Original
ISSN 2413-0516

Association of CTLA-4 (rs231775) and FOXP3 (rs3761548) Gene Polymorphisms with Type 1 Diabetes in Iraqi Kurdish Children

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(Submitted: 04 January 2026 – Revised version received: 22 January 2026 – Accepted: 07 February 2026 – Published online: 26 February 2026)

Abstract

Objective: The objective of the current research was to determine the correlation between FOXP3 (rs3761548) and CTLA-4 (rs231775) gene polymorphisms and Type 1 Diabetes susceptibility among children of the Iraqi-Kurdish population.

Methods: A total of 140 pediatric participants (70 with T1D and 70 age- and sex-matched controls) were recruited in this study. Whole blood taken at the periphery was subjected to genomic DNA extraction using the Qiagen DNA Extraction Kit (Qiagen, Hilden, Germany). Amplification of the specific genomic regions containing the target polymorphisms CTLA4 rs231775 and FOXP3 rs3761548 was performed through polymerase chain reaction (PCR). The presence of the PCR product and the amplicon size was confirmed using the QIAxcel Advanced System (QIAGEN GmbH, Hilden, Germany). The confirmed products were then sent for Sanger sequencing.

Results: The CTLA-4 rs231775 variant was statistically significant at the genotype level ($P = 0.039$) and under the dominant inheritance model ($P = 0.017$); the allele G seemed to have a higher frequency in the patients. In contrast, a sex-stratified analysis across the X-linked FOXP3 3761548 polymorphism showed no statistically significant association with T1D either female or male subjects.

Conclusion: The current study demonstrates that the CTLA 4 rs231775 polymorphism, specifically the AG genotype and G allele is correlated with the susceptibility to T1D in the children of Iraqi Kurdish population, but the X-linked FOXP3 variant is not statistically significant.

Keywords: Diabetes mellitus, Type 1, CTLA-4 antigen, FOXP3, polymorphism, genetic, autoimmunity

Introduction

Type 1 diabetes (T1D) is a chronic disease that is associated with the destruction of insulin-producing pancreatic β-cells by antibodies, leading to the total absence of insulin production and a lasting need for exogenous insulin replacement therapy.[1,2] The pathogenesis involves a combination of genetic predisposition and environmental exposures that lead to the breakdown of immunological tolerance to pancreatic antigens of the beta cells.[3] Although some cellular immunity mechanisms are involved, T-cell mechanisms are predominant in the pathogenesis of T1D, and they are characterized by the infiltration of pancreatic islands by auto-reactive CD8+ and CD4+ T lymphocytes and progressive degradation of β-cells.[4]

The growing data highlight a high level of heterogeneity of T1D due to differences in individual and population levels in the age of onset, rates of β-cell destruction, immunoinflammatory phenotypes and clinical manifestations. This heterogeneity suggests that T1D consists of several immunological endotypes, and not just a single homogeneous disease.[5,6] The human leukocyte antigen (HLA) is the most important determinant of predisposition to T1D. The contribution made by HLA genes to the familial aggregation of T1D has been estimated to be about 40–50%. However, even the presence of HLA susceptibility is not quite enough to explain the occurrence of the disease because only a portion of genetically susceptible people develop the disease, and significant inter-ethnic differences in the incidence of the disease still exist.[7]

In addition to the HLA genes, non-HLA genes that play a role in immune regulation have also been proven to play a role in the susceptibility to T1D. Specifically interesting are the proteins CTLA-4 (cytotoxic T-lymphocyte antigen-4)[8] and FOXP3 (forkhead box P3), which play central immunological roles in maintaining immune tolerance.[9] CTLA-4 is an inhibitory immune checkpoint receptor found on activated T lymphocytes and constitutively on regulatory T-cells, and it is at the core of T-cell-activation inhibition and immune homeostasis.[10] The genetic variation of CTLA-4 has thus been widely studied in autoimmune diseases, especially T1D. Globally, many investigations have linked the single-nucleotide polymorphism (SNP) of functional CTLA-4 rs231775 to changes in CTLA-4 expression and immune regulation.[11] Population studies and meta-analyses have identified an association with T1D and autoimmune phenotypes related to its prevalence and genotype.[12]

