CTLA-4 (+49A/G) Polymorphism in Type 1 Diabetes Children of Sudanese Population

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Introduction

Type 1 diabetes mellitus (T1DM) is an organ-specific T cell-mediated autoimmune disease, characterized by destruction of pancreatic islets. Cytotoxic lymphocyte antigen-4 (CTLA-4) is a negative regulator of T cell proliferation, thus conferring susceptibility to autoimmunity.

Aims

This study aimed to investigate the association of CTLA-4 (+49A/G (rs231775) polymorphism with a risk of T1DM in Sudanese children.

Methods

This a case–control study included 100 children with T1DM, referred to the pediatric clinic at referral pediatric teaching hospital in Gezira State-Sudan. Hundred unrelated healthy controls were recruited from departments in the same hospital. Genomic deoxyribonucleic acid (DNA) was extracted from Ethylenediaminetetraacetic Acid (EDTA)-preserved blood using QIAamp DNA Blood Mini Kit (QIAamp Blood) (QIAGEN; Valencia, CA). The polymerase chain reaction PCR restriction fragment length polymorphism (PCR-RFLP) and sequencing were applied for the CTLA-4 (+49A/G) genotyping. The changes accompanied the polymorphism were evaluated using relevant bioinformatics tools.

Results

The genotype and allele frequencies of the CTLA-4 (+49A/G) polymorphism were significantly different between the patients and controls (p = 0.00013 and 0.0002, respectively). In particular, the frequency of the G allele, GG homozygous genotype, and AG heterozygous genotype were significantly increased in patients than in controls ([28% versus 7%, odds ratio (OR) = 5.16, 95% confidence interval [CI] = 2.77–9.65, p = 0.00] [12% versus 2%, OR = 6.68, CI = 1.46–30.69, p = 0.01] [32% versus 10%, OR = 4.24, CI = 1.95–9.21, p = 0.00], respectively). The presence of the G allele (homozygous) showed an influence on the signal peptide polarity, hydrophobicity, and α-helix propensity of the CTLA-protein.

Conclusion

The results further support the association of CTLA-4 (+49A/G) polymorphism and the risk of T1DM in our study population.

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Keywords

► CTLA-4
► polymorphism
► type 1 diabetes Mellitus
► Sudanese

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associated with susceptibilities to a wide range of T cell-mediated autoimmune diseases.3

One of these polymorphisms was the CTLA-4 +49A/G single nucleotide polymorphism (SNP) that causes a threonine-to-alanine substitution in codon 17, which altered protein expression4 and T cell activation.5 Since the +49A/G SNP is located in the N-terminal of the signal peptide sequence of the CTLA-4, which is not a part of the mature protein, the substitution of threonine to alanine may affect the proper translocation of the growing CTLA-4 peptide from ribosome to endoplasmic reticulum (ER) lumen, as a result of alteration in signal peptide hydrophobicity and helix propensity,6 rendered possible evidence of defective CTLA-4 targeting to the cell surface.7

The association of CTLA-4 polymorphisms with the risk to develop T1DM has been investigated in different populations with conflicting data.8 Recent study has shown no association between the aforementioned SNPs and susceptibility to T1DM among Sudanese adults.9 Since Sudanese population is characterized by multiethnic groups, the search for further association between groups of different age and ethnicity could likely help pursuing conclusive remarks about this association. In the present study, we investigated the association of the CTLA-4 +49A/G SNP with the risk of T1DM among Sudanese children and the proposed effect of this polymorphism on the CTLA-4 signal peptide instability.

**Subjects and Methods**

**Patients and Sampling**

This is a case–control study that encompasses 100 Sudanese children with T1DM (48 males and 52 females; mean age, 11.49 ± 3.38), referred to the diabetic clinic at Wad Medani Pediatric Hospital in Gezira state, Sudan. The selected patients were clinically diagnosed with T1DM, their disease duration was more than 1 year, and they are dependent on insulin therapy. The patients were classified based on the hemoglobin A1c (HbA1c) levels into poor glycemic control (>8%) and well glycemic control ≤8%, as stated on the American Diabetes Association (ADA) and Japan Diabetes Society (JDS) guidelines.10,11 The demographic characteristics, clinical presentations, HbA1c levels, concomitant complications, and the presence of other autoimmune diseases were all reported in well-structured questionnaire. The control group includes 100 unrelated healthy children (44 males and 56 females; mean age, 11.49 ± 3.38) without or family history of T1DM or any other autoimmune diseases. The controls were recruited from departments in the same hospital, they lived in the same state, and they have ethnic background similar to the patients.

The study met the University of Gezira ethical committee review board requirements, and granted the permission to be performed from the hospital clinical directorate and the diabetic clinical staff as well. Signed written informed consent was obtained from the parents or guardians of all study subjects, after they informed about the study objectives and procedures.

