Detection of Cytotoxic T-Lymphocyte Associated Antigen-4 Gene Polymorphism in Type 1 Diabetes Mellitus

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Type 1 diabetes is one of the most common chronic childhood illnesses. Interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for the disease. It has been shown that more than 40 genetic loci are associated with T1DM. Important among these is the CTLA-4. This work aimed to detect Cytotoxic T Lymphocyte-associated antigen 4 (CTLA-4) gene polymorphism in patients with type 1 diabetes mellitus T1DM using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to clarify its role in the susceptibility to T1DM. The study was carried out on Forty unrelated Egyptian children with T1DM. Twenty unrelated healthy children were enrolled as a control group. Blood samples were collected from patients and control groups and subjected to CTLA-4 gene polymorphism analysis using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). CTLA-4 G allele and GG homozygous genotype were significantly increased in T1DM patients than in control group (P<0.001, P = 0.002 respectively). There was significant association between the three CTLA-4 genotypes (AA, AG, GG) and diabetic complications (p=0.002), AG and GG polymorphisms were associated with complications of diabetes with ratio 84.6% and 100% respectively. While no association was found with sex, weight, height, risk factors of diabetes or insulin treatment. It was concluded that there is a strong association between AG polymorphism and T1DM (P=0.002).

Type-1 diabetes mellitus (T1DM) is an insulin-dependent form of diabetes with high morbidity and mortality rates which usually begins in childhood and adolescence. Most cases are primarily due to T-cell mediated pancreatic islet β-cell destruction and the patient becomes clinically symptomatic when approximately 90% of pancreatic beta-cells are destroyed (Gepts, 1965).

Type-1 diabetes patients have increased susceptibility to develop multiple organ specific autoimmune diseases such as thyroid disorders, celiac disease and Addison’s disease (Lazzarott, 2003).

A variety of genetic predisposing factors and contributing factors are known to influence the pathogenesis of T1DM. There is some evidence suggesting that the susceptible genes to T1DM are associated with amplification of the immune response and rate of progression of the disease. The role of these genes appears to be more important during childhood than during adult life (Field, 2002). It has been shown that more than 40 genetic loci are associated with T1DM (Noble & Erlich, 2012).

Many of the susceptibility genes are located within the HLA locus on chromosome 6p21, known as insulin-dependent diabetes mellitus-1 (IDDM1) (Davies et al., 1994).

Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) is a member of the immunoglobulin superfamily that is expressed on the surface of activated T-cells and downregulates T-cell function. Binding of CTLA-4 to the B7 receptor limits the proliferation of T-cells and terminates the ongoing immune response (Alegre et al., 2001).
The most relevant non-HLA genes identified as susceptible for T1DM are those connected with the T-cell mediated immune response. The activity level of T-cells and their effector functions are determined by intracellular signaling pathways and related genes. These include PTPN22 and CTLA-4, both of which prevent spontaneous activation of auto-reactive cells and development of Autoimmunity (Mosaad et al., 2012). Viral infections and nutritional factors when superimposed on genetic factors may lead to the activation of T1DM (Sawka et al., 2007).

This study aimed to detect Cytotoxic T-Lymphocyte associated antigen 4 (CTLA-4) gene polymorphism in patients with type 1 diabetes mellitus using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to clarify its role in the susceptibility to type 1 diabetes mellitus.

**Subjects and Methods**

**Subjects**

This study was carried out at Microbiology and Immunology Department of Benha faculty of Medicine and Pediatric Department, at Benha University Hospital, in the period from October 2013 to June 2014. A written informed consent (in Arabic language) was obtained from the patients parents before Participation. Forty unrelated Egyptian children with T1DM (20 males and 20 females), age ranged from 4 to 18 years were enrolled in this study (ISPAD, 2007). They were chosen from Pediatric Endocrinology Outpatient Clinic of Benha University Hospital. Twenty unrelated healthy children matched with patients in age and sex were enrolled in this study as a control group; They had no family history of T1DM or other autoimmune disease.

