**	**Responsive; n (%)**	**Non-Responsive; n (%)**	***p* value**	**OR**	**95% CI**
TNF- α						
-238 G/A	GG	44 (88)	32 (89)	-	-	-
	GA	3 (6)	3 (9)	0.706	1.37	0.260 to 7.262
	AA	3 (6)	1 (2)	0.498	0.45	0.045 to 4.613
-308 G/A	GG	46 (90)	30 (90)	-	-	-
	GA	2 (4)	2 (5)	0.675	1.53	0.204 to 11.49
	AA	3 (6)	2 (5)	0.981	1.02	0.161 to 6.487
-863C/A	CC	33 (61)	20 (59)	-	-	-
	CA	12 (22)	4 (11)	0.384	0.55	0.155 to 1.941
	AA	9 (17)	10 (30)	0.285	1.83	0.636 to 5.283

p<0.05 considered significant, *p* value determined via chi square distribution. No significant associations were found at analyzed positions.

4.3.1.2. *HLA-DRB1* genotype distribution in responsive and non-responsive patients

4.3.1.2.1. *HLA-DRB1*6151, 6167 and 6192 positions genotype distribution in responsive and non-responsive patients

Genotype distribution in the *HLA-DRB1* gene 6151, 6167 and 6192positions for the therapy group showed no significant association with therapy outcome, data shown in Table. no. 4.5.

Table 4.5. *HLA-DRB1*6151, 6167 and 6192 genotype prevalence in responsive and non-responsive patients

Locus	Genotype	Responsive; n (%)	Non-Responsive; n (%)	*p* value	OR	95% CI
HLA-DRB1						
6151 (T/C)	TC	19 (36)	8 (24)	-	-	-
	TT	15 (28)	12 (35)	0.259	1.90	0.618 to 5.836
	CC	19 (36)	14 (41)	0.306	1.75	0.596 to 5.137
6167 (A/C/T)	AA	25 (50)	22 (65)	-	-	-
	AC	7 (14)	2 (6)	0.172	0.32	0.060 to 1.730
	AT	15 (30)	6 (18)	0.059	0.35	0.1164 to 1.065
	CT	2 (4)	2 (5)	-	-	-
	TT	1 (2)	2 (6)	-	-	-
6192 (T/C)	CC	48 (88.8)	34 (94.5)	-	-	-
	CT	6 (11.2)	2 (5.55)	0.364	0.47	0.089 to 2.475

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.1.2.2. *HLA-DRB1* 6228, 6231, 6236, 6251 genotype distribution in responsive and non-responsive patients

Data analysis for 6228, 6231, 6236 and 6251 position did not revealed any significant association for genotype prevalence in therapy patients. Comparisons were made showing *p* values, confidence intervals shown in Table 4.6.

Table 4.6. *HLA-DRB1* 6228, 6231, 6236, 6251 position genotype prevalence in R and NR patients

Locus	Genotype	Responsive; n (%)	Non-Responsive; n (%)	*p* value	OR	95% CI
HLA-DRB1						
6228 (C/T)	CC	30 (59)	14 (39)	-	-	-
	CT	4 (8)	3 (8)	0.565	1.60	0.316 to 8.172
	TT	17 (33)	19 (53)	0.050	2.39	0.962 to 5.959
6231 (T/C/G)	CC	12 (24)	7 (20)	-	-	-
	CG	8 (16)	3 (9)	0.592	0.64	0.127 to 3.255
	CT	8 (16)	7 (20)	0.563	1.50	0.378 to 5.950
	GG	5 (10)	3 (9)	0.974	1.02	0.186 to 5.679
	TA	1 (2)	0 (0)	-	-	-
	TG	9 (18)	8 (22)	0.534	1.52	0.401 to 5.779
	TT	8 (16)	7 (20)	0.563	1.50	0.371 to 5.950
6236 (A/C)	AA	37 (69)	28(82)	-	-	-
	AC	15 (28)	4 (12)	0.563	1.50	0.378 to 5.950
	CC	1 (2)	2 (6)	0.082	0.35	0.105 to 1.179
6251(A/T)	AA	45 (85)	28 (82)	-	-	-
	AT	7 (13)	6 (18)	0.596	1.37	0.419 to 4.521
	TT	1 (2)	0 (0)	-	-	-

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.1.3. *HLA-DQB1* genotype distribution in responsive and non-responsive patients

4.3.1.3.1. *HLA-DQB1* -8288, -8298 and -8307 genotypes distribution in R and NR patients

Distribution of genotypes at this position revealed that none of the observed genotype was significantly associated with therapy response in studied patients (Table 4.7).

Table 4.7. Distribution of genotypes in *HLA-DQB1* gene at-8288, -8298 and -8307 position in R and NR patients

Locus	**Genotype**	**Responsive n (%)**	**Non-Responsive n (%)**	***p* value**	**OR**	**95% CI**
HLA-DQB1						
-8288(G/C)	CC	39 (71)	23 (70)	-	-	-
	CG	13 (24)	7 (21)	0.865	0.91	0.318 to 2.619
	GG	3 (5)	3 (9)	0.534	1.69	0.315 to 9.113
-8298(G/C)	CC	34 (63)	17 (52)	-	-	-
	CG	13 (24)	11 (33)	0.296	1.69	0.627 to 4.564
	GG	7 (13)	5 (15)	0.585	1.42	0.394 to 5.175
-8307(A/G/C)	AA	20 (37)	12 (36)	-	-	-
	AC	3 (5)	5 (15)	0.200	2.77	0.560 to 13.77
	AG	5 (9)	1 (3)	0.323	0.33	0.034 to 3.206
	CC	5 (9)	1 (3)	0.323	0.33	0.034 to 3.206
	GC	3 (6)	4 (12)	0.338	2.22	0.422 to 11.6
	GG	19 (34)	10 (30)	0.806	0.87	0.307 to 2.503

p<0.05 considered significant, *p* value determined via chi square distribution.

4.3.1.3.2. *HLA-DQB1* -8331, -8344 and -8362 genotypes distribution in R and NR patients

Statistical analysis for genotype distribution at -8331, -8344 and -8362 positions in the *HLA-DQB1* gene revealed higher *p* values (p>0.05), and hence were not statistically significant. None of the genotype in these positions was responsible for the therapy outcome in studied patients (Table 4.8).

Table 4.8. Genotype prevalence at -8331, -8344 and -8362 positions in R and NR patients

Locus	Genotype	Responsive n (%)	Non-Responsive n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8331(T/G)	TT	26 (49)	21 (64)	-	-	-
	TG	14 (26)	7 (21)	0.379	0.61	0.211 to 1.813
	GG	13 (25)	5 (15)	0.213	0.47	0.146 to 1.552
-8344(C/T)	TT	37 (69)	21 (64)	-	-	-
	TC	10 (18)	8 (24)	0.529	1.41	0.482 to 4.122
	TT	37 (69)	21 (64)	-	-	-
-8362 (G/A/C)	AA	25 (47)	14 (44)	-	-	-
	AC	5 (9)	4 (13)	0.633	1.42	0.328 to 6.207
	AG	2 (5)	2 (6)	0.578	1.78	0.226 to 14.11
	CC	14 (26)	7 (22)	0.663	0.59	0.056 to 6.282
	CG	3 (6)	1 (3)	0.454	1.78	0.385 to 8.271
	GG	4 (7)	4 (12)	0.454	1.78	0.385 to 8.271

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.1.3.3. Genotype distribution at *HLA-DQB1* -8442, -8447, -8465, -8471 in R and NR patients.

Analysis of genotypes for the above mentioned positions were determined and once again it was confirmed that there was no significant association between genotypes and development of therapy response in studied patients. Data shown in Table no. 4.9.

Table 4.9. Genotype prevalence at -8442, -8447, -8465, -8471 positions in R and NR patients

Locus	Genotype	Responsive n (%)	Non-Responsive n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8442(G/C)	GG	32 (58)	17 (52)	-	-	-
	GC	17 (31)	15 (46)	0.272	1.66	0.668 to 4.127
	CC	6 (11)	1 (2)	0.279	0.31	0.034 to 2.825
-8447 (C/A/T/G)	CC	21 (39)	17 (52)	-		-
	CA	5 (9)	4 (12)	0.987	0.98	0.229 to 4.266
	CG	2 (4)	4 (12)	0.317	2.47	0.402 to 15.16
	TA	3 (6)	0 (0)	-	-	-
	TC	11 (20)	2 (6)	0.050	0.22	0.043 to 1.155
	TG	1 (2)	0 (0)	-		-
	TT	11 (20)	6 (18)	0.511	0.67	0.206 to 2.199
-8465(G/A)	GG	39 (72)	22 (67)	-	-	-
	GA	14 (26)	8 (24)	0.980	1.01	0.367 to 2.792
	AA	0 (0)	3 (9)	-	-	-
	GC	1 (2)	0 (0)	-	-	-
-8471 (A/G/C)	AA	31 (57)	13 (39)	-	-	-
	AG	7 (13)	5 (15)	0.425	1.70	0.455 to 6.364
	CA	0 (0)	0 (0)	-	-	-
	GC	0 (0)	1 (3)	-	-	-
	GG	16 (30)	14 (43)	0.133	2.08	0.793 to 5.486

$p<0.05$ considered significant, *p* value determined via chi square distribution. TC/CC genotypes were predictors for responsiveness to anti-HCV therapy

4.3.2. Genotype distribution in Chronically infected (CI) and Spontaneously recovered (SR) patients

4.3.2.1. *TNF-α* genotype distribution at -238, -308 and -863 position in SR and CI patients

Table number 4.10 represents the distribution of *TNF-α* gene variation at the above-mentioned positions and it was again revealed that none of the genotype was playing any kind of role in chronic infection or spontaneous recovery of studied patients.

Table 4.10. Genotype distribution at -238, -308and -863 positions in SR and CI patients

Locus	Genotype	Spontaneously recovered n (%)	Chronically infected n (%)	*p* value	OR	95% CI
TNF- α						
-283(G/A)	GG	20 (91)	58 (89)	-	-	-
	GA	2 (9)	3 (4)	0.480	0.51	0.080 to 3.324
	AA	0 (0)	4 (7)	-	-	-
-308(G/A)	GG	17 (81)	54 (86)	-	-	-
	GA	1 (5)	2 (3)	0.710	0.62	0.053 to 7.386
	AA	3 (14)	7 (11)	0.677	0.73	0.170 to 3.158
-863(C/A)	CC	15 (75)	35 (55)	-	-	-
	CA	3 (15)	18 (28)	0.164	2.57	0.657 to 10.06
	AA	2 (10)	11 (17)	0.290	2.35	0.464 to 11.96

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.2.2. *HLA-DRB1* genotype distribution in SR and CI patients

4.3.2.2.1. Genotype distribution in *HLA-DRB1* 6151, 6167 and 6192 positions in SR and CI patients

Association of genotypes at 6151, 6167 and 6192 positions with chronic HCV infection and spontaneous recovery was established and no significant association was found in the analyzed set of patients, data shown in Table number 4.11

Table 4.11. Genotype prevalence at 6151, 6167 and 6192 positions in SR and CI patients

Locus	Genotype	Spontaneously recovered n (%)	Chronically infected n (%)	*p* value	OR	95% CI
HLA-DRB1						
6151 (T/C)	TC	8 (35)	25 (38)	-	-	-
	TT	8 (35)	25 (38)	0.990	1.00	0.324 to 3.084
	CC	7 (30)	15 (24)	0.536	0.68	0.206 to 2.276
6167 (A/C/T)	AA	11 (53)	34 (53)	-	-	-
	AC	1 (5)	6 (9)	0.552	1.94	0.210 to 17.95
	AT	9 (42)	19 (30)	0.473	0.68	0.240 to 1.942
	CT	0 (0)	3 (5)	-	-	-
	TT	0 (0)	2 (3)	-	-	-
6192 (T/C)	CC	20 (91)	57 (88)	-	-	-
	CT	2 (9)	8 (12)	0.682	1.40	0.274 to 7.174

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.2.2.2. Genotype distribution in *HLA-DRB1* 6228, 6231, 6236 and 6251 positions in SR and CI patients

None of the analyzed genotypes at these positions was found to be significantly associated with HCV disease outcome in shape of CI and SR, data shown in Table 4.12.

Table 4.12. Genotype prevalence at 6228, 6231 and 6236 and 6251 positions in SR and CI patients

Locus	Genotype	Spontaneously recovered n (%)	Chronically infected n (%)	*p* value	OR	95% CI
HLA-DRB1						
6228 (C/T)	CC	11 (48)	32 (50)	-	-	-
	CT	3 (13)	5 (8)	0.487	0.57	0.117 to 2.802
	TT	9 (39)	27 (42)	0.952	1.03	0.372 to 2.858
6231 (T/C/G)	CC	2 (9)	12 (19)	-	-	-
	CG	5 (23)	13 (20)	0.359	0.43	0.070 to 2.670
	CT	6 (27)	10 (16)	0.151	0.27	0.045 to 1.693
	GG	3 (13)	10 (16)	0.273	0.36	0.058 to 2.293
	TA	0 (0)	0 (0)	-	-	-
	TG	5 (23)	11 (17)	0.825	1.33	0.102 to 17.29
	TT	1 (5)	8 (13)	0.825	1.33	0.102 to 17.29
6236 (A/C)	AA	14 (61)	48 (75)	-	-	-
	AC	8 (35)	15 (23)	0.253	0.54	0.192 to 1.554
	CC	1 (4)	1 (2)	-	-	-
6251(A/T)	AA	21 (91)	57 (89)	-	-	-
	AT	2 (9)	7 (11)	0.762	1.28	0.2477 to 6.712
	TT	0 (0)	0 (0)	-	-	-

p<0.05 considered significant, *p* value determined via chi square distribution.

4.3.2.3. *HLA-DQB1* genotype distribution in SR and CI patients

4.3.2.3.1. *HLA-DQB1* -8288, -8298 and -8307 genotype distribution in SR and CI patients

Distribution of genotypes at above mentioned positions were investigated but no significant associations were found among the HCV infection outcomes and patient genotypes (Table 4.13).

Table 4.13. Genotype prevalence at -8288, -8298 and -8307positions in SR and CI patients

Locus	**Genotype**	**Spontaneously recovered n (%)**	**Chronically infected n (%)**	***p* value**	**OR**	**95% CI**
HLA-DQB1						
-8288(G/C)	CC	17 (77)	47 (73)	-	-	-
	CG	5 (23)	10 (16)	0.598	0.72	0.216 to 2.423
	GG	0 (0)	7 (11)	-	-	-
-8298(G/C)	CC	18 (82)	45 (70)	-	-	-
	CG	4 (18)	14 (22)	0.593	1.44	0.405 to 4.831
	GG	0 (0)	5 (8)	-	-	-
-8307(A/G/C)	AA	6 (29)	23 (38)	-	-	-
	AC	3 (14)	8 (13)	0.652	0.69	0.140 to 3.456
	AG	5 (24)	10 (17)	0.358	0.52	0.128 to 2.116
	CC	2 (9)	3 (5)	0.347	0.39	0.052 to 2.89
	GC	1 (5)	5 (8)	0.822	1.30	0.127to 13.38
	GG	4 (19)	11 (19)	0.653	0.71	0.167 to 3.075

$p<0.05$ considered significant, p value determined via chi square distribution.

4.3.2.3.2. *HLA-DQB1* -8331, -8344 and -8362 genotype distribution in SR and CI patients

Genotype prevalence at -8331 and -8344 were not significantly associated with HCV infection outcome in studied patients. Genotype CC at position -8362 (G/A/C) was significantly associated with HCV infection outcome in studied patients with p value of 0.035, odd ratios of 0.12 and 95% confidence interval of 0.049 to 0.950 respectively. Data shown in Table 4.14.

Table 4.14. Genotype prevalence at -8331, -8344 and -8362positions in SR and CI patients

Locus	Genotype	Spontaneously recovered n (%)	Chronically infected n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8331(T/G)	TT	10 (45)	37 (62)	-	-	-
	TG	8 (36)	11 (18)	0.085	0.37	0.117 to 1.171
	GG	4 (19)	12 (20)	0.757	0.81	0.214 to 3.066
-8344(C/T)	TT	13 (57)	38 (63)	-	-	-
	TC	6 (26)	16 (27)	0.873	0.91	0.294 to 2.825
	CC	4 (17)	6 (10)	0.349	0.51	0.124 to 2.109
-8362 (G/A/C)	AA	8 (36)	21 (35)	**-**	**-**	**-**
	AC	2 (9)	4 (7)	0.776	0.76	0.115 to 5.009
	AG	4 (18)	7 (12)	0.588	0.66	0.152 to 2.912
	CC	**7 (32)**	**14 (23)**	**0.035**	**0.21**	**0.049 to 0.950**
	CG	1 (5)	2 (3)	0.831	0.76	0.060 to 9.617
	GG	0 (0)	12 (20)	-	-	-

p<0.05 considered significant, *p* value determined via chi square distribution. Genotypes AA and CC were associated with SR and CI in analyzed patients.

4.3.2.3.3. *HLA-DQB1* -8442, -8447, -8465 and -8471 genotype distribution in SR and CI patients

None of the genotype at the studied positions were significantly associated with CI and SR in analyzed set of patients. Data shown in Table 4.15.

Table 4.15 Genotype prevalence at -8442, -8447, -8465 and -8471 positions in SR and CI patients

Locus	Genotype	Spontaneously recovered n (%)	Chronically infected n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8442(G/C)	GG	9 (39)	32 (52)	-	-	-
	GC	12 (52)	21 (34)	0.171	0.49	0.176 to 1.372
	CC	2 (9)	8 (14)	0.893	1.12	0.202 to 6.266
-8447 (C/A/T/G)	CC	6 (26)	20 (34)	-	-	-
	CA	4 (17)	5 (8)	0.221	0.37	0.075 to 1.859
	CG	3 (13)	2 (3)	0.958	0.20	0.026 to 1.491
	TA	2 (9)	4 (8)	0.601	0.60	0.087 to 4.123
	TC	3 (13)	12 (21)	0.818	1.20	0.252 to 5.712
	TG	1 (4)	1 (1)	0.871	1.12	0.268 to 4.709
	TT	4 (18)	15 (25)	0.871	1.12	0.268 to 4.709
-8465(G/A)	GG	16 (73)	51 (84)	-	-	-
	GA	5 (23)	10 (16)	0.448	0.62	0.186 to 2.108
	AA	0 (0)	0 (0)	-	-	-
	GC	1 (4)	0 (0)	-	-	-
-8471 (A/G/C)	AA	13 (59)	16 (28)	-	-	-
	AG	0 (0)	15 (26)	-	-	-
	CA	0 (0)	1 (2)	-	-	-
	GC	0 (0)	0 (0)	-	-	-
	GG	9 (41)	25 (44)	0.127	2.27	0.784 to 6.493

p<0.05 considered significant, *p* value determined via chi square distribution.

4.3.3. Gender based genotypes distribution in studied patients

4.3.3.1. *TNF-α* genotypes distribution in gender

4.3.3.1.1. *TNF-α* -238, -308 and -863 genotype distributions in male and females

Gender based genotype distributions at 238, -308 and -863 positions revealed no significant associations, data shown in Table 4.16.

