



Novel Polymorphism in the Promoter Region of *HLA-DQB1* Is a Predictor of Anti-HCV Therapy Response

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Abstract

Background: Hepatitis C virus (HCV) is a major cause of chronic liver infection, which may lead to liver cirrhosis, fibrosis, and hepatocellular carcinoma while about one-fourth of the infected patients recover spontaneously. The host immune response remains decisive in the outcomes of antiviral treatment. Single nucleotide polymorphisms (SNPs) at the genes within the human leukocyte antigen (HLA) cluster are associated with differential immune response and treatment outcomes.

Objectives: This study aimed to determine the association of SNPs in Tumor necrosis factor-alpha (*TNF-α*) and HLA genes (*HLA-DRB1* and *HLA-DQB1*) with the outcomes of HCV infection and anti-HCV therapy.

Methods: The study included 245 HCV-infected patients visiting a tertiary care hospital, located in Islamabad, Pakistan. Viral quantification and genotyping were performed by real-time PCR. Twenty SNPs in *TNF-α*, *HLA-DRB1*, and *HLA-DQB1* were sequence-genotyped by the Sanger method in 180 patients. Multivariate logistic regression was performed to establish the association of SNPs with spontaneous clearance of the virus and response to anti-HCV therapy.

Results: Five out of 20 SNPs were novel. Allelic variants at three different locations (*HLA-DRB1*, rs2308802; *HLA-DQB1*,-8447, and *HLA-DQB1*,-8471) showed significant associations with two groups of HCV patients receiving anti-HCV treatment (responsive and non-responsive). Multivariate analyses disclosed that genotype A/A at *HLA-DQB1* (-8471) was a significant predictor of positive response to treatment although in the presence of the variant at *HLA-DRB1* (rs230880) ($P = 0.004$). However, no association was detected between the haplotypes constructed for the three sets of SNPs and different categories of patients.

Conclusions: This study established a novel SNP *HLA-DQB1*(-8471) as an important predictor of an effective response to anti-HCV therapy in HCV-infected Pakistani patients. Prescreening of this variant before therapy would benefit HCV patients.

Keywords: Hepatitis, Polymorphisms, SNPs, *TNF-α*, *HLA-DRB1*, *HLA-DQB1*, Haplotypes

1. Background

Infection caused by hepatitis C virus (HCV) is one of the main public health concerns across the globe. Hepatitis C virus is associated with consistently higher morbidity and mortality in various developing countries including Pakistan (1). Despite a wide variation across different geographical regions, the overall prevalence of HCV in Asia is > 2% (2). According to the estimates, approximately 77% of the individuals infected with HCV are unable to clear the virus spontaneously during the initial six months of infection (so-called acute phase). Eventually, these patients develop a chronic infection that may lead to other complications such as fibrosis, cirrhosis, hepatocellular carcinoma, and liver failure (2, 3). The exact mechanism by which the spontaneous clearance of HCV occurs remains obscure.

However, predisposition to the viral infection is dependent on both environmental and genetic factors (4). Single nucleotide polymorphisms (SNPs) at various loci, particularly the immune system genes, have been implicated in the host immune response, as well as treatment outcomes. For instance, studies have shown that variant rs4273729 at *HLA-DQB1* was associated with the clearance of HCV in European and African cohorts (5). On the other hand, SNPs in the intronic region of *HLA-DQB1* were found to be associated with chronic HCV infection (6).

Currently, HCV treatment comprises combination therapy with pegylated interferon (*peg-IFN*) for up to 48 weeks and ribavirin (RBV) for up to 24 weeks (7). After 24 weeks of the completion of therapy, the absence of the HCV RNA and evidence of a sustained virological response (SVR) indicates treatment success (8). The presence of HCV

RNA is indicative of non-clearance, which could be due to ineffective recognition of viral antigen by T cells or failure in the treatment (4, 8). Moreover, human leukocyte antigen (HLA) molecules such as HLA-DQ, -DR, and -DP trigger the immune response via CD4 T cells (9, 10). Similarly, *IFN-L4* genotypes (rs12979860 C/C and ss469415590 TT/TT) were described as the predictors of response to IFN and RBV therapy (11, 12). Allelic polymorphisms *HLA-DRB1*0301*, *DRB1*0701*, and *DRB1*11* showed to have significant roles in therapeutic response to HCV infection in Romanian patients (10). Likewise, *HLA-A01* and *HLA-B38* were associated with response to IFN-RBV therapy in HCV-infected Egyptian patients (13).

The roles of *HLA-DRB1* and *DQB1* allelic variations were investigated in Brazilian patients and *DRB1*11* along with *DQB1*03* was witnessed to have frequent occurrence among the responsive group of patients under IFN-RBV therapy (6, 14). The association of SNPs in *TNF- α* , *HLA-DRB1*, and *HLA-DQB1* with differential response to HCV infection and anti-HCV therapy has been established in many populations (10).

2. Objectives

In this study, we first established the distribution of SNPs in three key immunological factors, i.e., *TNF- α* , *HLA-DRB1*, *HLA-DQB1*, in a cohort of HCV-infected patients of Pakistani origin. Second, through regression modeling, we investigated the role of 20 different SNPs in spontaneous clearance and response to anti-HCV therapy among chronically infected patients. We reported five novel SNPs in the promoter region of *HLA-DQB1* and haplotypes of SNPs in association with patients' disease status. To the best of our knowledge, no previous study focused the Pakistani population for comprehensive characterization and analysis of polymorphisms in *TNF- α* , *HLA-DRB1*, and *HLA-DQB1* loci among HCV-infected patients.

3. Methods

3.1. Study Participants and Sampling

The blood samples were collected in the period of July 2015 to September 2015. Data on clinical presentation, risk factors, and anti-HCV treatment were collected. The infection status of the participants was confirmed by the immune chromatographic technique (ICT). Children, immunocompromised patients, pregnant women, and patients having HBV/HIV co-infection were excluded. HIV was detected using anti-HIV antibody by the third-generation ELISA kit (Saceae, Italy) whereas HBsAg was detected using the ICT-based method.