FOXP3 is an essential transcription factor that is needed in the maturation and suppressive activity of the regulatory T (Treg) cells, which are fundamental in the preservation of peripheral immune tolerance.[13] It is also linked to reduced immune regulation with diminished capacity of Treg cells, which can cause an increased number of autoreactive effector T lymphocytes. This opens the possibility of enhancing the pancreatic autoimmune response, such as immune-mediated destruction of pancreatic β-cells.[14] Therefore, genetic variation in the FOXP3 gene has received significant attention, specifically the type of promoter polymorphism, the variant of the regulatory region (rs3761548). It has been reported that the A allele of the rs3761548 position could alter transcription factor binding motifs in the FOXP3 promoter, which mediates the effect of lowering FOXP3 transcription and impairment of regulatory T-cell action.[9] Although the FOXP3 rs3761548 polymorphism has been widely linked with various autoimmune diseases, including T1D in different populations,[9,15–20] the role of the same polymorphism in T1D among the Middle Eastern population has not been well investigated.

Together, the evidence that is available supports the possibility of a biologically consistent hypothesis in which the non-HLA genetic variations can influence the susceptibility

to T1D. Nevertheless, the strength and orientation of such associations differ among the populations, which indicates a complex genetic structure and immunological diversity of the disease. Thus, the study of CTLA-4 rs231775 and FOXP3 rs3761548 polymorphisms in Iraqi Kurdish children is of special interest. Therefore, the current study aims to explore the relationship between FOXP3 rs3761548 and CTLA-4 rs231775 polymorphisms and T1D among children of the Iraqi Kurdish population.

Materials and Methods

Study Population and Clinical Characteristics

A total of 140 children were recruited for this study. Peripheral blood samples were taken from 70 healthy controls (37 males and 33 females) and 70 children with T1D (35 males and 35 females). The participants were categorized into 2–6, 7–11 and 12–16 years old. In order to following inclusion and exclusion criteria, the control individuals were chosen among those people who do not have any family history of T1D or any other autoimmune disease. All the participants were Kurdish, residing in the Kurdistan Region of Iraq. The collection of the samples was done in cooperation with Dr. Jamal Children's Hospital, Sulaymaniyah, Kurdistan Region, Iraq. The Ethics Committee of Koya University (Date 08/02/2025/ No. DBIO-7-26) approved the study protocol and the parents signed the informed consent before sampling. All the individuals were given a structured questionnaire including sociodemographic and clinical data, such as age, ethnicity, sex, age at the onset of the disease, lifestyle, vaccination, infections, consanguinity and family history.

Data and Sample Collection

The sociodemographic and clinical information related to the participants was collected through a structured questionnaire given to the parents. A sample of 5 mL of peripheral venous blood from each participant was aseptically taken and placed in ethylenediaminetetraacetic acid (EDTA)-coated tubes. The samples were then transferred in cold insulated containers to the Molecular Laboratory of Hiwa Hospital (Sulaymaniyah, Kurdistan Region, Iraq), where they were processed immediately to extract genomic DNA.

DNA Extraction, Quantification and PCR

Genomic DNA isolation from peripheral whole blood was performed by following the instructions of the manufacturer provided with the Qiagen DNA Extraction Kit (Qiagen, Hilden, Germany). The DNA samples obtained were then measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). In which the DNA concentration and purity was determined based on the calculation of A260/A280 ratios. Conventional PCR was used to amplify the target polymorphisms in the CTLA-4 (rs231775) and FOXP3 rs3761548 at positions +49 A/G and –3279 C/A, respectively. These were expected to yield amplicon lengths of 663 base pairs and 577 base pairs in CTLA-4 and FOXP3, respectively. PCRs were performed in a total of 30-µL reaction volume with the presence of 1 µL of ≈ 50 ng of genomic DNA, 12.5 µL of 2x PCR Master Mix (constituting Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer), 1 µL of forward and reverse primers (Table 1) and 14.5 µL of nuclease-free water.