Table 4.16. *TNF-α* -238, -308 and -863genotype distribution in male and female

Locus	Genotype	Male n (%)	Female n (%)	*p* value	OR	95% CI
TNF- α						
-283(G/A)	GG	77 (91)	77 (87)	-	-	-
	GA	6 (7)	5 (6)	0.770	0.83	0.244 to 2.847
	AA	2 (2)	6 (7)	0.167	3.00	0.586 to 15.34
-308(G/A)	GG	77 (91)	77 (87)	-	-	-
	GA	6 (7)	5 (6)	0.673	0.72	0.155 to 3.331
	AA	2 (2)	6 (7)	0.191	0.48	0.156 to 1.473
-863(C/A)	CC	47 (55)	56 (64)	-	-	-
	CA	21 (25)	16 (18)	0.245	0.63	0.299 to 1.364
	AA	17 (20)	15 (18)	0.458	0.74	0.334 to 1.641

p<0.05 considered significant, *p* value determined via chi square distribution.

4.3.3.2. *HLA-DRB1* genotypes distribution in males and females

4.3.3.2.1. *HLA-DRB1* 6151, 6167 and 6192 genotype distribution in males and females

Genotype distribution at 6151(T/C) position revealed that genotype TT (p=0.044, OR=0.47, 95%CI: 0.229-0.985) and CC (p=0.033, OR=0.446, 95% CI: 0.211-0.943) were significantly associated with gender based HCV infection in analyzed set of patients. Genotype frequencies for 6167 and 6192 were not significantly associated with gender based HCV infection. Data for these set of SNPs is shown in Table 4.17

Table 4.17. *HLA-DRB1* 6151, 6167 and 6192 genotype distribution in male and females

Locus	Genotype	Male n (%)	Female n (%)	*p* value	OR	95% CI
HLA-DRB1						
6151 (T/C)	TC	23 (26)	37 (43)	**-**	**-**	**-**
	TT	**34 (38)**	**26 (30)**	**0.044,S**	**0.47**	**0.229 to 0.985**
	CC	**32 (36)**	**23 (27)**	**0.033,S**	**0.44**	**0.211 to 0.943**
6167 (A/C/T)	AA	41 (48)	51 (61)	-	-	-
	AC	8 (9)	8 (10)	0.686	0.80	0.277 to 2.327
	AT	29 (34)	20 (24)	0.098	0.55	0.274 to 1.120
	CT	4 (5)	3 (3)	0.159	0.60	0.127 to 2.849
	TT	3 (4)	2 (2)	0.499	0.08	0.085 to 3.36
6192 (T/C)	CC	81 (90)	78 (90)	-	-	-
	CT	9 (10)	9 (10)	0.939	1.03	0.391 to 2.753

p<0.05 considered significant, *p* value determined via chi square distribution. S=Significant. Genotypes at 6151 (T/C) position were associated with HCV infection in both male and female patients.

4.3.3.2.2. *HLA-DRB1* 6228, 6231, 6236 and 6251 genotype distribution in males and females

Data for these positions revealed that genotype CC at 6231 position was significantly associated with HCV infection in both male and female patients (p=0.026, OR=0.326, 95% CI: 0.119-0.890). Genotype frequencies for the rest of positions were not significantly associated with infection in both male and female patient (Table 4.18).

Table 4.18. *HLA-DRB1* 6228, 6231, 6236 and 6251 genotype distribution in males and females patients

Locus	Genotype	Male n (%)	Female n (%)	*p* value	OR	95% CI
HLA-DRB1						
6228 (C/T)	CC	51 (58)	36 (42)	-	-	-
	CT	5 (6)	10 (11)	0.069	2.88	0.892 to 8.997
	TT	32 (36)	40 (47)	0.150	1.57	0.846 to 2.927
6231 (T/C/G)	CC	12 (14)	21 (24)	**-**	**-**	**-**
	CG	12 (14)	17 (20)	0.685	0.80	0.290 to 2.255
	CT	15 (17)	16 (20)	0.330	0.60	0.224 to 1.657
	GG	11 (13)	10 (12)	0.245	0.51	0.170 to 1.580
	TA	1 (1)	0 (0)	-	-	-
	TG	**21 (24)**	**12 (12)**	**0.026, S**	**0.32**	**0.119 to 0.890**
	TT	14 (17)	10 (12)	0.999	0.40	0.138 to 1.200
6236 (A/C)	AA	60 (68)	67 (78)	-	-	-
	AC	25 (28)	17 (20)	0.167	0.60	0.300 to 1.236
	CC	3 (4)	2 (2)	0.575	0.59	0.096 to 3.697
6251(A/T)	AA	72 (83)	79 (91)	-	-	-
	AT	15 (17)	7 (8)	0.0724	0.42	0.164 to 1.103
	TT	0 (0)	1 (1)	-	-	-

p<0.05 considered significant, *p* value determined via chi square distribution.
S=Significant. Genotype CC and TG at 6231 position were associated with HCV infection in both genders.

4.3.3.3. Gender based *HLA-DQB1* genotypes distribution

4.3.3.3.1. *HLA-DQB1* -8288, -8298 and -8307 positionsgenotypes distribution in males and females

Genotype prevalence in male and female patients at these above-mentioned positions revealed no significant association with gender based HCV infection. Statistical analysis is shown in the Table 4.19.

Table 4.19. Prevalence of genotypes at -8288, -8298 and -8307 positions in male and female patients

Locus	Genotype	Male n (%)	Female n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8288(G/C)	CC	61 (68)	65 (76)	-	-	-
	CG	21 (24)	14 (16)	0.225	0.62	0.292 to 1.340
	GG	7 (8)	6 (8)	0.709	0.80	0.255 to 2.529
-8298(G/C)	CC	58 (65)	56 (67)	-	-	-
	CG	23 (26)	19 (23)	0.666	0.85	0.420 to 1.740
	GG	8 (9)	9 (10)	0.769	1.16	0.419 to 3.234
-8307(A/G/C)	AA	29 (36)	32 (38)	-	-	-
	AC	7 (8)	12 (14)	0.413	1.55	0.538 to 4.48
	AG	10 (11)	11 (13)	0.995	0.99	0.369 to 2.691
	CC	6 (7)	5 (6)	0.668	0.75	0.208 to 2.741
	GC	7 (8)	6 (7)	0.679	.776	0.233 to 2.581
	GG	25 (30)	19 (22)	0.348	0.68	0.315 to 1.503

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.3.3.2 *HLA-DQB1* -8331, -8344 and -8362 positionsgenotypes distribution in males and females

Genotype prevalence at 8331, -8344 and -8362 positions showed no significant association with infection in both male and female patients. Statistical analysis is shown in Table number 4.20.

Table 4.20. Prevalence of genotypes at -8331, -8344 and -8362 positions in male and female patients

Locus	Genotype	Male n (%)	Female n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8331(T/G)	TT	46 (54)	48 (58)	-	-	-
	TG	23 (27)	17 (20)	0.364	0.70	0.335 to 1.494
	GG	16 (19)	18 (22)	0.851	1.07	0.491 to 2.365
-8344(C/T)	TT	56 (66)	53 (63)	-	-	-
	TC	16 (19)	24 (28)	0.218	1.58	0.759 to 3.308
	CC	13 (15)	8 (9)	0.376	0.65	0.249 to 1.694
-8362 (G/A/C)	AA	37 (44)	31 (38)	-	-	-
	AC	7 (8)	8 (10)	0.586	1.36	0.444 to 4.186
	AG	11 (13)	4 (5)	0.179	0.43	0.125 to 1.500
	CC	19 (22)	23 (28)	0.349	1.44	0.667 to 3.129
	CG	3 (3)	4 (5)	0.559	1.59	0.330 to 7.661
	GG	8 (9)	12 (15)	0.257	1.79	0.649 to 4.936

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.3.3.3. *HLA-DQB1* -8442, -8447, -8465 and -8471 positions genotypes distribution in males and females

These associations revealed that there is no significant association of genotypes at -8442, -8447, -8465 and -8471 positions with HCV infection in both male and female patients. Data for these positions is shown in Table 4.21.

Table 4.21. Genotype distribution at -8442, -8447, -8465 and -8471 positions and their role in infection in male and female patients

Locus	Genotype	Male n (%)	Female n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8442(G/C)	GG	47 (53)	43 (51)	-	-	-
	GC	35 (40)	30 (36)	0.841	0.93	0.494 to 1.776
	CC	6 (7)	11 (13)	0.200	2.00	0.682 to 5.886
-8447 (C/A/T/G)	CC	32 (38)	32 (38)	-	-	-
	CA	11 (13)	7 (8)	0.404	0.63	0.218 to 1.850
	CG	6 (7)	5 (6)	0.780	0.83	0.230 to 3.010
	TA	4 (5)	5 (6)	0.754	1.25	0.307 to 5.087
	TC	13 (15)	15 (18)	0.752	1.15	0.473 to 2.810
	TG	0 (0)	3 (4)	-	-	-
	TT	18 (22)	18 (20)	0.999	1.00	0.441 to 2.263
-8465(G/A)	GG	63 (73)	65 (78)	-	-	-
	GA	20 (23)	17 (20)	0.604	0.82	0.395 to 1.716
	AA	1 (1)	2 (2)	0.586	1.93	0.171 to 21.93
	GC	2 (3)	0 (0)	-	-	-
-8471 (A/G/C)	AA	38 (46)	35 (42)	-	-	-
	AG	16 (19)	11 (13)	0.521	0.74	0.305 to 1.826
	CA	0 (0)	1 (2)			
	GC	1 (1)	0 (0)	-	-	-
	GG	28 (34)	36 (43)	0.331	1.39	0.711 to 2.740

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.4. Allelic distribution in different groups of patients

Allelic distribution in the studied group of patients was carried out and the association of each allele with the disease outcome was determined. Chi square distributions were carried out to calculate probability values, odd ratios and confidence intervals. The allelic distribution in various group of patients are discussed below.

4.3.4.1. Allelic distribution in the therapy group

4.3.4.1.1. Allelic distribution of *TNF-α* in responsive (R) and non-responsive (NR) patients

Allelic variations were analyzed at three different positions in *TNF* gene, -238 (G/A), -308 (G/A) and -863 (C/A). Statistical analysis revealed no significant association between alleles observed and the anti-HCV therapy outcome. Data is shown in Table 4.22.

Table 4.22. Allelic distributions of *TNF-α* gene in the responsive and non-responsive patients

Locus	Allele	Responsive; n (%)	Non-responsive; n (%)	*p* value	OR	95% CI
TNF-α						
-238 G/A	G	91 (91)	67 (93)		-	
	A	9 (9)	5 (7)	0.626	0.75	0.242 to 2.355
-308 G/A	G	94 (92)	62 (91)		-	
	A	8 (8)	6 (9)	0.819	1.14	0.376 to 3.438
-863 C/A	C	78 (72)	44 (65)		-	
	A	30 (28)	24 (35)	0.292	1.42	0.739 to 2.722

$p<0.05$ considered significant, *p* value determined via chi square distribution.

4.3.4.1.2. Allelic distribution of *HLA-DRB1* gene in responsive (R) and non-responsive (NR) patients

Allelic distributions were analyzed at seven different positions in *HLA-DRB1* gene (Table 4.23). Only significant associations are discussed below, whereas distributions for other analyzed alleles are shown in Table 4.23. Allelic distribution at 6228 (C/T) position revealed that allele C was prevalent in n=64 (63%) of the R and n=31 (43%) of the NR patients. The Allele T was frequent in n=38 (37%) in R and n=41 (57%) of NR patients had T allele with probability of 0.010, odd ratio of 2.23 and 95% CI of 1.204 to 4.122. These alleles were found to be significantly associated with response to interferon therapy in infected patients.

Table 4.23. Therapy based distribution of alleles in observed SNPs in *HLA-DRB1* gene

Locus	**Allele**	**Responsive; n (%)**	**Non-responsive; n (%)**	***p* value**	**OR**	**95% CI**
HLA-DRB1						
6151 (T/C)	T	53 (50)	28 (41)		-	
	C	53 (50)	40 (59)	0.254	1.43	0.772 to 2.643
6167 (A/C/T)	A	72 (72)	52 (76)		-	
	C	9 (9)	4 (6)	0.435	0.62	0.179 to 2.107
	T	19 (19)	12 (18)	0.744	0.87	0.390 to 1.958
6192 (C / T)	C	102 (94)	70 (97)		-	
	T	6 (6)	2 (3)	0.375	0.49	0.095 to 2.477
6228 (C/T)	C	64 (63)	31 (43)		-	
	T	**38 (37)**	**41 (57)**	**0.010, S**	**2.23**	**1.204 to 4.122**
6231 (T/C/ G)	C	40 (39)	24 (34)		-	
	G	27 (26)	17 (24)	0.904	1.05	0.476 to 2.313
	T	34 (33)	29 (42)	0.329	1.42	0.700 to 2.887
	A	1 (1)	0 (0)		-	
6236 (A/C)	A	89 (84)	60 (88)		-	
	C	17 (16)	8 (12)	0.433	0.69	0.283 to 1.720
6251 (A/T)	A	97 (92)	62 (91)		-	
	T	9 (8)	6 (9)	0.939	1.04	0.353 to 3.075

$p<0.05$ considered significant, p value determined via chi square distribution. Allelic distribution at 6228 position was significantly associated with response to therapy in infected patients.

4.3.4.1.3. Allelic distribution of *HLA-DQB1 gene* in responsive (R) and non-responsive (NR) patients

A total of 10 SNPs at various positions were observed in both R and NR patients. Two novel SNPs were found to be associated with the predictor of anti-HCV therapy response in studied groups of patients. Each of the observed SNP was statistically analyzed in corresponding group of patients as shown in the Table number 4.24. Only significant associations are discussed below.

i. Allelic distribution at -8447 (C/A/G/T) position revealed that allele C was prevalent in n=49 (50%) of R and n=42 (66%) in NR patients. Allele A was prevalent in 8 (9%) of R and 4 (6%) in NR patients with P value of 0.401, odd ratio of 0.58, 95% CI of 0.163 to 2.076. Allele G was frequent in n=3 (3%) of R and n=4 (6%) of NR patients (p=0.574, OR=1.55, 95% CI;0.329 to 7.351). Allele T was prevalent in n=37 (38%) and n=14 (22%) in NR patients with significant association of T allele with interferon therapy (p=0.028, OR=0.44, 95% CI; 0.210 to 0.925).

ii. Distribution of -8471 (A/G/C) position polymorphism showed that allele A was frequent in n=69 (64%) of R and 31 (47%) of NR patients. Similarly allele G was prevalent in n=39 (36%) in R and 34 (51%) in NR patients. Significant associations were found as (p=0.036) between G allele and interferon therapy. Allele C was not prevalent in R and n=1 (2%) in NR.

Table 4.24. Allelic prevalence in R and NR patients in *HLA-DQB1* gene

Locus	**Allele**	**Responsive; n (%)**	**Non-responsive; n (%)**	***p* value**	**OR**	**95% CI**
HLA-DQB1						
-8288 (C/G)	C	91 (83)	53 (80)		-	
	G	19 (17)	13 (20)	0.686	1.17	0.537 to 2.570
-8298 (C/G)	C	81 (76)	45 (68)		-	
	G	27 (24)	21 (32)	0.328	1.4	0.711 to 2.755
-8307 (A/C/G)	A	48 (44)	30 (45)		-	
	C	16 (14)	11 (17)	0.834	1.1	0.450 to 2.687
	G	46 (42)	25 (38)	0.681	0.86	0.446 to 1.695
-8331 (T/G)	T	66 (62)	49 (74)		-	
	G	40 (38)	17 (26)	0.104	0.57	0.290 to 1.127
-8344 (T/C)	T	84 (78)	50 (76)		-	
	C	24 (22)	16 (24)	0.758	1.12	0.543 to 2.308
-8362 (A/C/G)	A	57 (53)	34 (53)		-	
	C	36 (34)	19 (30)	0.731	0.88	0.439 to 1.781
	G	13 (13)	11 (17)	0.449	1.41	0.571 to 3.519
-8442 (G/C)	G	81 (74)	49 (74)		-	
	C	29 (26)	17 (26)	0.929	0.96	0.483 to 1.944
-8447 (C/A/G/T)	C	49 (50)	42 (66)		-	
	A	8 (9)	4 (6)	0.401	0.58	0.163 to 2.076
	G	3 (3)	4 (6)	0.574	1.55	0.329 to 7.351
	T	**37 (38)**	**14 (22)**	**0.028**	**0.44**	**0.210 to 0.925**
-8465 (G/A/C)	G	93 (86)	52 (79)		-	
	A	14 (13)	14 (21)	0.158	1.78	0.791 to 4.041
	C	1 (1)	0 (0)		-	
-8471 (A/G)	A	69 (64)	31 (47)		-	
	G	39 (36)	34 (51)	0.036	1.94	1.038 to 3.627

p<0.05 considered significant, *p* value determined via chi square distribution.

Distribution of alleles at -8447 position in *HLA-DQB1* gene was related to response against anti-HCV therapy in studied patients.

4.3.4.2. Allelic distribution in spontaneously recovered (SR) and chronically infected (CI) patients

4.3.4.2.1. Allelic distribution of *TNF-α* in spontaneously recovered (SR) and chronically infected (CI) patients

No significant associations were found between *TNF-α* promoter polymorphism and HCVinfection or treatment response.Data shown in Table 4.25

Table 4.25. *TNF- α* genetic polymorphisms in SR and CI patients

Locus	**Allele**	**Spontaneously Recovered; n (%)**	**Chronically Infected; n (%)**	***p* value**	**OR**	**95% CI**
TNF-α						
-238 G/A	G	42 (96)	119 (92)		-	
	A	2 (4)	11 (8)	0.393	1.94	0.413 to 9.123
-308 G/A	G	35 (83)	110 (87)		-	
	A	7 (17)	16 (13)	0.517	0.72	0.276 to 1.912
-863 C/A	C	33 (82)	88 (69)		-	
	A	7 (18)	40 (31)	0.090	2.14	0.873 to 5.257

p<0.05 considered significant, *p* value determined via chi square distribution

4.3.4.2.2. Allelic distribution of *HLA-DRB1* in spontaneously recovered (SR) and chronically infected (CI) patients

None of the studied alleles in this gene were significantly associated with spontaneous recovery or chronic HCV infection in studied patients. Allelic distribution at the *HLA-DRB1* gene is shown in Table 4.26.

Table 4.26. Allelic distribution *HLA-DRB1* in spontaneously recovered (SR) and chronically infected patients

Locus	Allele	Spontaneously Recovered; n (%)	Chronically Infected; n (%)	*p* value	OR	95% CI
HLA-DRB1						
6151 (T/C)	T	24 (52)	75 (58)		-	
	C	22 (48)	55 (42)	0.516	0.81	0.407 to 1.572
6167 (A/C/T)	A	32 (76)	93 (73)		-	
	C	1 (2)	9 (7)	0.269	3.09	0.377 to 25.42
	T	9 (22)	26 (20)	0.989	0.99	0.421 to 2.345
6192 (C / T)	C	42 (96)	122 (94)		-	
	T	2 (4)	8 (6)	0.691	1.37	0.281 to 6.746
6228 (C/T)	C	25 (54)	69 (54)		-	
	T	21 (46)	59 (46)	0.958	1.01	0.517 to 2.002
6231 (T/C/ G)	C	15 (34)	47 (37)		-	
	G	16 (36)	44 (34)	0.753	0.87	0.388 to 1.984
	T	13 (30)	37 (29)	0.826	0.90	0.384 to 2.144
6236 (A/C)	A	36 (78)	111 (87)		-	
	C	10 (22)	17 (13)	0.174	0.55	0.231 to 1.312
6251 (A/T)	A	44 (96)	121 (94)		-	
	T	2 (4)	7 (6)	0.768	1.27	0.254 to 6.363

$p<0.05$ considered significant, *p* value determined via chi square distribution

4.3.4.2.3. Allelic distribution of *HLA-DQB1* in spontaneously recovered (SR) and chronically infected (CI) patients

Only significant associations are discussed below, remaining data is shown in Table 4.27.

i. Distribution of alleles at -8471 (A/G/C) position showed that allele A was frequent in n=69 (64%) of the SR patients and n=31 (47%) in CI patients, similarly allele G was prevalent in n=39 (36%) of SR and n=34 (51%) of the CI patients, with significant association (p=0.036), OR of 1.94 and 95% CI of 1.038 to 3.627. Allele C was prevalent not prevalent in SR patients and n=1 (2) in CI patients.