3.2. Quantitative PCR and HCV Genotyping

Hepatitis C virus quantification and genotypic analysis were carried out according to the methods described elsewhere (15, 16).

3.3. Patient Categories

In total, 245 patients (121 men, 124 women) were included in this study (Table 1). The mean age of the patients was 41.1 ± 11.8 years ($P = 0.964$), with the highest percentage of subjects in the age group of 31% - 40.9% (31%; data not shown). The study patients were divided into two groups, i.e., patients receiving anti-HCV therapy (therapy group) and patients not receiving anti-HCV therapy (non-therapy group). The therapy group was further divided into responsive and non-responsive categories. The non-therapy group was divided into two different sub-categories: chronically infected and spontaneously recovered. Hence, the patients in the therapy group were not treatment naïve since they had already received combination therapy (peg-IFN/RBV) for HCV infection. Patients under treatment were receiving antiviral combination therapy with peg-IFN (180 mcg) once a week and RBV (800 - 1200 mg) prescribed according to their body mass index.

3.4. Candidate Gene Selection

The candidate genes selected for the polymorphism study included tumor necrosis factor (*TNF- α* ; NM-000594), human leukocyte antigen-DRB1 (*HLA-DRB1*; NM-002124), and human leukocyte antigen-DQB1 (*HLA-DQB1*; NM-002123). In total, 20 different SNPs were studied, including 3 in the upstream region of *TNF- α* , 7 in exon 2 of *HLA-DRB1*, and other 10 in the upstream region of *HLA-DQB1* (Figure 1). This combination of genes and SNPs had not been previously studied in Pakistani HCV cohorts.

3.5. DNA Extraction and Genotyping

By using the standard phenol-chloroform method, genomic DNA was extracted from the whole blood. For PCR analysis, 50 μ L of the reaction mixture was prepared, consisting of 4 μ L of *ExTaq* polymerase, 5 μ L of both forward and reverse primers, 6 μ L of template DNA (40 ng/ μ L), and 35 V of PCR water. The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 2 minutes, annealing at 58°C for 30 seconds, extension at 72°C for 2 minutes (the cycle repeated from step 2 for 35 times), and a final extension at 72°C for 10 minutes. Amplified products were purified (Qiagen Purification kit). The DNA sequencing was performed on both strands by the Sanger method using both forward and reverse primers (sequences available on request).

Table 1. Features of Therapy Group and Non-Therapy Group

Characteristics	Therapy Group (N = 110)		Non-Therapy Group (N = 135)		Total (N = 245)
	Responsive (N = 68)	Non-Responsive (N = 42)	Spontaneously Recovered (N = 36)	Chronically Infected (N = 99)	
Gender^a					
Male	33 (49)	23 (55)	22 (61)	43 (43)	121 (49)
Female	35 (51)	19 (45)	14 (39)	56 (57)	124 (51)
Age category, year^a					
Up to 39	31 (46)	17 (40)	21 (58)	52 (53)	121 (49)
Above 39	37 (54)	25 (60)	15 (42)	47 (47)	124 (51)
Viral genotype^a					
1a	1 (5)	1 (4)	0	0	2 (2)
1b	1 (5)	1 (4)	0	0	2 (2)
3a	20 (90)	21 (84)	6 (100)	40 (100)	87 (94)
Untypable	0	2 (8)	0	0	2 (2)
ALT, IU/L^a	53 ± 53	79 ± 71	39 ± 21	58 ± 17	59 ± 54
Viral load, ×10⁵ IU/L					
< 8 ×10 ⁵ IU/L		17		33	50
> 8 ×10 ⁵ IU/L		19		56	75

^aDistributions between SR/CI and R/NR groups were not statistically significant.

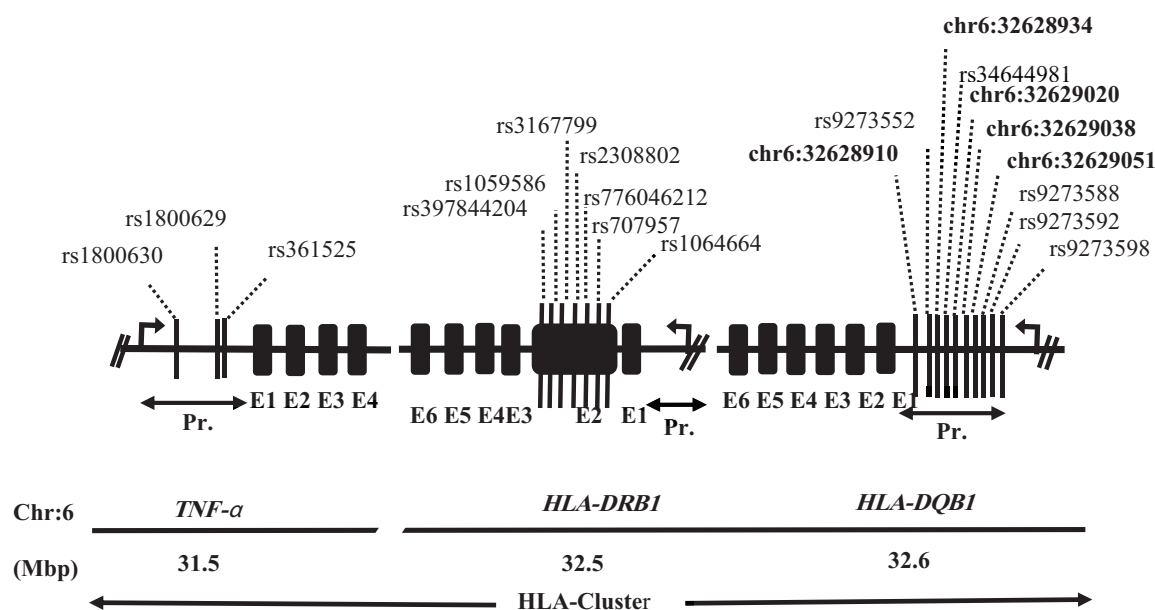


Figure 1. Genomic interval at chromosome 6 illustrating the map of SNPs in three genes investigated in this study. Arrows indicate the direction of the reading frame of the gene. SNPs are shown with rs number. Novel SNPs detected in *DQB1* are shown in boldface. E = exon; Pr. = promoter.

