**TGF-β1 Gene Polymorphism in Codon 10 +869*C/T and Codon 25 +915*G/C Positions in Iraqi Patients with Type 2 Diabetes Mellitus**

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Abstract  
This work aimed to explore association of TGF-β1 gene polymorphisms in codons 10 and 25 with Type 2 Diabetes Mellitus (T2DM) among Iraqis. Blood samples were collected from 50 patients with T2DM and 30 healthy individuals as control samples. The polymorphism results of TGF-β1 gene in codon 10 +869*C/T position by using amplification refractory mutation system (ARMS-PCR) showed that the T allele was suggested to have a protective effect, while C allele was associated with an increased risk of T2DM. The TT and CT were suggested to have a protective effect, while C allele was associated with an increased risk of T2DM. The GC genotype was suggested to have a protective effect, while GG and CC genotypes were associated with an increased risk of T2DM.

**Keywords:** TGFβ1 gene, Type 2 Diabetes Mellitus, codon 10: +869*C/T, codon 25: +915*G/C

Introduction  
The type 2 diabetes mellitus (T2DM) is a common, chronic, complex disorder of rapidly growing global importance. It accounts for 95% of diabetes worldwide is characterized by concomitant defects in insulin secretion (from the β-cells in the pancreatic islets) and insulin action (in fat, muscle, liver, and elsewhere); the latter being typically associated with obesity [1]. The maximal capacity of cytokine production in individuals has a major genetic component [2]. A potential mechanism was described involving polymorphisms within the coding regions or signal sequences of cytokine genes. These genetic polymorphisms were shown to affect the overall expression and secretion of cytokines in vivo [3]. TGF-β1 is an anti-inflammatory immune mediator, which inhibits or reverses the activation of macrophages by interfering with their activation pathways [4]. The highly polymorphic human TGF-β1 gene is located on chromosome 19q 13.1–13.3 [5]. There have been some known TGF-β1 gene polymorphisms such as 2988C/A, 2800G/A, 2509C/T and +869T/C; however, previous studies concerning association between 2509C/T polymorphism and risk of T2DM are limited and their results are rather conflicting [6-8]. The polymorphisms at codons 10 and 25 may be associated with higher or lower TGF-β1 synthesis in vitro [9]. Increases or decreases in the production of TGF-β1 have been linked to numerous diseases including atherosclerosis, and fibrotic diseases of the kidney, liver, and lung [10]. This work aimed to explore association between T2DM and TGF-β1 gene polymorphisms at +869 T>C and +915 G>C positions.
Materials and Methods

Subjects
Thirty T2DM patients and 20 control (healthy) persons were enrolled in the study. All samples were collected from Abu-Graeeb and Al-Shaheed Saif Saad hospitals in Baghdad-Iraq. The disease was diagnosed international criteria, by the consultant medical staff at both hospitals. All cases and controls were in the age range 35-65 years, and they were from the same geographical and socioeconomic conditions. The peripheral blood was collected at the time of diagnosis in anticoagulants EDTA tubes for DNA isolation.

Genotyping
DNA from venous blood was isolated using ReliaPrep™ Blood gDNA Miniprep System kit (Promega) and used for the genetic analysis. The TGF-β1 gene at positions codon 10 +869*C/T and codon 25 +915*G/C were genotyped using the amplification refractory mutation system (ARMS-PCR) approach. Primers for these positions were used depending on [11,12], and synthesized in Alpha DNA company (Canada). Primer sequences of the TGF-β1 gene at position codon 10 +869*C/T was 5′- TCCGTGGGATACTGAGACAC-3′ as generic primer and 5′- AGCACGGTAGCAGCAGCA-3′ for specific T primer and 5′- GCAGCGTTAGCAGCCG-3′ for specific C primers. Primer sequences of the TGF-β1 gene at positions codon 25 +915*G/C was 5′- GGCTCCGTTCTGCAC-3′ as generic primer and 5′- GTGCTGACGCCTGGCCG-3′ for specific G primer and 5′- GTGCTGACGCCTGGCC-3′ for specific C primers. TGF-β1 gene forward internal control primer was 5′- GCCCTCCCCACCATTCCTTA-3′ and the reverse primer was 5′- TCACGAGATTCTGTTGTGTTTC-3′.