Table 4.27. Allelic distribution *HLA-DQB1* in spontaneously recovered (SR) and chronically infected patients

Locus	**Allele**	**Spontaneously Recovered; n (%)**	**Chronically Infected; n (%)**	***p* value**	**OR**	**95% CI**
HLA-DQB1						
-8288 (C/G)	C	91 (83)	53 (80)		-	
	G	19 (17)	13 (20)	0.686	1.17	0.537 to 2.570
-8298 (C/G)	C	81 (76)	45 (68)		-	
	G	27 (24)	21 (32)	0.328	1.4	0.711 to 2.755
-8307 (A/C/G)	A	48 (44)	30 (45)		-	
	C	16 (14)	11 (17)	0.834	1.1	0.450 to 2.687
	G	46 (42)	25 (38)	0.681	0.86	0.446 to 1.695
-8331 (T/G)	T	66 (62)	49 (74)		-	
	G	40 (38)	17 (26)	0.104	0.57	0.290 to 1.127
-8344 (T/C)	T	84 (78)	50 (76)		-	
	C	24 (22)	16 (24)	0.758	1.12	0.543 to 2.308
-8362 (A/C/G)	A	57 (53)	34 (53)		-	
	C	36 (34)	19 (30)	0.731	0.88	0.439 to 1.781
	G	13 (13)	11 (17)	0.449	1.41	0.571 to 3.519
-8442 (G/C)	G	81 (74)	49 (74)		-	
	C	29 (26)	17 (26)	0.929	0.96	0.483 to 1.944
-8447 (C/A/G/T)	C	49 (50)	42 (66)		-	
	A	8 (9)	4 (6)	0.401	0.58	0.163 to 2.076
	G	3 (3)	4 (6)	0.574	1.55	0.329 to 7.351
	T	37 (38)	14 (22)	0.028	0.44	0.210 to 0.925
-8465 (G/A)	G	93 (86)	52 (79)		-	
	A	14 (13)	14 (21)	0.158	1.78	0.791 to 4.041
-8471 (A/G)	A	69 (64)	31 (47)		-	
	G	39 (36)	34 (51)	0.036	1.94	1.038 to 3.627

$p<0.05$ considered significant, p value determined via chi square distribution. None of the allele was significantly predicting the spontaneous recovery or chronic infection in analyzed set of patients for *HLA-DQB1* gene.

4.3.5. Gender based allelic distribution in studied patients.

4.3.5.1. *TNF- α* genetic polymorphisms in male and female patients

No significant associations were found in *TNF- α* promoter polymorphisms. Data shown in Table 4.28.

Table 4.28 *TNF- α* allelic polymorphisms in male and female patients

Locus	Allele	Male; n (%)	Female; n (%)	*p* value	OR	95% CI
TNF-α						
-238 G/A	G	160 (94)	159 (90)			
	A	10 (6)	17 (10)	0.190	1.71	0.759 to 3.851
-308 G/A	G	148 (86)	153 (92)			
	A	24 (14)	13 (8)	0.071	0.52	0.257 to 1.068
-863 C/A	C	115 (68)	128 (74)			
	A	55 (32)	46 (26)	0.228	0.75	0.471 to 1.197

$p<0.05$ considered significant, *p* value determined via chi square distribution

4.3.5.2. Gender based *HLA-DRB1* allelic variations in studied patients

Association of HLA-DRB1 allelic variation in both males and female patients is discussed only for significant associations, whereas data is presented in Table 4.29.

i. Allelic variation at 6151 (T/C) position showed that T allele was frequent in n=80 (45%) of the males and n=100 (58%) of the female patients. Similarly n=98 (55%) of the male patients and n=72 (42%) patients had allele A and is significantly associated with HCV infection (p=0.013, OR=0.58, 95% CI; 0.385 to 0.897)
ii. Allelic polymorphism at 6167 (A/C/T) revealed that allele A was prevalent in n=119 (70%) in males and n=130 (77%) of females. Similarly allele C was found to be prevalent in n=12 (7%) of males and n=11 (7%) of females (p=0.687, OR=0.83, 95% CI;0.356 to 1.974). Allele T was frequent in n=39 (23%) of males and n=27 (16%) of females (p=0.102, OR=0.63, 95% CI; 0.365 to 1.099).
iii. Association of allelic polymorphism at 6231 (T/C/G/A) position showed that allele T was represented in 69 (39%) of the male and 90 (52%) of the females patients. Allele G was prevalent in n=55 (32%) of the male and n=49 (28%) of the female patients (p=0.060, OR=0.60, 95% CI;0.358 to 1.023). Similarly allele T was frequent in n=65 (37%) of male n=48 (29%) of the female patients and the association was statistically

significant (p=0.008, OR=0.50, 95% CI;0.299 to 0.840). Allele A was prevalent in n=1 (1) of males and not in females.

Table 4.29. Gender based *HLA-DRB1* allelic distribution in studied patients

Locus	Allele	Male; n (%)	Female; n (%)	*p* value	OR	95% CI
HLA-DRB1						
6151 (T/C)	T	80 (45)	100 (58)			
	C	98 (55)	72 (42)	0.013	0.58	0.385 to 0.897
6167 (A/C/T)	A	119 (70)	130 (77)			
	C	12 (7)	11 (7)	0.687	0.83	0.356 to 1.974
	T	39 (23)	27 (16)	0.102	0.63	0.365 to 1.099
6192 (C/T)	C	171 (95)	165 (95)			
	T	9 (5)	9 (5)	0.941	1.03	0.401 to 2.676
6228 (C/T)	C	107 (61)	82 (48)			
	T	69 (39)	90 (52)	0.014	1.70	1.112 to 2.605
6231 (T/C/G/A)	C	51 (30)	75 (43)			
	G	55 (32)	49 (28)	0.060	0.60	0.358 to 1.023
	T	65 (37)	48 (29)	0.008	0.50	0.299 to 0.840
	A	1 (1)	0 (0)			
6236 (A/C)	A	145 (82)	151 (89)			
	C	31 (18)	21 (11)	0.157	0.65	0.357 to 1.184
6251 (A/T)	A	159 (91)	165 (95)			
	T	15 (9)	9 (5)	0.204	0.57	0.245 to 1.359

p<0.05 considered significant, *p* value determined via chi square distribution.

4.3.5.3. Allelic variation in *HLA-DQB1* gene in male and female patients

None of the studied alleles were significantly associated with HCV infection in male and female patients, data shown in Table 4.30.

Table 4.30. Gender based *HLA-DQB1* allelic distribution in studied patients

Locus	Allele	Male; n (%)	Female; n (%)	*p* value	OR	95% CI
HLA-DQB1						
-8288 (C/G)	C	143 (80)	144 (85)		-	
	G	35 (20)	26 (15)	0.284	0.73	0.422 to 1.289
-8298 (C/G)	C	139 (78)	131 (78.0)		-	
	G	39 (22)	37 (22.0)	0.979	1.00	0.604 to 1.675
-8307 (A/C/G)	A	75 (45)	87 (51)		-	
	C	26 (15)	28 (17)	0.813	0.92	0.501 to 1.720
	G	67 (40)	55 (32)	0.150	0.70	0.441 to 1.134
-8465 (G/A/C)	G	148 (86)	147 (87)		-	
	A	22 (13)	21 (13)	0.371	0.96	0.506 to 1.823
	C	2 (1)	0 (0.0)		-	
-8442 (G/C)	G	129 (73)	116 (69)	-	-	
	C	47 (27)	52 (31)	0.384	1.23	0.770 to 1.964
-8331 (T/G)	T	115 (68)	113 (68)		-	
	G	55 (32)	53 (32)	0.933	0.98	0.620 to 1.550
-8344 (T/C)	T	128 (75)	130 (77)		-	
	C	42 (25)	40 (23)	0.799	0.93	0.570 to 1.542
-8447 (C/A/G/T)	C	81 (53)	76 (49)		-	
	A	15 (10)	12 (8)	0.703	0.85	0.375 to 1.938
	G	6 (4)	8 (5)	0.531	1.42	0.471 to 4.287
	T	53 (33)	59 (38)	0.489	1.18	0.730 to 1.928
-8471 (A/G/C)	A	92 (54)	82 (50)		-	
	G	73 (44)	83 (49)	0.270	1.27	0.827 to 1.967
	C	1 (1)	1 (1)	0.935	1.12	0.069 to 18.24
-8362 (A/C/G)	A	92 (54)	74 (45)		-	
	C	48 (28)	58 (35)	0.102	1.50	0.920 to 2.452
	G	30 (18)	32 (20)	0.343	1.32	0.739 to 2.380

p<0.05 considered significant, *p* value determined via chi square distribution

4.3.6. Haplotypes observed in the studied patients

Haplotypes in the studied group of patients were generated for each gene. Haplotypes for *TNF-α, HLA-DRB1* and *HLA-DQB1* was generated using Haplo2ped online tool, along with their percentile in the studied patients were generated and shown in the Table below (Table 4.31).

Table 4.31. Most prevalent haplotypes detected in three of the studied genes.

Gene	Haplotype	Percentile
TNF-α	GGC	0.5873
	GGA	0.2414
	GAC	0.0726
	AGC	0.0318
	AGA	0.0280
	GAA	0.0208
	AAC	0.0150
	AAA	0.0032
HLA-DRB1	CACTCAA	0.1307
	TACCCAA	0.1194
	TACCTAA	0.0917
	TACTCAA	0.0811
	CACTTAA	0.0802
	CACCTAA	0.0685
	CACCCAA	0.0606
	TTCTCAA	0.0439
	TACTTAA	0.0371
	CACCCCA	0.0298
	TACCCCA	0.0289
	CTCCTAA	0.0260
HLA-DQB1	CCAGGTTCGA	0.0656
	CCAGGTTCAC	0.0482
	CCGGGTTCGA	0.0424
	CCGGGTTTAC	0.0276
	CCGGGTTTGA	0.0251
	CCAGCTTCAC	0.0237
	CCAGGTTTAA	0.0235
	CCGGCTTTGA	0.0223
	CGAGGTTTAA	0.0205
	CCAGGGTTAC	0.0186
	CCGGGTTCAC	0.0171
	CCAGGTTTAC	0.0159

4.3.7. Linkage disequilibrium analysis

4.3.7.1. Linkage disequilibrium analysis for *TNF-α* gene

Linkage disequilibrium analysis (LD) for three SNPs -238 G/A, -308 G/A, -863 C/A tagged as snp1, snp2, snp3 at *TNF-α* genewere analyzed. The D' statistics showed varying results whereas chi square distributions showed no significant associations for LD in all three SNPs studied in *TNF-α* gene. Table 4.32 shows the values and LD patterns in analyzed gene.

Table 4.32. Linkage disequilibrium analysis for SNPs observed in *TNF-α* gene

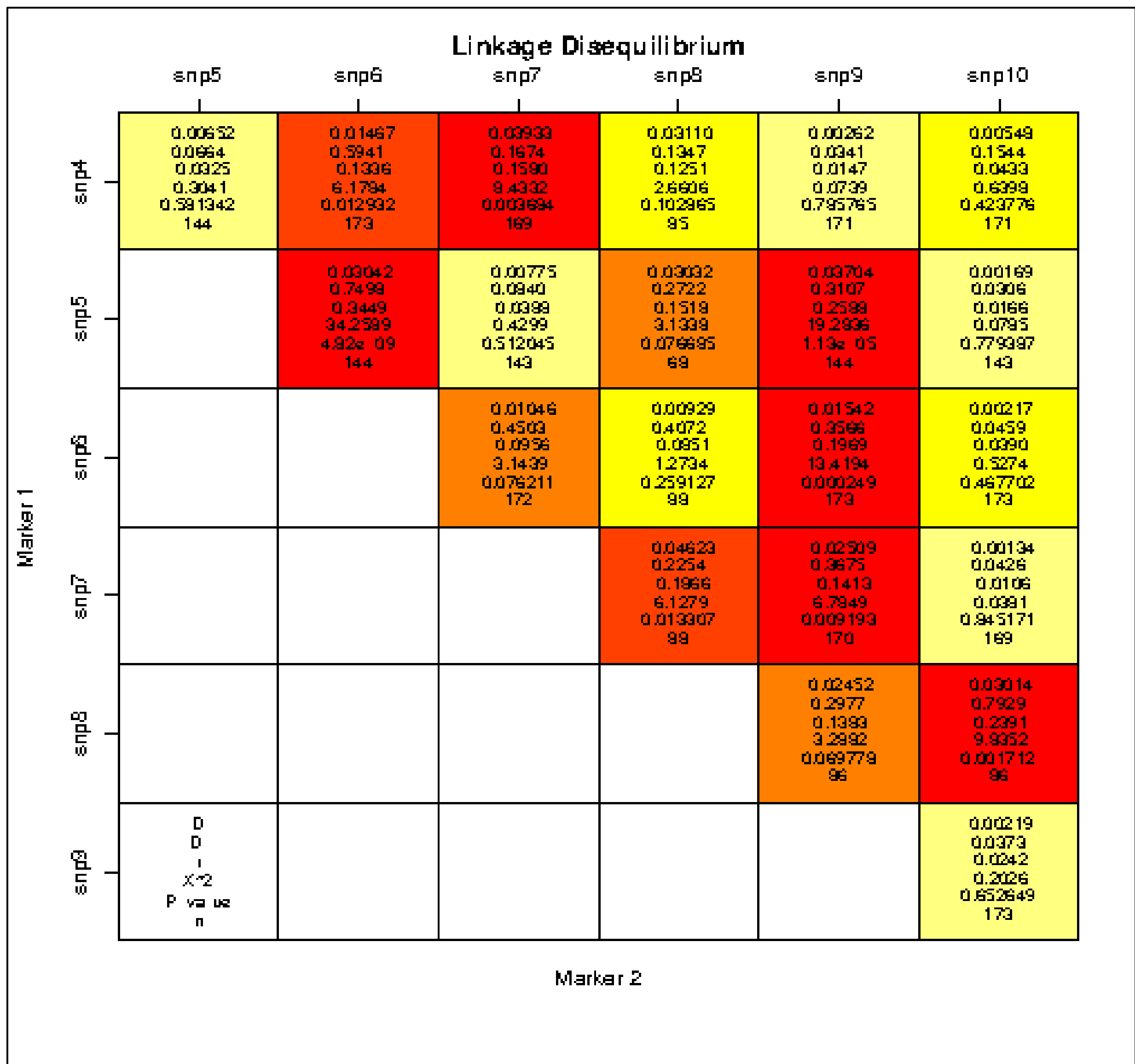
D statistic			
	-238 G/A (rs361525)	-308 G/A (rs1800629)	-863 C/A (rs1800630)
Sr.no	**snp1**	**snp2**	**snp3**
snp1	.	0.0087	0.0081
snp2	.	.	-0.0083
snp3	.	.	.
D' statistic			
	snp1	snp2	snp3
snp1	.	0.1258	0.1463
snp2	.	.	0.2592
snp3	.	.	.
r statistic			
	snp1	snp2	snp3
snp1	.	0.1044	0.066
snp2	.	.	-0.0586
snp3	.	.	.
***p* value**			
	snp1	snp2	snp3
snp1	.	0.0565	0.2305
snp2	.	.	0.2932
snp3	.	.	.

Linkage patterns were observed for *TNF-α* gene. Associations are listed in Table.

4.3.7.2. Linkage disequilibrium analysis for *HLA-DRB1* gene

LD analysis for SNPs observed in *HLA-DRB1* gene revealed that that there was linkage among certain SNPs as shown in the Figure below. Blocks having dark red color represents linkage among various SNPs based on the D, D' and p values. All the values are shown in the Figure (4.62) and Table (4.33) below.

Figure 4.27. Linkage disequilibrium heat map for SNPs observed in *HLA-DRB1* gene



The dark red cells shows the stronger linkage among the analyzed SNPs, whereas the light red color indicate weak linkage and white or yellow cells indicate no linkage among the analyzed SNPs for *HLA-DRB1* gene.

Table 4.33. Linkag disequilibrium analysis for all SNPs in *HLA-DRB1* gene

D statistic							
	6151 (T/C) (rs1064664)	6167 (A/C/T) (rs707957)	6192 (C / T) (rs776046212)	6228 (C/T) (rs2308802)	6231 (T/C/ G) (rs3167799)	6236 (A/C) (rs1059586)	6251 (A/T) (rs397844204)
Sr.no	**snp4**	**snp5**	**snp6**	**snp7**	**snp8**	**snp9**	**snp10**
snp4	.	-0.0065	-0.0147	0.0393	0.0311	0.0026	0.0055
snp5	.	.	0.0304	-0.0078	0.0303	0.037	0.0017
snp6	.	.	.	-0.0105	-0.0093	0.0154	0.0022
snp7	.	.	.	.	-0.0462	-0.0251	-0.0013
snp8	.	.	.	.	.	0.0245	0.0301
snp9	.	.	.	.	.	.	0.0022
snp10	.	.	.	.	.	.	
D' statistic							
Sr.no	**snp4**	**snp5**	**snp6**	**snp7**	**snp8**	**snp9**	**snp10**
snp4	.	0.0664	0.5941	0.1674	0.1347	0.0341	0.1544
snp5	.	.	0.7498	0.084	0.2722	0.3107	0.0306
snp6	.	.	.	0.4503	0.4072	0.3566	0.0459
snp7	.	.	.	.	0.2254	0.3675	0.0426
snp8	.	.	.	.	.	0.2977	0.7929
snp9	.	.	.	.	.	.	0.0373
snp10	.	.	.	.	.	.	.
r statistic							
Sr.no	**snp4**	**snp5**	**snp6**	**snp7**	**snp8**	**snp9**	**snp10**
snp4	.	-0.0325	-0.1336	0.158	0.1251	0.0147	0.0433
snp5	.	.	0.3449	-0.0388	0.1518	0.2588	0.0166
snp6	.	.	.	-0.0956	-0.0851	0.1969	0.039
snp7	.	.	.	.	-0.1866	-0.1413	-0.0106
snp8	.	.	.	.	.	0.1383	0.2391
snp9	.	.	.	.	.	.	0.0242
snp10	.	.	.	.	.	.	.
***p* values**							
Sr. no	**snp4**	**snp5**	**snp6**	**snp7**	**snp8**	**snp9**	**snp10**
snp4	.	0.5813	0.0129	0.0037	0.1029	0.7858	0.4238
snp5	.	.	0	0.512	0.0767	0	0.7794
snp6	.	.	.	0.0762	0.2591	2e-04	0.4677
snp7	.	.	.	.	0.0133	0.0092	0.8452

snp8	.	.	.	.	.	0.0698	0.0017
snp9	.	.	.	.	.	.	0.6526
snp10	.	.	.	.	.	.	.

4.3.7.3. Linkage disequilibrium analysis for *HLA-DQB1* gene

LD analysis for SNPs observed in this gene showed linkage pattern among certain SNPs as shown in the Figure 4.34. All the observed values for the corresponding SNPs are shown in the Table along with the level of significance and analyses.