3.6. SNP Typing and Mapping, Detection of Novel SNPs and Haplotype Generation

DNA sequence reads were analyzed for the typing of SNPs. Sequence electropherograms were inspected

using BioEdit Sequence Editor (TomHall) and Codon-Code Aligner (version 7.0.1). Minor allele frequencies (MAF) were observed in online databases such as ExAC,

1000 Genome, and SNPedia. Positions and maps of the SNPs were determined using the UCSC genome browser (GRCh37/hg19). Haplotypes were generated by online tool SNPstat (<https://www.snpstats.net/start.htm>) and linkage disequilibrium estimates were obtained.

3.7. Statistical Analysis

Allele and genotype frequencies of all the SNPs were compared between the responsive and non-responsive (R vs. NR) groups and between chronically infected and spontaneously recovered (CI vs. SR) groups. An exact test for Hardy-Weinberg equilibrium (HWE) was implemented for genotype frequencies observed in SR and CI patients, as well as in the R and NR groups. Statistical analyses were carried out using GraphPad Prism and STATA (11.0) software. The significance of the differences from random distribution was estimated by the chi-square and *t* tests. Yates' corrected chi-square and Fisher's exact tests were also employed. The level of significance was set at a *P* value of < 0.05. The magnitude of the effect was analyzed by calculating odds ratios (OR) and 95% confidence intervals (95% CI). For multivariate analyses, logistic regression was performed for 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 eliminating the non-significant ones.

4. Results

4.1. Sample Characteristics and HCV Prevalence

The distribution of subjects in the R/NR and CI/SR groups is presented in [Table 1](#). The tertiary care hospital, which was the sampling setting for the current study, is located in Federal Capital Islamabad and has a high influx of patients from the adjoining areas. Hence, the majority of the patients originated from Islamabad (33%) and Rawalpindi (27%) districts (*P* = 0.386). The most prevalent viral genotype detected in this cohort was 3a (94%). The alanine aminotransferase (ALT) ranges were higher in the NR group than in the R group and in the CI group than in the SR group, but the differences were statistically non-significant.

The total number of patients receiving anti-HCV therapy was 110 ([Table 1](#)). In this group, 62% (*n* = 68) of the patients were responsive (R) while 38% (*n* = 42) were non-responsive (NR). Moreover, 135 patients did not receive any anti-HCV therapy among whom, 73% (*n* = 99) were chronically infected (CI) and 27% (*n* = 36) recovered spontaneously (SR). Overall, 51% (*n* = 125) were positive for viral RNA. The average viral load was 41×10^5 IU/mL. The most

prevalent viral genotype was 3a (95%), followed by 1b (2%) and 1a (1%), while 2% (*n* = 2) of the isolates were untypable ([Table 1](#)).

4.2. Clinical Presentation and Risk Factors

A range of clinical symptoms was observed among the patients. The most common symptoms were fatigue (56%), jaundice (47%), fever (40%), abdominal discomfort (38%), and anorexia (36%) (*P* = 0.162) (Supplementary File Appendix 1). The most common risk factors associated with HCV infection were the use of injection (100%), followed by intravenous infusions (59%), skin piercing (51%), barber visits (47%), dentist visits (45%), surgery (33%), blood transfusion (24%), and having a family member with HCV infection (24%) (*P* < 0.0001).

4.3. Distribution of SNPs in Responsive (R) and Non-Responsive (NR) Groups

The allele frequencies at three of the 20 studied SNPs varied significantly among the R and NR patients ([Table 2](#)). At *HLA-DRB1* locus, the allele frequency of rs2308802 showed a significant variation among the patient groups (*P* = 0.010). At this locus, the C allele was more prevalent in the R group than in the NR group (63% vs. 43%) whereas the T allele was more frequently presented among the NR group than among the R group (56.9% vs. 37.2%; *P* = 0.01; OR = 2.23; 95% CI = 1.20 - 4.12). Likewise, the allele distribution of *HLA-DQB1* -8447 significantly varied among the patient groups (*P* = 0.028). At this locus, the T allele was nearly two times higher in the R group than in the NR group (38% vs. 22%; *P* = 0.028; OR = 0.44; 95% CI = 0.21 - 0.93). At *HLA-DQB1* -8471, the difference was statistically significant in the distribution of alleles between the R and NR groups (*P* = 0.036). Here, the G allele was more found in the NR group than in the R group (51% vs. 36%) while the A allele was more prevalent in the R group than in the NR group (64% vs. 47%; OR = 1.94; 95% CI = 1.04 - 3.63).

Further analyses showed that the genotype frequencies of these 20 SNPs did not vary significantly between the R and NR groups of patients. The genotype frequencies of 4 out of 20 SNPs were concordant with the assumptions of HWE ([Table 2](#)). For multivariate analyses, genotypes were coded and the most common genotype was used as the reference genotype. Interestingly, *HLA-DQB1* -8471 was detected to be a significant predictor of response to treatment though in the presence of *HLA-DQB1* rs230880 while the overall model was highly significant ([Table 3](#)).