The amplification process was conducted in a thermal cycler (Bio-Rad Laboratories, USA) under the following conditions: an initial denaturation cycle for 3 min at 94°C, followed by 35 cycles of three steps, including denaturation for 30 s at 94°C, annealing for 30 s at 58°C, extension for 45 s at 72°C and a final extension for 5 min at 72°C. The quality of the PCR product and the amplicon size was confirmed using the QIAxcel Advanced System (QIAGEN GmbH, Hilden, Germany) with the QIAxcel DNA High-resolution Kit (2400) (QIAGEN GmbH, Hilden, Germany). A 15 bp–6 kb QX Alignment Marker was employed alongside the DNA size marker 100 bp–2500 bp at a concentration of 30 ng/µL.

Sequencing and Genotype Analysis

Each PCR amplicon (15 µL) was aliquoted into labelled Eppendorf tubes, complemented with the respective forward primer, and sent to Macrogen Inc. (Seoul, South Korea) to have the Sanger sequencing done, using an automated DNA sequencer to determine the genotypes of the studied SNPs. Chromatogram sequencing results were analysed twice in FinchTV software by two investigators counting the genotypes independently.

Statistical Analysis

GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA) was used to perform statistical analysis. The genotype and allele frequency distributions were tested using Pearson chi-square and odds ratios (ORs), and a 95% confidence interval (CI) was tested using two-tailed tests under the genotype, dominant, and recessive models of inheritance. As a quality-control measure, in the control group, Hardy-Weinberg Equilibrium (HWE) was tested. Regarding sample size, a post-hoc statistical power was calculated using G Power v3.1.9.7 with an $\alpha = 0.05$. A *P*-value of <0.05 was considered to represent a statistically significant value.

Results

Demographic and Clinical Characteristics

The research population was a total of 140 children, 70 of whom were diagnosed with T1D and 70 healthy volunteer controls. The age distribution of the participants was similar throughout the categories, with the majority (47.1%) falling into the 7–11 age group, followed by the 12–16 age group (34.3%) and the 2–6 age group (18.6%). Patients and controls

Table 1. **Forward and Reverse primers for the CTLA-4 and FOXP3 genes containing the SNP site**

Gene	SNP	Forward-primer 5′ to 3′	Reverse-primer 5′ to 3′	PCR Amplicon size (bp)
CTLA-4	rs231775	5'-CCAGATCCTCAAAGTGAACATGA-3'	5'-CCCTACTAAATACCTGGCGC-3'	663
FOXP3	rs3761548	5'-TTCGCCAATACAGAGCCCAT-3'	5'-TATCTCTCAGCCAGTCCCCT-3'	577

did not differ statistically significantly in terms of age distribution ($P = 0.898$). Males made up 50.0% of patients and 52.9% of controls, while females made up 50.0% and 47.1%, respectively ($P = 0.866$). The distribution of genders was also identical across groups. Although this difference did not achieve statistical significance ($P = 0.069$), a positive family history of diabetes was recorded in 7.1% of patients, whereas it was not present in the controls. Parental consanguinity was found in 29 (20.7%) of the total cohort with 13 (18.6) and 16 (22.9) within the patient and the control group respectively. Regarding parental consanguinity, there was no statistically significant difference between the two groups ($P = 0.677$) (Table 2).

PCR Amplification and Quality Assessment

Both target genes containing the SNP sites were amplified successfully by PCR to give the desired amplicon of the correct size. The QIAxcel Advanced System was used to check the quality of the amplicons, and the length of the amplicons was confirmed to be 663 bp of CTLA-4 and 577 bp of FOXP3 (Fig. 1). In all negative controls, amplification was not observed and this was used to ensure that there was no contamination in the PCR reactions.

Genotype Determination

Sanger sequencing results of the genotypes of CTLA-4 rs231775 and FOXP3 rs3761548 were analysed using FinchTV (Fig. 2) which illustrates chromatograms of the homozygous and heterozygous allelic states of CTLA-4 and female-specific FOXP3, thus confirming the accuracy of determining genotypes. The allelic patterns of the CTLA-4 rs231775 had the genotypes of AA, AG, or GG and the allelic patterns of the FOXP3 rs3761548 had the genotypes of CC, CA and AA.

