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Association between TNF-α Serum level and TNF-α *308* Gene Polymorphisms in Sample of Diabetes Iraqi Patients

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ABSTRACT

Background: Diabetes mellitus (DM) is one of the most prevalent, chronic diseases affecting majority of world population.
Objective: The aim of this study was to explore the correlation between TNF-α serum levels and TNF-308 gene polymorphisms in a group of diabetic Iraqi patients.
Subjects and Methods: A blood sample was taken from fifty patients suffered from type2 diabetes (T2DM), and fifty healthy volunteers as a control. TNF-α serum level was detected by ELISA and *TNF-308G/A* (rs1800629) gene polymorphism was assessed by high resolution melting real time PCR technique.
Results: The results of estimation TNF-α level showed high elevation in patients' group (52.69±4.48 pg/ml) with high significant difference (P≤0.01) as compared with control group (23.35±1.67 pg/ml). While detection of TNF-α-308 polymorphism in patients revealed that the wild genotype GG was 1 (2%), heterogeneous genotype GA was 19 (38%), and homogeneous genotype AA was 30 (60%) with significant difference (P≤0.001) and even as in control GG genotype was 35(70%), GA genotype was 10 (20%), AA genotype was 5 (10%) with considerable difference (P≤0.01). The findings of correlation between TNFα level and TNFα-308 genotype in T2DM patients, revealed that a substantial increase at AA genotype patients (P≤0.01) in TNFα serum level (46.17±4.64 pg/ml) followed by GA patients genotype (29.21±2.74 pg/ml) and finally GG genotype (5.11±9.15 pg/ml). **Conclusion**: The result revealed that patients with (A) allele had a higher risk for T2DM; also the genotype of TNFα-308 and the serum level of TNF-α in people with T2DM and healthy control were significantly different.
Keywords: Type 2 diabetes mellitus; TNF-α; HRM; ELISA; Iraq.

INTRODUCTION

Due to its high and rising occurrence and the varied and widespread morbidity it causes, which has an impact on people, health systems, and national economies, diabetes mellitus is one of the most difficult public health issues in the world [(1)]. T2DM is a metabolic disease largely manifested by an inflammatory systemic condition along with insulin resistance (IR) or a diminished metabolic response to insulin in a variety of tissues, such as adipose tissue, the liver, and skeletal muscle, additionally, by inadequate pancreatic beta cell production of insulin [(2,3)]. It is a heterogeneous disease characterized by prolonged periods of elevated blood glucose levels, which result in long-term health problems [(4-6)]. Diabetes is directly responsible for about 1.6 million deaths annually [(7)]. In Iraq, T2DM affects almost 1.4 million Iraqis, varying in prevalence from 8.5 percent to 13.9 % [(8)]. The genetic susceptibility is essential for the development of T1DM, but inheritance does not follow simple Mendelian rules. The pathogenesis of type 2 diabetes is influenced by both environmental and genetic elements [(7,9)]. Tumor necrosis factor (TNF-α) is a cytokine that promotes inflammation. Numerous human disorders, like type 2 diabetes mellitus (T2DM), have been linked to its dysregulation. TNF-α is the first pro-inflammatory cytokine whose role in the establishment of insulin resistance and T2DM has been recognized [(10)]. The regulation of this cytokine's expression is linked to insulin resistance and highly influenced by genetic factors [(11)]. Tumor necrosis factor alpha has an impact on glucose metabolism and affects insulin transduction [(12,13)]. Impairment of β-cell function and reduced insulin secretion induced by glucose may both be mediated by the TNF- α. It has been demonstrated in vitro that TNF-α may reduce the glucose-induced insulin release from pancreatic β-cells [(14,15)]. Because of the considerable differences between the various studies that are now accessible, the relationship between the TNF-promoter genotypes and the chance of developing T2DM is still debatable. The TNF-α promoter region comprises a number of polymorphisms that are positioned adjacent to the transcriptional start point. One polymorphism at nucleotide position −308 has a direct impact on TNF- α expression; which guanine substituted by adenosine. Given the importance of the topic, this research sought to investigate the relationship between TNF-α serum levels and TNF-α *-308* gene single nucleotide polymorphisms in sample of diabetes Iraqi patients.