4.4. Distribution of SNPs in Spontaneously Recovered (SR) and Chronically Infected (CI) Patients

The overall distribution of allelic polymorphisms at the 20 SNPs did not differ significantly between the SR

Table 3. Genotype Frequencies of HLA-DRB1 rs2308802 and HLA-DQB1 -8471 in Responsive (R) and Non-Responsive (NR) Patients and the Result of Multivariate Logistic Regression for Treatment Response^a

Locus	Genotype	Responsive	Non-Responsive	P Value	Odds Ratio	95% CI
HLA-DRB1						
6228 (rs2308802)	C/C	30 (33)	14 (53)	-	Reference	
	C/T	4 (8)	3 (8)	0.565	1.67	0.3161 to 8.172
	T/T	17 (59)	19 (39)	0.058	2.40	0.9625 to 5.959
HLA-DQB1 (-8471)	A/A	31 (57)	13 (39)	-	Reference	
	A/G	7 (13)	5 (15)	0.426	1.70	0.456 to 6.364
	G/C	0 (0)	1 (3)	0.132	7.00	0.268 to 183.1
	G/G	16 (30)	14 (42)	0.133	2.09	0.794 to 5.486
Multivariate						
HLA-DRB1 (rs2308802)^b				0.088	0.66	0.405 to 1.065
HLA-DQB1 (-8471)^b				0.030 ^c	0.58	0.350 to 0.949
_cons				0.004 ^c	9.56	2.100 to 43.50

^aValues are expressed as No. (%).

^bHighly significant when adjusted for age but not significant when adjusted for gender.

^cStatistically significant.

and CI groups of patients (Supplementary File Appendix 2). However, at *HLA-DQB1* -8471, the distribution of the A allele was relatively higher among SR patients than among CI patients (59% vs. 42%; $P = 0.061$) while the G allele frequency was found to be 57% in CI patients compared to 41% in SR patients (OR = 1.96; 95% CI = 0.96 - 3.97). Notably, at this locus, the genotype frequencies varied significantly between the SR and CI groups (Table 4). The genotype frequencies at 7 out of 20 SNPs were concordant with the assumptions of HWE (Supplementary File Appendix 2). Overall, multivariate analyses did not show a significant association between different genotypes and disease status of the patients.

4.5. Distribution of SNPs in Male and Female Patients

Significant differences in the allele frequencies of three SNPs were witnessed among male and female patients. For instance, at *HLA-DRB1* (rs1064664), the T allele was more prevalent in female patients than in male patients (58% vs. 43%) while the C allele showed more frequencies among male patients than among female patients (52% vs. 42%) ($P = 0.0135$; OR = 0.59; 95% CI = 0.39 - 0.90). Similarly, at *HLA-DRB1* (rs2308802), the C allele was more prevalent among male patients than among female patients (61% vs. 48%) while the T allele was more prevalent in women than in men (52% vs. 39%) ($P = 0.014$; OR = 1.70; 95% CI = 1.11 - 2.61). At *HLA-DRB1* (rs3167799), the C allele was observed to be more frequent among female patients than among male patients (75% vs. 51%) while the G allele was more common among male patients (55% vs. 49%). The T allele was found to be more prevalent in males than in females (65% vs. 48%)

($P = 0.009$; OR = 0.50; 95% CI = 0.30 - 0.84) (Supplementary File Appendix 3).

4.6. Identification of Novel SNPs and Haplotype Construction

Five novel SNPs were detected at *HLA-DQB1*, including -8331(T/G), -8344(T/C), -8447(C/A/G/T), -8471(A/G/C), and -8362(A/C/G) (Figure 1). The differences in the distribution of haplotypes generated for the three sets of SNPs in three loci were not statistically significant between different groups of patients including the R and NR groups and the CI and SR groups (Supplementary File Appendix 4). In addition, there were no gender-specific differences in the distribution of haplotypes. The estimates of linkage disequilibrium between the SNPs of the three genes showed a rather mosaic pattern (Supplementary File Appendix 5 - 7). The MAF and allele frequency estimates in the South Asian population are presented in Supplementary File Appendix 8.

5. Discussion

This study investigated the distribution of 20 SNPs in *TNF- α* , *HLA-DRB1*, and *HLA-DQB1* among HCV infected patients. These immunological factors have critical roles in guiding immune responses by controlling the presentation of antigen to CD4 T-cells. The identification of polymorphisms associated with therapy responses could greatly help optimize virologic outcome and economic resources (6, 17). Gender plays an important role in the context of HCV infection and anti-HCV therapy outcomes. A number of studies have reported that men are much more

Table 4. Genotype Frequencies of HLA-DQB1 -8471 in Spontaneously Recovered (SR) and Chronically Infected (CI) Patients^a

Locus	Genotype	Spontaneously Recovered	Chronically Infected	P Value	Odds Ratio	95% CI
HLA-DQB1 (-8471)	A/A	13 (59)	16 (28)	-	Reference	
	A/G	0 (0)	16 (28)	0.002 ^b	27.0	1.479 to 493.0
	G/G	9 (41)	25 (44)	0.128	2.26	0.7845 to 6.493
	Total	22	57			

^aValues are expressed as No. (%).^bStatistically significant.

prone to developing chronic liver diseases than women are (18). It has been established that women tend to have better responses against HCV infection than men do. Studies have also reported that spontaneous clearance and response to anti-HCV therapy are more common in women than in men (19).

Conflicting observations have also been reported. For instance, Samimi-Rad et al. witnessed no difference in the spontaneous clearance between male and female patients recruited from the Iranian population (20). The present study, likewise, showed that the clinical presentation of male and female patients was rather similar and the signs and symptoms of HCV infection were not significantly different between them ($P = 0.162$) (Supplementary File Appendix 1). Furthermore, the differences in the average viral load, viral genotypes, and ALT levels were not statistically significant between male and female patients. There were also no statistically significant differences between male and female patients respecting SR/CI and R/NR ($P = 0.0691$ and $P = 0.525$, respectively).

