Original Article

CTLA-4 Gene Polymorphism in Pediatric Type 1 Diabetes Mellitus: A Case–Control Study

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Abstract

Objective: To analyze the relationship between *CTLA-4* (+49A/G) polymorphism and pediatric type 1 diabetes mellitus (T1DM). **Materials and Methods:** The observational case–control study was done for 18 months in 44 children (1–16 years of age), 22 of whom had T1DM and 22 controls, were included. *CTLA-4* (+49A/G) gene polymorphism was analyzed using polymerase chain reaction-restriction fragment length polymorphism and validated by Sanger sequencing. Various clinical, anthropometric, and biochemistry variables were documented. **Results:** The cases and controls were similar in terms of age, gender distribution, family history of diabetes, and consanguinity. The average hemoglobin A1c values were higher in case groups compared to controls (12.41% ±3.09% vs. 4.21 ± 0.5%; $P < 0.001$). The *CTLA4* (+49A/G) gene showed higher frequencies of the G/G genotype in cases compared to controls (54.55% vs. 22.73%; $P = 0.030$), whereas A/A was found to be higher in controls ($P = 0.014$). The frequency of the G allele in cases (0.66) was higher than in controls (0.32). The G/G genotype showed higher prevalence in the case group at a younger age, and siblings of offspring showed clustering of the G/G genotype. **Conclusion:** In particular, the G/G genotype of the *CTLA-4* + 49A/G polymorphism was found to be significantly associated with T1DM in children and to influence the age of onset.

Keywords: +49A/G polymorphism, *CTLA-4*, familial risk, genetic susceptibility, pediatric, Sanger sequencing, type 1 diabetes mellitus

Résumé

Objectif: Analyser la relation entre le polymorphisme *CTLA-4* (+49A/G) et le diabète sucré de type 1 (DT1) chez l'enfant. **Matériels et méthodes:** Cette étude observationnelle cas-témoins, menée sur une période de 18 mois, a inclus 44 enfants âgés de 1 à 16 ans, dont 22 atteints de DT1 et 22 témoins. Le polymorphisme du gène *CTLA-4* (+49A/G) a été analysé par réaction en chaîne par polymérase–polymorphisme de longueur des fragments de restriction (PCR-RFLP) et validé par séquençage de Sanger. Diverses variables cliniques, anthropométriques et biochimiques ont été recueillies. **Résultats:** Les cas et les témoins étaient comparables en termes d'âge, de répartition selon le sexe, d'antécédents familiaux de diabète et de consanguinité. Les valeurs moyennes de l'hémoglobine glyquée (HbA1c) étaient significativement plus élevées chez les cas que chez les témoins (12.41 % ± 3.09 % contre 4.21 % ± 0.5 %; $P < 0.001$). Le polymorphisme du gène *CTLA-4* (+49A/G) a montré une fréquence plus élevée du génotype G/G chez les cas que chez les témoins (54.55 % contre 22.73 %; $P = 0.030$), tandis que le génotype A/A était plus fréquent chez les témoins ($P = 0,014$). La fréquence de l'allèle G était plus élevée chez les cas (0.66) que chez les témoins (0.32). Le génotype G/G était plus fréquent chez les patients présentant un début plus précoce de la maladie, et un regroupement de ce génotype a été observé chez les frères et sœurs. **Conclusion:** Le génotype G/G du polymorphisme *CTLA-4* +49A/G est significativement associé au DT1 pédiatrique et semble influencer l'âge d'apparition de la maladie.

Mots-clés: Polymorphisme +49A/G, *CTLA-4*, risque familial, susceptibilité génétique, pédiatrique, séquençage de Sanger, diabète sucré de type 1

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Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease occurring among children and is the result of an immune-mediated destruction of the pancreatic β-cells, thereby resulting in an absolute deficiency of insulin and the need for lifelong therapy with exogenous insulin.[1,2]

Although various environmental factors such as viral infections, diet, and immune modulation in early life have been shown to contribute to the onset of the disease, genetic susceptibility remains the most important risk factor.[3,4] Human leukocyte antigen (HLA) genes are responsible for the greatest share, that is, about 40%–50% of the risk, although non-HLA genes are also important.[5] Of the various non-HLA genes, the CTLA-4 gene has been shown to be very important, as maintaining peripheral immune tolerance is its main function.[6]