Table 2. **Demographic characteristics of the groups**

Parameters	Total ($N = 140$)	Patients ($N = 70$)	Control ($N = 70$)	*P*-value
Age (years)				0.898
2–6	26 (18.6%)	12 (17.1%)	14 (20.0%)	
7–11	66 (47.1%)	34 (48.6%)	32 (45.7%)	
12–16	48 (34.3%)	24 (34.3%)	24 (34.3%)	
Gender				0.866
Male	72 (51.4%)	35 (50.0%)	37 (52.9%)	
Female	68 (48.6%)	35 (50.0%)	33 (47.1%)	
Family History				0.069
Positive	5 (3.6%)	5 (7.1%)	0 (0.0%)	
Negative	135 (96.4%)	65 (92.9%)	70 (100.0%)	
Parental Consanguinity				0.677
Yes	29 (20.7%)	13 (18.6%)	16 (22.9%)	
No	111 (79.3%)	57 (81.4%)	54 (77.1%)	

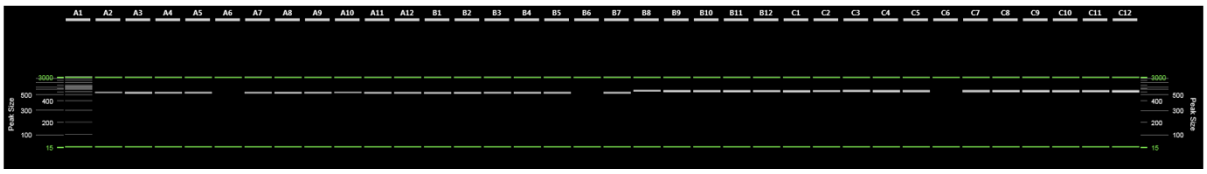

Fig. 1 **QIAxcel digital gel of FOXP3 (rs3761548) and CTLA-4 (rs231775) amplicons. A1: 100–2500 bp DNA marker. A2-A5, A7-B5, B7: FOXP3 products. B8-C5, C7-C12: CTLA-4 products. A6, B6, C6: Negative controls.**

