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Molecular Medicine Communications
DOI: 10.55627/mmc.004.001.0561

Research Article

Association of Human Leukocyte Antigen (HLA) Class II Alleles of DQB1 *(DQB1*03:02) (DQB1*06:02)* and *DRB1 (DRB1*04:01)* with Type-1 Diabetes Patients of Khyber Pakhtunkhwa, Pakistan

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Abstract

Type-1 diabetes (T1D), an autoimmune disease, significantly impacts global health, with varying prevalence across different geographies. This study investigates the association of specific Human Leukocyte Antigen (HLA) Class II alleles, namely *DQB1*03:02, DQB1*06:02*, and *DRB1*04:01*, with T1D in the Khyber Pakhtunkhwa (KP) population of Pakistan. Conducted as a 1:1 matched case-control study, this research involved 33 T1D patients and an equal number of age- and gender-matched healthy controls. Our findings reveal a weak association of alleles *DRB1*04:01* and *DQB1*03:02* with T1D (OR = 2.065 and 1.393, respectively, $p > 0.05$). *DQB1*06:02* allele, which is considered to have a protective effect against T1D was more prevalent in control as compared to cases (93.9% vs 90.9%), however, the association was non-significant (OR 0.645, $p > 0.05$). These insights contribute to understanding genetic influences on T1D in the KP region and underscore the need for further research with larger, diverse cohorts to refine the genetic markers of T1D risk and protection.

Keywords: Type 1 Diabetes, HLA Class II, Genomic Ethnicity, Genetic Association, Polymorphism

1. Introduction

Type 1 diabetes mellitus (T1D), also referred to as insulin-dependent diabetes mellitus (IDDM), is a chronic autoimmune condition characterized by the destruction of insulin-producing beta cells in the pancreas (IBRAHIM, FATHY, GHADA, & Nancy, 2021). This leads to insulin deficiency and resultant hyperglycemia. Although T1D constitutes roughly 10% of all diabetes cases globally, it significantly affects younger demographics, often manifesting in childhood and adolescence(Kahkoska, Dabelea, & Clinics, 2021). This early onset makes T1D a critical concern within pediatric and endocrine healthcare(Chiang et al., 2018).

The incidence and demographic distribution of T1D vary worldwide. It is less prevalent in East Asia and among Native American populations, while countries like Finland, Sardinia, and Sweden report higher rates(Tuomilehto, 2013). This geographical variance highlights the influence of both genetic and environmental factors on the disease's development(Borchers, Uibo, & Gershwin, 2010).

Recent data underscores a concerning rise in T1D prevalence in Pakistan (Abbas et al., 2018). The country was ranked 10th worldwide in terms of diabetes prevalence by the International Diabetes Federation's 9th edition (Sun et al., 2022). More specifically, emerging studies indicate that the Khyber Pakhtunkhwa region is

experiencing a significant increase in cases, particularly among its younger population (Ahmad, Afzal, Rauf, & Sustainability, 2021). This trend points to the urgent need for enhanced research and targeted diabetes prevention strategies within the region.

To combat the rising prevalence of T1D, Pakistan's health sector has ramped up its diabetes prevention programs, emphasizing early diagnosis and public education on recognizing early symptoms(Barry-Menkhaus, Stoner, MacGregor, & Soyka, 2020). Despite these efforts, challenges persist, particularly in rural and underserved areas. Recent statistics from the Ministry of Health indicate that approximately 40,000 children in Pakistan suffer from T1D, with new cases increasing by about 5% annually(M. H. Khan & Khalid, 2016; M. T. R. Khan, 2014). This alarming trend highlights the critical need for targeted interventions that incorporate genetic research to improve prevention and management strategies.

Environmental factors such as viral infections (e.g., coxsackievirus B and Helicobacter pylori) and early-life nutritional exposures are known to precipitate the autoimmune destruction of beta cells(Lemos, Hirani, & von Herrath, 2024). However, genetic predispositions play a more substantial role, contributing approximately 30-50% of the risk (Wu, Ding, Tanaka, & Zhang, 2014). Among these genetic factors, Human Leukocyte Antigen (HLA) genes on chromosome 6p21—specifically Class II alleles like *DRB1**04:01 and *DQB1**03:02—show a strong association with increased susceptibility to T1D (Hsu et al., 2012). Conversely, the HLA-*DQB1**06:02 allele has been identified as providing a protective effect in various ethnic groups.

This study aims to explore the association of specific HLA Class II alleles with Type 1 diabetes mellitus (T1D) in a focused subset of the Khyber Pakhtunkhwa population. By understanding these genetic correlations, we aim to contribute to the global effort in T1D research and aid in developing more effective preventive measures tailored to Pakistan's genetic and environmental context.