The *CTLA-4* gene, found on chromosome 2q33, codes for a receptor with co-inhibitory function and is primarily expressed on T-lymphocytes activated during immune responses.[7,8] Abnormal CTLA-4 function associated with immune checkpoint disruption leads to autoimmunity and susceptibility to Graves' disease, lupus erythematosus, and T1DM.[9]

Several single-nucleotide polymorphisms within this gene have been studied for their potential link with T1DM. Of these, the + 49A/G polymorphism, also known as rs231775, positioned within the exon 1 region, has received the most attention.[10,11] However, the extent to which such associations are strong may vary between populations.[12-15] Meta-analyses suggest that this association is more significant in the Asian population.[16,17] Therefore, the present study aimed to evaluate the relationship between T1DM in children and the + 49A/G polymorphism of the *CTLA-4* gene.

Materials and Methods

The present research was conducted as an observational case–control study at a tertiary care center. Data collection was carried out for 18 months, starting March 2024 and ending in September 2025. Study participants were children with T1DM seen in the pediatric outpatient clinic or hospitalized in the pediatric ward within the study duration. Age- and gender-matched individuals were chosen as healthy controls, and they were not having any history of diabetes or autoimmune diseases. Written informed consent was obtained from the parents or legal guardians of all participating children prior to enrolment in the study.

The sample size calculation was performed using G*Power software version 3.1.9.4 (Heinrich Heine University Düsseldorf, Düsseldorf, North Rhine-Westphalia, Germany), with estimated frequency of G allele of 37.3% in cases with T1DM and 25.6% in controls, significance level of 5%, and power of 80%. The minimum sample size required was calculated to be 44 participants, with 22 cases and 22 controls.

The inclusion criteria included children aged 1–16 years and cases diagnosed with T1DM. The exclusion criteria included a history of chronic infections such as tuberculosis and/or hepatitis, malignancies, cases with immunodeficiency, on chronic immunosuppressive medication, and other autoimmune conditions.

Peripheral venous blood samples (1–2 mL) were aseptically drawn from all participants using EDTA-coated vacutainer tubes and chilled at 4°C before processing. A portion of this was further submitted to routine biochemical analyses; another portion was allocated for genomic deoxyribonucleic acid (DNA) extraction. Genomic DNA was extracted from 200 µL aliquots of whole venous blood using a G Sure Blood DNA Mini Kit as per the manufacturer's guidelines. The isolated DNA was then dissolved in nuclease-free water and held at either −20°C or −80°C before downstream analyses.

The concentration and purity of the DNA were analyzed using the TECAN multimode microplate reader by measuring absorbency at 260 nm, and the A260/A280 ratio was determined. A ratio of between 1.5 and 1.8 with concentrations of between 20 and 100 ng/µL was considered acceptable for further processing. The integrity of the DNA was checked using agarose gel electrophoresis run in a tris–acetate–EDTA (TAE) buffer. The gels were detected using the ultraviolet (UV) gel documentation system.

Exon-specific intronic primer pairs for the *CTLA-4* gene were designed using Primer3 software, with the specificity of the primers tested using *in silico* polymerase chain reaction (PCR). The complementary sequence of the reverse primer began with the third base from the 5' ends of the first and second exons of the gene, respectively, and the third base of the 5' ends of the second and third exon, respectively, and proceeded backward using the sequence of the gene in the National Center for Biotechnology Information's GenBank database; the sequence of the forward primer began with the third base from the 5' ends of the third, second, and first exon, respectively, and proceeded backward using the sequence of the gene in the National Center for Biotechnology Information's GenBank database.

The sequence of the forward primer, therefore, was 5′-GCT CTA CTT CCT GAA GAC CT-3′, while that of the reverse primer was 5′-AGT CTC ACT CAC CTT TGC AG-3′. PCR amplification of the gene using the primers was performed using Takara Readymade PCR Master Mix (2X) in a final volume of 20 µL, consisting of the master mix, the forward primer, the reverse primer, the DNA, and nuclease-free water.

Cycling conditions for PCR were set as follows: an initial Restriction fragment length polymorphism analysis was performed to identify the + 49A/G polymorphism in the CTLA-4 gene utilizing the BseXI (BbvI) restriction enzyme [Figure 1].