ARMS-PCR
ARMS-PCR approach was used for detecting polymorphisms at TGF-β1 gene positions. AccuPower® PCR PreMix (Bionner, Korea) was prepared depending on [11,12] with some modifications. Twenty microliter of PCR reaction was used: 12.5 µl master mix (1x), 1 µl of each primer (1 µM) and 2 µl of DNA template (100 ng). Total volume of reaction was completed with nuclease-Free water. The specific T and generic primers were used for detecting T allele and the specific C and generic primers were used for detecting C allele for the TGF-β1 gene at position codon 10 +869*C/T. The specific G and generic primers were used for detecting G allele and the specific C and generic primers were used for detecting C allele for the TGF-β1 gene position codon 25 +915*G/C. The reaction mixers were placed in Thermo cycler (Esco, Singapore). PCR conditions for the all reaction mixers was: template denature at 95°C for 1 min, first initial denaturation at 95°C for 15 sec, first annealing at 65°C for 15 sec and first extension at 72°C for 40 sec. The last three steps were 10 cycles. The second initial denaturation is at 95°C for 15 sec, second annealing at 56°C for 20 sec and second extension at 72°C for 50 sec. The last three steps were 20 cycles and the final extension at 72°C for 7 min. The PCR products were resolved on 2% agarose gels prepared in 1x TBE buffer (Bioner, Korea). The DNA ladder (2000 bp) with intervals 100 bp was also loaded on the agarose gel (Bioner, Korea). Three microliter of Bromophenol blue dye was loaded with the all reaction mixers. The gel electrophoresis was done by using 75V for 3-4 hrs in 1x Tris-borate buffer (TBE). The gel was stained with ethidium bromide (Promega, USA) for 20 min and documented with gel documentation system (Biocom, USA).

Statistical analysis
Data were given as percentage frequencies and significant differences between patients and controls were by Fisher’s exact test. The Odds ratio (OR) and Confidence Intervals (CI) were analyzed by using Compare 2 Ver.3.04 program designed by J. H. Abramson/2003-2013. Deviations from Hardy–Weinberg were tested using an exact test available at http://www.had2know.com/academics.

Results and Discussion
The polymorphism results of TGF-β1 gene in codon 10: +869*C/T position by using ARMS-PCR showed the presence of T and C alleles and three genotypes (TT, CT and CC) by using the specific T, specific C and generic primers. Presence of one band in T lane and absence this band in C lane refers to the genotype TT. In contrast, presence of one band in C lane and absence of this band in T lane refer to the genotype CC. Presence of two bands in both lanes refer to the genotype is CT Figure (1).
The frequency of T allele in control sample was higher in comparison with the same allele in patients (57.5% vs. 33.3%). In contrast, the C allele in patient sample (66.7%) was higher as compared with control sample (42.5%) Table (1). The odds ratio (OR) for the T and C alleles were 0.37 and 2.71, respectively; therefore the T allele was suggested to have a protective effect, while C allele was associated with an increased risk of T2DM Table (1). The frequencies of TT and CT genotypes in control sample were higher in comparison with the same genotypes in patients and the frequencies were 35% and 45% for the TT and CT genotypes, respectively in control and 23.3% and 20% for the TT and CT genotypes, respectively in patients. In contrast, the CC genotype in patient sample (56.7%) was higher as compared with control sample (20%). The odds ratio for the TT, CT and CC genotypes were 0.57, 0.31 and 5.23, respectively. The TT and CT were suggested to have a protective effect, while CC genotype was associated with an increased risk of T2DM.

The polymorphism results of TGF-β1 gene in codon 25: +915*G/C position in samples by using ARMS-PCR showed the presence of G and C alleles and three genotypes (GG, GC and CC) by using the specific G, specific C and generic primers. Presence one band in G lane and absence this band in C lane refers to the genotype GG. In contrast, presence one band in C lane and absence of this band in G lane refer to the genotype GG. Presence two bands in both lanes refer to the genotype GG Figure (2).

The frequency of G allele in control sample was higher in comparison with the same allele in patients (72.5% and 70%). In contrast, the C allele in patient sample (30%) was higher as compared with control sample.

Table (1): Allele frequencies of TGF-β1 codon 10 +869*C/T and Codon 25 +915*G/C genotypes in patient and control samples

<table>
<thead>
<tr>
<th>Gene position</th>
<th>Allele</th>
<th>T2DM No. (%)</th>
<th>Control No. (%)</th>
<th>OR(95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 codon 10 +869*C/T</td>
<td>T</td>
<td>20 (33.3)</td>
<td>23 (57.5)</td>
<td>0.37(0.16-0.84)</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>40 (66.7)</td>
<td>17 (42.5)</td>
<td>2.71(1.20-6.12)</td>
<td></td>
</tr>
<tr>
<td>TGF-β1 codon 25 +915*G/C</td>
<td>G</td>
<td>42 (70.0)</td>
<td>29 (72.5)</td>
<td>0.89(0.37-2.13)</td>
<td>0.485</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>18 (30.0)</td>
<td>11 (27.5)</td>
<td>1.13(0.47-2.72)</td>
<td></td>
</tr>
</tbody>
</table>

OR=Odds ratio, CI=Confidence Intervals, #=Significant differences at P<0.05 level by using Fisher’s test.