2. Materials and Methods

2.1. Design and Study Sample

A case-control study was designed, and the sample size was calculated by using Table 10 from WHO's sample size calculation handbook (No. WHO/HST/ESM/86.1) with a 5% level of significance, 90% power, and odds ratio equals to 4 as assumptions. 33 T1D patients were recruited from different tertiary care hospitals of Peshawar Khyber Pakhtunkhwa-matched with healthy controls with no history of Diabetes type 1 were recruited in the general local population. The Research study was approved after obtaining ethical approval from the ethical committee of the Department of Pharmacy University of Peshawar [306/EC/F.LIFE/UOP-2020 Dated November 3, 2020].

2.2. Case Ascertainment

The following criteria were used to diagnose T1D patients: fasting glycemia 126 mg/dL, unexplained weight loss, indicators of hyperglycemia (polyuria, polydipsia, polyphagia, and asthenia), and absolute insulin dependence. The American Diabetes Association's recommendations were utilized to develop these criteria (Goyal et al., 2020).

2.3. Participant Eligibility

2.3.1. Inclusion Criteria & Identification of Cases and Controls

This study specifically targeted the Pashtun population in Khyber Pakhtunkhwa, Peshawar, Pakistan. Participants diagnosed with Type 1 diabetes mellitus (T1D) served as cases, and age- and gender-matched healthy individuals from the same demographic were selected as controls.

a) Diagnostic Criteria for T1D Cases

1. **Fasting Glycemia:** A fasting blood glucose level of 126 mg/dL or higher on two separate occasions.
2. **Insulin Dependence:** Required insulin therapy from the time of diagnosis, confirmed by medical records.
3. **Age:** All diabetic participants were younger than 30 years old at the time of diagnosis.
4. **Autoimmune Markers:** Presence of T1D-related autoantibodies, such as islet cells, insulin autoantibodies, and glutamic acid decarboxylase antibodies.
5. **Family History:** A documented family history of T1D.

b) Control Group Criteria

1. **Fasting Glycemia:** A fasting blood glucose level below 100 mg/dL.
2. **Glycated Hemoglobin (HbA1c):** Normal HbA1c levels indicate no chronic hyperglycemia.

c) Exclusion Criteria for All Participants

1. **Co-existing Conditions:** Individuals with any chronic medical conditions that could influence insulin sensitivity or glycemic control were excluded.
2. **Medications:** Those currently undergoing treatment with medications known to affect glucose metabolism, such as corticoids and growth hormones, were also excluded.
3. **Acute Diseases:** Anyone in the acute phase of a major disease requiring active treatment was excluded from participation.

d) Assessment of Insulin Dependence

Insulin dependence was determined by reviewing treatment histories documented in the participant's medical records. All T1D cases were confirmed to have been prescribed and to be actively using insulin therapy daily to manage their diabetes from the time of their initial diagnosis.

2.4. Participant Characteristics

A total of 66 volunteers were signed up for the study between November 2020 and December 2022, comprising 15 males and 18 females. Among them, 33 were found to be healthy, while the remaining 33 were diagnosed with T1D.

2.5. HLA Class II Genotyping

2.5.1. DNA Extraction:

Blood samples were collected from all participants using EDTA tubes to prevent coagulation. DNA was extracted from these samples using a standard DNA extraction kit (WizPrep™ gDNA Mini Kit). The integrity and concentration of the extracted DNA were assessed by measuring absorbance at 260 nm and 280 nm using a UV spectrophotometer.

2.5.2. Genotyping:

Genotyping of the HLA Class II alleles—specifically *DRB1**04:01, *DQB1**03:02, and *DQB1**06:02—was performed using the Polymerase Chain Reaction with Sequence-Specific Primers (PCR-SSP) technique. Each reaction was prepared with the following components: master mix, primers specific to the target alleles, and an appropriate volume of extracted DNA. The thermal cycling conditions were set for an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes.

2.6. Gel Electrophoresis

Following PCR, the amplified fragments were separated by gel electrophoresis. A 2% agarose gel was prepared, and

Table 1: Table 1 shows the sequence of the primers used in this study.