Table 4.34. Linkage disequilibrium analysis for all SNPs in *HLA-DQB1* gene.

D statistic										
	-8471 (A/G/C) (chr6:32628910)	-8465 (G/A/C) (rs9273552)	-8447 (C/A/G/T) (chr:32628934)	-8442 (G/C) (rs34644981)	-8362 (A/C/G) (chr6:32629020)	-8344 (T/C) (chr6:32629038)	-8331 (T/G) (chr6:32629051)	-8307 (A/C/G) (rs9273588)	-8298 (C/G) (rs9273592)	-8288 (C/G) (rs9273598)
Sr.no	**snp11**	**snp12**	**snp13**	**snp14**	**snp15**	**snp16**	**snp17**	**snp18**	**snp19**	**snp20**
snp11	.	0.0312	-0.0259	0.0042	0.0284	0.0402	0.0573	-0.0115	-0.0117	-0.0031
snp12	.	.	-3e-04	0.0124	0.0111	0.0372	0.05	0.0047	-0.0135	-0.0394
snp13	.	.	.	0.0052	0.0045	0.0221	-0.014	0.0486	0.0266	-0.015
snp14	.	.	.	.	-0.0042	0.003	0.0085	0.004	0.013	-0.01
snp15	.	.	.	.	.	0.029	0.0253	0.0198	0.012	-0.0137
snp16	.	.	.	.	.	.	0.0565	0.0096	-0.0252	0.0036
snp17	.	.	.	.	.	.	.	-0.0175	-0.0177	0.0054
snp18	.	.	.	.	.	.	.	.	-0.033	-0.0291
snp19	.	.	.	.	.	.	.	.	.	-0.0701
snp20	.	.	.	.	.	.	.	.	.	.
D' statistic										
Sr.no	**snp11**	**snp12**	**snp13**	**snp14**	**snp15**	**snp16**	**snp17**	**snp18**	**snp19**	**snp20**
snp11	.	0.2281	0.3416	0.0397	0.2275	0.3383	0.431	0.1686	0.1415	0.0444
snp12	.	.	0.0035	0.1246	0.0709	0.2495	0.3001	0.035	0.1301	0.4526
snp13	.	.	.	0.0713	0.0276	0.1212	0.1341	0.2193	0.1168	0.0876
snp14	.	.	.	.	0.1132	0.0349	0.0871	0.0511	0.1929	0.1966
snp15	.	.	.	.	.	0.1485	0.1475	0.1128	0.0793	0.1201

snp16	.	.	.	.	.	.	0.345	0.0491	0.1656	0.0184
snp17	.	.	.	.	.	.	.	0.1857	0.1555	0.037
snp18	.	.	.	.	.	.	.	.	0.1785	0.188
snp19	.	.	.	.	.	.	.	.	.	0.3748
snp20	.	.	.	.	.	.	.	.	.	.
r statistic										
Sr.no	**snp11**	**snp12**	**snp13**	**snp14**	**snp15**	**snp16**	**snp17**	**snp18**	**snp19**	**snp20**
snp11	.	0.1982	-0.1375	0.033	0.165	0.2266	0.3525	-0.0623	-0.0617	-0.0166
snp12	.	.	-0.0016	0.09	0.0592	0.1924	0.2824	0.0232	-0.0654	-0.1944
snp13	.	.	.	0.0313	0.0201	0.0956	-0.066	0.2011	0.1077	-0.0619
snp14	.	.	.	.	-0.0276	0.0194	0.0592	0.0244	0.0781	-0.061
snp15	.	.	.	.	.	0.1372	0.1308	0.0895	0.0533	-0.0618
snp16	.	.	.	.	.	.	0.2826	0.0422	-0.1079	0.0157
snp17	.	.	.	.	.	.	.	-0.0838	-0.083	0.0258
snp18	.	.	.	.	.	.	.	.	-0.1353	-0.1219
snp19	.	.	.	.	.	.	.	.	.	-0.2873
snp20	.	.	.	.	.	.	.	.	.	.
***p* values**										
Sr.no	**snp11**	**snp12**	**snp13**	**snp14**	**snp15**	**snp16**	**snp17**	**snp18**	**snp19**	**snp20**
snp11	.	2e-04	0.0304	0.5467	0.0023	0	0	0.3231	0.2665	0.7943
snp12	.	.	0.9799	0.1012	0.2753	5e-04	0	0.7142	0.2409	0.0023
snp13	.	.	.	0.6252	0.7515	0.1387	0.3026	0.0058	0.0966	0.4167
snp14	.	.	.	.	0.6134	0.7287	0.2838	0.6992	0.1666	0.3467
snp15	.	.	.	.	.	0.0133	0.0168	0.1536	0.3391	0.3342

snp16	.	.	.	.	.	.	0	0.5076	0.0536	0.8075
snp17	.	.	.	.	.	.	.	0.185	0.134	0.6858
snp18	.	.	.	.	.	.	.	.	0.0377	0.102
snp19	.	.	.	.	.	.	.	.	.	0
snp20	.	.	.	.	.	.	.	.	.	.

4.3.8. Dominancy models for genotypes in analyzed genes

4.3.8.1. *TNF-α* (-238) genotype dominancy model

Dominancy models for all genotypes observed on the respective SNPs were generated. These models were generated according to gender response in therapy. In each model genotype prevalence was compared with gender, odd ratios and probability values. Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) were also calculated for the analyzed genotypes. The lower the values of AIC and BIC the more chances of significant associations for genotypes models. Only one model for each gene is shown here whereas all the remaining models are presented in the Appendix portion.

Table 4.35. Dominancy model for *TNF-α* (-238) genotype in gender response

Model	Genotype	Female	Male	OR (95% CI)	*p* value	AIC	BIC
Codominant	G/G	77 (87%)	77 (90%)	1.00	0.34	243.6	253.1
	G/A	5 (5%)	6 (7%)	1.20 (0.35-4.10)			
	A/A	6 (6%)	2 (2%)	0.33 (0.07-1.70)			
Dominant	G/G	77 (87%)	77 (90%)	1.00	0.52	243.4	249.7
	G/A-A/A	11 (12%)	8 (9%)	0.73 (0.28-1.91)			
Recessive	G/G-G/A	82 (93%)	83 (97%)	1.00	0.15	241.7	248
	A/A	6 (6%)	2 (2%)	0.33 (0.06-1.68)			
Overdominant	G/G-A/A	83 (94%)	79 (92%)	1.00	0.71	243.6	249.9
	G/A	5 (5%)	6 (7%)	1.26 (0.37-4.30)			
Log-additive	---	---	---	0.71 (0.37-1.36)	0.29	242.7	249

$p<0.05$: Sig, No dominant model was depicted for gender

4.3.8.2. *HLA-DRB1* (6151) genotype dominancy model

The dominancy model for this particular SNP revealed significant association for Over-dominant and Codominant expression in the analyzed set of patients ($p<0.05$). The odd ratios, confidence intervals and AIC, BIC values are represented in the Table 4.36.

Table 4.36 Dominancy models for *HLA-DRB1* 6151 SNP in gender response

Model	Genotype	Female	Male	OR (95% CI)	*p* value	AIC	BIC
Codominant	**T/T**	**26 (30%)**	**34 (38%)**	**1.00**	**0.050**	242	252
	T/C	**37 (43%)**	**23 (25%)**	**0.48 (0.23-0.99)**			
	C/C	**23 (26%)**	**32 (36%)**	**1.06 (0.51-2.23)**			
Dominant	T/T	26 (30%)	34 (38%)	1.00	0.27	245	251
	T/C-C/C	60 (69%)	55 (61%)	0.70 (0.37-1.31)			
Recessive	T/T-T/C	63 (73%)	57 (64%)	1.00	0.19	244	251
	C/C	23 (26%)	32 (36%)	1.54 (0.81-2.93)			
Overdominant	**T/T-C/C**	**49 (57%)**	**66 (74%)**	**1.00**	**0.016**	240	247
	T/C	**37 (43%)**	**23 (25%)**	**0.46 (0.24-0.87)**			
Log-additive	---	---	---	1.02 (0.71-1.47)	0.92	246	252

$p<0.05$: Sig. co-dominant model was significantly prevalent in both male and female patients in this study

4.3.8.3. Dominancy model for *HLA-DQB1* -8288 genotypes

Models for all the analyzed SNPs were generated. Only one model for 8288 position is shown here. Dominancy model for genotypes showed no significant association with gender response. Data for genotype model is shown in Table 4.37.

Table 4.37. Genotype models for *HLA-DQB1* 8288 position.

Model	Genotype	Female	Male	OR (95% CI)	*p* value	AIC	BIC
Codominant	C/C	65 (76%)	61 (68%)	1.00	0.47	245.6	255.1
	C/G	14 (16%)	21 (23%)	1.60 (0.75-3.42)			
	G/G	6 (7%)	7 (7%)	1.24 (0.40-3.91)			
Dominant	C/C	65 (76%)	61 (68%)	1.00	0.24	243.7	250.1
	C/G-G/G	20 (23%)	28 (31%)	1.49 (0.76-2.92)			
Recessive	C/C-C/G	79 (92%)	82 (92%)	1.00	0.84	245.1	251.4
	G/G	6 (7%)	7 (7%)	1.12 (0.36-3.49)			
Overdominant	C/C-G/G	71 (83%)	68 (76%)	1.00	0.24	243.7	250.1
	C/G	14 (16%)	21 (23%)	1.57 (0.74-3.33)			
Log-additive	---	---	---	1.26 (0.77-2.06)	0.35	244.2	250.6

$p<0.05$: Sig

5. Discussion

Chronic hepatitis C (CHC) caused by hepatitis C virus (HCV) is a serious health concern affecting 71 million people globally (McPhee, 2019; Blach *et al*., 2015). Owing to preventive measures, incidence of HCV has decreased considerably in developed countries; however, it still remains a leading cause of morbidity and mortality in developing countries (Arshad *et al*., 2007; Petalruzziello *et al*., 2016).

HCV is predominantly responsible for liver diseases and profound increased risk of hepatocellular carcinoma and liver failures (Farahnaz *et al*., 2007; Westbrook and Dusheiko, 2014). Worldwide prevalence estimation of HCV revealed an average prevalence rate of 2.5% in adults: ranging from 1.3% in US and 2.9% in Africa. Based on genotype prevalence, genotype 1 is ranked the most prevalent type with a percentage prevalence of 49.1%, followed by genotype 3, 4 and 2 with 17.9%, 16.8% and 11.0% prevalence rates, respectively. Genotypes 5 and 6 have the lowest combined prevalence i.e. < 5%, whereas, genotype 1 and 3 are known for a worldwide distribution; genotype 4 and 5 are found mostly in low-income countries (Hanafiah *et al*., 2013; Umer and Iqbal, 2016). Chronic diseases are slow progressive diseases characterized by hepatic inflammation resulting in development of cirrhosis in 10-20% of patients (Pol *et al.,* 2018). Cirrhosis leads to annual risk of 1-5% of HCC and 3–6% of hepatic decompensation, which afterwards can increase the risk of death by 15% and 20% (van de Laar *et al*., 2010). The high burden of chronic hepatitis C (CHC) together with absence of vaccine, reflects treatment as part of disease control stratagem but the effectiveness, impact, and outcome of treatment in various groups remain unclear (Umer and Iqbal, 2016). Several meta-analysis studies demonstrated that HCV eradication with antiviral therapy reduces the risk of hepatocellular carcinoma (HCC) in patients infected with chronic hepatitis C but not eliminated completely (Umer and Iqbal, 2016; Waheed, 2015). Prevalence of HCV in Pakistan reported to be 6%, which makes it the second highest after Mongolia, which has the prevalence rate of 10% (Umer and Iqbal, 2016; Arshad and Ashfaq, 2018). The major goal of anti-HCV therapy is to attain sustained virological response (SVR) in infected patients. SVR is the absence of detectable HCV RNA in patient serum confirmed by real time PCR (Akhtar, 2015).Therapy for HCV infection started two decades earlier, when interferon/ribavirin were started to be used as a combinatorial therapy for the treatment of

HCV infection. Later on were replaced by pegylated interferon/ribavirin combination therapy (Akhtar, 2015; Qureshi *et al*., 2015). Direct-acting antivirals (DAAs) regimens have been approved for HCV therapy and initial phase protease inhibitors known as boeceprevir (BOC) along with telaprevir (TVR) were also approved in combination with IFN and RBV (Cortez *et al*., 2015). This triple therapy resulted in achievement of higher SVR but is also associated with severe side effects. Both the European Association for the study of Liver (EASL) and American Association for the study of Liver Diseases (AASLD) do not recommend the anti-HCV triple therapy consisting of BOC, TVR for anti-HCV therapy (AASLD, 2014; EASL, 2015).

United Nations (UN) estimates as of July 2019, Pakistan has population of 204,571,621 patients, which makes number 6th in the rank in terms of the most populated countries of the world. Estimates for the land area revealed that there is a total area of 297,638 sq. miles in the whole country, whereas the population density of 265 patients per kilometer square (UN, 2019). Household situation in Pakistan tends to be bigger with about 6.5 persons eating and living together (Census 2017). The main working force in Pakistan is constituted mostly by males as 82.5% of the working labor, whereas 24.8% of the labor force is consisted by females although women constitute 49% of the total Pakistani population (ILO, 2017).

Pakistan has the lowest literacy rates in South Asia with only 58% population being literate, which is one of the main reasons for endemic rates of HCV infection in Pakistani population (Census 2017). The per capita income in Pakistan is US $1,629 and about 40% of the population lives below the poverty line, such conditions already indicate that effective and updated anti-HCV treatment is not affordable for Pakistani patients (Gissel *et al*., 2015). The pegylated interferon/ribavirin therapy for the period of six months (Rs. 100,000 approximately) is beyond an average Pakistani's health budget. (Gissel *et al*., 2015; Tang *et al*., 2016). In Pakistani patients, receiving anti-HCV interferon/ribavirin combination therapy, David et al estimated the incremental cost effectiveness ration (ICER) for quality adjusted life years as Rs. 144673, respectively. However, government sector strategy reportedly had a net cost lesser than the strategy discussed above. Still internationally reported costs for DAA therapy for boeceprevir (€1,447.69) and Sofosbuvir/Simeprevir (€1,560.13) are not affordable in Pakistan (Abdelwahab, 2016).

Due to such high cost of the DAA therapy most of the patients had to rely on the classical therapy comprising of IFN/RBV or peg-IFN/PBV combinatorial therapy for the treatment of HCV infection.

We believe that population genetics may show a significant character in defining outcome of HCV infection and therapy in Pakistani population. Interestingly various studies have reported response rates to IFN/RBV combination therapy in HCV infected patients, as Aziz et al reported sustained virological response to PEG-IFN-alpha-2b/ribavirin (Aziz *et al*., 2011). Similarly another study reported SVR rates in 89% of ETR patients followed by 80% RVR rates reported in another study (Iqbal *et al*., 2014; Akram *et al*., 2011). These studies suggest a critical part of host immune genetic features determining the consequences of HCV infection in Pakistani population.

This detailed cross-sectional study was designed to investigate the association of polymorphisms in *TNF-α*, *HLA-DRB1* and *HLA-DQB1* genes and their possible outcome in patient's receving anti-HCV combinatorial therapy. No control group was included due to the fact that crossectional studies are based on the detailed investigation of the exposure and outcome variables in the targeted population and mutual comparisions of the variables. Cross-sectional study designs often don't need any control group for comparison and as this study is looking into the prevalence of genetic polymorphisms in three set of genes, so no control group was included for comparison (Setia *et al*., 2016). Similarly the comparison for the association of SNPs were made between the responsive (R) and non-responsive (NR) patients in therapy group along with chronically infected (CI) and spontaneously recovered (SR) patients and therefore control group was not included in this study.

A large number of patients were recruited in this study in order to have a clear picture of the role of host immunogentic polymorphisms in the outcomes of response to anti-HCV therapy and infection in Pakistani population. Demographic and clinical information of the studied patients in this study were also analyzed. Gender based differentiation of HCV infection showed that both genders were almost equally distributed, males (49%) and females (51%). Comparing literacy rates in gender revealed that there was a statistically significant association between gender and literacy rates ($p<0.05$), odd ratio of 4.321 and 95% CI (1.797 to 10.39). Most of the patients included in this study are literate as this sampling is carried out in Islamabad, the capital city of Pakistan, where the literacy rates

are high as compared to the rural parts of the coutry It is believed that women tend to have better responses against HCV infection than men however; various factors are responsible for the outcomes of HCV infection in both male and female patients (Kumar *et* al., 2017). Different studies across the globe had reported varying percentages for the prevalence of male and female patients as a study reported 51% of male and 49% of female patients in a study conducted in Wuhan city of China (Sarin *et al*., 2012). Similarly another study also reported a higher percentage (53%) of female infected patients in comparison to males (47%), which is in concordance, with our study (Thimme *et al*., 2001).

Signs and symptoms associated with acute HCV infections are mostly infrequent and the infection is asymptomatic, however development of signs and symptoms in certain acutely infected patients as well as chronically infected patients begin to appear within 3 to 12 weeks post infection; which mainly include jaundice, anorexia, malaise and weakness (Syhavong *et al*., 2010; Jalil *et al*., 2016).The most common symptoms observed in this study were jaundice and fatigue in both male and female patients followed by fever and abdominal discomfort. In literate and illiterate patients the common symptoms were fever and abdominal discomfort followed by anorexia. Other symptoms like dark urine and malaise were also detected in studied patients. Signs and symptoms of HCV infection can vary in a group of patients or populations as many genetic and environmental factors can affect the development of symptoms in infected patients (Xu *et al*., 2014).

Studies have reported different symptoms associated with HCV infection like a study reported that the most common symptom associated with HCV-HBV co-infection were abdominal discomfort and fatigue, which is in concordance with our study (Kumar *et al*., 2017). Similarly jaundice was also associated with hepatitis, A, C, and E infection in majority of the studied patients, which also co-relates with our study (Jalil *et al*., 2016). Demographic analysis of the patients revealed that most of the included patients in this study belonged to Islamabad and Rawalpindi region of the Pakistan.

Gender based differentiation showed that both male and female patients mostly belonged to Islamabad and Rawalpindi districts and similar scenario was observed for the literacy based distribution. Patients from other parts of the country like central Punjab, Khyber Pakhtunkhwa (KPK) were also distributed among analyzed patients. Various studies have

targeted different populations across the country which depends on the type of study and ethnicity of the populations (Jilani *et al*., 2017; Khan *et al*., 2011; Attaullah *et al*., 2011).
Age based distribution of the infection in patients revealed that the most infected age group reported in this study was 31-40 years in both gender and literacy based distributed patients, which is a major issue regarding HCV infection in working class to the general health of a society. Different studies have reported different age groups for HCV infection, like a study reported that 16-45 years was the most infected age group. Another study reported the mean age of 42 years in patients who were chronically infected with HCV, which are not in coherence with our study (Rehman *et al*., 2018; Patil *et al*., 2017; Khan *et al*., 2011).
Risk factors like surgery, blood transfusions, hospitalizations and injection use, associated with HCV infection were also analyzed for the studied group of patients and it was found that the most common risk factor in studied patients was injection use followed by intra-venous infusions. Gender based differentiation of risk fator in in male and female patients were barber visits and skin piercing followed by blood transfusions and injection use. The other prevalent risk factors were also found to be associated with HCV infection in studied patients. Significant associations were found to be present between studied risk factors and gender as ($p<0.05$). In the literacy group the common risk factors in literate patients were avoiding use of boiling water followed by spouse status and infected father, whereas in illiterate patients the associated risk factors were skin piercing, illegal injections and hospitalizations. These risk factors were found to be significantly associated with HCV infection ($p<0.05$).

