The codon 17 polymorphism of the $\text{CTLA4}$ gene in type 1 diabetes mellitus in the Baghdad population

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The aim of this work was to study the polymorphism in $\text{CTLA4}$ gene in insulin-dependent diabetes mellitus (IDDM) type 1 patients in Baghdad population. To achieve this goal, blood samples were collected from 80 IDDM (40 males and 40 females) and 20 samples of healthy, DNA was isolated and the $\text{CTLA4}$ gene (A 152 bp fragment) were amplified by using specific primers for exon1 of this gene, and then found the sequence of this region. The DNA sequencing results of flank sense of $\text{CTLA4}$ gene from healthy patients was found to be compatible (100%) with wild type of $\text{Homo sapiens}$ from the Gene Bank, while 99% compatibility were found for the gene from 70 IDDM cases patients with wild type of gene. The difference may be attributed to one transition mutations, A/G at position 49 of the $\text{CTLA4}$ gene (from AGC to AAC). It is a missense mutation that leads to changes in amino acid from serine (S) to asparagine (N). Our results showed that the incidence of A/G mutation at nucleotide position 49 and diabetes was highly significant ($X^2 = 100$, $P < 0.01$). In total, 12% of patients with IDDM (10 cases) had two transition mutation +49 A/G and +47 C/T single nucleotide polymorphism from total cases, 98% compatibility were found for that gene from 10 IDDM cases patients with wild type of gene. The +47 C/T SNP was silent mutation which resulted in change of codon from GGT to GGC but no changes translated to amino acid (glycine to glycine). However, there was no significant correlation between diabetes and incidence of C/T at nucleotide 47 ($X^2 = 0.055$, $P > 0.05$). In conclusion, our case study suggests that the +49 A/G SNP of the $\text{CTLA4}$ gene is strongly associated with genetic susceptibility to type 1 diabetes mellitus in the Baghdad/Iraqi population.

Key words: $\text{CTLA4}$ gene, insulin-dependent diabetes mellitus, A/G polymorphism.

INTRODUCTION

Cytotoxic T-lymphocyte antigen 4 ($\text{CTLA4}$) also known as cluster of differentiation 152 (CD152) is a protein that plays an important role in the immune system regulations. $\text{CTLA4}$ is a member of the immunoglobulin superfamily, which is expressed on the surface of Helper T cells and transmits an inhibitory signal to T cells. The $\text{CTLA4}$ encodes the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis (Yanagawa et al., 1997; Larsen et al., 1999). The receptor protein is a specific T lymphocyte surface antigen that is detected on cells only after antigen presentation. Thus, $\text{CTLA4}$ is directly involved in both immune and autoimmune responses and may be involved in the pathogenesis of multiple T cell-mediated autoimmune disorders. The human $\text{CTLA4}$ gene is located at chromosome 2q33 (Nistico et al., 1996; Donner et al., 1997a). This gene is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. The protein contains a V domain of 116 amino acids, a
transmembrane domain, and a cytoplasmic tail. Alternate transcriptional splice variants, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer which is interconnected by a disulfide bond, while the soluble isoform functions as a monomer (Kristiansen et al., 2000).

An A-to-G substitution at nucleotide 49 in exon 1 results in an amino acid substitution (Thr/Ala) in the leader peptide of the protein (Donner et al., 1997b). The Ala allele has been shown to predispose the individual carrying it to the development of various immune diseases including insulin-dependent diabetes mellitus, Graves disease, Hashimoto thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, and other autoimmune diseases (Anjos and Polychronakos, 2004).

Mutations and polymorphisms in this gene results in alteration of the CTLA4 activity and are believed to play an important role in the risk of developing autoimmunity (Anjos and Polychronakos, 2004). The CTLA4 (49+) GG homozygous genotype is associated with Type 1 diabetes in Egyptian children especially with younger age of onset and in younger patients and not associated with grades of diabetic control or diabetic complication (Hatem et al., 2008; Mosaad et al., 2012). The aim of this study was to assess the contribution of this CTLA4 polymorphism to the susceptibility to type 1 diabetes in the Baghdad population.

MATERIALS AND METHODS

Samples and DNA extraction

Whole blood samples were obtained from 80 Baghdad patients affected by insulin-dependent diabetes mellitus (IDDM) (40 males and 40 females, age ranged from 4 to 25 years). Samples from 20 healthy individuals were used as a control group. In total, 4 ml whole blood was collected into an Ethylenediaminetetraacetic acid (EDTA) tube. The samples were stored at -20°C until further processing. DNA was extracted by DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer’s protocol.

Amplification of exon 1 of CTLA4 gene

A 152 bp fragment containing the +49 A/G polymorphism in exon 1 of CTLA4 was amplified using a forward primer (CTLA4: 5'-AAGGCTCAGCCTAATGCCT-3') and a reverse primer (CTLA4: 5'-CTGCTGAACAAATGAAACC-3') (Alpha DNA Company, Canada) (Marron et al., 1997). The polymerase chain reaction (PCR) amplification was performed in a total volume of 25 µl containing 5 µl DNA, 12.5 µl Go Taq green master mix 2X (Promega corporation, USA), 1 µl of each primer (50 pmol). The thermal cycling conditions were as follows: Denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 1 min with final incubation at 72°C for 7 min using a thermal Cycler (Gene Amp. PCR system 9700; Applied Biosystem) (Genc et al., 2004; Hatem et al., 2008). The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet light (302 nm).