S/No	Primers		Sequence 5′-3′	length	GC	GC%	Tm °C
1	HLA-*DQB1**06:02	For	CGTGCGTCTTGTGACCAGAT	20	11	55	62
		Rev	GCTGTTCCAGTACTCGGCAT	20	11	55	62
2	HLA-*DQB1**03:02	For	GACGGAGCGCGTGCGTTA	18	12	66.67	60
		Rev	AGTACTCGGCGTCAGGCG	18	12	66.67	60
3	*DRB1**04:01	For	CAGGTTAAACATGAGTGTCATTTCTTC AAC	30	10	33.3	80
		Rev	GCTGTCGAAGCGCACGTACTCCTCTTG GTG	30	18	60	96
Annealing temperature=4(G+C) +2(A+T)-5							

GC; Guanin, Cytosine

electrophoresis was conducted to resolve the PCR products. The gels were stained with ethidium bromide and visualized under UV light. The specific fragment sizes corresponding to each HLA allele were as follows:

I. *DRB1**04:01: 250 bp
II. *DQB1**03:02: 200 bp
III. *DQB1**06:02: 150 bp

These distinct fragment sizes helped to confirm the presence or absence of each allele in the samples. [Fig 1, 2].

2.7. Statistical Analysis

The statistical analysis was designed to evaluate the association between specific HLA Class II alleles and the prevalence of Type 1 diabetes mellitus (T1D) among participants. The binary Logistic Regression model was employed to assess the odds ratios (ORs) and 95% confidence intervals (CIs) for the presence of HLA alleles in T1D cases compared to controls. The logistic regression analysis helped to determine the likelihood of disease presence associated with specific genetic markers, adjusting for potential confounders such as age and gender. The Chi-Square Test was used to compare the frequency of each allele between the case and control groups. This test helped to identify significant differences in allele distribution, providing a basic measure of association. All statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). This software facilitated complex statistical computations and ensured the accuracy of our results.

3. Results

The odds ratio (OR), confidence interval (CI), and P-value of alleles and their combinations are presented in Tables 2 and 3. Results demonstrate that all three studied alleles and their possible combinations are weakly associated with the prevalence of T1D (odds ratio) as well as it is nonsignificant too as suggested by (OR ≤ 1; P-value = ≥ 0.5).

According to the findings of the study, the most powerful markers of T1D vulnerability are HLA genes of class II. In the study population alleles of HLA class II *DRB1**04:01, and *DQB1**03:02 having OR (2.065,1.393), 95% C. I (0.178-23.942, 0.449-4.324, 0) and P-value (0.562, 0.566,) indicate positive association with TID in the Khyber Pakhtunkhwa population [Table 2].

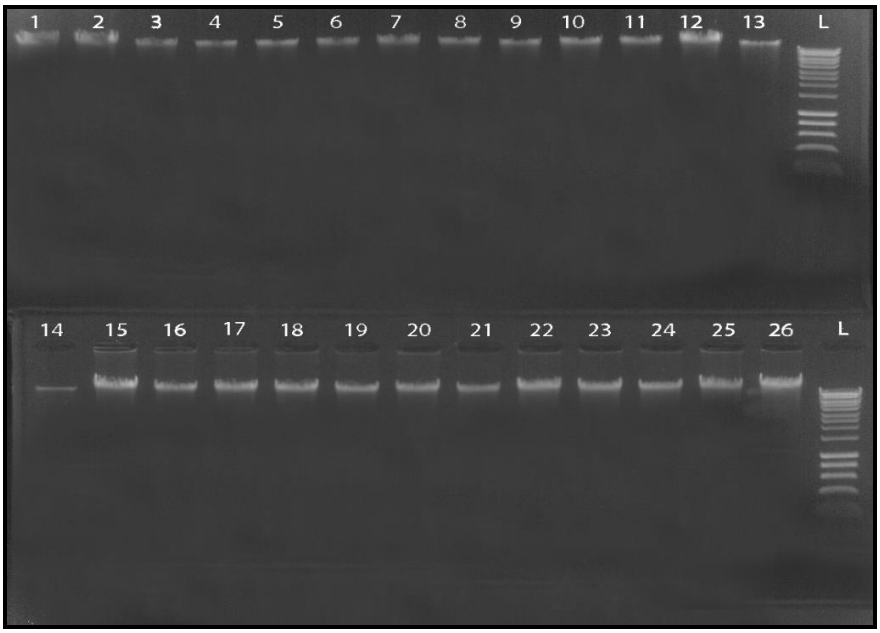

Figure 1: Showing Electropherogram of DNA (Participants). L indicates DNA ladder (1000 bp)

