Evaluation of CTLA-4 Gene polymorphism SNP 49 G/ A Association with Diabetes Mellitus Type 1 in Egyptian Population

Ali Salim Al-shehmany1, Ahmad A. El- Kafoury2, Medhat A. Haroun1, Amira M. Embaby1

1Department of Biotechnology, IGSR, Alexandria University
2 Department of Pediatric, Faculty of Medicine, Alexandria University

Abstract

The (CTLA-4) encodes of the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis. The contribution of CTLA-4 gene variants to type 1 diabetes has been analyzed in several ethnic groups. In this study, the association of CTLA-4 +49 A/G polymorphism with type 1 diabetes was investigated in Egyptian patients. Sixty type 1 diabetic patients (25 males and 35 females) and 60 healthy individuals (33 males and 27 females) subjects formed the studied populations. CTLA-4 A/G polymorphism at position 49 in exon 1 was identified using allele specific methods. Patient numbers with A/G, A/A and G/G genotypes were 45 (75.0 %), 6 (10.0 %) and 9 (15.0 %) while in healthy controls, these were 48 (80.0 %), 2 (3.3 %) and 10 (16.7 %), respectively. In conclusion, the results of this study showed that CTLA-4 +49 A/G polymorphism were not associated with susceptibility to type 1 diabetes in Egyptian population.

Keywords: Type 1 diabetes; CTLA-4 polymorphism; susceptibility

تقيم ارتباط التباین الجیني للجین CTTLA-4+49 A/G مع مرض السکری من النوع الأول في المجتمع المصري

علي الشحمانی 1, احمد الكافوري 2, مدبحة هارون 1, أميرة محمد 1
1 قسم التکنولوژیا الحیویة معهد الدراسات الغلیة والبحوث, جامعة الاسکندریة, مصر
2 زمیل بقسم الاطفال, كلیة الطب, جامعة الاسکندریة, مصر

الخلاصة:

يشغل CTTLA-4 تحالفاً والارتباط في السيطرة والتنظيم التکاثر للخلايا التائية. أيضاً التباین CTTLA-4 لمرض السکری من النوع الأول في العديد من المجموعات العریقة. تم تحری في هذه الدراسة عن ارتباط CTTLA-4 A/G +49 G مع مرض السکری من النوع الأول في المجتمع المصري. تم فحص 60 عینة من المصابین بداء السکری من النوع الأول (25 ذكور و35 نانث) مقابل مجموعة ضابطة المتكونة من 60 عيّنة (33 ذكر و 27 أنثى). وقد تم تحديد التباین الجیني CTTLA-4 بواسطة طريقة allele specific باستخدام عينة من المجموعات السکری من النوع الأول. وكانت توزیع عدد المصابین بالنسبة للتربیک CTTLA-4 G/G (75.0 %), 6 (10.0 %), A/A, A/G و G/G (15.0 %) و G/A (10.0 %). بينما توزیع مجموعة الإصابة بالنسبة للتربیک G/G كان كالالي (3.3 %) و A/A, A/G و G/G (10.0 %) على (80.0 %), (20.0 %) و (16.7 %) على (10.0 %) و (15.0 %) على

*Email: alisalim883@yahoo.com
Introduction

Type 1 DM results from β-cell destruction, usually leading to absolute insulin deficiency. Testing for islet-cell antibodies (ICA) or other autoantibodies (antibodies to glutamic acid decarboxylase [anti-GAD], insulin, and to the tyrosine phosphatase IA-2) in serum may be helpful if establishing the diagnosis is important; a positive result is indicative of immune-mediated or type 1 diabetes [1]. Genetic susceptibility to T1D is conferred by more than 20 putative loci [2-4]. The cytotoxic T lymphocyte-associated antigen 4 gene (CTLA-4) is considered one of the most important candidate genes for autoimmunity and it has been reported to be associated with several endocrine disorders including type 1 diabetes, Graves' disease and autoimmune hypothyroidism [5]. The CTLA-4 encodes the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis [6]. The human CTLA-4 gene was mapped to chromosome 2q33 [7]. Polymorphisms have been identified in the CTLA-4 gene and have been associated with different susceptibilities to a wide range of T-cell-mediated autoimmune disorders [8]. Most molecular epidemiology studies have evaluated the role of the +49A/G (rs#231775) single nucleotide polymorphism (SNP) that causes a threonine-to-alanine substitution in codon 17 and is associated with altered protein expression [9]. This polymorphism seems to be associated with the genetic susceptibility to type 1 diabetes in several populations, although conflicting data also exist in populations of different ethnic backgrounds [10].

The aim of this study was to assess the contribution of this CTLA4 (SNP 49 G / A rs#231775) polymorphism to the susceptibility to type 1 diabetes in the Egyptian children.