Studies have reported different risk factors associated with HCV infection in different populations, like a study reported that dental procedures and blood transfusions were the main risk factors associated with HCV infection, which is in coherence with our study (Jilani *et al*., 2017; Jalil *et al*., 2016). Similarly another study also reported blood transfusions and intravenous infusion as the main risk factors for HCV infection in studied patients, while another study reported injections the risk factor associated with HCV infection which also co-insides with our study (Arshad and Ashfaq, 2017; Kumar *et al*., 2018).

Genotype detection for the analyzed patients in this study revealed that HCV genotypes 3a, 1a, 1b and un-typable were detected in the total 100 studied patients. Overall prevalence of

3a genotype was 93% in the studied patients and it makes sense because HCV genotype 3a is the most prevalent genotype in Pakistani population. A large number of studies have confirmed the fact that HCV genotype 3a is the most prevalent genotype in Pakistan, which confirms our findings (Attaullah *et al*., 2011; Westermann *et al*., 2015; Henderson *et al*., 2003). Data regarding the occupation of the studied patients was also acquired and it was found that the major professions of male gender included; driving, laborer and private business, whereas, among female patients, the major occupations were; housewife, teacher and students.

Statistical correlation was significant for gender based occupation in studied patients ($p<0.05$). Occupation of the patients have significant contribution to HCV infection if the individual is working in a healthcare sector or laboratory associated medical staff and hence more prone to HCV infection (Ndjoyi-Mbiguino *et al*., 2018; Aguinaga *et al*., 2014). Awareness and education regarding HCV infection is very important to prevent HCV infection. Literacy rates in studied patients were considered a parameter in this study and it was revealed that most of the male patients had intermediate, matriculate and middle school education, most of the female patients had bachelor and primary education. Education levels in the studied patients were significantly associated ($p<0.05$) with HCV disease outcome. Viral RNA detection in the studied patients showed that viral RNA was detected in total of 51% of the studied patients. Similarly 47% of the male patients were found to be positive for HCV RNA while 53% of female patients were found to have viral RNA. Females are more likely to clear HCV infection, however presence of viral RNA is regardless of gender, similarly a study in Spain reported that HCV RNA was detected in 53% of patients which is in-coherence with our study (Gul *et al*., 2016).

Studies reported that the prevalence of viral RNA in studied patients was 78% and 82% (Bakr *et al*., 2006; Sasaki *et al*., 2018). The viral load distribution in the patients depicted that the lowest viral load in both male and female patients were 12 IU/ml and 15 IU/ml respectively, whereas the highest viral load observed in males was 51,809,739 IU/ml and 31,874,791 IU/ml in females respectively. The average viral load in both male and female patients was estimated to be 5,315,791 IU/ml and 3,180,912 IU/ml, indicating that the average viral load is higher in males when compared to females, which may point out the fact that women respond better to HCV infection in comparison to men (Ali *et al*., 2011).

HCV viral load is predictor of patient response to therapy or defining the chronicity of infection (Ali *et al*., 2016). Different viral loads have been reported in the analyzed patient that depends on ethnicity, gender along with immunogenetic factors (Ali *et al*., 2011).

Patients analyzed in this study were categorized mainly into two groups, patients that were receiving interferon/ribavirin for the past 24 weeks and patients that were not receiving any therapy. Therapy group comprised of patients receiving antiviral combination therapy with Pegylated IFN (180lg) once a week and ribavirin (800–1200 mg) according to the patient's body mass index. The standard recommended therapy for HCV treatment is comprised of a subcutaneous intake of 3 MU/ml of Interferon replicating three times in a week plus oral intake of 10mg/day/kg of the body weight of ribavirin continued for period of 24 weeks (Shiffman *et al*., 2011; Tsertsvadze *et al*., 2016). Therapy group was further divided into two groups; responsive (R) to interferon/ribavirin and non-responsive (NR) to interferon/ribavirin therapy group. Those patients having anti-HCV present in their serum were included in this study. These groups were defined on the basis of presence or absence of viral RNA. Patients that had detectable HCV RNA after completing IFN/RBV therapy were categorized as non-responsive (NR), whereas those patients that did not have any detectable viral RNA after completion of IFN/RBV therapy were categorized into responsive (R) group. The non-therapy group was further divided into two types that included, chronically infected (CI) and spontaneously recovered (SR) group. Chronically Infected group was defined by the presence of viral RNA for at least six months with presence or absence of clinical symptoms (Westbrook and Dusheiko, 2016). Spontaneously recovered patients were defined by the fact that these patients were positive for anti-HCV antibody, however they were negative for HCV RNA via real time PCR and studies have suggested that approximately 25% of the patients usually clear the virus, whereas the remaining 75% progress towards chronic infection (Gunal *et al*., 2017; Danilau *et al*., 2017).

A total of three genes known as *TNF-α, HLA-DRB1* and *HLA-DQB1* were targeted for presence of genetic polymorphism in all the patient groups. Post-clinical and demographic analysis included the peak analysis of the obtained sequences from Macrogen, New York, followed by the mapping of the identified single nucleotide polymorphism (SNPs) in the studied patients which are described in the results section. After mapping of the identified

SNPs in the studied genes, genotypes for each set of SNPs were established. Genotypes for the studied patients were determined by analyzing the nucleotide on both the strands using the BioEdit software and the determined genotypes were then compared for the association with HCV infection outcome in studied group of patients. The first targeted group of patients was therapy group which comprised of Responsive (R) and non-responsive (NR) group. Distribution of various genotypes at the three positions in the promoter region of *TNF-α* gene showed that none of the analyzed genotypes at these particular positions (-238, -308, -863) were associated with response to anti-HCV therapy. The statistical associations revealed that all these genotypes had probability values of more than ($p>0.05$) and hence were not involved in developing any response to interferon/ribavirin therapy in the studied group of patients.

Developing response to anti-HCV therapy is relied on various sociodemographic factors such as age, gender, ethnicity and many other factors. A number of other studies have also investigated the association of genotypes at these positions and found varying results like a study in Turkish population revealed that genotype GG at -308 position was associated with higher fibrosis and ALT levels and hence may be associated with better treatment outcomes (Yu *et al*., 2003). Similarly another study reported that the prevalence of GG, GA and AA genotypes in the observed patients was found to be 74%, 23% and 3% in studied patients, these analysis are in coherence with our analysis (Yenigün *et al*., 2003).

A study also reported no association between *TNF-α* genotype variation and anti-HCV therapy in shape of interferon/ribavirin, which confirms our results and also in the pathogenicity of HCV infection (Yue *et al*., 2015). Genotype frequency at *HLA-DRB1* gene were analyzed and it was found that none of the genotype combination was significantly associated with response to interferon therapy in HCV infected patients ($p>0.05$). There are few studies conducted in regard to the genotype distribution of *HLA-DRB1* alleles in patients receiving anti-HCV therapy.

Importance of *HLA-DRB1* genotypes in defining the outcome of HCV infection cannot be neglected (Xu *et al*., 2014). Genotype distribution of *HLA-DQB1* in therapy group patients were also analyzed in this study and it was found that all the genotypes observed at the analyzed position in the *HLA-DQB1* were not significantly associated with the treatment outcomes in HCV infected patients. As mentioned above that host genetic and

sociodemographic factors mediate immune responses and hence the response can be attributed to the complex series of events (Pasha *et al*., 2014; Corchado *et al*., 2013). We also came to know that data regarding genotype polymorphism of *HLA-DQB1* with HCV treatment outcomes is scarce especially in Pakistani population and this is a first study confirming the association of genetic variation with treatment outcomes in Pakistani population.

Associations of genotype variations in spontaneously cleared (SR) and chronically infected (CI) patients were also analyzed in the three genes. Data revealed that genotype prevalence at all positions in the *TNF-α* promoter regions were not significantly associated with development of SR or CI in the infected patients. A study reported that *TNF-α* AG and GG at -308 position were associated with susceptibility to HCV infection, which is not in concordance with our study (Nowakowska *et al*., 2004).

Similarly another study reported that genotype GG at -238 position of *TNF-α* gene was an independent factor for development of liver cirrhosis (Cangussu *et al*., 2011). Genotype distribution at studied loci of *HLA-DRB1* gene also revealed that none of the observed genotype was associated with the development of SR or CI in the studied group of patients in this study. Studies have reported that genotype polymorphism in the *HLA-DRB1* gene may be responsible for the spontaneous clearance of HCV infection and is dependent on the patient ethnicity (Zeisel *et al*., 2013). Genotype distributions in gender revealed that the distributions in both *TNF-α* and *HLA-DQB1* genes were not significantly associated with HCV infection (p>0.05) and therefore are not described here. The genotype prevalence at two position in the *HLA-DRB1* were found to be significantly associated with HCV infection in both male and female patients. Genotype distribution at 6151(T/C) position showed that genotypes TT and CC were significantly distributed in male and female patients with p= 0.044, odd ratio of 0.475 and 95% CI of 0.2292 to 0.9857 and p= 0.033, odd ratio of 0.446 and 95% CI of 0.2117 to 0.9431 respectively. Similarly genotype TG at 6231 position in *HLA-DRB1* gene was also significantly associated with HCV infection in studied patients (p= 0.026, OR= 0.3265, 95% CI; 0.1197 to 0.890).

It is assumed that genotypes at these positions may be play a role in the selection of epitopes and presenting these epitopes to CD4T cells, which may lead to an efficient immune response against the virus (Xu *et al*., 2013; He *et al*., 2011; Rosen *et al*., 2002).The

current study also investigated the role of *TNF-α* and *HLA* class II alleles with the outcomes of interferon/ribavirin therapy along with the spontaneous recovery and chronic infection in the studied group of patients. Allelic distribution in the *TNF-α* gene showed no significant association in any of the observed group. Polymorphism in gene regulating region of *TNF alpha* can affect the overall expression and secretion of cytokine.

Studies have reported that polymorphism in these cytokine genes affect the process of inflammation, autoimmune response, infectious diseases and allograft rejection (Yen *et al*., 2008; Schneider *et al*., 2004). SNP present in the respective genes that are responsible for the both pro and anti- inflammatory effects may help in the antiviral therapy responses. *TNF-α*and *IL-10* performs a major role in regulation of cellular and immune responses to HCV infection (Dogra *et al*., 2011). Polymorphisms at -238G/A, -308G/A and-863C/A positions were analyzed in this study in order to associate the effect of SNPs in *TNF-α* gene with anti-HCV therapy and HCV infection outcomes in Pakistani population. Allelic polymorphisms at -238G/A, -308G/A and-863C/A positions in the promoter region of *TNF* gene were found to have no effect on the response to therapy or infection outcome in case of HCV infection. In addition these allelic variations also had no association in the gender based allelic prevalence in the studied patients. A large number of studies have focused on the association of *TNF-α* promoter region polymorphisms and their outcomes in HCV infected populations. A meta-analysis study reported no association of *TNF-α-* 238, -308 gene polymorphisms with HCV susceptibility and infection (Corchado *et al*., 2013). Similarly another study failed to associate any link between these polymorphisms and response to interferon therapy, which is in coherence with our study (Dai *et al*., 2006). Similarly other studies also reported no association of *TNF-α* promoter region polymorphisms in HCV infection outcomes (Gunal *et al*., 2017; Williams *et al*., 2004; Danzer *et al*., 2017).

Major Histocompatibility Complex (MHC) is regarded as the most highly polymorphic genes in the human genome and it is thought that this extreme allelic polymorphism could be a mechanism to counteract a broad range of antigens and pathogens (Nicole and Elahi, 2017; Robinson *et al.,* 2014). Polymorphism in these genes can result in the change of amino acid sequence, which could further result in the altered peptide binding (NCBI). The

α and β domains of the MHC complex are responsible for the antigen presentation and processing (Wagner *et al.,* 2012).

In this study SNPs in the exon 2 of the *HLA-DRB1* gene were analyzed, exon 2 codes for the extracellular domain of the MHC complex (NCBI). It was revealed that in R and NR patients only one SNP at 6228 (C/T) (rs2308802) was significantly associated with response to antiviral therapy in studied patients (*p*=0.010, OR=2.23, 95% CI; 1.02 to 4.12). Different studies have been carried out to analyze this polymorphism like a study carried out in Caucasian population showed the distribution of rs2308802 haplotype in studied patients (Logar *et al*., 2002; Andersen *et al*., 1991).

Gender based allelic differentiation showed that a novel SNP at position 6231 (T/C/G) showed significant association with HCV infection in both male and female patients (*p*=0.008, OR=0.50, 95% CI; 0.29 to 0.840). This polymorphism was reported for the first time in HCV infected patients in Pakistani population. Allelic distribution of SNPs in the *HLA-DRB1* gene in SR and CI groups showed no significant association with disease outcome in studied patients in this study, as many sociodemographic factors may be related, which elaborates the need of further research and analysis in this context of immunogenetics.

Allelic polymorphism at the promoter region of *HLA-DQB1* gene was analyzed in the studied group of patients. Analysis at -8447 (C/A/T/G) position polymorphism revealed that allelic polymorphism at this position was significantly associated with response to interferon therapy in therapy group (*p*=0.028, OR=0.44, 95% CI; 0.210 to 0.925). Similarly SNPs at -84471 (A/G/C) position was also significantly associated with response to interferon therapy in studied group of patients (*p*=0.036, OR=1.84, 95% CI; 1.038 to 3.627).

These polymorphisms at the promoter region of *HLA-DQB1* gene were first time reported to be associated with response to IFN/RBV therapy. The promoter region of this gene is known to be associated with the transcriptional control of the whole gene. Various studies have investigated the role of *HLA-DQB1* promoter region association with different disease outcomes, like systemic lupus erythematosus, autoimmune hepatitis and transcriptional regulation of *DQB* alleles and reported various associations of SNP with disease outcomes

in various groups of patients (Fernandez *et al*., 1998; Zajacova *et al*., 2015; Huang *et al*., 2015; Sammi *et al*., 2015).

Multivariate logistic regression analysis of *HLA-DRB1* rs2308802 and *HLA-DQB1* -8471 SNPs in responsive and non-responsive patients revealed that these SNPs were predictors for anti-HCV therapy response in studied patients. The *p* values and odd ratios were highly significant in developing response to anti-HCV therapy. The multivariate logistic regression analyses were significantly associated when adjusted for age, whereas non-significantly associated when adjusted for gender in studied patients. Haplotypes for the studied group of genes were constructed and percentiles for the each haplotype were calculated. The most prevalent haplotype in each gene were determined and showed in the results section.

A number of studies have described haplotypes for these genes and their respective prevalence rates (Hydes *et al*., 2015; Ansari *et al*., 2015; Sedighimehr *et al*., 2017). A large number of studies have focused on the association of single nucleotide polymorphisms (SNPs) in the immune system genes and their outcomes in the context of interferon-ribavirin therapy for HCV infection (Chen *et al*., 2014). A study conducted in Iranian patients revealed that *HLA* SNPs rs4273729, along with *IFNL4* rs469415590 and *IL28B* rs12979860 were influential predictors for rapid virological response in patients treatedwith peg-interferon and ribavirin (Chen *et al*., 2016). Similarly another study also reported a significant association of *HLA*, SNP rs4273729 and *IL-28B* rs12980275 with the therapy outcomes in infected patients (Susser *et* al., 2014). SNPs in the *HLA-DM* region of class II gene (rs23544, rs1063478) were found to be associated with anti-HCV therapy outcomes in Chinese Han population (Aziz *et al*., 2015).

In-addition *IFN-L4* and *IL28B* genotypes were found to be predictors of response to interferon and ribavirin therapy HCV infected patients (Huang *et al*., 2014; Gheorghe *et al*., 2014). Candidate gene analysis of 9 HLA genes revealed that *HLA-DOB* rs7383287, *HLA-DMA* rs1063478 and *HLA-DOA* rs2284191 were the novel loci involved in the development of HCV infection (Nozawa *et al*., 2013). Allelic polymorphism in the *HLA-DRB1* gene among Romanian patients revealed that *HLA-DRB1*0301, DRB1*0701* and *DRB1*11* may have a significant role in developing a therapeutic response against HCV infection (Farag *et al*., 2013). A study conducted in HCV genotype 1b infected Japanese

patients reported that *HLA-B/KIR3* and *HLA-C1/KIR2* were significantly associated with response to interferon-ribavirin therapy (Marangon *et al*., 2012).

HLA-A01 and *HLA-B38* allelic distribution is responsible or may have an impact in generating response to peg-IFN-ribavirin therapy in HCV infected Egyptian patients (Shaker *et al*., 2013). Role of *HLA-DRB1* and *DQB1* allelic variations were investigated in Brazilian patients and they found that *DRB1* alleles *DRB1*11* along with *DQB1*03* were more frequent in the in the interferon-ribavirin therapy responsive group, similarly another study also reported a positive association of *HLA-DRB1* alleles with the development of response to interferon-ribavirin therapy (De Rueda *et al*., 2011). These studied suggest the role of multiple host genetic factors and their possible outcomes in defining the outcomes for HCV infection in both therapy and non-therapy groups. Similarly these studies also elaborates the HCV infection outcomes in patients groups like chronically infected and spontaneously recovered groups.

This study demonstrated that allele frequencies at 3 of the 20 SNPs were varying significantly among the R and NR patients, i.e., *HLA-DRB1* rs2308802, *HLA-DQB1* -8447, and *HLA-DQB1* -8471. Two of these SNPs were novel. However, in the multivariate analyses, *HLA-DQB1* -8471 was detected to be a significant predictor of response to treatment, in the presence of *HLA-DQB1* rs230880. Further analyses of locus *HLA-DQB1*-8471 depicted that G allele was highly prevalent in the NR group compared to R (51% vs. 36%, respectively), while allele A was more pronounced among the R group compared to NR (64% vs. 47%; OR: 1.94; 95% CI: 1.038-3.627). A review of published studies reveals that different combinations of SNPs in HLA cluster appear to be associated with HCV clearance or treatment response. Further, a certain SNP associated with a patient group in one population may appear to be non-significant with the patient group in other population. For instance, Ksiaa et al. showed that in patients of Tunisian origin, DRB1*08 was associated with HCV clearance, DRB1*15 appeared to be a risk marker for viral persistence (Ksiaa *et al*., 2006). Similarly, in a study carried out in Spain, de Rueda et al. observed favorable treatment response among the HCV infected patients harboring *DQB**0301 allele. Other studies however, showed no significant differences in the allelic polymorphisms at *DQBI* among the spontaneously recovered and chronically infected

patients.Similarly Romero et al. reported no association of HLA polymorphism DQB1*0301 and the SR and CI patients (Romero *et al*., 2015).

Furthermore, the distribution of allelic polymorphisms at 20 SNPs showed no significant differences among the SR and CI groups. At *HLA-DQB1* (-8471), the genotype frequencies were scientifically varying between SR and CI. Multivariate analyses did not reveal any significant association between genotypes and disease status. This discrepancy between the univariate and multivariate results could be due to ethnic heterogeneity in patients and small sample size. In addition to observing the association of SNPs with the disease status, we constructed haplotypes for each gene. SNPs were analysed in three categories. Haplotypes were generated by manually reading the sequence traces and their relative frequencies were calculated. Our analyses showed that the differences in the distribution of haplotypes were statistically not significant between R and NR, and between CI and SR patients.