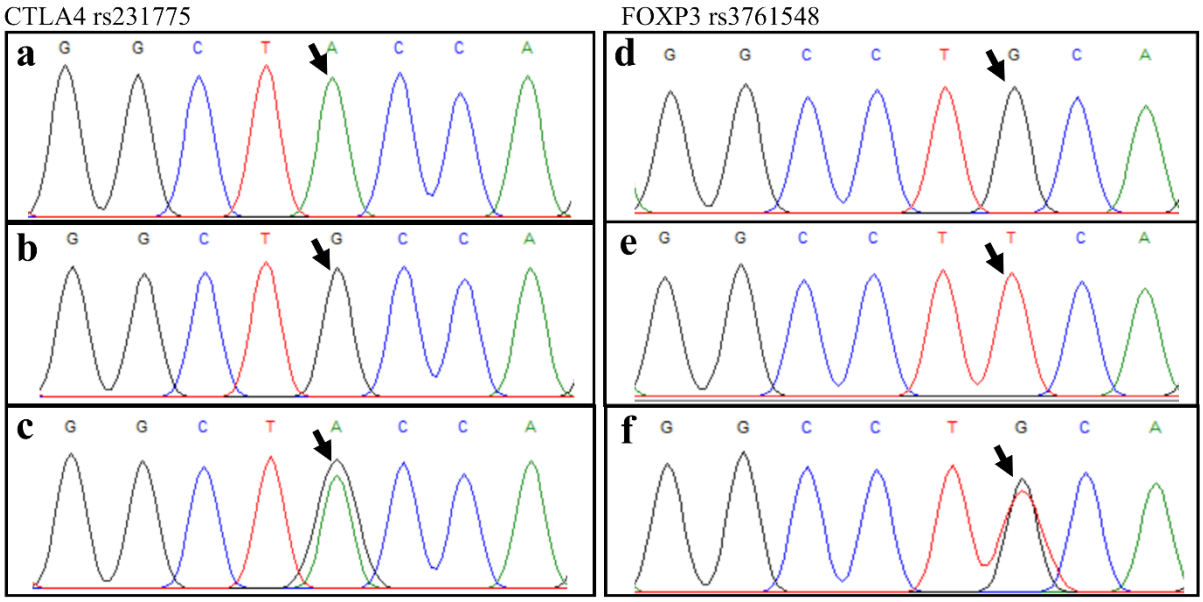

Fig. 2 **Representative Sanger sequencing chromatograms of CTLA-4 rs231775 and FOXP3 rs3761548. Left panel (CTLA-4): (a) AA wild-type, (b) GG homozygous mutant, (c) AG heterozygous mutant. Right panel (FOXP3): (d) CC wild-type, (e) AA homozygous mutant, (f) CA heterozygous mutant. Arrows indicate SNP site. Note: Genotypes for FOXP3 rs3761548 are reported according to the reverse-strand orientation (CA).**

Association of CTLA-4 rs231775 Polymorphism with T1D

The genotype and allele distributions of the CTLA-4 rs231775 polymorphism agreed with the Hardy-Weinberg Equilibrium HWE (P = 0.997), confirming that the allele segregation among the study population is independent. As shown in Table 3, patients' and controls' overall genotype frequencies were different (P = 0.039). The AG genotype was more common among the patients (51.4%) compared to the controls (32.9%). While, the GG genotype did not significantly differ between the groups (P = 0.27). Patients and controls had different genotype frequencies under the dominant model (AG+GG versus AA) (P = 0.017), whereas under the recessive model (P = 0.718), there was no difference. Allele analysis with a P-value (P = 0.0305) showed that patients had a higher G allele frequency (32.9%) than the controls (20.7%). The strength of these results was tested by a post hoc power assessment: The power was found to be 93.1% and justified that the sample size was enough to find the genetic impact.

Association of FOXP3 rs3761548 Polymorphism with T1D

As FOXP3 is an X-linked gene, HWE was tested in females only, and showed no significant departure (P = 0.731). Considering the X-linked characteristics of FOXP3, the analyses were sex stratified to consider male hemizygosity. Table 4 shows the distribution of the genotype and allele of the FOXP3 polymorphism at the rs3761548. Comprehensively, there was no statistically significant difference in the frequencies of the genotypes among the female patients and controls (P = 0.243). Although CA genotype was more common among the female patients (65.7%) than the controls (45.4%), the difference was not found to be significant (P = 0.171). There were no significant associations with the dominant model (CA+AA vs. CC; P = 0.289), or the recessive model (AA vs. CA+CC; P = 0.506). Similar prevalence of the A allele was described (37.1% vs. 32.0%), and allelic analysis did not indicate any significant difference of the allele between female patients and controls (P = 0.469). In males, the percentage of the hemizygous A

Table 3. **Association of CTLA-4 rs231775 (+49 A/G) polymorphism with T1D**

Genotype/Allele	T1D cases (n = 70)	Controls (n = 70)	OR (95% CI)	P-value
Genotype distribution (3x2 χ^2)	*n* (%)	*n* (%)		**0.039***
AA	29 (41.4%)	44 (62.9%)	Reference	–
AG	36 (51.4%)	23 (32.9%)	2.37 (1.18–4.79)	0.022*
GG	5 (7.1%)	3 (4.3%)	2.53 (0.56–11.40)	0.27
Genetic models				
Dominant (AG+GG vs AA)	41 (58.6%)	26 (37.1%)	2.39 (1.21–4.72)	0.017*
Recessive (GG vs AG+AA)	5 (7.1%)	3 (4.3%)	1.72 (0.39–7.48)	0.718
Allele	*n* (freq)	*n* (freq)		
A	94 (67.1%)	111 (79.3%)	Reference	–
G	46 (32.9%)	29 (20.7%)	1.87 (1.09–3.21)	0.0305*