Five-milliliter (5 mL) blood sample was taken in the morning (before breakfast) in EDTA container. Serum was separated to measure the HbA1c by chromotographic-spectrophotometer ion exchange (BioSystems, United States), and the pellet used for the deoxyribonucleic acid (DNA) analysis of CTLA-4 +49A/G genotypes.

**DNA Extraction and CTLA-4 Amplification**

Genomic DNA was extracted using QIAamp DNA Blood Mini Kit (QIAamp Blood) (Qiagen, Valencia, CA). The desired fragment of the CTLA-4 gene was amplified by polymerase chain reaction (PCR) using CTLA-4 gene-specific forward (5'-gCTCTACTTGGAgGCTTGAAGCCT-3') and the reverse (5'-AgTCTCAGAGCAGCTTtgCAG-3') primers, which amplified 207 fragments of CTLA-4 gene.

Approximately, 0.2 mg genomic DNA was amplified in 25 mL PCR reaction containing 10 mM of each dNTPs (i-StarTaq, iNtRon Biotech, Korea), 5 U of Taq DNA polymerase (i-StarTaq, Korea), 2.5 mL of 10X PCR buffer, and 100 pmol/mL of each primer (Sinagen, Iran). Reaction conditions were performed in PCR thermocycler (Eppendorf, Germany), starting with initial denaturation at 94°C for 4 minutes followed by 34 cycles of denaturation at 94°C for 30 second annealing at 60°C for 30 seconds elongation at 72°C for 2 minutes, and final extension at 72°C for 5 minutes. Then 5 mL of the PCR product was run in 2% agarose gel electrophoresis to check the target PCR product at 207 bp length (Fig. 1A).

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**Fig. 1** (A) Confirmation of the genomic deoxyribonucleic acid (DNA) in patients and controls. Lane 1: DNA ladder 100 bp. The band appeared in lane 2 to 7 showed the polymerase chain reaction (PCR) product length of 207 bp for the CTLA-4 target gene in exon 1. (B) Fragment size for CTLA-4 (+49A/G) polymorphism in diabetic patients and controls by FastDigest BbvI (LSP11091, Germany). Lane 1: DNA ladder 100 bp; the bands in lane 3, 4, 7, and 8 represent allele A at 207 bp, whereas fragments of band G at 168 and 49 bp appear in lane 2, 5, and 6. Lanes 5, 6, 7: heterozygous (AG) genotype. Lanes 3, 4, 7, 8: homozygous (AA) genotype. Lane 2 homozygous (GG). CTLA-4, cytotoxic T-lymphocyte antigen-4.
Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) analysis was conducted using FastDigest BbvI (Fermentas, Germany) in 30 μL total volume that includes 10 μL amplification product, 1.0 μL (10 U/μL) of the restriction enzyme, 2.0 μL 10X fast digest green buffer, and 17 μL nuclease-free water. All components were mixed gently, spun down, and incubated for 10 minutes at 37°C followed by heating at 65°C for 10 minutes. DNA fragments were visualized in 2.0% agarose gels exposed to UV light in a gel documentation system (Ingenus, United States). The restriction cut showed two fragments (49/168 bp) for the G allele and one fragment (207bp) for A allele (Fig. 1B). Direct DNA sequencing by Sanger method on an ABI 3730 sequencer (Macrogen, South Korea) was performed for 20 patients and 20 control by using 25 μL of each PCR result independently, to validate the RFLP results. The sequencing results were analyzed using BLAST and Clustal X at the NCBI webpage.

Bioinformatics Analysis Tools
I-mutant version 3 (http://gpcr2.biocomp.unibo.it/cgi/predictors/1-Mutant3.0/) was used to predict the effect of the CTLA-4 +49A/G SNP in proteins stability.12