It is, nonetheless, pertinent to mention that in the current study, gender-based differences were evident in the allele distribution at three SNPs in *HLA-DRB1*. In SNP rs1064664, allele T was more prevalent in female patients than in male patients (58.1% vs. 42.6%) while allele C was more prevalent in males than in females (52.1% vs. 41.9%) (OR = 0.587). In SNP rs2308802, allele C was more prevalent among males than among females (60.8% vs. 47.7%) while allele T was more prevalent in females (52.3% vs. 39.2%; $P = 0.014$) (OR = 1.702). At SNP rs3167799, allele C was more prevalent in females while allele T was more prevalent in males ($P = 0.008$). Even though the clinical presentation, spontaneous clearance, and treatment response were not significantly different between male and female patients, it is quite likely that these SNPs had associations with other certain traits/infections or susceptibilities. This research area warrants further investigations.

In the current study, the allele frequencies at 3 out of 20 different SNPs varied significantly between the R and NR patients, including *HLA-DRB1* rs2308802 (C/T), *HLA-DQB1* -8447 (C/A/G/T), and *HLA-DQB1* -8471. However, in the pres-

ence of *HLA-DRB1* rs2308802, SNP *HLA-DQB1* -8471 was witnessed as a significant predictor of response to treatment. Furthermore, analyses of locus *HLA-DQB1* -8471 showed that allele G was more prevalent in the NR group than in the R group (51% vs. 36%) while allele A was more pronounced in the R group than in the NR group (64% vs. 47%; OR: 1.94; 95% CI: 1.04 - 3.63). It was further observed that the differences in the distribution of alleles at 20 different SNPs were not statistically significant between SR and CI patients. However, at locus *HLA-DQB1* (-8471), genotypic frequencies varied significantly between the SR and CI groups of patients. A review of the published studies disclosed that different combinations of SNPs in the HLA cluster appear to be associated with HCV clearance or treatment response.

A certain SNP significantly associated with a patient group in a specific population may be non-significant in a patient group from another population. For instance, Ksaa et al. (21) showed that in patients of Tunisian origin, DRB1*08 was associated with HCV clearance and DRB1*15 appeared to be a risk marker for viral persistence. Similarly, in a study carried out in Spain, de Rueda et al. (22) observed favorable treatment responses among the HCV-infected patients harboring the *DQB1**0301 allele. Other studies, however, showed no significant differences in the allelic polymorphisms at DQB1 between spontaneously recovered and chronically infected patients. Romero et al. (23) reported no association of HLA polymorphism DQB1*0301 with SR and CI groups. In a Romanian cohort of chronic HCV patients, Gheorghe et al. observed that *HLA-DRB1**0301 had a high frequency (14.8%) in NR patients while alleles DRB1*0701 (11.1%), DRB1*11 (22.2%), and DRB1*0101 (16.7%) were prevalent in sustained virologic responders (SVR) (10). On the other hand, it was interesting that the SNPs in *TNF- α* were not associated with any patient category. Hence, the research findings in one population cannot be generalized to other population.

Single nucleotide polymorphisms in the promoter region are responsible for the differential expression of HLA genes, immunological outcome, and response to therapy. Here, for the first time, SNPs in *HLA-DQB1* are reported to be associated with response to IFN/RBV therapy in a Pakistani

population. Various other studies have investigated the association of SNPs in the promoter region of *HLA-DQB1* with differential disease outcomes, such as systemic lupus erythematosus and autoimmune hepatitis (24). In the current study, the most common risk factors associated with HCV infection was injection use, intravenous infusion, and skin piercing. These findings are in concordance with other studies reported from Pakistan and demonstrate the importance of proper public awareness programs to reduce the prevalence of HCV infection in Pakistan (25-27). In a recent study carried out in Faisalabad, Pakistan, the most prominent risk factor was injecting drug use observed in 51% of HCV patients (27). Reker and Islam showed that surgery, transfusion, and age were the most important risk factors of HCV in an Egyptian population (28). In another study carried out in Italy, intravenous drug abuse and age of ≤ 60 years were associated with HCV (29).

In our study, multivariate analyses did not show any significant association between genotypes and infection status. This discrepancy between the univariate and multivariate results could be attributed to the small sample size and ethnic heterogeneity among patients. Further, one of the limitations of the study is that information was missing regarding lifestyle, smoking, dietary habits, and other physiological variables that could be confounding factors of morbidity. Additionally, haplotype analyses showed that the differences in the distribution of haplotypes were not significant between R/NR and CI/SR groups.

5.1. Conclusions

This study reported the 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. Prescreening of this variant before therapy would benefit HCV patients.

Supplementary Material

Supplementary material(s) is available [here](#) [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: Javid Iqbal Dasti designed the study. Muhammad Arshad and Ismail Jalil conducted the sample collection and all the experiments. Abida Raza

helped in providing the samples and data. Sajid Malik carried out all the extensive statistical analyses.

Conflict of Interests: The authors declare none.

Ethical Approval: The Institutional Review Board of Quaid-i-Azam University, Islamabad, granted ethical approval for this study (Micro/2016/1027). The ethical guidelines of the Helsinki Declaration were followed, informed consent was obtained from patients visiting the Nuclear Medicine Oncology and Radiotherapy Institute, Islamabad.