SUBJECTS AND METHODS

Four ml of venous blood was taken from 50 patients suffered from T2DM (22 female and 28 male) with age ranged between (35-65years) who attended Ashnuna Laboratory for Pathological and Hormonal Analyzes in Diyala, Baquba, and from 50 apparently healthy volunteers as a control group. Each blood sample was divided up into two halves; first 2 ml of blood was put in gel tube, left for 10-30 min to allow clotting, afterwards

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centrifuged at 3000 rpm for 10 minutes, and kept at (-20 °C) until use for evaluation of TNF-α serum level by ELISA. The rest 2 ml was put into EDTA anticoagulant tubes, mix gently and preserved at (-20º C) till DNA extraction was applied for detection TNF- α 308 (rs1800629) by HRM RT-PCR method. This study was carried out during the period from 5th of December 2020 to 26th of June 2022.

Evaluation TNF-α serum level

Evaluation of TNF-α serum level was done by employing the ELISA technique and TNF-α ELISA kit (Sunlong Biotech, China) according to **Mohammed [16]**.

Recognition of *TNF-α* −308G/A Polymorphism

DNA purification kit (Biotech, China) was used to extract genomic DNA from fresh whole blood according to **Mohammed [17]**, DNA purity in ng/ µl and concentration within the accepted ratio 260 nm /280 nm were estimated by Quantus Fluorometer (Promega/USA). Recognition of TNF-α- 308 (rs1800629) gene polymorphisms was done by high resolution melting method real time PCR (HRM RT-PCR) technique using specific primer supplied by (Macrogen, (Korea), based on NCBI as displayed in the table (1). Component of PCR mixture reactions with a total volume of 20 µl comprising EVA Green Master mix (Promega, USA) as displayed in table (2)

Table (1): The TNF-α- 308 (rs1800629) primers sequences

Primer	5 ' ➡ 3 '
F	CCCCAAAAGAAATGGAGGCAATAGG
R	GTAGGACCCTGGAGGCTGAAC

Table (2): Component of the HRM RT-PCR reactions

Master-mix components	Volume 20 µl
EVA Master Mix	4
Forward primer	0.4
Reverse primer	0.4
Nuclease Free Water	9.2
DNA	6

HOT FIREPol® EvaGreen® HRM Mix, Solis BioDyne, Estonia was selected for doing qPCR-HRM. The cycling regimen was programmed for the following optimal cycles and in accordance with the thermal profile provided in table (3). Normalized melting curves (NMC) and differential curves (DC) were constructed using the HRM tool included in the integrated package, and triple synthetic controls were examined by qPCR-HRM to identify allelic differences. Data analysis was conducted by Rotor-Gene Q Series Software, which contained recording Ct values of each amplification reaction, amplification plots as well as HRM curves.

Table (3): program of qPCR-HRM

Step	Temperature	Duration	Cycles
Enzyme-activation	95º C	(5) min	1
Denaturing	95º C	(15) sec	40
Annealing	60º C	(15) sec	
Extension.	72º C	(30) sec	
HRM	65- 95	0.2sec for 1 degree	

Ethical approval:

The research approved by Iraqi Ministry of Health's Ethics Committee, Institute for Genetic Engineering and Biotechnologies for Post-graduate Study, Baghdad University, Iraq. A consent document was signed by all those involved. The World Medical Association's Declaration of Helsinki was strictly adhered to in all human subjects' studies.

Statistical Analysis

To determine how various factors affected the study parameters, the statistical analysis system program was employed. Least significant difference (LSD) test was used as post-hoc test with ANOVA test and T-test for comparison between means. Chi-square test was utilized to significant comparison between percentages in this study. Odds ratio (ORs) with a 95% confidence interval (CI) was done to assess the potential associations among genetic variants. P value less than 0.05 was considered significant.

RESULTS

The results of comparison between serum TNFα level in T2DM patients and control groups, revealed a noteworthy increased in the patients' serum level as compared to healthy control with highly significant differences, as shown in the table (4). The result of distribution of TNFα -308 (rs1800629) polymorphism and allele frequency in patients and control groups revealed that there was significant difference between the 2 studied groups regarding the wild genotype GG, heterogeneous genotype GA, homogeneous genotype AA as well as the frequency of G allele as shown in table (5).

Table (4): Comparison between serum TNFα level in T2DM patients and control groups

Group	Mean ± SE of serum TNFα (pg/ml)
Patients N=50	52.69±4.48 pg /ml
Control N=50	23.35±1.67 pg /ml
T-test	10.085**
P- value	<0. 0001
** (P≤ 0.01)	

SE: Standard error, **: Highly significant

Table (5): Genotype distribution plus allele frequency of TNFα308 (rs1800629) in patients and control groups

Genotype G/A	Patients No. (%)	Control No. (%)	P-value	O.R. (C.I.)
Wild: GG	1 (2)	35 (70%)	<0.0001**	(1) Reference
Hetero : GA	19 (38%)	10 (20%)	0.048*	1.330 (0.09-0.82)
Mutant: AA	30 (60%)	5 (10%)	<0.0001**	1.52 (0.89-2.76)
Total	50 (100%)	50 (100%)		
Allele	Frequency			
G	0.22	0.77	OR =0.1814 (95% CI-0.0865 to 0.3803)	
A	0.78	0.23	OR= 5.5125 (95% CI-2.6295 to 11.5564)	
** (P≤ 0. 01)				

*: Significant, **: Highly significant.