Linkage disequilibrium (LD) is the non-random association of alleles at particular loci having the ability to inherit together (Houldsworth *et al*., 2015). A number of factors can influence the patterns of LD, factors like population ethnicity, genetic drift, mutation rate and non-random mating (Ardlie *et al*., 2002). Genome wide linkage analysis for the identification genetic basis of diseases has not yet al been promising and effective strategies would be needed (Freimer and Sabatti, 2004; Risch, 2000). One of the major issue in HapMap and disease association mapping is the varying pattern of LD across different populations. New genotype and haplotype maps are required to confirm disease and linkage associations in various populations (Cavalli-Sforza *et al*., 1994; David *et al*., 2005).

LD analyses were carried out for the genotypes observed in this study. Linkage analysis for *TNF-α* gene revealed no significant association for the identified set al of genotypes in studied patients. Linkage disequilibrium analysis (LD) for three SNPs -238 G/A, -308 G/A, -863 C/A tagged as snp1, snp2, snp3 at *TNF-α* genewere analyzed. The D’ statistics showed varying results whereas chi square distributions showed no significant associations for LD in all three SNPs studied in *TNF-α* gene. A combination of haplotypes in *TNF-α* and HLA genes can have a progressive role in HCV infection and therapy outcome, however

genotypes interplay can also have a significant role in defining HCV disease outcome (Tibbs *et al*., 1996).

LD analysis for *HLA-DRB1* gene revealed some linkage patterns as shown in the Figure 4.27 in results section. The heat map showed that there is linkage between various genotypes at different SNPs across the gene. In this gene genotype at SNP 4 (6151 (T/C) rs 1064664) was in LD with SNP 7 (6228 (C/T) rs 2308802). Similarly SNP 5 (6167 (A/C/T) rs 7079657) genotype was also in linkage with SNP 6 (6192 (C/T) rs 776046212). SNP 5 (6167 (A/C/T) rs 7079657) and SNP 6 6192 (C/T) rs 776046212) along with SNP7 (6228 (C/T) rs 2308802) were also found to be in linkage with SNP 9 (6236 (A/C) rs 1059586). Another genotype found to be in linkage with SNP 8 (6231 (T/C/G) rs 3167799) was SNP 10 (6251 (A/T) rs 397844204).

The most linked SNP in these analyses is SNP 9, which is linked to three SNPs. All the genotypes analyzed on these SNPs were found to be in linkage with each other and rightly so because these genotypes are located very close to each other on same fragment and gene, so there are higher chances that there is LD in these sets of SNPs. Further analyses is required regarding these genotypes and SNPs to conform their importance and role in HCV immunogenetics.

HLA-DQB1 linkage patterns were also analyzed; tables and maps were generated and shown in the results section. Linkage analysis of different SNPs at this gene also showed linkage disequilibrium. SNP 11 (-8471 (A/G/C)rs unknown) and SNP 12 (-8465 (G/A/C) rs9273552) were found to be in LD in this analysis. Similarly SNP 11 (-8471 (A/G/C) 6:32628910) was also linked with SNP 15 (-8362 (A/C/G) rs unknown), along with SNP 16 (-8344 (C/T) rs unknown) and SNP 17 (-8331 (T/G) rs unknown). SNP 12 (-8465 (G/A/C) rs9273552) was found to be linked with both 16 (-8344 (C/T) rs unknown) and SNP 17 (-8331 (T/G) rs unknown). Similarly SNP 12 was also found to be linked with SNP 20 (-8288 (C/G) rs 9273598). SNP 13 (-8447 (C/A/G/T) rs unknown) was found to be linked with SNP 18 (-8307 (A/C/G) rs 9273588), whereas SNP 16 (-8344 (C/T) rs unknown) and SNP 17 (-8331 (T/G) rs unknown). Similarly SNP 19 (-8298 (C/G) rs9273592) was found to be linked with SNP 20 (-8288 (C/G) rs 9273598).

The most common linkage patterns were observed in SNP 11, 12 and 20. As we are aware of the fact that due to the occurrence of linkage disequilibrium, SNPs are segregated in

non-random method, which results in the identification and explanation for the haplotype blocks. In the region of haplotype block information regarding a locus can result in prediction of the genotype at the corresponding connected polymorphic loci (Doyle *et al*., 2012). Such information regarding a tagged SNPs can be widely used as a shortcut for genotyping in GWAS and further may result in the identification of multiple genotypes from a single SNP. A project entitled human HapMap resulted in creation of tag SNPs database that can help in the analysis of similar variations in the genome and determination of linkage disequilibrium in analyzed set of patients (HapMap consortium, 2005).

A number of studies have concluded LD in genotypes and haplotypes and its association with HCV therapy outcome. A study reported LD in *HLA-DRB1*11* and *HLA-DQB1*0301* alleles as predictor of therapy response in studied patients; similarly haplotypes were also found to be predictors of response in Pakistani population (Lubna *et al*., 2010). Another study reported that DRB1*11 and DQB1*0301 haplotypes were in LD and were responsible for clearance of HCV infection (Minton *et al*., 1996).

Another study conducted by a Chinese group reported that HLA allele's rs3077 and rs2395309 were in close and highly significant LD and were responsible for natural susceptibility to HCV infection (Ming *et al*., 2015). Similarly DRB1*11 and DQB1*03 alleles were associated with presentation of viral peptides into CD4T cells and hence efficient immune response against HCV infection, these haplotypes were in LD and play an important role in modeling the anti-HCV response (Cangussu *et al*., 2005). A study reported that close LD among alleles DQB1*0301 andDRB1*1101 of HLA class II regionwere providing protection against HCV infection in studied group of patients (Corghi *et al*., 2010). The HLA class C alleles Cw*04 and B*5301 were in strong linkage disequilibrium can result in increased persistent HCV infections (Thio *et al*., 2002).

5.1. Conclusion

Conclusively, epidemiological aspects and association of host genetic factors in determining the outcome of anti-HCV therapy were deciphered. This study reported allelic variations at 20 different SNPs in *TNF-α, HLA-DRB1*, and *HLA-DQB1* in a cohort of HCV infected patients of Pakistani origin. This cohort study also reported the distribution of five novel SNPs in the promoter region of *HLA-DQB1*. Further, a novel SNP *HLA-DQB1* (-8471) emerged as a predictor of positive response to anti-HCV therapy in HCV-infected Pakistani patients. Mapping analysis revealed that three SNPs in the *TNF-α* gene mapped into promoter of the gene, similarly seven SNPs observed in *HLA-DRB1* gene mapped into exon two of the *HLA-DRB1* gene, whereas ten SNPs observed in *HLA-DQB1* gene mapped into promoter region of gene. Patient genotype analysis showed that none of the genotype at *TNF-α* gene was associated with therapy outcome or HCV disease outcome. Genotypes at -8362 and 6151 (rs1064663) and 6231 (rs3167799) at *HLA-DQB1* and *HLA-DRB1* gene were significantly associated with infection and therapy outcome. Haplotype analysis revealed none of the generated haplotypes were known to have a statistically significant association with therapy response or HCV infection outcomes in studied group of patients. Analysis of linkage disequilibrium in studied patients showed that there was linkage among various SNPs of *HLA-DRB1*, *DQB1* genes. Dominancy models for all the genotypes at studied SNPs were generated and varying associations were found among the prevalent genotypes and study groups. This study reveals that prescreening of this variant before therapy would benefit patients with HCV. These findings are vital in describing new therapeutic formulations for the treatment of HCV infection in Pakistani population. Genotyping of these identified variants can help in development of personalized medicines or pan-genotype DAAs in future. Mainstream patients belonged to Islamabad and Rawalpindi, whereas jaundice, fatigue, abdominal discomfort, anorexia and malaise were the prevalent symptoms. Age group 31-40 years was frequent among studied patients and injections, intravenous infusions, barber visits and skin piercing were the prevalent risk factors. Average HCV viral load was 4,137,337 IU/ml in studied patients whereas the most prevalent genotype was genotype 3a (86%) in all patients. These demographic and patient history information reveal that solid steps should be taken by Government of Pakistan to raise awareness among the people regarding the transmission and preventive measures for

HCV infection. Awareness campaigns on Television, Radio and social media can help in controlling the spread of this silent killer in general population of Pakistan.

5.2. Future prospects

A number of genes are involved in mediating the immune responses against HCV infection. In addition to the role of SNPs in *TNF-α, HLA-DRB1* and *HLA-DQB1* in response to interferon/ribavirin therapy, the association of polymorphism in other genes like *IFNL3/4*, *KIR*, and *TLRs* can be investigated in the studied population. Linkage disequilibrium studies describing the association of the above mentioned genes can provide an insight to the genetic basis of response to anti-HCV therapy and inheritance patterns in Pakistani population. Similarly more studies confirming the association of identified SNPs in these genes with more aggressive regimens such as pan-genotype DAAs can be conducted. Similarly docking and simulation studies of the identified SNPs in this study can further elaborate the molecular associations among the variant and therapy response. Knock out mice models for these identified SNPs can be generated in order to confirm the association of response to therapy and genetic polymorphism in the respective group of patients. Association of interferon lambda with anti-HCV therapy in Pakistani population has been investigated; a combinatorial analysis of analyzed genes in this study and interferon lambda can be executed. Ethnic based analysis of these identified SNPs can also be carried out in Pakistani population and compared for anti-HCV therapy response. GWAS analyzing the HCV immunogenetics can also be conducted in Pakistani population. Similarly in addition to genotype 3a, association of other HCV genotypes with disease progression and therapy response can also be carried out. Prevalence studies confirming HCV prevalence along with prevalent genotypes and associated risk factors can also be carried out in Pakistani population preceded by more immunogenetic analysis.

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Appendix

Table A.1.1 Gender and literacy based distribution of symptoms in patients

Symptoms	Literate (n)	Illiterate (n)	Males (n)	Females (n)
Jaundice	101	16	70	47
Abdo. D	77	14	39	52
Anorexia	76	13	46	43
Fatigue	117	19	58	78
Fever	83	16	45	54
Malaise	59	13	34	38
Dark Urine	43	1	24	20
Other	55	6	32	29
***p* value**	0.377, NS		0.1505, NS	
Mean	76.38	12.25	43.50	45.13
SD	24.42	5.898	14.85	17.55

$p<0.05$= Significant, NS= non-significant

Table A.1.2 Demographic distribution of patients on gender and literacy

Demography	Literate (n)	Illiterate (n)	Males (n)	Females (n)
Islamabad	70	11	40	41
KP	24	5	10	19
Punjab	28	2	17	13
Rawalpindi	77	13	46	44
Other	13	2	8	7
***p* value**	0.8053, NS		0.4911, NS	
Mean	42.40	6.600	24.20	24.80
SD	29.02	5.128	17.61	16.74

$p<0.05$= Significant, NS= non-significant

Table A.1.3 Gender and literacy based distribution of age intervals in studied patients

Age intervals	Literate (n)	Illiterate (n)	Males (n)	Females (n)
19-30	52	5	30	27
31-40	69	7	34	42
41-50	48	6	24	30
51-60	34	9	23	20
61-70	9	6	10	5
***p* value**	0.008, S		0.479, NS	
Mean	42.40	6.600	24.20	24.80
SD	22.46	1.517	9.121	13.63

$p<0.05$= Significant, NS= non-significant, S= significant

Table A.1.4 Gender and literacy based distribution of HCV genotypes in studied patients

Genotype	Literate (n)	Illiterate (n)	Males (n)	Females (n)
3a	70	17	42	45
1a	2	0	1	1
1b	2	0	2	0
Untypable	2	0	1	1
***p* value**	0.697, NS		0.553, NS	
Mean	19.00	4.250	11.50	11.75
SD	34.00	8.500	20.34	22.17

$p<0.05$= Significant, NS= non-significant

Table A.1.5 Risk factors distribution on gender and literacy basis in studied patients

Risk factors	Literate (n)	Illiterate (n)	Males (n)	Females (n)
Boil Water	31	0	31	13
Family member infected	50	10	60	24
Father Status	8	0	8	5
Mother Status	8	1	9	5
Parental Consanguinity	44	2	46	23
Injections	211	33	244	120
Surgery	69	11	80	36
Blood Transfusion	54	6	60	22
IV Infusion	123	22	145	67
Dentist Visit	93	18	111	58
Barber Visit	109	7	116	114
Skin piercing	100	24	124	10
Illegal Injections	1	1	2	1
Spouse status	9	0	9	3
Hospitalization	37	9	46	19
***p* value**	0.023, S		0.001, S	
Mean	63.13	9.600	72.73	34.67
SD	56.72	10.29	66.22	38.54

$p<0.05$= Significant, S= significant

Table A.1.6 Occupational information of male and female patients

Occupation	Male	Female
Business	10	1
Driver	12	0
Labor	25	0
Servant	36	7
Shopkeeper	13	1
Student	8	4
Teacher	8	7
Nil	0	2
House wives	0	102
***p* value**	0.001, S	
Mean	12.44	13.78
SD	11.56	33.20

$p<0.05$= Significant, S= significant

Table A.1.7 Literacy levels in male and female patients

Education	**Male**	**Female**
Madrasa	8	7
Primary	15	22
Middle	18	14
Metric	29	19
Intermediate	20	8
Bachelors	13	19
Master's	11	9
Nil	7	26
Total	121	124
***p* value**	0.003, S	
Mean	15.13	15.50
SD	7.200	7.071

$p<0.05$= Significant, S= significant

Single SNP analysis

Table A.2.1Hardy-Weinberg equilibrium analysis for *TNF-α* -238 position

snp1 exact test for Hardy-Weinberg equilibrium (n=173)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	154	11	8	319	27	**<0.0001***
sex=Female	77	5	6	159	17	**<0.0001**
sex=Male	77	6	2	160	10	**0.021**

*$p<0.05$= Significant observed

Table A.2.2Dominancy models for *TNF-α* -238 position genotypes in studied patients

snp1 association with response sex (n=173, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	G/G	77 (87.5%)	77 (90.6%)	1.00	0.34	243.6	253.1
	G/A	5 (5.7%)	6 (7.1%)	1.20 (0.35-4.10)			
	A/A	6 (6.8%)	2 (2.4%)	0.33 (0.07-1.70)			
Dominant	G/G	77 (87.5%)	77 (90.6%)	1.00	0.52	243.4	249.7
	G/A-A/A	11 (12.5%)	8 (9.4%)	0.73 (0.28-1.91)			
Recessive	G/G-G/A	82 (93.2%)	83 (97.7%)	1.00	0.15	241.7	248
	A/A	6 (6.8%)	2 (2.4%)	0.33 (0.06-1.68)			
Overdominant	G/G-A/A	83 (94.3%)	79 (92.9%)	1.00	0.71	243.6	249.9
	G/A	5 (5.7%)	6 (7.1%)	1.26 (0.37-4.30)			
Log-additive	---	---	---	0.71 (0.37-1.36)	0.29	242.7	249

$p<0.05$= Significant

Table A.2.3Hardy-Weinberg equilibrium analysis for *TNF-α* -308 position

snp2 exact test for Hardy-Weinberg equilibrium (n=169)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	147	7	15	301	37	**<0.0001**
sex=Female	75	3	5	153	13	**<0.0001**
sex=Male	72	4	10	148	24	**<0.0001**

p<0.05= Significant

Table A.2.4Dominancy models for *TNF-α* -308 position genotypes in studied patients

snp2 association with response sex (n=169, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	G/G	75 (90.4%)	72 (83.7%)	1.00	0.4	238.4	247.8
	G/A	3 (3.6%)	4 (4.7%)	1.39 (0.30-6.42)			
	A/A	5 (6%)	10 (11.6%)	2.08 (0.68-6.39)			
Dominant	G/G	75 (90.4%)	72 (83.7%)	1.00	0.2	236.6	242.8
	G/A-A/A	8 (9.6%)	14 (16.3%)	1.82 (0.72-4.61)			
Recessive	G/G-G/A	78 (94%)	76 (88.4%)	1.00	0.2	236.6	242.8
	A/A	5 (6%)	10 (11.6%)	2.05 (0.67-6.29)			
Overdominant	G/G-A/A	80 (96.4%)	82 (95.3%)	1.00	0.73	238.1	244.4
	G/A	3 (3.6%)	4 (4.7%)	1.30 (0.28-6.00)			
Log-additive	---	---	---	1.44 (0.84-2.46)	0.17	236.4	242.6

p<0.05= Significant

Table A.2.5Hardy-Weinberg equilibrium analysis for *TNF-α* -863 position

snp3 exact test for Hardy-Weinberg equilibrium (n=172)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	103	37	32	243	101	**<0.0001**
sex=Female	56	16	15	128	46	**<0.0001**
sex=Male	47	21	17	115	55	**0.00011**

p<0.05= Significant

Table A.2.6 Dominancy models for *TNF-α* -863 position genotypes in studied patients

snp3 association with response sex (n=172, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	C/C	56 (64.4%)	47 (55.3%)	1.00	0.46	242.9	252.3
	C/A	16 (18.4%)	21 (24.7%)	1.56 (0.73-3.33)			
	A/A	15 (17.2%)	17 (20%)	1.35 (0.61-2.99)			
Dominant	C/C	56 (64.4%)	47 (55.3%)	1.00	0.22	240.9	247.2
	C/A-A/A	31 (35.6%)	38 (44.7%)	1.46 (0.79-2.70)			
Recessive	C/C-C/A	72 (82.8%)	68 (80%)	1.00	0.64	242.2	248.5
	A/A	15 (17.2%)	17 (20%)	1.20 (0.56-2.59)			
Overdominant	C/C-A/A	71 (81.6%)	64 (75.3%)	1.00	0.31	241.4	247.7
	C/A	16 (18.4%)	21 (24.7%)	1.46 (0.70-3.03)			
Log-additive	---	---	---	1.21 (0.83-1.78)	0.32	241.4	247.7

$p<0.05$= Significant

Table A.2.7 Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6151 position

snp4 exact test for Hardy-Weinberg equilibrium (n=175)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	60	60	55	180	170	**<0.0001**
sex=Female	26	37	23	89	83	0.2
sex=Male	34	23	32	91	87	**<0.0001**

$p<0.05$= Significant

Table A.2.8Dominancy models for *HLA-DRB1* 6151 position genotypes in studied patients

snp4 association with response sex (n=175, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	T/T	26 (30.2%)	34 (38.2%)	1.00	0.055	242.8	252.2
	T/C	37 (43%)	23 (25.8%)	**0.48 (0.23-0.99)**			
	C/C	23 (26.7%)	32 (36%)	1.06 (0.51-2.23)			
Dominant	T/T	26 (30.2%)	34 (38.2%)	1.00	0.27	245.3	251.6
	T/C-C/C	60 (69.8%)	55 (61.8%)	0.70 (0.37-1.31)			
Recessive	T/T-T/C	63 (73.3%)	57 (64%)	1.00	0.19	244.8	251.2
	C/C	23 (26.7%)	32 (36%)	1.54 (0.81-2.93)			
Overdominant	T/T-C/C	49 (57%)	66 (74.2%)	1.00	0.016	240.8	247.1
	T/C	37 (43%)	23 (25.8%)	**0.46 (0.24-0.87)**			
Log-additive	---	---	---	1.02 (0.71-1.47)	0.92	246.5	252.9