MATERIALS AND METHODS

Study populations

A total of 60 type 1 diabetic patients (25 males and 35 females) with an age mean ± SD 11.2±3.7, and 60 healthy individuals (33 males and 27 females) with an age mean ± SD 27.2± 6.4, family history (25 positive/ 35 negative to family history, disease onset (years) mean±SD 5.3±3.5, were enrolled in this study and recruited at the El-Shatby University Hospital, Faculty of Medicine Alexandria University. Egypt. Patients diagnosed according to WHO criteria [11]. Patients were diagnosed on the basis of classical clinical presentation, first-degree family history of diabetes, history of chronic diabetes complications, and treatment of diabetes. Healthy controls had no personal or first-degree history of diabetes and were free from T1DM. The Ethics Committees of participating universities and university hospitals approved the study, and informed consent was obtained from all participants.

Blood sampling (one ml of venous blood) was collected in EDTA tubes from each individual (patient or healthy control) and was stored as whole blood at -20°C for subsequent DNA isolation. Genomic DNA was isolated from whole blood according to Sambrook et al 1989 [12].

Genotyping of CTLA-4 gene polymorphism

One SNP (SNP 49 G/A rs#231775) in CTLA-4 gene was genotyped among the participants groups in this study. The CTLA-4 A/G polymorphic region (rs#231775) was amplified by polymerase chain reaction (PCR) using allele specific PCR technique as shown in Table 1. Four primers (two allele specific primers, forward control and common reverse primer) were designed based on the nucleotide sequence of a partial fragment (retrieved from the online dbSNP) of the gene containing the target SNP. The polymorphism was visualized by separating the DNA fragments in a 2% agarose gel that was stained with ethidium bromide and illuminated by UV. To validate the PCR- allele specific results to validate the PCR-allele specific results. All primers used in this study were newly designed using Primer Blast online programme (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
Table 1- Primers sequences, PCR conditions, length of PCR products.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Primers sequences</th>
<th>PCR Conditions</th>
<th>Size of PCR Products digestion products</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>A-allele specific primer: F1: 5-GCTCAGCTGACCTGGCTA</td>
<td>An initial denaturation at 95°C for 5 min -Then, 30 cycles each cycle consisted of denaturation at 94°C for 60s, annealing at 50°C for 30s and extension at 72°C for 30 s. -A final extension at 72°C for 10 min.</td>
<td>Allele A: 189 bp Allele G: 189 bp Control fragment: 390 bp</td>
</tr>
<tr>
<td>SNP49</td>
<td>G-allele specific primer: F2: 5-GCTCAGCTGACCTGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/A</td>
<td>Forward control primer: 5-GCTTTCTATTCAGTGCC TTCT-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs#231775)**</td>
<td>Common reverse primer: 5-CTTTAACTTCTGGCTTTGCTATA-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis of data

Statistical analysis of data was done to correlate genotype distribution and allele frequencies were performed by SPSS package version 11. The frequencies of alleles, genotypes in different groups were compared using the Chi-squared test ($X^2$), $t$-test and Mann Whitney test were used to test the significance of results of quantitative variables. Odds ratio and 95% confidence interval (95% CIs) were calculated for different studied parameters. The confidence interval (CI) at 95% was used to describe the amount of uncertainty associated with the samples [13, 14]. A 95% confidence level means that 95% of the intervals would include the parameter. The significance of the results was taken at the $P<0.05$ level of significance.

Results And Discussion

This study demonstrates that $CTLA-4$ gene polymorphisms are associated with a risk of T1DM in Egyptian population. The Results revealed that the allele and genotypic distributions did not significantly differ between the two groups ($P>0.05$) for the SNP 49 G/A (rs#231775) as shown in table 2.

Table 2- $CTLA-4$ gene polymorphism and allele frequencies among diabetic patients and control

<table>
<thead>
<tr>
<th>Gene polymorphism</th>
<th>Cases No. =60</th>
<th>Control No. =60</th>
<th>Significance</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>49 G/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>6</td>
<td>10.0</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>GG</td>
<td>9</td>
<td>15.0</td>
<td>10</td>
<td>16.7</td>
</tr>
<tr>
<td>AG</td>
<td>45</td>
<td>75.0</td>
<td>48</td>
<td>80.0</td>
</tr>
</tbody>
</table>

allele frequencies

<table>
<thead>
<tr>
<th></th>
<th>Cases No.</th>
<th>Control No.</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>60 0.18</td>
<td>60 0.12</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.82</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

$X^2$: Chi-Square test
Figure 1 Shows 2% agarose gel electrophoresis for allele specific PCR for CTLA-4 SNP 49 G>A (rs#231775). M: 100 bp DNA ladder from GeneDireX®. Lane1: PCR product upon using controls forward primer, Lanes 2 and 3: PCR products upon using allele specific G primer and allele specific A primer, respectively. Heterozygous genotype will give positive reaction upon using both allele specific primers. However, homozygous genotype will give positive reaction upon using only one of these allele specific primers.