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References

1. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2015;**385**(9963):117-71. doi: [10.1016/S0140-6736\(14\)61682-2](https://doi.org/10.1016/S0140-6736(14)61682-2). [PubMed: [25530442](https://pubmed.ncbi.nlm.nih.gov/25530442/)]. [PubMed Central: [PMC4340604](https://pubmed.ncbi.nlm.nih.gov/PMC4340604/)].
2. Webster DP, Klenerman P, Dusheiko GM. Hepatitis C. *Lancet*. 2015;**385**(9973):1124-35. doi: [10.1016/S0140-6736\(14\)62401-6](https://doi.org/10.1016/S0140-6736(14)62401-6). [PubMed: [25687730](https://pubmed.ncbi.nlm.nih.gov/25687730/)]. [PubMed Central: [PMC4878852](https://pubmed.ncbi.nlm.nih.gov/PMC4878852/)].
3. van der Meer AJ, Veldt BJ, Feld JJ, Wedemeyer H, Dufour JF, Lammer F, et al. Association between sustained virological response and all-cause mortality among patients with chronic hepatitis C and advanced hepatic fibrosis. *JAMA*. 2012;**308**(24):2584-93. doi: [10.1001/jama.2012.144878](https://doi.org/10.1001/jama.2012.144878). [PubMed: [23268517](https://pubmed.ncbi.nlm.nih.gov/23268517/)].
4. Westbrook RH, Dusheiko G. Natural history of hepatitis C. *J Hepatol*. 2014;**61**(1 Suppl):S58-68. doi: [10.1016/j.jhep.2014.07.012](https://doi.org/10.1016/j.jhep.2014.07.012). [PubMed: [25443346](https://pubmed.ncbi.nlm.nih.gov/25443346/)].
5. Hudson LE, Allen RL. Leukocyte Ig-like receptors - a model for MHC class I disease associations. *Front Immunol*. 2016;**7**:281. doi: [10.3389/fimmu.2016.00281](https://doi.org/10.3389/fimmu.2016.00281). [PubMed: [27504110](https://pubmed.ncbi.nlm.nih.gov/27504110/)]. [PubMed Central: [PMC4959025](https://pubmed.ncbi.nlm.nih.gov/PMC4959025/)].
6. Miki D, Ochi H, Takahashi A, Hayes CN, Urabe Y, Abe H, et al. HLA-DQB1*03 confers susceptibility to chronic hepatitis C in Japanese: A genome-wide association study. *PLoS One*. 2013;**8**(12): e84226. doi: [10.1371/journal.pone.0084226](https://doi.org/10.1371/journal.pone.0084226). [PubMed: [24376798](https://pubmed.ncbi.nlm.nih.gov/24376798/)]. [PubMed Central: [PMC3871580](https://pubmed.ncbi.nlm.nih.gov/PMC3871580/)].
7. Sarrazin C, Hezode C, Zeuzem S, Pawlotsky JM. Antiviral strategies in hepatitis C virus infection. *J Hepatol*. 2012;**56** Suppl 1:S88-100. doi: [10.1016/S0168-8278\(12\)60010-5](https://doi.org/10.1016/S0168-8278(12)60010-5). [PubMed: [22300469](https://pubmed.ncbi.nlm.nih.gov/22300469/)].
8. Nguyen LH, Nguyen MH. Systematic review: Asian patients with chronic hepatitis C infection. *Aliment Pharmacol Ther*. 2013;**37**(10):921-36. doi: [10.1111/apt.12300](https://doi.org/10.1111/apt.12300). [PubMed: [23557103](https://pubmed.ncbi.nlm.nih.gov/23557103/)].
9. Huang P, Dong L, Lu X, Zhang Y, Chen H, Wang J, et al. Genetic variants in antigen presentation-related genes influence susceptibility to hepatitis C virus and viral clearance: A case control study. *BMC Infect Dis*. 2014;**14**:716. doi: [10.1186/s12879-014-0716-8](https://doi.org/10.1186/s12879-014-0716-8). [PubMed: [25528575](https://pubmed.ncbi.nlm.nih.gov/25528575/)]. [PubMed Central: [PMC4279674](https://pubmed.ncbi.nlm.nih.gov/PMC4279674/)].
10. Gheorghie L, Rugina S, Dumitru IM, Franciu I, Martinescu A, Balas I. HLA class II alleles in Romanian patients with chronic hepatitis C. *Germes*. 2015;**5**(2):44-9. doi: [10.11599/germs.2015.1070](https://doi.org/10.11599/germs.2015.1070). [PubMed: [26097834](https://pubmed.ncbi.nlm.nih.gov/26097834/)]. [PubMed Central: [PMC4472190](https://pubmed.ncbi.nlm.nih.gov/PMC4472190/)].