Table 4. **Sex-stratified association of FOXP3 rs3761548 with Type 1 Diabetes**

Genotype/Allele	T1D cases	Controls	OR (95% CI)	P-value
FEMALES	**(n = 35)**	**(n = 33)**		
Genotype	*n* (%)	*n* (%)		0.243
CC	8 (22.9%)	12 (36.4%)	Reference	–
CA	23 (65.7%)	15 (45.4%)	2.30 (0.76–6.95)	0.171
AA	4 (11.4%)	6 (18.2%)	1.00 (0.21–4.71)	1.00
Genetic models				
Dominant (CA + AA vs CC)	27 (77.1%)	21 (63.6%)	1.93 (0.67–5.57)	0.289
Recessive (AA vs CA + CC)	4 (11.4%)	6 (18.2%)	0.58 (0.15–2.28)	0.506
Allele	n = 70	n = 66		
C	66 (62.9%)	70 (68.0%)	Reference	–
A	39 (37.1%)	33 (32.0%)	1.25 (0.71–2.22)	0.4685
MALES	**(n = 35)**	**(n = 37)**		
Hemizygous Genotype	*n* (%)	*n* (%)		0.559
C	27 (77.1%)	31 (83.8%)	Reference	–
A	8 (22.9%)	6 (16.2%)	1.53 (0.47–4.97)	0.558

genotype among the patients (22.9%) was greater than the one among the controls (16.2%), but it was not statistically significant ($P = 0.558$).

Discussion

The current study examined polymorphisms (rs231775 and rs3761548), in two important immune-regulatory genes CTLA-4 and FOXP3 respectively to determine the genetic susceptibility of Iraqi Kurdish children to T1D. The results of this study indicate a potential association between the CTLA-4 rs231775 and susceptibility to T1D, especially when the dominant genetic model is used. In contrast, there was no significant association between T1D and FOXP3 rs3761548 in either female or hemizygous male subjects. These data indicate that this variant may not be a contributor to the disease vulnerability of the population under research.

Our demographic study of the cohort indicated that most of the participants with T1D were between the ages of 7–11 years (48.6%), with the highest prevalence lying in the mid-childhood stage, which is also in line with other epidemiological studies carried out across the globe that show a strong peak prevalence of the condition in the same age group. Even though the proportion of patients who reported a family history of T1D was higher in comparison with controls (7.1% versus 0%), the difference between them was not statistically significant ($P = 0.069$), and therefore cannot be considered concrete evidence of a hereditary factor in this cohort. However, T1D has always shown family clustering with the first-degree family relatives having a significantly higher risk as compared to the general population.[21] Since paternal consanguinity was prevalent in 20.7% of the entire sample (18.6% in patients vs. 22.9% in controls), the results are similar to the ones reported in the Kurdish region of north Iraq 24.3% to 36.9%.[22]

In our study, the analysis of CTLA-4 rs231775 data statistically demonstrated that there was a marked difference in the genotype of the cases and the controls ($P = 0.039$). The occurrence of the AG genotype was associated with possibility of developing T1D (OR = 2.37, $P = 0.022$). In addition, the dominant genetic model (AG + GG versus AA) showed that carriers of the G allele were more exposed to the disease (OR = 2.39, $P = 0.017$). The G allele was significantly ($P = 0.0305$) more prevalent in the case group (32.9%) than in the control group (20.7%).

The results are in line with the significant amount of data that the CTLA-4 rs231775 is a risk factor for autoimmune diabetes. For instance, the results of a study on Sudanese children were consistent as the prevalence of the G allele and the AG/GG genotype were significantly higher among diabetic children compared to controls.[23] It has also been reported that there exists a relationship between the CTLA-4 rs231775 and T1D in the Ethiopian population with a higher prevalence of AG and GG genotypes in the patients,[24] which supports a dominant risk effect of the G allele and agrees with our results. Our findings are further supported by a meta-analysis, which combined the data of various groups of people. The study concluded that the rs231775 polymorphism in CTLA-4 gene has a strong connection with the increased risk of having T1D, especially in the case of childhood-onset diabetes and that the G allele was the risk allele in both Asian and Caucasian ethnic groups.[11] This enabled us to make the conclusion that the polymorphism that we studied is a possible predictor of the risk of T1D in the target population.