The reaction was conducted at 65°C for 1–16 h, and the digests were resolved on a 2% agarose gel and detected under UV light. For validation of the polymorphic alleles, selectively

Figure 1: Confirmation of restriction fragment length polymorphism product of type 1 diabetes mellitus patients and control group

amplified PCR products were analyzed by Sanger sequencing. Purification of the products was accomplished using the Qiagen Gel Extraction Kit, and the sequencing reaction was conducted using the BigDye Terminator v3.1 Cycle Sequencing Kit. DNA Baser software was employed for interpretation of the electropherograms, and polymorphic variations were determined by analyzing the sequences relative to the reference sequence of the *CTLA-4* gene [Figure 2].

Statistical analysis

The analysis of the data was done using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and Statistical Package for the Social Sciences (SPSS) software, version 26.0 (IBM Corp., Armonk, NY, USA). Continuous data were presented as mean and standard deviation and analyzed using independent samples *t*-test/Mann–Whitney *U*-test, while categorical data as frequencies and percentages and analyzed using Chi-square test/Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

The majority of the children with T1DM belonged to the age group of 11–16 years (72.73%), while the control group had a relatively even distribution ($\chi^2 = 3.68$, $P = 0.158$). The gender ratio was similar in both the groups ($P = 0.365$). A larger percentage of the cases had a positive family history of diabetes (27.27%) compared to the control group (13.64%), while the percentage of consanguinity in cases (13.64%) was lower than the control group (27.27%), although not statistically significant ($P = 0.262$ for both). The mean hemoglobin A1c (HbA1c) values were significantly higher in cases [Table 1].

There were no significant differences in the anthropometric parameters while systolic blood pressure (BP) values were significantly more in the cases ([110 ± 11.55 mmHg vs. 100 ± 11.13 mmHg]; $P = 0.006$).

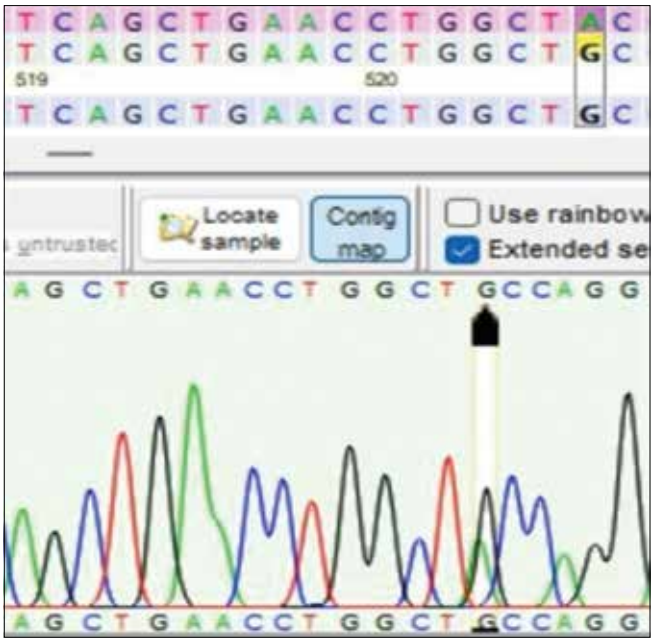

Figure 2: Sanger sequencing electropherogram of the *CTLA-4* gene revealing a heterozygous A/G substitution at nucleotide position +49 (rs231775)

The A/A genotype was found to have a significantly reduced frequency in the case group (22.73%) than the controls (59.09%) ($P = 0.014$); however, the G/G genotype was more frequently present in cases (54.55%) than the controls (22.73%) ($P = 0.030$); there was no significance in the A/G genotype. The frequency of alleles was higher in the case group with the G allele (0.66) than the controls with the G allele (0.32). Sibling genotype distribution indicated the relative prevalence of the G/G genotype alone in the case siblings, with the A/A genotype being predominant in the control sibling ($P = 0.028$) [Table 2].

Discussion

In the current case–control study, both the groups had similar age and gender composition, thus eliminating demographic confounders. The preponderance of adolescents with T1DM is also supported by the literature, with the evidence showing that despite the possibility of the disorder manifesting at any age, most patients are actually diagnosed or placed on registries in the later stages of childhood or during the course of adolescence.[14,18] A lack of gender disparity in type 1 diabetes is also afforded face validation by the evidence showing the disorder is more or less equally distributed between the genders.[12,14,18]

There were more instances where the cases reported a positive family history of diabetes than the controls, although this was not significant. This phenomenon is in agreement with the observation that there is familiarity and genetic predisposition to T1DM, as suggested by earlier literature.[14] Consanguineous marriages were not shown to have any significant effect regarding the development of the disease, as suggested