11. Susser S, Herrmann E, Lange C, Hamdi N, Muller T, Berg T, et al. Predictive value of interferon-lambda gene polymorphisms for treatment response in chronic hepatitis C. *PLoS One*. 2014;**9**(11):e112592. doi: [10.1371/journal.pone.0112592](https://doi.org/10.1371/journal.pone.0112592). [PubMed: [25393304](https://pubmed.ncbi.nlm.nih.gov/25393304/)]. [PubMed Central: [PMC4231027](https://pubmed.ncbi.nlm.nih.gov/PMC4231027/)].
12. Aziz H, Raza A, Ali K, Khattak JZ, Irfan J, Gill ML. Polymorphism of the IL28B gene (rs8099917, rs12979860) and virological response of Pakistani hepatitis C virus genotype 3 patients to pegylated interferon therapy. *Int J Infect Dis*. 2015;**30**:91-7. doi: [10.1016/j.ijid.2014.09.021](https://doi.org/10.1016/j.ijid.2014.09.021). [PubMed: [25462177](https://pubmed.ncbi.nlm.nih.gov/25462177/)].
13. Farag RE, Arafa MM, El-Etreby S, Saady NS, Eldeek BS, El-Alfy HA, et al. Human leukocyte antigen class I alleles can predict response to pegylated interferon/ribavirin therapy in chronic hepatitis C Egyptian patients. *Arch Iran Med*. 2013;**16**(2):68-73. [PubMed: [23360626](https://pubmed.ncbi.nlm.nih.gov/23360626/)].
14. Shaker O, Bassiony H, El Raziky M, El-Kamary SS, Esmat G, El-Ghor AM, et al. Human leukocyte antigen class II alleles (DQB1 and DRB1) as predictors for response to interferon therapy in HCV genotype 4. *Mediators Inflamm*. 2013;**2013**:392746. doi: [10.1155/2013/392746](https://doi.org/10.1155/2013/392746). [PubMed: [23576852](https://pubmed.ncbi.nlm.nih.gov/23576852/)]. [PubMed Central: [PMC3612450](https://pubmed.ncbi.nlm.nih.gov/PMC3612450/)].
15. Ali A, Nisar M, Ahmad H, Saif N, Idrees M, Bajwa MA. Determination of HCV genotypes and viral loads in chronic HCV infected patients of Hazara Pakistan. *Virology*. 2011;**8**:466. doi: [10.1186/1743-422X-8-466](https://doi.org/10.1186/1743-422X-8-466). [PubMed: [21982599](https://pubmed.ncbi.nlm.nih.gov/21982599/)]. [PubMed Central: [PMC3198714](https://pubmed.ncbi.nlm.nih.gov/PMC3198714/)].
16. Din G, Malik S, Ali I, Ahmed S, Dasti JI. Prevalence of hepatitis C virus infection among thalassemia patients: A perspective from a multi-ethnic population of Pakistan. *Asian Pac J Trop Med*. 2014;**7**(1):S127-33. doi: [10.1016/S1995-7645\(14\)60218-2](https://doi.org/10.1016/S1995-7645(14)60218-2). [PubMed: [25312106](https://pubmed.ncbi.nlm.nih.gov/25312106/)].
17. Dogra G, Chakravarti A, Kar P, Chawla YK. Polymorphism of tumor necrosis factor-alpha and interleukin-10 gene promoter region in chronic hepatitis C virus patients and their effect on pegylated interferon-alpha therapy response. *Hum Immunol*. 2011;**72**(10):935-9. doi: [10.1016/j.humimm.2011.06.008](https://doi.org/10.1016/j.humimm.2011.06.008). [PubMed: [21756957](https://pubmed.ncbi.nlm.nih.gov/21756957/)].
18. Narciso-Schiavon JL, Schiavon Lde L, Carvalho-Filho RJ, Sampaio JP, Batah PN, Barbosa DV, et al. Gender influence on treatment of chronic hepatitis C genotype 1. *Rev Soc Bras Med Trop*. 2010;**43**(3):217-23. doi: [10.1590/S0037-86822010000300001](https://doi.org/10.1590/S0037-86822010000300001). [PubMed: [20563484](https://pubmed.ncbi.nlm.nih.gov/20563484/)].
19. Fernandez-Arquero M, Caldes T, Casado E, Maluenda C, Figueredo MA, De La Concha EG. Polymorphism within the HLA-DQB1*02 promoter associated with susceptibility to coeliac disease. *Eur J Immunogenet*. 1998;**25**(1):1-3. doi: [10.1046/j.1365-2370.1998.00083.x](https://doi.org/10.1046/j.1365-2370.1998.00083.x). [PubMed: [9587738](https://pubmed.ncbi.nlm.nih.gov/9587738/)].
20. Samimi-Rad K, Sadeghi F, Amirzargar A, Eshraghian MR, Alavian SM, Rahimnia R. Association of HLA class II alleles with hepatitis C virus clearance and persistence in thalassemia patients from Iran. *J Med Virol*. 2015;**87**(9):1565-72. doi: [10.1002/jmv.24211](https://doi.org/10.1002/jmv.24211). [PubMed: [25970464](https://pubmed.ncbi.nlm.nih.gov/25970464/)].
21. Ksiai L, Aayed-Jendoubi S, Sfar I, Gorgi Y, Najjar HA, Abdallah TB, et al. Clearance and persistence of hepatitis C virus in a Tunisian population: Association with HLA class I and class II. *Viral Immunol*. 2007;**20**(2):312-9. doi: [10.1089/vim.2006.0060](https://doi.org/10.1089/vim.2006.0060). [PubMed: [17603847](https://pubmed.ncbi.nlm.nih.gov/17603847/)].
22. de Rueda PM, Lopez-Nevot MA, Saenz-Lopez P, Casado J, Martin-Casares A, Palomares P, et al. Importance of host genetic factors HLA and IL28B as predictors of response to pegylated interferon and ribavirin. *Am J Gastroenterol*. 2011;**106**(7):1246-54. doi: [10.1038/ajg.2011.82](https://doi.org/10.1038/ajg.2011.82). [PubMed: [21670772](https://pubmed.ncbi.nlm.nih.gov/21670772/)].
23. Romero V, Azocar J, Zuniga J, Clavijo OP, Terreros D, Gu X, et al. Interaction of NK inhibitory receptor genes with HLA-C and MHC class II alleles in Hepatitis C virus infection outcome. *Mol Immunol*. 2008;**45**(9):2429-36. doi: [10.1016/j.molimm.2008.01.002](https://doi.org/10.1016/j.molimm.2008.01.002). [PubMed: [18289678](https://pubmed.ncbi.nlm.nih.gov/18289678/)]. [PubMed Central: [PMC2387047](https://pubmed.ncbi.nlm.nih.gov/PMC2387047/)].
24. Huang P, Zhang Y, Lu X, Xu Y, Wang J, Zhang Y, et al. Association of polymorphisms in HLA antigen presentation-related genes with the outcomes of HCV infection. *PLoS One*. 2015;**10**(4):e0123513. doi: [10.1371/journal.pone.0123513](https://doi.org/10.1371/journal.pone.0123513). [PubMed: [25874709](https://pubmed.ncbi.nlm.nih.gov/25874709/)]. [PubMed Central: [PMC4395248](https://pubmed.ncbi.nlm.nih.gov/PMC4395248/)].
25. Qureshi H, Bile KM, Jooma R, Alam SE, Afridi HU. Prevalence of hepatitis B and C viral infections in Pakistan: Findings of a national survey appealing for effective prevention and control measures. *East Mediterr Health J*. 2010;**16** Suppl:S15-23. doi: [10.26719/2010.16.Supp.15](https://doi.org/10.26719/2010.16.Supp.15). [PubMed: [21495584](https://pubmed.ncbi.nlm.nih.gov/21495584/)].
26. Gul A, Ahmed J, Zahir F, Ali Khan I, Ali I. New patterns of HCV subtypes distribution in the Khyber Pakhtunkhwa province of Pakistan. *Braz J Infect Dis*. 2016;**20**(1):107-8. doi: [10.1016/j.bjid.2015.09.007](https://doi.org/10.1016/j.bjid.2015.09.007). [PubMed: [26626168](https://pubmed.ncbi.nlm.nih.gov/26626168/)].
27. Arshad A, Ashfaq UA. Epidemiology of hepatitis C infection in Pakistan: Current estimate and major risk factors. *Crit Rev Eukaryot Gene Expr*. 2017;**27**(1):63-77. doi: [10.1615/CritRevEukaryotGeneExpr.2017018953](https://doi.org/10.1615/CritRevEukaryotGeneExpr.2017018953). [PubMed: [28436333](https://pubmed.ncbi.nlm.nih.gov/28436333/)].
28. Reker C, Islam KM. Risk factors associated with high prevalence rates of hepatitis C infection in Egypt. *Int J Infect Dis*. 2014;**25**:104-6. doi: [10.1016/j.ijid.2014.02.003](https://doi.org/10.1016/j.ijid.2014.02.003). [PubMed: [24865321](https://pubmed.ncbi.nlm.nih.gov/24865321/)].
29. Buonomo AR, Scotto R, Pinchera B, Coppola N, Monari C, Macera M, et al. Epidemiology and risk factors for hepatitis C virus genotypes in a high prevalence region in Italy. *New Microbiol*. 2018;**41**(1):26-9. [PubMed: [29112765](https://pubmed.ncbi.nlm.nih.gov/29112765/)].