Each patient was subjected to:
- Complete medical history taking including age, sex, regimen of insulin treatment, associated complications if present (macro vascular or micro vascular complications, diabetic ketoacidosis, hypoglycemic attacks or psychic troubles)
- General examination including weight and height.
- Laboratory investigations: Measurement of fasting and postprandial blood glucose levels.
- Detection of CTLA-4 gene polymorphism by (PCR-RFLP).

**Methods**

- **Samples**
  The skin over the vein was sterilized by 70% alcohol and 2ml whole venous blood sample was collected into tube containing EDTA. The samples were stored at -20°C until further processing.

- **DNA extraction**
  DNA was extracted by DNA extraction kit (GeneJET Whole Blood Genomic DNA Purification Mini Kit, Fermentas, Germany) according to the manufacturer’s instructions.

- **Genotyping**
  Genotyping of polymorphic restriction sites in the CTLA-4 gene exon 1 position 49 which encodes a threonine to alanine substitution at codon 17 was done using PCR amplification followed by the Restriction Fragment Length Polymorphism (RFLP) method.

- **Amplification of exon 1 of CTLA4 gene**
  Genomic DNA was amplified using PCR with both primers (forward and reverse) (Biolegia, BV Nijmegen) as described by Mosaad et al., 2012. Sequence of used primers were: Forward primer: 5’ GCT CTA CTTCCTGAA GAC CT 3’, Reverse primer: 5’AGT CTC ACT CTC ACT CAC TTT TGC AG 3’.

For each 25μl reaction volume the following components were added: 2μl of template DNA + 12.5μl DreamTaq Green master mix + 0.5 μl of each primer (forward and reverse) + 9.5μl nuclease-free water. The reaction volume containing tubes were then gently vortexed and briefly centrifuged to collect all drops to the bottom of the tube. The samples were placed in the thermal cycler: Thermal cycling conditions were carried out in thermocycler PTC-100 (Biorad, USA) as described by Mosaad et al., 2012: Initial denaturation by one cycle at 94°C for 5 min. 40 cycles of Denaturation at 94°C for 30 seconds and annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds. Final extension by one cycle at 72°C for 5 min. 10μl of PCR products were then loaded into2% agarose gel to check the PCR products at 162bp fragment.
- Restriction fragment length polymorphism (RFLP)
  RFLP analysis was done using FastDigest BbvI (Fermentas, Germany) in 30μl total volume: 17μl Water, nuclease-free + 2 μL10X FastDigest Buffer + 10 μl DNA (PCR product) + 1 μl (10 units) FastDigest BbvI enzyme (total volume: 30 μl). All components were mixed gently and spinned down. Then the mixture was incubated at 37°C in a heat block (Stuart, UK) for 3hours. The enzyme was inactivated by heating for 5 min at 65°C.

- Agarose gel electrophoresis
  DNA fragments were loaded into 2% agarose gel and visualized by ethidium bromide staining. The A allele does not create restriction site (162 bp), while the G allele creates restriction site producing two fragments (88 bp and 74 bp) as shown in figure (1).

![Figure 1](image)

**Figure 1.** The BbvI restriction profiles of the 49A/G polymorphic sites.
Lane 1: DNA ladder (50-1000bp). Lane 2: PCR product at 162bp. Lane 3: AA genotype (homogenous- normal), Lane 4: AG genotype (heterozygous- polymorphic), Lane 5: GG genotype (homogenous – polymorphic), *88 and 74bp fragments of heterozygous- polymorphic are very close to each other.

Statistical Analysis

The collected data were summarized as Mean ± Standard deviation (M±SD) and range for quantitative data and proportions for qualitative data. Differences between the study groups regarding the studied parameters were tested using the test of proportion (Z-test) and the student t-test to compare two groups. The Analysis of Variants (ANOVA) test and Chi-squared (X2) test to compare more than two groups. After the calculation of each of the test statistics, the corresponding distribution tables were consulted to get the “P” (probability value). Statistical significance was accepted at P value <0.05. All statistical analyses were carried out in STATA/SE version 11.0 for Windows.

Results

The results of the study are represented in the following tables and figure.