$p<0.05$= Significant

Table A.2.9Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6167 position

snp5 exact test for Hardy-Weinberg equilibrium (n=146)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	92	49	5	233	59	0.8
sex=Female	51	20	2	122	24	1
sex=Male	41	29	3	111	35	0.75

p<0.05= Significant

Table A.2.10Dominancy models for *HLA-DRB1* 6167 position genotypes in studied patients

snp5 association with response sex (n=146, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	A/A	51 (69.9%)	41 (56.2%)	1.00	0.23	205.4	214.4
	A/T	20 (27.4%)	29 (39.7%)	1.80 (0.89-3.64)			
	T/T	2 (2.7%)	3 (4.1%)	1.87 (0.30-11.70)			
Dominant	A/A	51 (69.9%)	41 (56.2%)	1.00	0.086	203.4	209.4
	A/T-T/T	22 (30.1%)	32 (43.8%)	1.81 (0.92-3.57)			
Recessive	A/A-A/T	71 (97.3%)	70 (95.9%)	1.00	0.65	206.2	212.2
	T/T	2 (2.7%)	3 (4.1%)	1.52 (0.25-9.38)			
Overdominant	A/A-T/T	53 (72.6%)	44 (60.3%)	1.00	0.11	203.9	209.9
	A/T	20 (27.4%)	29 (39.7%)	1.75 (0.87-3.50)			
Log-additive	---	---	---	1.64 (0.90-2.99)	0.1	203.7	209.7

p<0.05= Significant

Table A.2.11Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6192 position

snp6 exact test for Hardy-Weinberg equilibrium (n=177)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	159	18	0	336	18	1
sex=Female	78	9	0	165	9	1
sex=Male	81	9	0	171	9	1

p<0.05= Significant

Table A.2.12Dominancy models for *HLA-DRB1* 6192 position genotypes in studied patients

snp6 association with response sex (n=177, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
---	C/C	78 (89.7%)	81 (90%)	1.00	0.94	249.3	255.7
	C/T	9 (10.3%)	9 (10%)	0.96 (0.36-2.55)			

$p<0.05$= Significant

Table A.2.13Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6228 position

snp7 exact test for Hardy-Weinberg equilibrium (n=174)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	87	15	72	189	159	**<0.0001**
sex=Female	36	10	40	82	90	**<0.0001**
sex=Male	51	5	32	107	69	**<0.0001**

$p<0.05$= Significant

Table A.2.14Dominancy models for *HLA-DRB1* 6228 position genotypes in studied patients

snp7 association with response sex (n=174, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	C/C	36 (41.9%)	51 (58%)	1.00	0.076	242	251.5
	C/T	10 (11.6%)	5 (5.7%)	0.35 (0.11-1.12)			
	T/T	40 (46.5%)	32 (36.4%)	0.56 (0.30-1.06)			
Dominant	C/C	36 (41.9%)	51 (58%)	1.00	0.033	240.7	247
	C/T-T/T	50 (58.1%)	37 (42%)	**0.52 (0.29-0.95)**			
Recessive	C/C-C/T	46 (53.5%)	56 (63.6%)	1.00	0.17	243.3	249.7
	T/T	40 (46.5%)	32 (36.4%)	0.66 (0.36-1.21)			
Overdominant	C/C-T/T	76 (88.4%)	83 (94.3%)	1.00	0.16	243.2	249.5
	C/T	10 (11.6%)	5 (5.7%)	0.46 (0.15-1.40)			
Log-additive	---	---	---	0.75 (0.54-1.02)	0.069	241.9	248.2

$p<0.05$= Significant

Table A.2.15Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6231 position

snp8 exact test for Hardy-Weinberg equilibrium (n=88)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	33	31	24	97	79	0.0092
sex=Female	21	16	10	58	36	0.065
sex=Male	12	15	14	39	43	0.12

p<0.05= Significant

Table A.2.16Dominancy models for *HLA-DRB1* 6231 position genotypes in studied patients

snp8 association with response sex (n=88, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	C/C	21 (44.7%)	12 (29.3%)	1.00	0.25	124.8	132.2
	T/C	16 (34%)	15 (36.6%)	1.64 (0.60-4.46)			
	T/T	10 (21.3%)	14 (34.1%)	2.45 (0.83-7.20)			
Dominant	C/C	21 (44.7%)	12 (29.3%)	1.00	0.13	123.3	128.3
	T/C-T/T	26 (55.3%)	29 (70.7%)	1.95 (0.81-4.73)			
Recessive	C/C-T/C	37 (78.7%)	27 (65.8%)	1.00	0.18	123.8	128.7
	T/T	10 (21.3%)	14 (34.1%)	1.92 (0.74-4.97)			
Overdominant	C/C-T/T	31 (66%)	26 (63.4%)	1.00	0.8	125.5	130.5
	T/C	16 (34%)	15 (36.6%)	1.12 (0.47-2.68)			
Log-additive	---	---	---	1.57 (0.92-2.68)	0.096	122.8	127.8

p<0.05= Significant

Table A.2.17Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6236 position

snp9 exact test for Hardy-Weinberg equilibrium (n=174)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	127	42	5	296	52	0.55
sex=Female	67	17	2	151	21	0.6
sex=Male	60	25	3	145	31	0.72

p<0.05= Significant

Table A.2.18Dominancy models for *HLA-DRB1* 6236 position genotypes in studied patients

snp9 association with response sex (n=174, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	A/A	67 (77.9%)	60 (68.2%)	1.00	0.35	245.1	254.6
	A/C	17 (19.8%)	25 (28.4%)	1.64 (0.81-3.33)			
	C/C	2 (2.3%)	3 (3.4%)	1.68 (0.27-10.37)			
Dominant	A/A	67 (77.9%)	60 (68.2%)	1.00	0.15	243.1	249.4
	A/C-C/C	19 (22.1%)	28 (31.8%)	1.65 (0.83-3.24)			
Recessive	A/A-A/C	84 (97.7%)	85 (96.6%)	1.00	0.67	245	251.3
	C/C	2 (2.3%)	3 (3.4%)	1.48 (0.24-9.10)			
Overdominant	A/A-C/C	69 (80.2%)	63 (71.6%)	1.00	0.18	243.4	249.7
	A/C	17 (19.8%)	25 (28.4%)	1.61 (0.80-3.26)			
Log-additive	---	---	---	1.51 (0.84-2.72)	0.17	243.3	249.6

p<0.05= Significant

Table A.2.19Hardy-Weinberg equilibrium analysis for *HLA-DRB1* 6251 position

snp10 exact test for Hardy-Weinberg equilibrium (n=174)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	151	22	1	324	24	0.57
sex=Female	79	7	1	165	9	0.2
sex=Male						

p<0.05= Significant

Table A.2.20Dominancy models for *HLA-DRB1* 6251 position genotypes in studied patients

snp10 association with response sex (n=174, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	A/A	79 (90.8%)	72 (82.8%)	1.00	0.096	242.5	252
	A/T	7 (8.1%)	15 (17.2%)	2.35 (0.91-6.09)			
	T/T	1 (1.1%)	0 (0%)	0.00 (0.00-NA)			
Dominant	A/A	79 (90.8%)	72 (82.8%)	1.00	0.11	242.7	249
	A/T-T/T	8 (9.2%)	15 (17.2%)	2.06 (0.82-5.14)			
Recessive	A/A-A/T	86 (98.8%)	87 (100%)	1.00	0.24	243.8	250.1
	T/T	1 (1.1%)	0 (0%)	0.00 (0.00-NA)			
Overdominant	A/A-T/T	80 (92%)	72 (82.8%)	1.00	0.065	241.8	248.1
	A/T	7 (8.1%)	15 (17.2%)	2.38 (0.92-6.17)			
Log-additive	---	---	---	1.72 (0.73-4.04)	0.2	243.6	249.9

p<0.05= Significant, NA= not availabale.

Table A.2.21Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8471 position

snp11 exact test for Hardy-Weinberg equilibrium (n=174)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	126	35	13	287	61	**0.00022**
sex=Female	65	14	6	144	26	**0.003**
sex=Male	61	21	7	143	35	**0.036**

p<0.05= Significant

Table A.2.22Dominancy models for *HLA-DQB1* -8471 position genotypes in studied patients

snp11 association with response sex (n=174, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	C/C	65 (76.5%)	61 (68.5%)	1.00	0.47	245.6	255.1
	C/G	14 (16.5%)	21 (23.6%)	1.60 (0.75-3.42)			
	G/G	6 (7.1%)	7 (7.9%)	1.24 (0.40-3.91)			
Dominant	C/C	65 (76.5%)	61 (68.5%)	1.00	0.24	243.7	250.1
	C/G-G/G	20 (23.5%)	28 (31.5%)	1.49 (0.76-2.92)			
Recessive	C/C-C/G	79 (92.9%)	82 (92.1%)	1.00	0.84	245.1	251.4
	G/G	6 (7.1%)	7 (7.9%)	1.12 (0.36-3.49)			
Overdominant	C/C-G/G	71 (83.5%)	68 (76.4%)	1.00	0.24	243.7	250.1
	C/G	14 (16.5%)	21 (23.6%)	1.57 (0.74-3.33)			
Log-additive	---	---	---	1.26 (0.77-2.06)	0.35	244.2	250.6

p<0.05= Significant

Table A.2.23Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8465 position

snp12 exact test for Hardy-Weinberg equilibrium (n=173)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	114	42	17	270	76	**0.00026**
sex=Female	56	19	9	131	37	**0.003**
sex=Male						

p<0.05= Significant

Table A.2.24Dominancy models for *HLA-DQB1* -8465 position genotypes in studied patients

snp12 association with response sex (n=173, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	C/C	56 (66.7%)	58 (65.2%)	1.00	0.85	245.4	254.8
	C/G	19 (22.6%)	23 (25.8%)	1.17 (0.57-2.38)			
	G/G	9 (10.7%)	8 (9%)	0.86 (0.31-2.38)			
Dominant	C/C	56 (66.7%)	58 (65.2%)	1.00	0.84	243.6	249.9
	C/G-G/G	28 (33.3%)	31 (34.8%)	1.07 (0.57-2.01)			
Recessive	C/C-C/G	75 (89.3%)	81 (91%)	1.00	0.7	243.5	249.8
	G/G	9 (10.7%)	8 (9%)	0.82 (0.30-2.24)			
Overdominant	C/C-G/G	65 (77.4%)	66 (74.2%)	1.00	0.62	243.4	249.7
	C/G	19 (22.6%)	23 (25.8%)	1.19 (0.59-2.39)			
Log-additive	---	---	---	0.99 (0.64-1.56)	0.98	243.7	250

$p<0.05$= Significant

Table A.2.25Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8447 position

snp13 exact test for Hardy-Weinberg equilibrium (n=126)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	61	21	44	143	109	**<0.0001**
sex=Female	32	11	19	75	49	**<0.0001**
sex=Male	29	10	25	68	60	**<0.0001**

$p<0.05$= Significant

Table A.2.26Dominancy models for *HLA-DQB1* -8447 position genotypes in studied patients

snp13 association with response sex (n=126, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	A/A	32 (51.6%)	29 (45.3%)	1.00	0.61	179.7	188.2
	A/G	11 (17.7%)	10 (15.6%)	1.00 (0.37-2.71)			
	G/G	19 (30.6%)	25 (39.1%)	1.45 (0.67-3.17)			
Dominant	A/A	32 (51.6%)	29 (45.3%)	1.00	0.48	178.1	183.8
	A/G-G/G	30 (48.4%)	35 (54.7%)	1.29 (0.64-2.59)			
Recessive	A/A-A/G	43 (69.3%)	39 (60.9%)	1.00	0.32	177.7	183.3
	G/G	19 (30.6%)	25 (39.1%)	1.45 (0.69-3.03)			
Overdominant	A/A-G/G	51 (82.3%)	54 (84.4%)	1.00	0.75	178.5	184.2
	A/G	11 (17.7%)	10 (15.6%)	0.86 (0.34-2.19)			
Log-additive	---	---	---	1.20 (0.81-1.77)	0.36	177.8	183.5

$p<0.05$= Significant

Table A.2.27Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8442 position

snp14 exact test for Hardy-Weinberg equilibrium (n=168)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	128	37	3	293	43	0.74
sex=Female	65	17	2	147	21	0.61
sex=Male	63	20	1	146	22	1

p<0.05= Significant

Table A.2.28Dominancy models for *HLA-DQB1* -8442 position genotypes in studied patients

snp14 association with response sex (n=168, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	G/G	65 (77.4%)	63 (75%)	1.00	0.74	238.3	247.7
	G/A	17 (20.2%)	20 (23.8%)	1.21 (0.58-2.53)			
	A/A	2 (2.4%)	1 (1.2%)	0.52 (0.05-5.83)			
Dominant	G/G	65 (77.4%)	63 (75%)	1.00	0.72	236.8	243
	G/A-A/A	19 (22.6%)	21 (25%)	1.14 (0.56-2.32)			
Recessive	G/G-G/A	82 (97.6%)	83 (98.8%)	1.00	0.56	236.6	242.8
	A/A	2 (2.4%)	1 (1.2%)	0.49 (0.04-5.55)			
Overdominant	G/G-A/A	67 (79.8%)	64 (76.2%)	1.00	0.58	236.6	242.8
	G/A	17 (20.2%)	20 (23.8%)	1.23 (0.59-2.56)			
Log-additive	---	---	---	1.05 (0.56-1.99)	0.87	236.9	243.1

p<0.05= Significant

Table A.2.29Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8362 position

snp15 exact test for Hardy-Weinberg equilibrium (n=172)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	90	65	17	245	99	0.35
sex=Female	43	30	11	116	52	0.13
sex=Male	47	35	6	129	47	1

p<0.05= Significant

Table A.2.30Dominancy models for *HLA-DQB1* -8362 position genotypes in studied patients

snp15 association with response sex (n=172, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	G/G	43 (51.2%)	47 (53.4%)	1.00	0.37	242.4	251.8
	G/C	30 (35.7%)	35 (39.8%)	1.07 (0.56-2.02)			
	C/C	11 (13.1%)	6 (6.8%)	0.50 (0.17-1.47)			
Dominant	G/G	43 (51.2%)	47 (53.4%)	1.00	0.77	242.3	248.6
	G/C-C/C	41 (48.8%)	41 (46.6%)	0.91 (0.50-1.66)			
Recessive	G/G-G/C	73 (86.9%)	82 (93.2%)	1.00	0.17	240.4	246.7
	C/C	11 (13.1%)	6 (6.8%)	0.49 (0.17-1.38)			
Overdominant	G/G-C/C	54 (64.3%)	53 (60.2%)	1.00	0.58	242	248.3
	G/C	30 (35.7%)	35 (39.8%)	1.19 (0.64-2.20)			
Log-additive	---	---	---	0.82 (0.53-1.30)	0.4	241.6	247.9

p<0.05= Significant

Table A.2.31Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8344 position

snp16 exact test for Hardy-Weinberg equilibrium (n=170)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	109	40	21	258	82	**<0.0001**
sex=Female	53	24	8	130	40	0.065
sex=Male	56	16	13	128	42	**<0.0001**

p<0.05= Significant

Table A.2.32Dominancy models for *HLA-DQB1* -8344 position genotypes in studied patients

snp16 association with response sex (n=170, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	T/T	53 (62.4%)	56 (65.9%)	1.00	0.24	238.8	248.2
	T/C	24 (28.2%)	16 (18.8%)	0.63 (0.30-1.32)			
	C/C	8 (9.4%)	13 (15.3%)	1.54 (0.59-4.01)			
Dominant	T/T	53 (62.4%)	56 (65.9%)	1.00	0.63	239.4	245.7
	T/C-C/C	32 (37.6%)	29 (34.1%)	0.86 (0.46-1.61)			
Recessive	T/T-T/C	77 (90.6%)	72 (84.7%)	1.00	0.24	238.3	244.6
	C/C	8 (9.4%)	13 (15.3%)	1.74 (0.68-4.44)			
Overdominant	T/T-C/C	61 (71.8%)	69 (81.2%)	1.00	0.15	237.6	243.8
	T/C	24 (28.2%)	16 (18.8%)	0.59 (0.29-1.21)			
Log-additive	---	---	---	1.05 (0.68-1.61)	0.83	239.6	245.9

p<0.05= Significant

Table A.2.33Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8331 position

snp17 exact test for Hardy-Weinberg equilibrium (n=168)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	94	40	34	228	108	**<0.0001**
sex=Female	48	17	18	113	53	**<0.0001**
sex=Male	46	23	16	115	55	**0.00092**

p<0.05= Significant

Table A.2.34Dominancy models for *HLA-DQB1* -8331 position genotypes in studied patients

snp16 association with response sex (n=168, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	T/T	48 (57.8%)	46 (54.1%)	1.00	0.59	237.8	247.2
	T/G	17 (20.5%)	23 (27.1%)	1.41 (0.67-2.98)			
	G/G	18 (21.7%)	16 (18.8%)	0.93 (0.42-2.03)			
Dominant	T/T	48 (57.8%)	46 (54.1%)	1.00	0.63	236.6	242.9
	T/G-G/G	35 (42.2%)	39 (45.9%)	1.16 (0.63-2.14)			
Recessive	T/T-T/G	65 (78.3%)	69 (81.2%)	1.00	0.64	236.7	242.9
	G/G	18 (21.7%)	16 (18.8%)	0.84 (0.39-1.78)			
Overdominant	T/T-G/G	66 (79.5%)	62 (72.9%)	1.00	0.32	235.9	242.1
	T/G	17 (20.5%)	23 (27.1%)	1.44 (0.70-2.95)			
Log-additive	---	---	---	1.01 (0.69-1.48)	0.94	236.9	243.1

p<0.05= Significant

Table A.2.35. Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8307 position

snp18 exact test for Hardy-Weinberg equilibrium (n=128)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	64	28	36	156	100	**<0.0001**
sex=Female	32	15	18	79	51	**<0.0001**
sex=Male	32	13	18	77	49	**<0.0001**

p<0.05= Significant

Table A.2.36Dominancy models for *HLA-DQB1* -8307 position genotypes in studied patients

snp18 association with response sex (n=128, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	C/C	32 (49.2%)	32 (50.8%)	1.00	0.95	183.3	191.9
	T/C	15 (23.1%)	13 (20.6%)	0.87 (0.36-2.11)			
	T/T	18 (27.7%)	18 (28.6%)	1.00 (0.44-2.26)			
Dominant	C/C	32 (49.2%)	32 (50.8%)	1.00	0.86	181.4	187.1
	T/C-T/T	33 (50.8%)	31 (49.2%)	0.94 (0.47-1.88)			
Recessive	C/C-T/C	47 (72.3%)	45 (71.4%)	1.00	0.91	181.4	187.1
	T/T	18 (27.7%)	18 (28.6%)	1.04 (0.48-2.26)			
Overdominant	C/C-T/T	50 (76.9%)	50 (79.4%)	1.00	0.74	181.3	187
	T/C	15 (23.1%)	13 (20.6%)	0.87 (0.37-2.01)			
Log-additive	---	---	---	0.99 (0.66-1.48)	0.96	181.4	187.1

p<0.05= Significant

Table A.2.37Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8298 position

snp19 exact test for Hardy-Weinberg equilibrium (n=164)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	73	27	64	173	155	**<0.0001**
sex=Female	35	11	36	81	83	**<0.0001**
sex=Male	38	16	28	92	72	**<0.0001**

p<0.05= Significant

Table A.2.38Dominancy models for *HLA-DQB1* -8298 position genotypes in studied patients