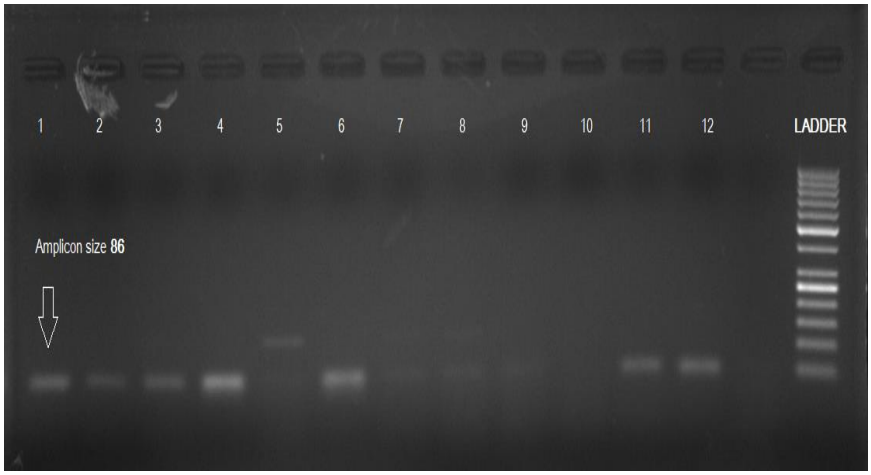

Figure 2: Electropherogram of DNA (Case T1D) for allele *DQB103:02 using 50 bp Ladder**

Aside from *DRB1*04:01* and *DQB1*03:02*, another allele of HLA class II, *DQB1*06:02*, with an OR of 0.645, 95% C.I (0.101-4.13) and P-value 0.644, did not show a clear protective effect against Type 1 Diabetes (T1D) [Table 2], although its percentage was higher in controls as compared to cases. Results also suggest that the combination of HLA class II alleles *DQB1*06:02* with *DQB1*03:02* having OR (1.314), 95% CI (0.417-3.669), and P-value (0.602) has no significant association with TID followed by *DRB1**04:01 with *DQB1**06:02 having OR (1.295), 95% CI (0.315-5.322) and P-value (0.720) and that of *DRB1**04:01 with *DQB1**03:02 OR (1.043), CI (0.352-3.093) and P-value(0.939) [Table 3]. This may be because of the small sample size; significance may be more evident in the present study with a higher sample size.

Table 2: Table 2 shows OR* C.I*, P-value and Frequency of *DRB104:01, *DQB1**06:02 and *DQB1**03:02**

Alleles	Odds Ratio	C.I 95%				P value
		Lower	Upper	Cases	Controls	
***DRB1**04:01**	2.065	0.178	23.942	32(97.0%)	31 (93.9%)	0.562
***DQB1**06:02**	0.645	0.101	4.13	30(90.9%)	31 (93.9%)	0.644
***DQB1**03:02**	1.393	0.449	4.324	26(78.8%)	24 (72.2%)	0.566

OR*; Odds Ratio, C.I*; Confidence Interval

4. Discussion

Our study's findings on the association of HLA Class II alleles *DRB1*04:01 and DQB1**03:02 with Type 1 diabetes (T1D) in the Khyber Pakhtunkhwa population reveal a complex picture. While these alleles demonstrated ORs of 2.065 and 1.393 respectively, their associations with T1D were not statistically significant (95% CI of 0.178-23.942 and 0.449-4.324; P-values of 0.562 and 0.566). This contrasts with findings from other populations where these alleles show a more pronounced correlation with T1D prevalence, such as in Tunisian Arabs where the alleles were significantly more prevalent among T1D patients (Sayed & Nabi, 2021), and other diverse ethnic groups showing similar trends (Mayer-Davis et al., 2017). The variance in our findings might be attributed to genetic, environmental, or methodological differences, highlighting the need for further studies in the region to refine these associations.

Beyond T1D, alleles *DRB1*04:01* and *DQB1*03:02* have been implicated in a range of other autoimmune and chronic diseases, underscoring their importance in immune system functioning. For example, *DRB1**04:01 is associated with an increased risk of rheumatoid arthritis, particularly in Caucasian populations, suggesting a shared pathogenic pathway with T1D that involves autoimmune destruction of specific tissues (Xie, Chang, Zhou, & immunology, 2014). Similarly, *DQB1**03:02 is linked with celiac disease, which, like T1D, involves an inappropriate immune response to specific antigens, such as gluten (Lerner, Benzvi, & Vojdani, 2023).

*DQB1**06:02 allele showed a lower prevalence in diabetics. This data might point towards validation of its possible protective effect. This finding aligns with results from studies in other regions, such as Cameroon and Madeira Island, where it has been shown to be less prevalent in diabetic subjects, suggesting a protective effect. (Qi et al., 2024). The consistency of this allele's protective nature across diverse populations warrants further investigation into its potential as a target for preventive strategies in T1D.