Table 1 Demographic characteristics of the patients and the controls

| Characteristics                              | T1DM (n = 100) | Controls (n = 100) |
|---------------------------------------------|----------------|-------------------|
| Gender                                      | Male           | 48                | 56                |
|                                             | Female         | 52                | 44                |
| Age group/yrs.                              | Mean ± SD      | 11.49 ± 3.38      | 10.24 ± 4.31      |
|                                             | <5             | 2                 | 24                |
|                                             | 5–9.9          | 26                | 38                |
|                                             | 10–14.9        | 50                | 30                |
|                                             | ≥15            | 22                | 8                 |
| Residence                                    | Rural          | 80                | 54                |
|                                             | Urban          | 2 (2.0)           | 46                |
| Insulin dependent                           | All            | None              |                   |
| Age at disease onset (years)                | 3–16           | NA                |                   |
| T1DM duration                               | 4.35 ± 3.02    | NA                |                   |
| Family history of diabetes                  | Negative       | 64                | NA                |
|                                             | Positive       | 36                | NA                |
| Glycemic control                            | HbA1c mean level | 10.68 ± 2.17   | 6.05 ± 1.40*      |
|                                             | HbA1c <8%      | 12                | 94                |
|                                             | HbA1c >8%      | 88                | 6                 |
| Breast feeding                              | Yes            | 45                | NA                |
|                                             | No             | 55                | NA                |
| BMI (kg/m²)                                 | Range          | 16.74 ± 2.90      | 19.4 ± 4.74*      |
|                                             | Underweight (<18.5 kg/m²) | 80 | 52 |
|                                             | Normal (18.5–24.9 kg/m²) | 18 | 28 |
|                                             | Overweight (25–29.9 kg/m²) | 1 | 16 |
|                                             | Obese (≥30 kg/m²) | 1 | 4 |
| On treatment                                | Regular        | All               | NA                |
|                                             | Not regular    | –                 | NA                |
| Disease complications                       | Eye complication | 9 | NA |
|                                             | Renal complication | 1 | NA |
|                                             | Hypoglycemia   | 45                | NA                |
|                                             | Ketoacidosis   | 63                | NA                |
| Autoimmune diseases                         | No             | 92                | NA                |
|                                             | Celiac disease | 8                 | NA                |

Abbreviations: BMI, body mass index; HbA1c, hemoglobin A1c; NA, not applicable; SD, standard deviation; T1DM, type 1 diabetes mellitus. *p < 0.05.
Sorting Intolerant from Tolerant (SIFT) was used to predict whether an amino acid substitution affects protein function or not, based on the degree of amino acids conservation residues in sequence alignments derived from closely related sequences. The main underlying principle of this program is that it generates alignments with large number of homologous sequences, and assigns scores to each residue ranging from zero to one. Score close to zero indicates evolutionary conservation of the gene and intolerance to substitution, while score close to one indicates only

Table 2  Genotypes and alleles frequencies of CTLA-4 +49A/G polymorphism in T1DM patients and controls

| CTLA-4 variants | T1DM (n=100) | Controls (n=100) | OR  | 95% CI  | p-Value |
|-----------------|--------------|------------------|-----|---------|---------|
| Genotype frequencies<sup>a</sup> | | | | | |
| AA (normal)     | 56 (56)      | 88 (88)          | 0.17| 0.08–0.36| 0.00   |
| AG (heterozygous) | 32 (32)   | 10 (10)          | 4.24| 1.95–9.21| 0.00   |
| GG (homozygous) | 12 (12)      | 2 (2)            | 6.68| 1.46–30.69| 0.01   |
| Allele frequencies<sup>b</sup> | | | | | |
| °A allele        | 144 (72)     | 186 (93)         | 1.94| 0.10–0.36| 0.00   |
| °G allele        | 56 (28)      | 14 (7)           | 5.16| 2.77–9.65| 0.00   |

Abbreviations: CI, confidence interval; CTLA-4, cytotoxic T-lymphocyte antigen-4; OR, odds ratio; T1DM, type 1 diabetes mellitus.

<sup>a</sup>p = 0.00013.
<sup>b</sup>p = 0.0002.
°Adenine.
°Guanine.

Table 3  The SIFT score for the CTLA-4 SNP (rs231775)

| SNP ID | Organism/Build | Ref allele | Alt allele | Amino acid change | Gene name | SIFT score | SIFT prediction |
|--------|----------------|------------|------------|-------------------|-----------|------------|----------------|
| rs231775 | Homo_sapiens/GRCh37.74 | A        | G   | T17A  | CTLA-4     | 0.06       | Tolerated      |

Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen-4; SIFT, Sorting Intolerant from Tolerant; SNP, single nucleotide polymorphism.
tolerance to substitution. The algorithmic calculation methods of the hydrophobicity and α-helix propensity that accompanied the CTLA-4 T17A change were also determined to help in predicting the alteration in the CTLA-4 signal peptide function.

**Data Analysis**

Statistical analysis was performed using SPSS software package, version 23 (SPSS, Inc.; Chicago, Illinois, United States). The mean difference between study group was assessed by using the Student’s t-test. Qualitative data were presented as number of (%), the comparisons of genotypes and alleles frequencies between patients and controls were assessed using the chi-squared (χ²) test and the Fisher’s exact tests, and levels of risk for genotypes and alleles were expressed as odds ratio (OR) with a 95% confidence interval (95% CI). Deviation from Hardy-Weinberg equilibrium was performed by applying the equation (p² + 2pq + q²) to compare the observed frequencies with the expected frequencies of the different genotype distribution in patients and controls by using Pearson’s χ² test of independence in SPSS. Statistical significance was considered at \( p < 0.05 \).

**Results**

The study included 100 children with T1D (48 males and 52 females) and 100 unrelated healthy controls (44 males and 56 females).