Table 1: Baseline characteristics

Variable	Cases ($n=22$), n (%)	Controls ($n=22$), n (%)	Test value	P
Age group (years)				
1–5	1 (4.55)	5 (22.73)	$\chi^2=3.68$	0.158
6–10	5 (22.73)	6 (27.27)		
11–16	16 (72.73)	11 (50.00)		
Gender				
Male	12 (54.55)	9 (40.91)	$\chi^2=0.82$	0.365
Female	10 (45.45)	13 (59.09)		
Family history of diabetes	6 (27.27)	3 (13.64)	$\chi^2=1.26$	0.262
Consanguinity	3 (13.64)	6 (27.27)	$\chi^2=1.26$	0.262
HbA1c (%), Mean±SD	12.41±3.09	4.21±0.56	$t=5.63$	<0.001

SD=Standard deviation, HbA1c: Glycated haemoglobin

Table 2: CTLA-4 (+49A/G) Genotype and allele distribution

Variable	Cases ($n=22$), n (%)	Controls ($n=22$), n (%)	Test value	P
Genotype				
A/A	5 (22.73)	13 (59.09)	$\chi^2=6.02$	0.014
A/G	5 (22.73)	4 (18.18)	$\chi^2=0.14$	0.709
G/G	12 (54.55)	5 (22.73)	$\chi^2=4.70$	0.030
Allele frequency				
A allele	0.34	0.68	-	
G allele	0.66	0.32	-	
Sibling genotype distribution				
A/A	0	3 (75)	$\chi^2=4.8$	0.028
G/G	4 (100)	1 (25)		

by literature in certain populations where consanguineous marriages have not been shown to have much effect regarding the development of autoimmune diabetes mellitus.[12]

However, there were no significant differences in the anthropometric variables, even though the cases had a lower body mass index, as reported in previous studies of T1DM in the pediatric population, and this can be explained by the catabolic effects of insulin deficiency and impaired glucose metabolism.[14,19] Among the vital variables, the systolic B*P* values were found to be higher in the cases, indicating the onset of vascular or autonomic changes due to chronic hyperglycemia, and findings have also been reported in previous studies in the pediatric T1DM population, emphasizing the importance of BP monitoring in the management of T1DM to prevent the onset of cardiometabolic complications in later life.[19] The excessively high levels of HbA1c in the cases indicate the poor glycemic control in the cases, emphasizing the importance of strict metabolic management in the diabetic cases.

The key finding obtained from this study was the significant association among the CTLA-4 + 49A/G polymorphism and childhood T1DM. The G/G genotype and G allele were found to be significantly more common within the cases, while the A/A genotype was largely restricted to the controls, indicating a possible protective effect imparted by the A allele. The findings were in accordance with the previous studies carried out among the Indian, Sudanese, Egyptian, Ethiopian, and Lebanese populations, who identified the G allele G/G genotype to be associated with a higher risk of developing T1DM.[14,18-23] In addition, the findings obtained from the current study, wherein the G/G genotype was found exclusively within the siblings, support the previous findings indicating a younger age at the time of presentation within the cases carrying the G/G genotype, probably indicating the potential for accelerated autoimmune destruction of the beta-cells and the earlier effect exerted on the disease onset.[18,20]

Limitations

Despite these results, the present research had some limitations. The number of participants could have reduced statistical power, especially in multiple subgroup analyses, and may be partly responsible for the nonsignificance of variables such as family history and consanguinity. As a single-centered study, it may be difficult to generalize its findings to pediatric populations with different ethnic and environmental contexts. It is a cross-sectional study, and it is not possible to infer any causal relationship and disease progression. Second, only one *CTLA-4* gene polymorphism was studied, and other genes involved in the regulation of immune functions, and the HLA haplotypes, which have been shown to affect the risk of T1DM, have not been studied. Finally, due to the small number of participants included in the sibling analysis, these findings should be considered with caution and require validation in further family-centered research.

Conclusion

The present study proves that, although there was equality between the two groups regarding the baseline factors, there was poor glycemic control in children with T1DM, establishing the need for early cardiovascular screening and proper diabetes care. A strong link was established between the CTLA-4 (+49A/G) polymorphism and the presence of pediatric T1DM, with an elevated distribution of the G/G genotype and the G allele, an early onset in the G/G carriers, and a clustering of the G/G genotype in the affected siblings. These results establish the association between the presence of the G allele, specifically the G/G genotype, and the susceptibility to pediatric T1DM.

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Conflicts of interest

There are no conflicts of interest.

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