The age of the studied cases ranged from 4 to 18 years. They were 50% males (20 cases) and 50% females (20 cases). Their weight ranged from 18-79 kg , while their height ranged from 104-181 cm. Thirty cases out of 40 were suffering from diabetic complications (diabetic ketoacidosis, diabetic coma, psychic troubles, others) while 10 cases were free from complications. Also, it was found that 26 patients had a history of insulin treatment three times daily with a higher percentage (65%) than those treated with two, four and five doses daily (25%, 2.5% and 7.5% respectively), (Table 1).

As regards CTLA-4 GG homogenous genotype there was insignificant statistical difference (P=0.07) between the diabetic cases and the control group While There was significant statistical difference (P=0.01) and significant statistical difference (P=0.001) between the diabetic cases and controls as regards CTLA-4 AG heterozygous genotype CTLA-4 AA genotype respectively, Table(2).

There is significant statistical value (P <0.001) between diabetic cases and control group as regards A and G alleles of CTLA-4, (Table3).
Table 1. Personal and medical data of the studied diabetic cases:

<table>
<thead>
<tr>
<th>Personal data</th>
<th>Diabetic cases N=40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years): Range (Mean ± SD)</td>
<td>4-18 (12.1±4.1)</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>20 (50%)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>20 (50%)</td>
</tr>
<tr>
<td>Weight (kg): Range (Mean ± SD)</td>
<td>18-79 (40.1 ±14.1)</td>
</tr>
<tr>
<td>Height (cm): Range (Mean ± SD)</td>
<td>104-181 (141.8 ± 18.0)</td>
</tr>
<tr>
<td>Complications of diabetes:</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>30 (75%)</td>
</tr>
<tr>
<td>No</td>
<td>10 (25%)</td>
</tr>
<tr>
<td>Insulin treatment* (times/day):</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10 (25%)</td>
</tr>
<tr>
<td>3</td>
<td>26 (65%)</td>
</tr>
<tr>
<td>4</td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>5</td>
<td>3 (7.5%)</td>
</tr>
</tbody>
</table>

*Doses of insulin ranges from 2 to 5

Table 2. Frequency of CTLA-4 genotypes in the studied groups:

<table>
<thead>
<tr>
<th>CTLA-4 Genotypes</th>
<th>Diabetic cases N=40</th>
<th>Controls N=20</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (%) Homogenous</td>
<td>6 (15%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>AG (%) Heterozygous</td>
<td>26 (65%)</td>
<td>6 (30%)</td>
<td>0.01</td>
</tr>
<tr>
<td>AA (%) Normal genotype</td>
<td>8 (20%)</td>
<td>14 (70%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

P >0.05 is not significant (NS).

Table 3. Frequency of Adenine and Guanine alleles in CTLA-4 gene of the studied groups:

<table>
<thead>
<tr>
<th>Allele</th>
<th>Diabetic cases</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (%)</td>
<td>42 (52.5)</td>
<td>34 (85%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>G (%)</td>
<td>38 (47.5)</td>
<td>6 (15%)</td>
<td></td>
</tr>
</tbody>
</table>

P <0.05 is significant.

There is a significant association between CTLA-4 genetic polymorphisms (AG and GG) with different age groups. (5-16y, 4-18y and 4.5-15y) (P=0.002) and diabetic complications (P=0.002); while there is insignificant statistical association between the three CTLA-4 genotypes (AA, AG, GG) and sex, weight, height or insulin treatment, Table(4) and figure(2).

There is a significant association between CTLA-4 genetic polymorphisms (AG and GG) and diabetic complications (diabetic ketoacidosis, diabetic coma, psychic troubles, others) (P=0.002)
Table 4. Distribution of different CTLA-4 genotypes among the diabetic studied cases in relation to their personal data.