The findings of correlation between TNFα serum level and TNFα-308 genotype in T2DM patients, revealed a substantial increase at AA genotype patients in TNFα serum level followed by GA patients genotype and finally GG genotype patients. Additionally, individuals with at least one copy of (A) allele had a greater risk of developing T2DM as well as there was a relationship between the existence of the mutant (A) allele heterozygous (GA) and homozygous (AA) with the serum TNFα in T2DM patients as illustrated in table (6).

Table (6): Correlation between TNFα serum level and TNFα-308 genotype in T2DM patients

Genotype	Mean ± SE
	TNFα serum (pg/ml)
GG	5.11 ± 9.15
GA	29.21 ± 2.74
AA	46.17 ± 4.64
P- value	<0. 0001
** (P≤ 0.01)	

SE: Standard error, **: Highly significant

DISCUSSION

Cytokines regulate immune system response and play crucial roles in T2DM. Pro-inflammatory cytokines, inflammation, and immune system activation have all been shown to play a substantial role on the development and pathophysiology of T2DM [18,19]. In this study, T2DM patients had a highly marked increase of serum TNF-α. Similar findings were reported in prior studies by **Mirza *et al.*** [20], and **Farhan** [21].

Meta-analyses in two different studies by **Liu *et al.*** [22] and **Qiao *et al.*** [23] revealed a substantial increase in serum TNF-α, which positively correlated with insulin resistance, in both type 1 and type 2 DM patients. **Bertin *et al.*** [24] reported that in obese T2DM patients, when blood glucose levels are suddenly lowered in poorly controlled diabetic patients, the TNF- α plasma level, which is relevant to the amount of visceral fat, is not immediately changed. **Wondmkun** [25] reported that the research on the relationship between TNF-α and diabetes is still ongoing and not conclusively understood, because the previous studies on the relationship between diabetes and TNF- α level produced conflicting results.

The results of the current investigation suggested that the TNF-promoter polymorphisms (rs1800629) might serve as potential predictive biomarkers for T2DM. There are numerous SNPs in the promoter region of *TNF-α* gene that altered the expression, and consequently elevating the serum level of TNF-α [26,27].

TNF-α *−308G/A* polymorphism exhibits enhanced gene transcription. Allele A has been shown to be a considerably more potent transcriptional activator, increasing TNF-α transcription levels by 6-7-fold, which is related to increased TNF-α production [28].

The findings of this research indicated that there was risk factor in patients group compared to control group with genotype AA had (O.R 1.52). This indicated that *TNF-α rs1800629* gene was related to T2DM risk. At the same time A allele had O.R (5.5125), which was considered as a risk factor for T2DM and G allele had O.R (0.1814) that was considered as a protective factor for T2DM. This result agrees with **Shi *et al.*** [29] who showed that the *TNF -α* rs1800629 polymorphism was highly correlated with the risk for T2DM.

Many association studies on the G-308 variant have been carried out, with contradicting results. **Fernández-Real *et al.*** [30] has revealed a critical relationship between the G-308A mutation and increased insulin resistance. The results of the current study showed that *TNF- α rs1800629* gene polymorphism led to increase the TNF α serum level. The previous study by **Aller *et al.*** [31] demonstrated that G-308A polymorphism of TNF-alpha is related with insulin resistance. The polymorphism at position 308 (TNF -308 G->A) causes an increase in the rate of TNF alpha gene transcription, which is followed by an increase in TNF alpha concentrations and a reduction in insulin sensitivity [32].

Nevertheless, the results of other research by **Mustapic *et al.*** [33] disproved the assumption that the A allele of G-308A TNF- α gene polymorphism and T2D are related.

CONCLUSION

It can be concluded that TNF- α played a critical role in immune responses then subsequently the T2DM susceptibility, also individuals with at least one (A) allele copy had a greater risk to T2DM as well as there was a relation between the existence of the mutant (A) allele, heterozygous (GA) and homozygous (AA) with the serum TNF-α status in Iraqi T2DM patients.

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CONFLICES OF INTREST

There are no conflicts of interest declared by the authors.

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