snp19 association with response sex (n=164, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	A/A	35 (42.7%)	38 (46.3%)	1.00	0.36	231.3	240.6
	A/G	11 (13.4%)	16 (19.5%)	1.34 (0.55-3.28)			
	G/G	36 (43.9%)	28 (34.1%)	0.72 (0.36-1.41)			
Dominant	A/A	35 (42.7%)	38 (46.3%)	1.00	0.64	231.1	237.3
	A/G-G/G	47 (57.3%)	44 (53.7%)	0.86 (0.47-1.60)			
Recessive	A/A-A/G	46 (56.1%)	54 (65.8%)	1.00	0.2	229.7	235.9
	G/G	36 (43.9%)	28 (34.1%)	0.66 (0.35-1.25)			
Overdominant	A/A-G/G	71 (86.6%)	66 (80.5%)	1.00	0.29	230.2	236.4
	A/G	11 (13.4%)	16 (19.5%)	1.56 (0.68-3.62)			
Log-additive	---	---	---	0.85 (0.61-1.19)	0.35	230.5	236.7

p<0.05= Significant

Table A.2.39Hardy-Weinberg equilibrium analysis for *HLA-DQB1* -8288 position

snp20 exact test for Hardy-Weinberg equilibrium (n=125)						
	N11	**N12**	**N22**	**N1**	**N2**	***p*-value**
All subjects	68	15	42	151	99	**<0.0001**
sex=Female	31	8	23	70	54	**<0.0001**
sex=Male	37	7	19	81	45	**<0.0001**

p<0.05= Significant

Table A.2.40Dominancy models for *HLA-DQB1* -8288 position genotypes in studied patients

snp20 association with response sex (n=125, crude analysis)							
Model	**Genotype**	**sex=Female**	**sex=Male**	**OR (95% CI)**	***p*-value**	**AIC**	**BIC**
Codominant	A/A	31 (50%)	37 (58.7%)	1.00	0.62	178.3	186.8
	A/C	8 (12.9%)	7 (11.1%)	0.73 (0.24-2.25)			
	C/C	23 (37.1%)	19 (30.2%)	0.69 (0.32-1.50)			
Dominant	A/A	31 (50%)	37 (58.7%)	1.00	0.33	176.3	182
	A/C-C/C	31 (50%)	26 (41.3%)	0.70 (0.35-1.42)			
Recessive	A/A-A/C	39 (62.9%)	44 (69.8%)	1.00	0.41	176.6	182.3
	C/C	23 (37.1%)	19 (30.2%)	0.73 (0.35-1.54)			
Overdominant	A/A-C/C	54 (87.1%)	56 (88.9%)	1.00	0.76	177.2	182.8
	A/C	8 (12.9%)	7 (11.1%)	0.84 (0.29-2.49)			
Log-additive	---	---	---	0.83 (0.56-1.22)	0.34	176.4	182

p<0.05= Significant

Figure A.3.1 Alligned sequences of the SNPs observed in *HLA-DRB1*gene

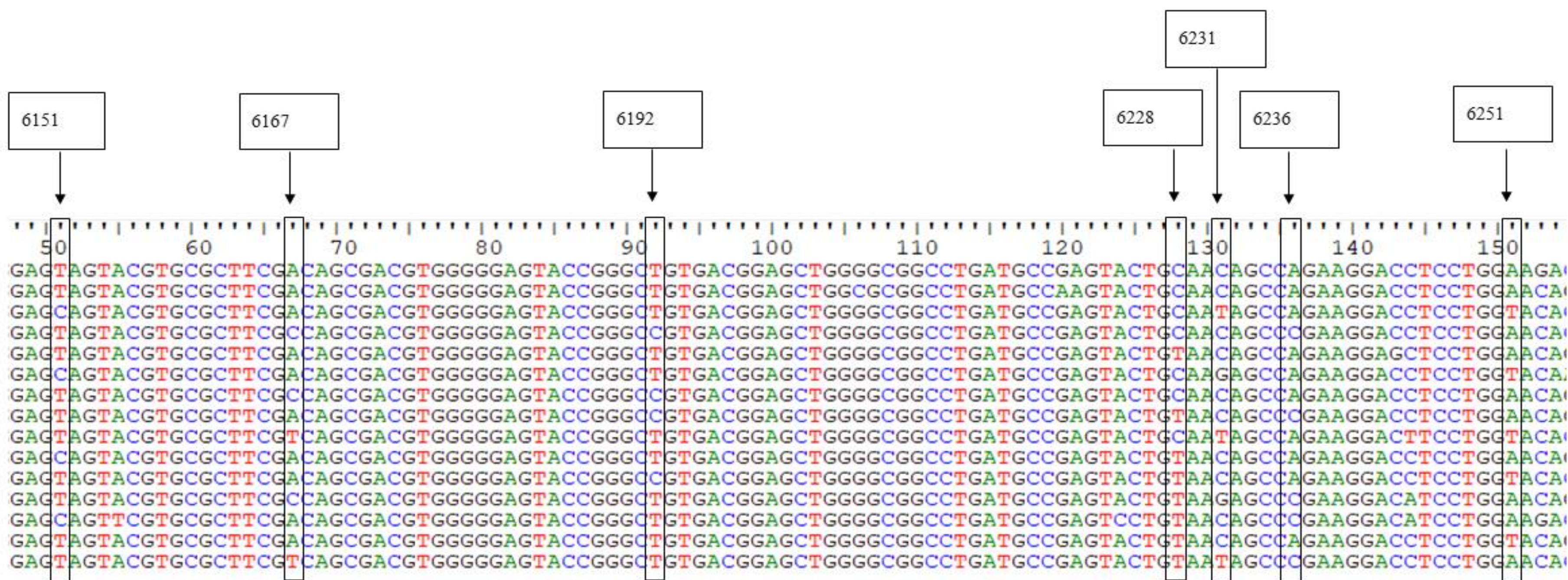

Figure A.3.2 Alligned sequences of the SNPs observed in *HLA-DQB1* gene (1-6)

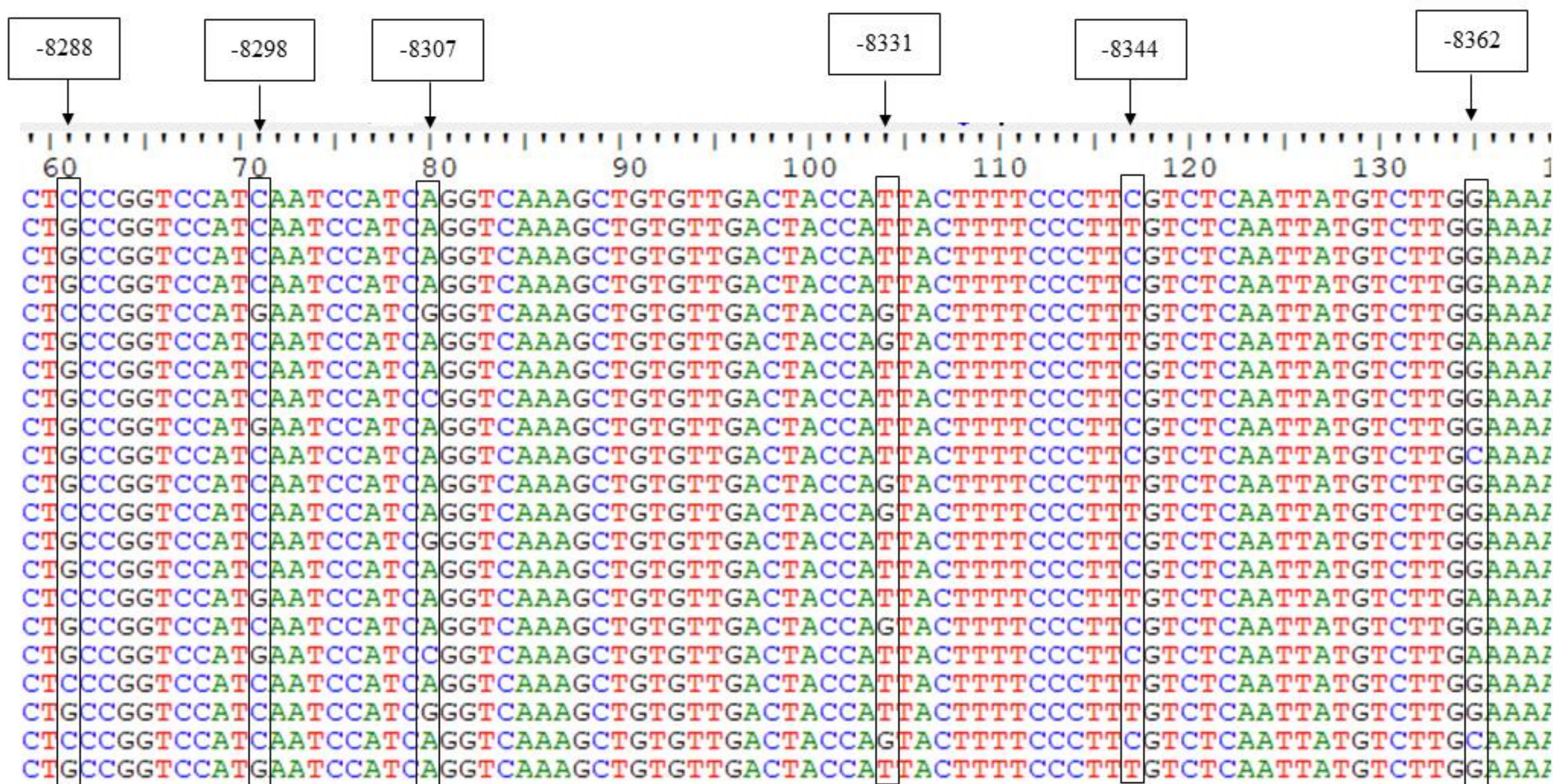

Figure A.3.2 Alligned sequences of the SNPs observed in *HLA-DQB1* gene (7-10)

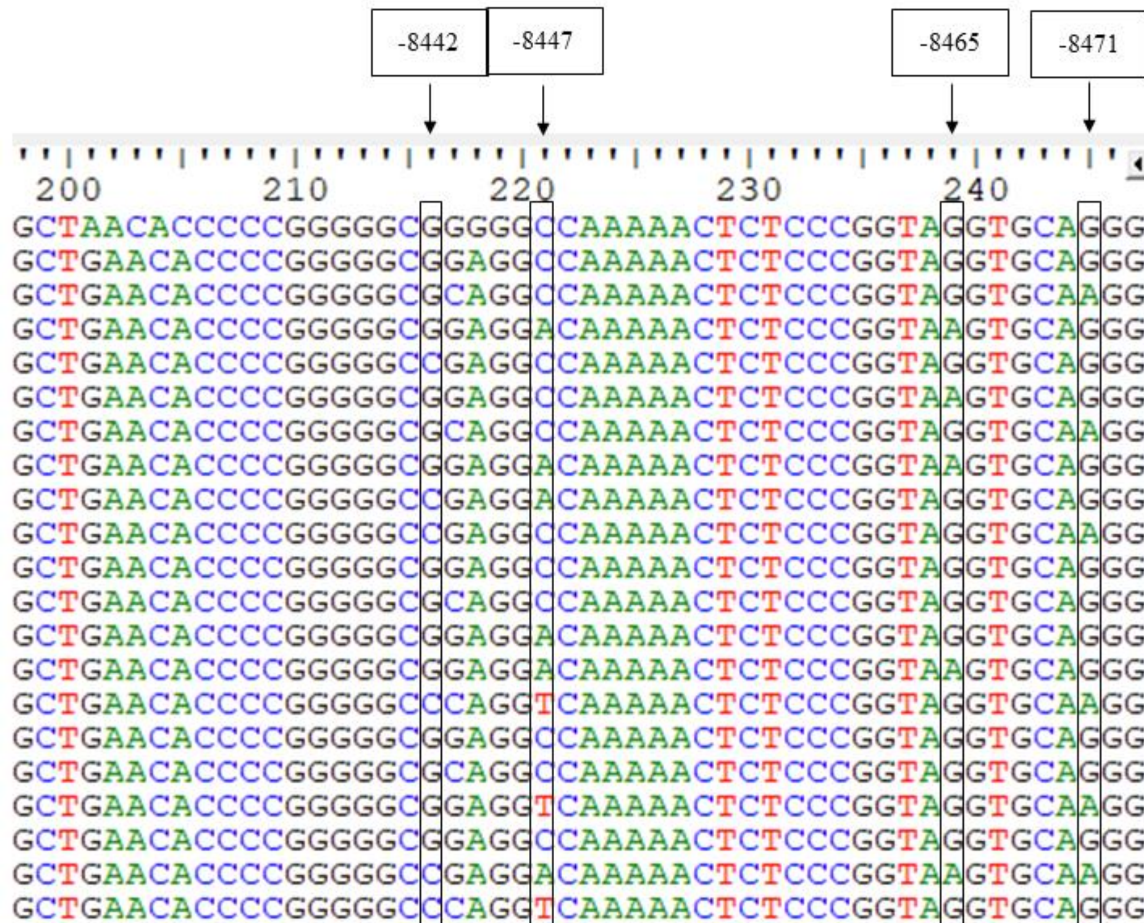

Figure A.3.3 Alligned sequences of the SNPs observed in *TNF-α* gene (-238/-308) positios

Figure A.3.3 Alligned sequences of the SNPs observed in *TNF-α* gene (-863) position

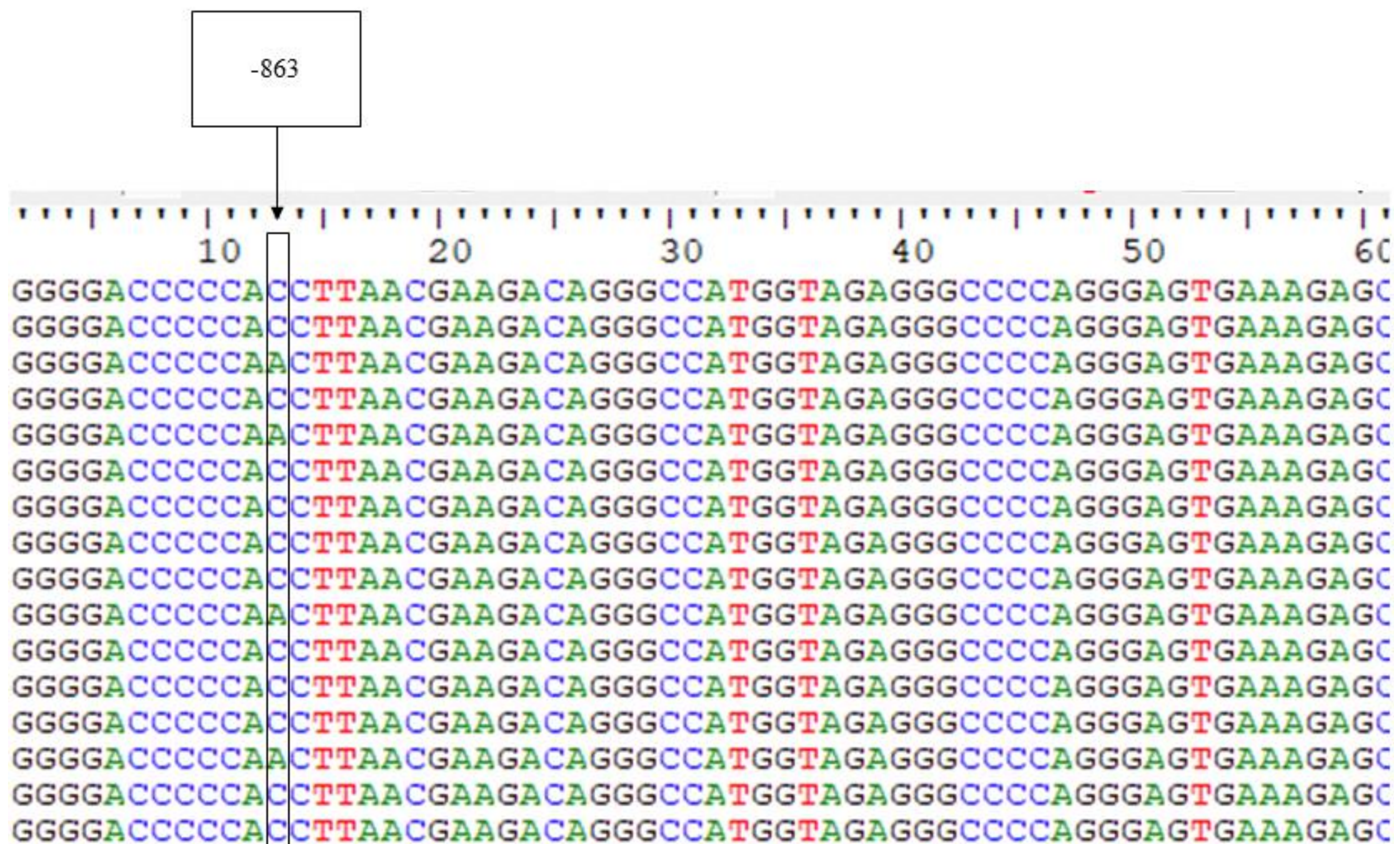

Table 3.4 Minor allele frequencies and allelic estimates in South Asian population for the studied SNPs

rs number	Reference Allele	Variant Allele	Allele frequency UCSC hg-19	MAF dnSNP	Allele frequency in South Asian population Ensemble h38
rs361525	G	A	G: 93.91%,A: 6.09%	A=0.0609/305 (1000 Genomes) A=0.0495/6212 (TOPMED)	G=89% A=11%
rs1800629	G	A	G: 85.24%, A: 14.73%	A=0.1616/3247 (ExAC) A=0.0903/452 (1000 Genomes) A=0.1414/803 (GO-ESP) A=0.1431/17974 (TOPMED)	G=95% A=5%
rs1800630	C	A	C: 84.58%, A: 15.41%	A=0.1542/772 (1000 Genomes) A=0.1413/17746 (TOPMED)	C=76% A=24%
rs1064664	T	C	T: 70.39%, C: 29.60%	G=0.2954/31059 (ExAC) G=0.3095/1550 (1000 Genomes) G=0.2867/3703 (GO-ESP) G=0.3190/40052 (TOPMED)	A=68% G=32%
rs707957	C	A/G	C: 26.92%, A: 60.76%, T: 12.31%	G=0.2693/29939 (ExAC)	A=11% G=37% T=52%
rs776046212	C	T	C: 99.998%, T:0.002%	T=0.00002/2 (ExAC)	C=100% T=0%
rs2308802	C	T/G	C: 54.31%, T: 45.62% G: 0.058%	A=0.4562/45680 (ExAC) A=0.4692/58916 (TOPMED)	A=31% C=0% G=69%
rs3167799	T	G/C	N/A	A=0.2676/33605 (TOPMED)	A=68% C=3% G=29%
rs1059586	A	C	A: 94.96%, C: 5.03%	G=0.0503/4812 (ExAC) G=0.0786/9867 (TOPMED)	G=1% T=99%
rs397844204	A	A	N/A	N/A	N/A
rs9273598	C	G	N/A	NA	N/A
rs9273592	G	C/T	G: 99.96%, T: 0.032%	T=0.0002/8 (ExAC) T=0.0012/6 (1000 Genomes)	G=100% T=0%

				T=0.0011/13 (GO-ESP) T=0.0047/584 (TOPMED)	
rs9273588	G	A/C	G: 99.35%, A: 0.642%	A=0.00003/1 (ExAC) A=0.0551/276 (1000 Genomes) A=0.0126/1584 (TOPMED)	G=84% A=16%
rs9273552	G	A	G: 87.14%, A: 12.85%	A=0.1286/644 (1000 Genomes) A=0.0355/4457 (TOPMED)	G=90% A=10%
rs34644981	G	C	G:87.89%, C: 12.10%	G=0.1210/606 (1000 Genomes) G=0.0928/11658 (TOPMED)	C=91% G=9%