Our results agree with Mosaad, et al. [15]. Proved that CTLA-4 +49 GG homozygous genotype is associated with T1D in Egyptian children especially with younger age of onset and in younger patients, and not associated with grades of diabetic control or diabetic complication.

Kamel, et al. [16]. Proved that no significant differences were encountered between the different groups with regard to CTLA4 +49 A/G genotype or allele frequencies. Neither was there a relation between the various genotypes and age of onset or the mode of presentation.

Saleh, et al. [17]. Found that an association of the C-819T and A+49G SNPs in the CTLA-4 gene with T1D patients ($P=0.0047$) and ($P=0.000575$), respectively. Moreover, this association was stratified by gender and age to female patients with age at onset 0-5 years old ($P=0.0186$) and ($P=0.00115$) more than male patient with the age at onset 0-5 years old ($P=0.3120$) and ($P=0.345161$), respectively.

Mochizuki, et al. [18]. In Type 1 Diabetes and Autoimmune Thyroid Disease in Japanese Children have demonstrated that a distinct association exists between the G allele of CTLA-4 and high values of GAD Ab, residual β-cell function, and the absence of HLA-DRB1*0405.

Present results disagree with Chistiakov, et al. [19]. In Russian population they found that the CTLA-4 gene SNP 49 G/A is strongly associated with IDDM in a Russian population. While Lemos, M. C. et al. [20]. Investigated that no significant differences were observed, suggesting that this polymorphism is not strongly associated with T1DM in the Portuguese population.

Yanagawa, et al. [21]. Investigated the distribution of a CTLA-4 gene SNP G>A polymorphism in 110 Japanese patients with IDDM and 200 control subjects. In 84 patients, they also investigated associations between this CTLA-4 gene polymorphism and GAD65 antibody positivity. An A/G transition at position 49 of exon 1, there was no significant difference in the distribution of CTLA-4 alleles in patients and controls and no difference was observed in prevalence of CTLA-4 alleles when GAD65 antibody-positive and -negative individuals in the IDDM groups were compared. This study did not support an association between the CTLA-4 gene and IDDM in the Japanese population.

Ma, et al. [22]. In Han Chinese proved that CTLA-4 49 AA is protective from diabetes mellitus, whereas, CTLA-4 49 G allele (both as homozygotes and as heterozygotes) confers an increased risk of diabetes mellitus. In the same population Lee, et al. [23]. Noted that CTLA4 49 A-G polymorphism is associated with T1DM in Han Chinese children. The CTLA4 49 G allele confers an increased risk of type 1
diabetes. Mojtabahedi, et al. [24]. Showed that CTLA-4 +49 A/G polymorphism confers genetic susceptibility to T1DM, particularly in younger individuals in Iranians population.

Present findings provide no significant effect on the susceptibility to T1DM among the studied group. The results do not support the involvement of the CTLA4 gene in the pathogenesis of type 1 diabetes in our population. The difference in the association of the aforementioned above studies variants with T1DM among different populations may be attributed to the presence of multiple susceptibility alleles at the aforementioned genes variants, the genetic heterogeneity among the populations studied, different interactions with environmental factors involved in the pathogenesis of type 1 diabetes, racial/ethnic differences in the distribution of these variants and multiple hypothesis testing. Furthermore, the CTLA4+ 49A/G SNP may not be the true disease-associated variant, but rather a marker in linkage disequilibrium with the causal variant, and the discrepant findings may reflect variable strengths of linkage disequilibrium in different populations. In conclusion, our case-control study suggests that the + 49A/G SNP of the CTLA4 gene is not strongly associated with type 1 diabetes mellitus in the Egyptian population.

Acknowledgement
First and foremost we would like to thank Allah the beneficent and the merciful, Who inspired with strength and patience on the alienation and facilitate all things and complications. We gratefully acknowledge and big appreciation and gratitude to Dr. Zafir Hassan Ghali, Head of Department of Biology, Faculty of Education, Iraq For providing scientific advice and Dr. Ali. H. Shuua. Al.taie, Head of Department of Mathematics, Faculty of Computer and Mathematics, Iraq and Fadhl Abdali, Department of Accounting, Faculty of Economics and Administration, Iraq and my friend Eng. Muntazar Jabar to help me in statistical analysis.

References