Table (2): Frequency distribution of the TGF-β1 codon 10 +869*C/T and codon 25 +915*G/C genotypes in patient and control samples

<table>
<thead>
<tr>
<th>Gene position</th>
<th>Genotype</th>
<th>T2DM No. (%)</th>
<th>Control No. (%)</th>
<th>OR(95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 gene codon 10 +869*C/T</td>
<td>TT</td>
<td>7 (23.3)</td>
<td>7 (35.0)</td>
<td>0.57(0.17-1.91)</td>
<td>0.280</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>6 (20.0)</td>
<td>9 (45.0)</td>
<td>0.31(0.09-1.04)</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>17 (56.7)</td>
<td>4 (20.0)</td>
<td>5.23(1.45-18.86)</td>
<td>0.010</td>
</tr>
<tr>
<td>TGF-β1 gene codon 25 +915*G/C</td>
<td>GG</td>
<td>19 (63.3)</td>
<td>12 (60.0)</td>
<td>1.5(0.37-3.59)</td>
<td>0.522</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>4 (13.4)</td>
<td>5 (25.0)</td>
<td>0.46(0.11-1.93)</td>
<td>0.247</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>7 (23.3)</td>
<td>3 (15.0)</td>
<td>1.72(0.04-7.40)</td>
<td>0.365</td>
</tr>
</tbody>
</table>

OR=Odds ratio, CI=Confidence Intervals, #=Significant differences at P<0.05 level by using Fisher’s test.
(27.5%) Table (1). The odds ratio (OR) for the G and C alleles were 0.89 and 1.13, respectively; therefore the G allele was suggested to have a protective effect, while C allele was associated with an increased risk of T2DM Table (1). The genotypes of TGF-β1 gene codon 25 +915*G/C position are showed in Table (2). The frequencies of GC genotype in control sample were higher in comparison with the same genotypes in patients and the frequencies were 25% and 13.4%, respectively. In contrast, the GG and CC genotypes in patient sample (63.3% and 23.3%, respectively) were higher as compared with control sample (60%) and (15%), respectively. The odds ratio for the GG, GC and CC genotypes are 1.5, 0.46 and 1.72, respectively. The GC genotype was suggested to have a protective effect, while GG and CC genotypes were associated with an increased risk of T2DM.

Fig. (2) Gel electrophoresis for the TGF-β1 gene codon 25 +915*G/C showing the G and C alleles in some T2DM patients
(Gel electrophoresis was done by using 1.5%agarose gel concentration, 75 Volt for 2 hours)

Type 2 diabetes (T2D) is the most common form of diabetes and an increasingly prevalent metabolic disease. It is associated with microvascular and macrovascular complications and is considered one of the major causes of morbidity and mortality [13]. There is mounting evidence that the ability of an individual to produce high or low levels of TGF-β1 may be genetically predetermined [10,14]. Polymorphisms at codons 10 and 25 have been linked with several diseases including autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, infectious, and fibrotic diseases [15]. Respectively, [16,7] studied TGF-β1 codon 10 polymorphism on Polish and Chinese populations. They reported different distribution of alleles, and genotypes compared with those in Egyptians. The differences between the two studies could be due to differences in the population genetic background. Because of the variation in cytokine allele frequency in different populations, it is not surprising that the results of this study of TGF-β1 genetic polymorphisms associated with T2D are conflicting in different ethnic groups. However, the small sample size is a limiting factor to have conclusive results, which suggested that TGF-β1 might play an important role in the etiology in T1D and T2D [17]. Association between diabetic control and change in genotypes of TGF-β1 codons 10 and 25 was performed to demonstrate if the TGF-β1 codons 10/25 genotypes had any relation in control of diabetes. In codon 10, most of T2D patients with TT genotype had moderate grade of control where the bad grade was associated with the TC. The same results were obtained for TGF-β1 codon 25 [18]. Also, [19] found same relationship between the gene and the disease and also, found significant differences in TT genotype frequencies of TGF-β1 gene codon 10, which related with the disease. In contrast, the same researchers found significant differences in GG genotype frequencies of TGF-β1 gene codon 25, which related with this disease. Role of the transforming growth factor-β1 (TGF-β1) gene polymorphisms located at codons 10 and 25 in the genetic predisposition to type 2 diabetes (T2D) and in diabetic nephropathy (DN) in Egyptian patients was investigated by [11]. They found that TGF-β1 (T869C) C allele, TC and TC+CC genotypes were significantly higher in patients; the T allele and TT genotype were significantly higher in controls (Pc<0.001). They also found non-significant differences were detected between T2D patients and controls in the frequencies of TGF-β1 (G915C) alleles and genotypes. The results of this study agreed with the other results especially in codon 10, which done by other researchers [7,11,20] and in codon 25, which done by [7,20] and disagreed with the study, which done by [11].
References