Some populations have, however, reported conflicting results. A recent study conducted in the Jordanian population observed that there was no significant correlation between CTLA-4 polymorphisms and T1D.[25] Our finding is also inconsistent with a study that performed an analysis of the CTLA-4 rs231775 in a cohort of Southern Brazilians and found that the polymorphism did not have a statistically significant association with either pediatric or adult-onset T1D.[26] Moreover, another study discovered that the CTLA-4 rs231775 polymorphism does not statistically correlate with T1D in the Northern Han Chinese population.[27] The potential association between the CTLA4 rs231775 and T1D in our cohort is consistent with some ethnic populations and inconsistent with others, underscores the importance of genetic background and heterogeneity of population.

The sex stratified analysis of the X-linked FOXP3 rs3761548 variant showed no statistically significant association with T1D. The CA genotype proportion of individuals in the female cohort was 65.7% among patients, versus 45.4% and among healthy controls ($P = 0.171$). Both dominant model (CA + AA vs. CC; $P = 0.289$) and recessive model (AA vs. CA + CC; $P = 0.506$) showed no significant associations and the proportions of the A allele were similar across groups (37.1% vs. 32.0%, $P = 0.469$). Hemizygous A genotype was found in 22.9% of the patients and 16.2% of controls in males ($P = 0.558$).

Limited intensive studies have been done on the association between the FOXP3 rs3761548 polymorphism and type 1 diabetes mellitus (T1D). In the current study, statistically significant correlation was not found, and the analysis was stratified based on sex to take hemizygous status of FOXP3 in males into account. The current data available concerning the variant is limited and gives inconclusive outcomes.

There was an early report which was carried out within a Japanese cohort indicating that the polymorphism (rs3761548) in the FOXP3 promoter region affected gene expression and was linked to increased risk of T1D.[19] This finding has not however been consistently reproduced by later studies. Our findings agree with the two only European studies in Sardinian and Norwegian subjects that have been conducted so far[28,29] and neither of which has shown a statistically significant relationship between FOXP3 polymorphisms and T1D susceptibility.

Together, the rest of the evidence base fails to give consistent support of any substantial association between the FOXP3 rs3761548 polymorphism and T1D. The heterogeneity of populations can be due to genetic and environmental differences or small sample sizes. It is interesting to note that the post-hoc power analysis showed that sex-stratified X-linked FOXP3 rs3761548 locus attained insignificant power levels (23.2% in females and 11.0% in males). In turn, the lack of statistically significant associations may be attributable to the lack of a sufficiently large sample. This, recommends the need to conduct large and well-powered studies in diverse cohorts.

Conclusion

This is the first study to examine the relationship between CTLA-4 (rs231775) and FOXP3 (rs3761548) polymorphisms and T1D in the Iraqi Kurdish community. The current research

shows there is a possibility of some association between the CTLA4 rs231775 polymorphism and T1D susceptibility, especially in the context of the genotype and dominant models of genetics. On the other hand, a sex-stratified analysis of the X-linked FOXP3 rs3761548 polymorphism did not find any statistically significant association with T1D in either the female or male subgroups.

The overall results of these studies indicate that the CTLA4 rs231775 variant is a possible contributor to susceptibility to T1D in this group of the population, but the variant of FOXP3 rs3761548 did not demonstrate a noticeable impact in our cohort. However, considering the small sample size used and the limited statistical power, the results given above should be interpreted with caution. To describe the possible role of the variants in the genetic architecture of type 1 diabetes in the Iraqi Kurdish population, it is necessary to conduct larger and sufficiently powered studies with functional studies.

Acknowledgments

The authors would like to thank the Dr. Jamal Children's Hospital for their cooperation in sample collection, and Hiwa Cancer Hospital for giving access to the Molecular Biology laboratory in Sulaymaniyah, Kurdistan Region, Iraq, we also thank all the children and their families who participated in this study.

Availability of Data and Materials

All data supporting the findings of this study are available from the corresponding author upon request.

Funding

The authors confirm that this work was conducted as part of a PhD thesis and that no funding, grants, or other support was received to carry out the study; the university provided laboratory facilities only.

Conflict of Interests

The authors declare that they have no conflicts of interests in this study.

AI Use and Originality

We clarify that only the Free version of "Grammarly" was used for checking grammar and spelling errors. ■

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