<table>
<thead>
<tr>
<th>Personal data</th>
<th>AA(%) n=8</th>
<th>AG(%) n=26</th>
<th>GG(%) n=6</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range(Mean ± SD)</td>
<td>5-16 (9.4 ±3.9)</td>
<td>4-18 (13.2 ± 3.8)</td>
<td>4.5-15 (10.8 ± 4.3)</td>
<td>0.002*</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>5 (62.5%)</td>
<td>11 (42.3%)</td>
<td>4 (66.7%)</td>
<td>NS**</td>
</tr>
<tr>
<td>Female (%)</td>
<td>3 (37.5%)</td>
<td>15 (57.7%)</td>
<td>2 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range(Mean ± SD)</td>
<td>19-56 (36.3 ± 12.2)</td>
<td>18-79 (42.2 ± 14.9)</td>
<td>18-50 (35.8 ± 12.6)</td>
<td>NS*</td>
</tr>
<tr>
<td>Height (cm):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (Mean ± SD)</td>
<td>107-154 (135.9 ± 16.3)</td>
<td>105-181 (145.1 ± 18.2)</td>
<td>104-156 (135.7 ± 18.3)</td>
<td>NS*</td>
</tr>
<tr>
<td>Complications:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2 (25%)</td>
<td>22 (84.6%)</td>
<td>6 (100%)</td>
<td>0.002**</td>
</tr>
<tr>
<td>No</td>
<td>6 (75%)</td>
<td>4 (15.4%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>Insulin treatment (times/day):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3 (37.5%)</td>
<td>6 (23.1%)</td>
<td>1 (16.7%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4 (50%)</td>
<td>18 (69.2%)</td>
<td>4 (66.7%)</td>
<td>NS**</td>
</tr>
<tr>
<td>≥4</td>
<td>1 (12.5%)</td>
<td>2 (7.7%)</td>
<td>1 (16.7%)</td>
<td></td>
</tr>
</tbody>
</table>

*= calculated by ANOVA test. **= Fisher exact test (FET). P > 0.05 is not significant (NS).

Figure 4. Percent Complication in diabetic cases with different CTLA-4 genotypes.
Detection of CTLA-4 Gene Polymorphism in T1DM

Discussion

Type 1 diabetes mellitus is one of the most common childhood illnesses. The incidence of T1DM has been increasing at an average rate of about 3% per year in almost all countries (Karvonen et al., 2000; Devendra et al., 2004).

In Egypt, T1DM is the most common endocrinial metabolic disease in childhood and its prevalence is varying between 0.7 and 1.9 in children aged from 10-18 years in Egypt (Azza et al., 2013). Susceptibility to T1DM is determined by complex interactions between several genetic loci and environmental factors. Alleles at the HLA locus on chromosome number 6 accounts for 50% of the familial clustering of T1DM through a large variety of protective and predisposing haplotypes. The remainder is contributed to multiple loci such as CTLA-4 on long arm (q) of chromosome number 2 at position 33(2q33) (Haller et al., 2007).

At present, the development of type 1 diabetes mellitus is a life sentence to a difficult therapeutic regimen that is only partially effective in preventing acute and chronic complications of the disease. Knowledge of the genetics of type 1 diabetes mellitus in our community would allow better disease definition and improved ability to identify individuals at risk of diabetes and its associated disorders (Azza et al., 2013). The association of CTLA-4 gene polymorphism with T1DM was first reported by Nistico’ et al. (1996).

We investigated the +49A/G polymorphism because it has been the most widely analyzed CTLA-4 variant in T1D patients from several ethnic populations. In addition, it is known to cause an amino acid change (threonine to alanine) and so it is associated with altered protein expression and T-cell activation. Our results support the involvement of CTLA-4 gene polymorphism in the pathogenesis of T1D. This is in agreement with Marron et al., 1997) who have been reported the positive associations for the +49A/G polymorphism in populations including Spanish, French, Korean, Italian, Mexican and American and Saleh et al., 2008; Mosaad et al., 2012 in Egyptian. On the other hand, no association has been reported in many populations including Chilean (Angel et al., 2009), Chinese and British (Marrom et al., 1997), Japanese (Awata et al., 1998).

Azza et al., (2013) claimed that there is Lack of association of CTLA-4 +49 A/G polymorphism with predisposition to T1DM in a cohort of Egyptian families.