Sequencing and sequence alignment

Sequencing of exon 1 of CTLA4 gene was performed by Macro gen company, USA. Homology search was conducted using Basic local alignment search tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit program. The results were compared with data obtained from Gene Bank published ExPASY program which is available at the NCBI online.

Statistical analysis

The statistical analysis is a very important final step in the research to analyses and evaluates the obtained results. Medical statistics of this study was conducted via computer based statistical program which was: X² for Windows computer package. The statistical analysis tests used in this were as follows: P value < 0.01 is considered a significant correlation.

RESULTS AND DISCUSSION

CTLA4 gene was successfully amplified using specific PCR primers for exon 1. Figure 1 showed PCR amplification of exon 1 of the CTLA4 where a specific product at 152 bp was observed. Our result is in agreement with other studies (Hatem et al., 2008; Waterhouse et al., 1995). Sequencing of this gene was performed to detect variant +49A/G which related to development of diabetes. Sequences alignment using BLAST and BioEdit showed the 100% similarity or homology of healthy sample with wild type of the CTLA4 gene of H. sapiens from the Gene Bank (Figure 2). The CTLA4 gene from 70 diabetes patients shows 99% compatibility with the wild type sequences of CTLA4 gene from Gene Bank as shown in Figure 3A, there are one transition at position +49 A/G single nucleotide polymorphism that cause a serine to asparagine substitution in codon 17, there is a high significance between diabetes and incidence of +49 A/G position in exon 1 of CTLA4 gene (X² = 100, P > 0.01), Table 1 shows the type of mutation and the effect of these mutations and Table 2 shows the translation of CTLA4 gene of all groups (healthy and patient) to a protein sequence, and two transition mutation at position +47 C/T and +49 A/G of CTLA4 gene from 10 diabetes patients was identified.

The sequence shows 98% compatibility with wild type CTLA4 gene as shown in Figure 3B, single nucleotide polymorphism at position 47+ C/T that silent mutation, no change translate amino acid (Glycine to Glycine), there is lower significant correlation between type 1 diabetes and incidence of this SNP, (X² = 0.055, P > 0.05). Most molecular epidemiology studies have evaluated the role of the +49A/G single nucleotide polymorphism that causes a threonine to alanine substitution in codon 17 and associated with altered protein expression (Anjos et al., 2002) and T-cell activation (Maurer et al., 2002). Gribben et al. (1995) have suggested that this may be through antigen specific induction of the apoptotic pathway. The mentioned study investigated the A49G polymorphism in
Homo sapiens chromosome 2 genomic contig, features in this part of subject sequence:

**cytotoxic T-lymphocyte protein 4**

Score = 150 bits (81), Expect = 2e-34, Identities = 81/81 (100%), Gaps = 0/81 (0%)

```plaintext
Query 16 CTTTGCAGAACAGGGATGAAGAGAAAGAAAAACAGGAGAGTGCAGGGCCAGGTCTCGG
Sbjct 264 CTTTGCAGACAGGGATGAAGAGAAAGAAAAACAGGAGAGTGCAGGGCCAGGTCTCGG
```

```plaintext
Query 76 TAGCCAGGTTCAGCTGAGCCT
Sbjct 204 TAGCCAGGTTCAGCTGAGCCT
```

**Figure 1.** Agarose gel electrophoresis for detection of amplified **CTLA4** gene. Bands were fractionated by electrophoresis on a 1.5% agarose gel (2 h., 5 V/cm, 1× Tris-acetic buffer) and visualized under UV light after staining with ethidium bromide.

Lane: 12 (M: 100 bp ladder); Lane: N (negative control); Lane: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (PCR product).

**Figure 2.** Sequencing of sense flanking the partial **CTLA4** gene for healthy as compared with wild type **CTLA4** obtained from Gene Bank.

exon 1 of **CTLA4** gene in 40 Lebanese and 46 controls from the same ethnic background.

An increase in the frequency of the G allele was discovered in patients when compared to control subjects, this difference was statistically significant, despite the small sample size. Wafai et al. (2011) showed an association of **CTLA4** with type 1 diabetes in Lebanese population. An association was detected between the **CTLA4** gene polymorphism and younger-onset type 1 diabetes with autoimmune thyroid disease (AITD) (Gough, et al., 2005). The G variant was suggested to be genetically linked to AITD-associated type 1 diabetes of younger onset in this Japanese population (Mochizuk, et al., 2003). The defect in these patients presumably lies in a T-cell mediated autoimmune mechanism (Takara et al., 2000). Chistiaakov et al. (2001) reported that the **CTLA4** gene is strongly associated with insulin-dependent diabetes mellitus (IDDM) in a fifty-six families each consisting of two siblings (one affected with IDDM diagnosed before the age of 18 years and one non-diabetic sibling).