The different HLA alleles were also evident in our analysis of allele combinations. The combinations of *DQB1*06:02 with DQB1**03:02, and *DRB1*04:01 with either DQB1**06:02 or *DQB1*03:02*, showed ORs of 1.314, 1.295, and 1.043 respectively. These combinations did not exhibit significant associations with T1D (P-values of 0.602, 0.720, and 0.939). This suggests that the mere presence of these alleles does not confer a higher risk or protection, but rather their interaction might be influenced by additional genetic or environmental factors. The variation in allele

Table 3: Table 3 shows OR* C.I*, P-value and Frequency of all three Possible Combinations

Combination of alleles	Odds Ratio	C.I 95% (Lower)	C.I 95% (Upper)	P value	Cases	Control
Combination of *DRB1*04:01* *with* *DQB1*06:02						
Either one is present			Reference		4 (12.1%)	4 (12.1%)
Both are present	1.295	0.315	5.322	0.720	29 (87.9%)	29 (87.9%)
Both are absent	0	0	0	0	0	0
Combination of *DRB1*04:01* *with* *DQB1*03:02						
Either one is present			Reference		9 (27.3%)	9 (27.3%)
Both are present	1.043	0.352	3.093	0.939	24 (72.2%)	23 (69.7%)
Both are absent	0	0	0	1.000	0	1 (3.0%)
Combination of *DQB1*06:02* *with* *DQB1*03:02						
Either one is present			Reference		10 (30.3%)	11 (33.3%)
Both are present	1.314	0.471	3.669	0.602	23 (69.7%)	22 (66.7%)
Both are absent	0	0	0	0	0	0

C.I*; Confidence Interval, OR*; Odds Ratio

effects across different populations and regions—as seen in studies from Arab countries, Brazil, and Iran—underscores the potential influence of ethnic-specific genetic backgrounds on T1D susceptibility (Sivaprasad, Gupta, Crosby-Nwaobi, & Evans, 2012).

Our findings emphasize the complexity of genetic factors in T1D and suggest that while some HLA alleles may contribute to the disease's pathogenesis, their impact can be significantly modulated by other genetic and environmental factors. The geographical and ethnic variations observed in allele associations with T1D suggest that personalized medical approaches could be crucial for effective prevention and treatment strategies. Future research should focus on larger, multi-ethnic cohorts to validate these findings and explore the gene-environment interactions that contribute to T1D. Additionally, longitudinal studies could provide insights into the temporal dynamics of these genetic associations, offering further understanding of the disease's onset and progression.

4. Conclusion

In conclusion, this study assessed the association of HLA Class II alleles *DRB1*04:01, *DQB1*03:02, and *DQB1*06:02 with Type 1 diabetes (T1D) in the Khyber

Pakhtunkhwa population. Our results demonstrated that while alleles *DRB1**04:01 and *DQB1**03:02 exhibit weak and non-significant associations with T1D, suggesting limited influence under the study conditions. Protective allele *DQB1**06:02 was more prevalent in healthy controls.

These findings underline the complex nature of genetic influences on T1D and highlight the variability of these influences across different populations. The research points to the need for further studies incorporating larger and more diverse cohorts to confirm these associations and to explore the underlying mechanisms in greater depth. Such studies are crucial for developing targeted interventions that can effectively address the genetic and environmental factors contributing to T1D.

Future research should focus on expanding these preliminary insights by exploring gene-environment interactions and leveraging larger, multi-ethnic datasets to provide a more comprehensive understanding of T1D predisposition and protection mechanisms. This approach will enhance our ability to develop personalized medicine strategies for preventing and managing T1D, particularly in regions with high disease prevalence.

Acknowledgments

The authors wish to thank volunteers (control participants and diabetic patients) who agreed to take part in this study. We appreciate the endocrinologists at Peshawar's several tertiary care hospitals for their assistance in gathering patient information and blood samples.

Data Availability

All the data related to this study is available with the authors.

Study Approval

The Research study was approved after obtaining ethical approval from the ethical committee of the Department of Pharmacy University of Peshawar [306/EC/F.LIFE/UOP-2020 Dated November 3, 2020].

Consent Forms

Consent forms were signed by the particiapnats and are available with authors.

Funding

This study was funded for sample collection and laboratory analysis by the Higher Education Research endowment fund (HEREF), Government of Khyber Pakhtunkhwa, under **[PMU1-22/HEREF/2014-15/Vol-IV/3408].**

Disclosure Statement

The authors declare that they have no competing interests.

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