The differences in CTLA-4 (49 A/G) genetic susceptibility to T1DM between different populations and sometimes in the same population as in Japanese & Egypt may be due to ethnic differences, difference in the number of subjects in each study or the nature of disease such as age of patients or age of onset of disease (Saleh et al., 2008).

Also those contradictory results can be explained by the genetic heterogeneity among the studied populations, the different environmental factors (as dietary factors and toxic chemical exposure) involved in the pathogenesis of T1DM, the limitations of the studied cases or other methodological issues (Lemos et al., 2009).

In this study, subjects with homozygous GG genotype were younger in age and presented with T1DM at an earlier age. This result coincides with previous results of Takara et al., 2000 and Zalloua et al., 2004. One possible explanation is that CTLA-4 exerts less profound inhibitory effects on proliferation of T cells carrying G49/G49 genotype rather than for cells carrying A49/A49 genotype, leading to appearance of T1DM at earlier age than other polymorphisms (Kouki et al., 2000).

In the present study, frequency of CTLA-4 AG genotype was higher in female (57.7%) when compared to male patients (42.3%) but
the difference was statistically insignificant \((P=0.46)\).

The specific mechanism whereby the +49A/G polymorphism of the CTLA-4 gene promote autoimmunity in females and lead to the development of T1DM is unknown. Steroid hormones are considered the most common factor that triggers the onset of female autoimmune diseases. Sex hormones may act as critical modulatory factors that can induce disease expression. These modifiers could directly or indirectly target steroid receptors that act as transcription factors for the susceptibility genes associated with T1DM (Whitacre et al., 2001).

In the present study diabetic complications (diabetic ketoacidosis (DKA), coma, psychic troubles and others) were found in 30(75%) out of 40 cases; In Mosaad et al., 2012 study 95% of their T1DM patients were free from diabetic complications while in Fatih et al., 2013 study DKA presented in 47.3% of their T1DM cases. Because CTLA-4 is involved in the regulation of T cell function, it was reported that CTLA-4 not only confers genetic susceptibility to T1DM but also has an influence on clinical features of T1DM (Pankewycz et al., 1995).

Abe et al., 1999 reported that 49A/G polymorphism was associated with presence of DKA in their studied patients. Balic et al., 2009 reported that 49A/G polymorphism could confer a genetic risk for T1DM, particularly with G allele in younger individuals, their data also suggested that the association of CTLA-4 with T1DM is more striking in patients carrying the G allele of +49A/G polymorphism, with higher episodes of ketoacidosis and higher glycemic levels at diagnosis. This coincides with the results of the present study where the diabetic complications constitutes 84% of AG heterozygous genotype and 100% in GG homozygous genotype. However these findings disagree with Lemos et al., (2009) study in Portuguese & Fatih et al., (2013) study in Turkey; they found no association between CTLA-4 (+49A/G) polymorphism and the clinical characters of patients with T1DM.

In this study the distribution of the heterozygous AG genotype is differed significantly between T1DM patients and controls \((P<0.01)\).while for homozygous GG genotype there is insignificant association between the patients and controls \((P=0.07)\), this may be due to the limited number of cases in the present study.

In this study, the frequency of G allele was higher with high significant value in T1DM patients than in controls \((P<0.001)\). This result is in agreement with those of Mojtahedi et al., 2005 in Iran, Bennmansour et al., (2010) in Tunisia & Wafai et al., 2011 in Lebanon, where a higher frequency of the G allele in T1DM in children was reported.

In conclusions, G allele was significantly increased in diabetic cases than in control group. There was a strong association between AG polymorphism and T1DM \((P=0.002)\). Both AG and GG polymorphisms were significantly associated with complications of diabetes with ratio 84.6% and 100% respectively. The present study reinforces the need for more large scale researches on the relationship between CTLA-4 gene polymorphism and various potential risk factors of T1DM and other autoimmune diseases. Future studies should be conducted on larger sample size and on study populations obtained from different geographic regions.

References


polymorphism with type 1 diabetes mellitus in Egyptian children. Immunol Invest 41:28–37