It was reported that the **CTLA4** 49 (A/G) mutation conferred a risk of type 1 diabetes in the Chinese children but not in the West African children. On the other hand, the novel **CTLA4** 159 (C/G) mutation conferred a risk of type 1 diabetes in the West African children but not in the Chinese type 1 diabetic children (Hyiaman et al., 2001). Donner et al. (1997b) showed that an alanine at codon 17 of **CTLA4** is associated with genetic susceptibility to Graves disease as well as to IDDM. Lemos et al. (2009) states that the **CTLA4** +49 A/G polymorphism is not associated with susceptibility to type 1 diabetes mellitus in the Portuguese population. This contrasts with positive associations that have been reported for the +49A/G polymorphism in case control studies in populations from Belgium, Germany, Poland, France, Japan, China, Italy, the Philippines, Lebanon, Estonia and Iran (Zalloua et al., 2004; Kavvoura and Ioannidis, 2005; Mojtabehdi et al., 2005). However, lack of association for the +49A/G polymorphism has also been reported in populations from the USA, Japan, Ghana, UK, France, Czech Republic, Morocco, Argentina, Brazil and Azerbijan (Marron et al., 1997; Caputo et al., 2005; Hauache et al., 2005; Kavvoura...
A: Sense of the partial CTLA4 gene, shown one transition mutation.

Score = 172 bits (93), Expect = 4e-41, Identities = 95/96 (99%), Gaps = 0/96 (0%)

Query 1
AAAAGTCTCCTACACTTTTGCAGAAGACAGGGATGAAGAGAAGAAAAAACAGGAGAGTGC

Sbjct 54942207
AAAAGTCTCCTACACTTTTGCAGAAGACAGGGATGAAGAGAAGAAAAAACAGGAGAGTGC

Query 61
AGGGCCAGGTCCTGGAACCAGGTTACGCTGAGCCT

Sbjct 54942147
AGGGCCAGGTCCTGGAACCAGGTTACGCTGAGCCT

B: Sense of the partial CTLA4 gene, shown two transition mutation.

Homo sapiens chromosome 2 genomic contig, features in this part of subject sequence:
cytotoxic T-lymphocyte protein 4

Score = 165 bits (89), Expect = 7e-39, Identities = 93/95 (98%), Gaps = 0/95 (0%)

Query 1
AAAAGTCTCCTACACTTTTGCAGAAGACAGGGATGAAGAGAAGAAAAAACAGGAGAGTGCA

Sbjct 54942206
AAAAGTCTCCTACACTTTTGCAGAAGACAGGGATGAAGAGAAGAAAAAACAGGAGAGTGCA

Query 61
GGGGCCAGGTCCTGGTAGCCAGGTTCAGCTGAGCCT

Sbjct 54942146
GGGGCCAGGTCCTGGTAGCCAGGTTCAGCTGAGCCT

Figure 3. Sequencing of sense flanking the CTLA4 gene for diabetes as compared with wild type CTLA4 obtained from Gene Bank. (A: 70 diabetes patients have one mutation; B: 10 diabetes patients have two mutation)

Table 1. Types of mutations detected in partial CTLA4 gene of diabetes patients.

<table>
<thead>
<tr>
<th>No.</th>
<th>Location of gene bank</th>
<th>Nucleotide change</th>
<th>No. of sample</th>
<th>Amino acid change</th>
<th>Predicted effect</th>
<th>Type of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/G 49+</td>
<td>AGC &gt; AAC</td>
<td>70</td>
<td>Serine (S)/Asparagine (N)</td>
<td>Missense</td>
<td>Transition Single nucleotide polymorphism</td>
</tr>
<tr>
<td>2</td>
<td>C/T 47+</td>
<td>GGT &gt; GGC</td>
<td>10</td>
<td>Glycine (G) / Glycine (G)</td>
<td>Silent</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Amino acid sequences of healthy and patient group.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequencing of amino acid</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KVSLTFEDREKKQQESAGPGPGSQQVQLSLK</td>
<td>20 healthy</td>
</tr>
<tr>
<td>2</td>
<td>KVSLTFEDREKKQQESAGPGPGNQVQLSLK</td>
<td>70 Patient</td>
</tr>
<tr>
<td>3</td>
<td>KVSLTFEDREKKQQESAGPGPGNQVQLSLK</td>
<td>10 patient</td>
</tr>
</tbody>
</table>

K: lysine; V: Valine; S: Serine; L: Lysine; T: Threonine; F: Phenylalanine; A: Alanine; E: Glutamic acid; D: Asparagine; R: Arginine; Q: Glutamine; G: Glycine; P: Proline.

and Ioannidis, 2005; Ahmedov et al., 2006).

Conclusion

Our study showed that there was significant correlation between diabetes and incidence of A/G +49 position in exon 1 of CTLA4 gene, despite the limited size of our sample, our results together with population studies show an association of CTLA4 with type 1 diabetes mellitus, on the other hand, the novel of +47 C/T silent mutation was no significant correlation between type 1 diabetes in
Baghdad population.

REFERENCES