Table 2. Allele Distribution of SNPs in the Studied Loci Among Responsive (R) and Non-Responsive (NR) Patients^a

Locus/SNP	Allele	Responsive	Non-Responsive	P Value	Odd Ratio	95% CI
TNF-α						
-238 G/A (rs361525)	G	91 (91)	67 (93)		Reference	
	A	9 (9)	5 (7)	0.626	0.75	0.242 to 2.355
-308 G/A (rs1800629)	G	94 (92)	62 (91)		Reference	
	A	8 (8)	6 (9)	0.819	1.14	0.376 to 3.438
-863 C/A (rs1800630)	C	78 (72)	44 (65)		Reference	
	A	30 (28)	24 (35)	0.292	1.42	0.739 to 2.722
HLA-DRB1						
6151 (T/C) (rs1064664)	T	53 (50)	28 (41)		Reference	
	C	53 (50)	40 (59)	0.254	1.43	0.772 to 2.643
6167 (A/C/T) (rs707957)	A	72 (72)	52 (76)		Reference	
	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 (rs776046212)	C	102 (94)	70 (97)		Reference	
	T	6 (6)	2 (3)	0.375	0.49	0.095 to 2.477
6228 (C/T) (rs2308802)	C	64 (63)	31 (43)		Reference	
	T	38 (37)	41 (57)	0.010 ^b	2.23	1.204 to 4.122
	C	40 (39)	24 (34)		Reference	
6231 (T/C/G) (rs3167799)	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)		-	
	C	89 (84)	60 (88)		Reference	
6236 (A/C) (rs1059586)	C	17 (16)	8 (12)	0.433	0.69	0.283 to 1.720
	A	97 (92)	62 (91)		Reference	
6251 (A/T) ^c (rs397844204)	A	97 (92)	62 (91)		Reference	
	T	9 (8)	6 (9)	0.939	1.04	0.353 to 3.075
HLA-DQB1						
-8471 (A/G/C) (chr6:32628910)	A	69 (64)	31 (47)		Reference	
	G	39 (36)	34 (51)	0.036 ^b	1.94	1.038 to 3.627
	C	0 (0)	1 (2)		-	
-8465 (G/A/C) (rs9273552)	G	93 (86)	52 (79)		Reference	
	A	14 (13)	14 (21)	0.158	1.78	0.791 to 4.041
	C	1 (1)	0 (0)		-	
-8447 (C/A/G/T) (chr:32628934)	C	49 (50)	42 (66)		Reference	
	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 ^b	0.44	0.210 to 0.925
-8442 (G/C) ^c (rs34644981)	G	81 (74)	49 (74)		Reference	
	C	29 (26)	17 (26)	0.929	0.96	0.483 to 1.944

-8362 (A/C/G) ^c (chr6:32629020)	A	57 (53)	34 (53)		Reference	
	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
-8344 (T/C) (chr6:32629038)	T	84 (78)	50 (76)		Reference	
	C	24 (22)	16 (24)	0.758	1.12	0.543 to 2.308
-8331 (T/G) (chr6:32629051)	T	66 (62)	49 (74)		Reference	
	G	40 (38)	17 (26)	0.104	0.57	0.290 to 1.127
-8307 (A/C/G) (rs9273588)	A	48 (44)	30 (45)		Reference	
	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
-8298 (C/G) (rs9273592)	C	81 (76)	45 (68)		Reference	
	G	27 (24)	21 (32)	0.328	1.4	0.711 to 2.755
-8288 (C/G) (rs9273598)	C	91 (83)	53 (80)		Reference	
	G	19 (17)	13 (20)	0.686	1.17	0.537 to 2.570

^aValues are expressed as No. (%).

^bDifferences in the distribution were statistically significant.

^cGenotype frequencies of SNPs were concordant with the assumption of HWE.