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TRANSFORMING GROWTH FACTOR-B1 T869C (CODON 10) GENE POLYMORPHISM IN TYPE 2 DIABETES IRAQI PATIENTS

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ABSTRACT

Background: Diabetes mellitus viewed as a wide spectrum of signs and symptoms which occur all in response to hyperglycemia that arises as a consequence to the disease pathology in which insulin secretion or action or both are defective. The fact that increased cellular resistance to insulin may be the causative factor to develop DM is considerable. The disease is associated with different types of complication that is attributed to morbidity and mortality. Transforming growth factor-β1 (TGF-β1), a pleiotropic cytokine, is a key player in immune regulation and plays an important role in the activation of inflammation and the resolution of inflammatory responses in a variety of autoimmune diseases and its levels was elevated in hyperglycemia. **Objective:** The aim of the presented study is to estimate the associations between transforming growth factor-beta1 (TGF-β1) gene polymorphism located in the chromosome 19q13.1–13.3 by (ARMS-PCR) with type 2 diabetes mellitus disease in patient of Kerbala population: Iraq. **Materials and Methods:** A case control study was performed on 200 male and female subjects, 100 of them with type 2 diabetes and 100 of them were apparently healthy as control group with matched are range between 45- 65 years at disease onset, and the mean duration of diabetes was 9.13±5.24 years. Blood samples were collected between Dec., 2017 to Oct., 2018. Gene polymorphism was genotyped by using tetra-primer ARMS–PCR procedure, HbA1c and fasting blood sugar was measured in patients and control groups. The odds ratio, t-test, and P-value at 95% confidence interval (CI) were measured, and the Hardy-Weinberg equilibrium was tested. **Results:** The frequencies of the alleles and genotypes for diabetic patient were as follows: 0.49 for TT, 0.44 for TC and 0.07 for CC, and 0.71 for TGF-β1 (T) and 0.29 TGF-β1 (C) and control: 0.69 for TT, 0.27 for TC and 0.04 for CC ,and 0.82 for TGF-β1(T) and 0.17 TGF-β1 (C). This preliminary study indicated that TGF-β1 T869C (codon 10) C allele, and C allele-containing genotypes may be susceptible, and the T allele / TT genotype may be protective factors for T2DM. In order to confirm the results obtained it would be advisable to conduct studies in a larger group of Iraqi population. **Conclusion:** Tetra primer-ARMS PCR technique which developed in our study an effective, robust assay and time saving for genotyping(T/C) of TGFβ1 gene, Our results were concluded TGFβ1T869C (codon 10) C allele, and C allele-containing genotypes could increase the risk of T2D,. In order to confirm the results obtained it would be advisable to conduct studies in a larger group of Iraqi population.

KEYWORDS: TGF-β1 gene, gene polymorphism, type 2 diabetes mellitus, ARMS-PCR.

INTRODUCTION

Diabetes mellitus viewed as a wide spectrum of signs and symptoms which occur all in response to hyperglycemia that arises as a consequence to the disease pathology in which insulin secretion or action or both are defective. The fact that increased cellular resistance to insulin may be the causative factor to develop DM is considerable. The disease is associated with different types of complication that is attributed to morbidity and mortality.[1] It is affected by multiple genetic and environmental factors. Extensive efforts have been made to identification of the disease-affecting genes to get better understanding of the disease pathogenesis, find new targets for clinical therapy and allow prediction of the disease.[2] These are the main cause of death in diabetic patients. Apart from the conventional risk factors such as obesity, dyslipidemia, and arterial hypertension, hyperglycemia are the independent risk factors for the development of (IHD), and for long-term leads to vascular damage through several mechanisms.[3,4] Also HDL-C level was low in all patients group but there are no significant differences and disagreement with other study which found that triglyceride and HDL-C are in highly significant, and the highest value of TG was in diabetic patients with CAD than in non-diabetic, while the level of HDL-C was low in patient groups.[5]

The maximal capacity of cytokine production in individuals has a major genetic component.[6] A potential mechanism was described involving polymorphisms within the coding regions or signal sequences of cytokine genes.[7] These genetic polymorphisms were shown to affect the overall expression and secretion of cytokines both in vitro and sporadically in vivo systems. Associations among polymorphisms in cytokine genes and inflammation, allograft rejection, autoimmune, and infectious diseases have been reported.[8] In diabetes mellitus, cytokine gene polymorphism may reflect or control the severity and progression of various immunological phenomena associated with the disease.[9]