- Table 1 showed the sociodemographic and clinical characteristics of study groups. There were no significant differences in the age and gender between patients and controls \( (p > 0.05) \). Compared with the controls, the patients showed significant high mean levels of HbA1c \( (p = 0.0003) \) and low mean level of body mass index \( (p = 0.01) \).

As shown in - Table 2, the frequency distribution of CTLA-4 +49 A/G genotypes and alleles showed significant differences between the patients and the controls \( (p = 0.00013 \text{ and } 0.0002) \). The genotypic distribution was not deviated from the Hardy-Weinberg equilibrium in patients \( (\chi^2 = 5.19, \text{ df} = 2, \ p = 0.08) \) and controls \( (\chi^2 = 4.82, \text{ df} = 2, \ p = 0.09) \).

The GG homozygous and AG heterozygous genotypes were more frequent in patients than in controls \( (12 \text{ vs. } 2\% \text{ and } 32 \text{ vs. } 10\%, \text{ respectively}) \). This difference was statistically significant \( (p = 0.01, \text{ OR} = 6.68, 95\% \text{ CI} = 1.46–30.69 \text{ vs. } p = 0.00, \text{ OR} = 4.24, 95\% \text{ CI} = 1.95–9.21, \text{ respectively}) \). At the same time, the CTLAAla\(^+\) (G) allele was significantly high in frequency in patients than in controls \( (28 \text{ vs. } 7\%, \text{ OR} = 5.16, 95\% \text{ CI} = 2.77–9.56, \ p = 0.00) \).

SIFT analysis score for the CTLA-4 SNP (rs231775) position at codon 17 (T17A) indicates evolutionary conservation of the gene and intolerance to substitution (− Table 3) that may decrease the CTLA-4 protein stability as predicted by the I mutant analysis (− Fig. 2), most likely by affecting the polarity (from polar threonine to nonpolar alanine) of the CTLA-4 signal peptide chain (− Fig. 3). The threonine to alanine substitution at position 17 also leads to increase in the hydrophobicity and α-helix propensity, two properties known to be important in the CTLA-4 signal peptide function (− Fig. 4).

**Discussion**

T1DM is one of the most frequent chronic diseases in children, and has become health problem in developing countries. Recently, associated studies have been conducted to address the association of polymorphisms in the CTLA-4 gene as a candidate gene with several autoimmune
The presence of the +49A/G-at-risk (G) allele in the CTLA-4 molecule has been shown to be effective in inhibiting the activated T cell proliferation in vitro.32 This perception coincides with the high frequency of G allele among our patients, and found to be consistent with results from other populations including Egyptians,16,31 Iranian,25 Turkish,34 Croatian,35 Belgian,36 Mexican-American, and Korean,37 and meta-analysis study in Asian population.38

However, lack of association between the aforementioned polymorphism and T1DM has been also reported in populations from Sudan,9 Czech,31 Turkey,34 Korea,38 Chile,39 Portugal,40 and Azerbaijan.41 The discrepancy in results between ethnic groups may be attributed to genetic heterogeneity relevant to ethnic diversity, to polymorphism co-players (environmental factors, etc.), or to differences in methodologies and sample size used.

The CTLAAla16 (G) allele (homozygotes) located at the N-terminal of conserved position in the loop region of the signal peptide sequence introduces the hydrophobic amino acid alanine instead of threonine in the signal peptide sequence. This introduction, and based on our bioinformatics analysis, was somewhat associated with evolutionary conservation of the gene and intolerance that may decrease the CTLA-4 protein stability, affecting the polarity, and increase hydrophobicity and α-helix propensity. These properties are collectively known to be important in signal peptide function. The consequences of these alterations may result in an aberrantly glycosylated product, alteration in proteins folding, and/or interaction with ER chaperones, which may finally lead to less functional expression of CTLA-4 at the cell surface of their T cells than the normal Thr49 allele.6 It is most likely that the one-third less expression of the mutant homozygous (GG) on the cell surface of T cells than the normal homozygous (AA) can lower the affinity of CTLA-4 for B7 molecule, skewing the negative balance exerted for damping T cell activation.7,42

The small sample size in this study, concomitant with the large discrepancy in Sudanese ethnic groups, makes the power of association between the CTLA-4 +49 A/G (rs2317775) polymorphism and T1DM relatively weak and the overall data are not fully conclusive.

**Conclusion**

The study supported the proposition that CTLA-4 +49 A/G polymorphism is associated with the risk of T1DM in Sudanese children, and the presence of the CTLA-4 Thr16 (G) allele (homozygous) represents an evolutionary change predisposed the risk for T1DM. This data warrant further studies with larger study population to verify our findings.

**Ethical Approval**

The informed consent was obtained from all the subjects and the study was approved by the University of Gezira.
Ethics Committee (UGE) and was performed in accordance with Helsinki Declaration of 1975.

Conflict of Interest
None declared.

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