Transforming growth factor-β1 (TGF-β1), a pleiotropic cytokine, is a key player in immune regulation.[10] TGF-β1 plays an important role in the activation of inflammation and the resolution of inflammatory responses in a variety of autoimmune diseases.[11] High glucose induces the increase of TGF-β1.[12] TGF-β1 also stimulates glucose uptake by enhancing the expression of glucose transporter 1 (GLUT1) in mesangial cells that leads to intracellular metabolic abnormalities in DM.[13] TGF-β1 regulates the production of almost every molecule of the extracellular matrix (ECM).[14] Glucose intolerance is the hallmark of DM. The central feature of DM is an alteration in the composition of the ECM, including thickening of the glomerular basement membrane (GBM) and expansion of the mesangial matrix.[15] In terms of abovementioned evidence, TGF-β1 expression may be associated with the risk of DM. Genetic polymorphisms were proven to affect the overall expression and secretion of cytokines.[16] For TGF-β1, the polymorphism at codon 10 has been reported to be associated with higher or lower TGF-β1 synthesis.[17] In this sense, TGF-β1 polymorphisms may be associated with the susceptibility of DM.

The aim of the presented study is to estimate the associations between transforming growth factor-beta1 (TGF-β1) gene polymorphism located in the chromosome 19q13.1–13.3 by (ARMS-PCR) with type 2 diabetes mellitus disease in patient of Kerbala population: Iraq.

MATERIALS AND METHODS

This study was conducted on 200 subjects,100 of them with T2DM and the other 100 were apparently healthy control. All blood samples were taken from Al-Hussein Teaching Hospital, in Kerbala city .T2DM was selected according to the criteria of American Diabetes Association 2010.[18] The age of patients and control groups was matched and ranged between 45- 65 years at disease onset, and the mean duration of diabetes was 9.13 ± 5.24 years. Informed consent was obtained from all the study subjects. All investigations were done in accordance with the Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethical committee approved the study protocol.

Blood samples were drawn for measuring fasting and postprandial blood glucose and Glycated hemoglobin (HbA1c).

DNA was extracted from each blood using a Mini Kit with from whole blood by applying a protocol (Geneaid / Korea), according to the manufacture company, DNA concentration was quantified by using NanoDrop™ Spectrophotometer and hold in 1% gel agarose electrophoresis.

We used National Center for Biotechnology Information NCBI website (http://www.ncbi.nlm.nih.gov) to design Pit1 sequences and annotation. For special set of primers was designed using web-based software accessible from the website (http://primer1.soton.ac.uk/public-html/primer1.html). The ARMS-primers used for detection of mutation screening PIT1 were provided in Table 1.

The reactions were optimized for 25 μl final volume using 50-100 ng genomic DNA, 200μM each dNTP, 15 mM $MgCl_2$; 1μlfrom 7 picomole of each primer (four different primers), 5X green *Taq* reaction buffer, 0.5 U of *Taq* DNA Polymerase (Promega) and completed with nuclease-free water.

Table 1: Primer sequences with melting and annealing temperature.

SNP	Primer sequence (5' to 3')	Melting Temp	Annealing Temp.	Expected products
TGF-β1 (T/C)	Forward outer primer 5'-CCACACCAGCCCTGTTCGCG-3'	63°C	59°C	Common primer 220bp
	Reverse outer primer 5'- TTGGACAGGATCTGGCCGCG-3'	61°C		
	Forward inner primer (C allele) 5'-CGGGCTGCGGCTGCTT<u>C</u>**C**-3'*	60°C		157bp
	Reverse inner primer (T allele) 5'-CCACAGCAGCGGTAGCAGCA<u>T</u>**C**A-3'*	69°C		104bp

*Underline letter represented inducer mismatch nucleotide's, bold letter represented the mutant and wild type neucleotides for C allele and T allele, respectively.

The PCR product for TGF-β1 gene was first denatured for 2 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 1 minute. Then the product was annealed at 59°C for 1 minute and extension at 72°C for 1 min, and final extension at 72°C for 2 min. The amplification products were hold through 1.5% agarose gel stained with and ethidium bromide, the procedure involved all the four different primers in one reaction tube, the reaction tube have detect the mutant-type (denoted by the letters C) and the wild-type denoted as (T).

All of the statistical analysis was performed on a personal computer using the Statistical Package for Social Science (SPSS) version 24 Data are presented as the mean +SD. Comparisons among different groups of patients were performed by one-way analysis of variance .The Frequencies of the PIT1 polymorphisms TT, TC and CC were expressed in numbers and percentages for wild-type, heterozygosity and recessive genotypes. χ2 test was used to evaluate consistency of genotype distributions with Hardy-Weinberg equilibrium as the following formula:

$$p = f(\mathbf{AA}) + \frac{1}{2}f(\mathbf{AB}) = \text{frequency of A}$$

$$q = f(\mathbf{BB}) + \frac{1}{2}f(\mathbf{AB}) = \text{frequency of B}$$

$$p + q = f(\mathbf{AA}) + f(\mathbf{BB}) + f(\mathbf{AB}) = 1$$

$$q = 1 - p \text{ and } p = 1 - q$$

RESULTS

The frequency of genotypes for TGF-β codon 10 (T869C) did not deviate significantly from the Hardy–Weinberg equilibrium. The values predicted by assumption of the Hardy–Weinberg equilibrium. The distribution of the genotypes and alleles in controls and diabetic patients are shown in Table 2. A statistically significant difference was detected between the control group and patients with T2D in the frequencies of the TGF-β1 codon 10 (P, 0.001). As shown in Table 2, T2D patients had increased frequency of TGF-β1 CC and TC genotypes (TT, TC, CC 49%, 44%, 7%, respectively) vs. controls (TT, CT, CC 69%, 27%, 3%, respectively).

Table 2: Hardy–Weinberg equilibrium examination of TGFβ1 gene polymorphism in T2DM and control individuals.

Study group	Genotype			Chi – Square	P value	Frequency	
	TT	**TC**	**CC**			**T**	**C**
T2DM	49% (0.49)	44% (0.44)	7% (0.07)	0.488	0.001	0.71	0.29
Control	69% (0.69)	27% (0.27)	4 %(0.04)	0.484		0.825	0.175

As shown in Table 3, the TC/CC vs. TT genotype had OR of 2.31 (95% CI ¼ 1.30 -4.12) under a dominant model (p value, 0.004) indicating to association of T/C SNP (either homo or hetero) with T2D. The TC vs. TT genotype had OR of 2.29 (95% CI 1.25 - 4.19) under a Co-dominant model (p= 0.006) indicating to association of T/C SNP (hetero) with T2D.

Table 3: Distribution of genotype and allele frequency of TGFβ1 gene polymorphism in DM and control groups.

TGFβ1	Control N =100	D.M N =100	Unadjusted OR (95% CI)	P value
Co-dominant				
TT(Reference)	69	49		
TC	27	44	2.29 (1.25 - 4.19)	0.006
CC	4	7	2.46 (0.68 -8.88)	0.16
Dominant				
TC + CC	31	51	2.31 (1.30 -4.12)	0.004
Recessive				
TT+TC (Reference)	96	93		
CC	4	7	1.80 (0.51 – 6.37)	0.35
Additive				
2(CC)+TC	35	58		

DISCUSSION

Several facts may account for the association between TGF-β1 gene polymorphism with the risk of T2DM. TGF-β1 plays a role of both pro-inflammation and anti-inflammation in many pathophysiological conditions. TGF-β1 inhibits and reverses the activation of macrophages and down regulates central effector mechanisms of the innate immunity.[19] The innate immune system modulates the effects of many factors, such as genes, fetal programming, nutrition, and age on the later development of metabolic sequel associated with insulin resistance.[20] On the other hand, TGF-β1 can also positively regulate immune responses. For example, TGF-β1 supports the differentiation of T-helper

17 (Th17) cells that are activated in many pro inflammatory conditions in the presence of Interleukin-6 (IL-6). Of note, IL-6 levels were increased before the onset of T2DM.[21] TGF-β1 could possibly prevent or slow down the autoimmune-mediated destruction of pancreatic Langerhans islets, leading to an absolute lack of insulin production.[22] In this sense, the activation of the innate immune system and the development of a systemic low-grade chronic inflammation are closely involved in the development of T2DM. In terms of above-mentioned evidence, TGF-β1 is closely associated with the susceptibility of DM. Second, the ability of an individual to produce high or low levels of TGF-β1 may be genetically predetermined. For example, the inflammatory and anti-inflammatory activities of TGF-β1 and its signaling pathway is often inactivated by mutation or altered expression of its components.[23] Gene polymorphisms can influence cytokine production or function; they may contribute to genetic predisposition to the disease. Polymorphism at codon 10 may be associated with higher or lower TGF-β1 synthesis.[24] SNPs in codon 10 of TGF-β1 alter the amino acid sequence and also affect TGF-β1 level. The C allele was repeatedly associated with increased TGF-β1 production, resulting from a leucine-to proline substitution in the signal amino-acid sequence of the protein, which indicated that certain allele/genotype may affect the risk of T2DM.

We found that codon 10 TC genotype increased the risk of T2DM, which was consistent with the notion that C allele was linked to an increased production of TGF-β1. The results of our study suggest that TC genotype at the TGF-β1 codon 10 sites may be an indicator for the risk of T2DM.

CONCLUSION

Tetra primer-ARMS PCR technique which developed in our study an effective, robust assay and time saving for genotyping (T/C) of TGFβ1 gene, Our results were concluded TGFβ1T869C (codon 10) C allele, and C allele-containing genotypes could increase the risk of T2D, In order to confirm the results obtained it would be advisable to conduct studies in a larger group of Iraqi population**.**

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