

# Characterization of a Polymorphism in the Stimulatory Region of the TNF Gene in Type 2 Diabetic Patients in Iraq

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## Abstract

The current study aims to analyze the distribution of the TNF- $\alpha$  gene and TNF- $\beta$  polymorphism (-308G/A) in type 2 diabetes mellitus (T2DM) patients. According to world health organization the prevalence of diabetes is rising exponentially. Tumor necrosis factor alpha and beta are members of the tumor necrosis factor (TNF) super-family considered as pro inflammatory cytokine which play major role in DM pathogenesis. single nucleotide polymorphism reported to have associated with type 2 DM. Frequency of TNF- $\alpha$  G308A polymorphism was determine in different ethnic groups and was found variable highly. Few studies recorded appositive association between DM and TNF- $\alpha$  polymorphism; including meta-analysis failed to find such association. Our finding revealed that the TNF- $\alpha$  gene polymorphism (-308G/A) had 38 (12.50%) of G/G genotype, 44 (45.83%) of G/A genotype and 40 (41.67%) A/A genotype showed in this population. This preliminary result indicated that G/A genotype was a common in the TNF- $\alpha$  gene polymorphism (-308G/A) in T2DM patients while healthy control group does not exhibit AA and GA genotype.

**Keywords:** TNF- $\alpha$ , G/A308 Gene polymorphism, Type 2 Diabetes, Insulin resistance .

## INTRODUCTION

Diabetes mellitus (DM) is metabolic syndrome conditions with an increasing prevalence of patients every year in the world. Chronic DM can cause complications such as hypertension, cardiovascular disease, kidney problems, and death. The American Diabetes Association (ADA) recommends the examination of HbA1c (glycated Hemoglobin) as the long-term glycemic control in DM patients [1]. Glycated Hemoglobin describes the history of blood glucose control in the previous 60-90 days. The glycated Hemoglobin test is recognized as the gold standard in assessing the development of DM [2].

TNF- $\alpha$  plays a central role in the pathogenesis of obesity-induced insulin resistance as evidenced by the augmented levels of TNF- $\alpha$  in systemic circulation, liver and adipocytes [3]. The TNF- $\alpha$ -induced insulin resistance is dependent on the intracellular and molecular mechanisms that involve the activation of stress-related protein kinase.

TNF-alpha has multiple mechanisms. it stimulate IL-6 release and directly inhibit signaling by phosphorylation at serine 307 residue of IRS-1. it also induce transcription factor nuclear factor (NF-kappa B) which causes apoptosis of beta cell pancreas. TNF-alpha also increase elevated serum free fatty acid levels. Local inflammation in obese adipose tissue was first reported in 2003. Elevated serum level of TNF - alpha are found in type 2 DM patients and in obese healthy persons who subsequently developed type 2 DM in a span of 2-5 years indicating the future risk of metabolic syndrome and type 2 DM. Adipocytes contribute almost all of TNF-alpha and explain the association of high TNF -alpha level with obesity and raised body mass index. Indirect studies also indicate exercise and weight loss can lead to decreased concentration of TNF alpha. High level of TNF has been reported to be associated with insulin resistance. TNF renders insulin resistance by interfering with insulin signaling adipocyte and hepatocyte. TNF alpha cause insulin resistance in skeletal muscle too by impairing glucose uptake and translocation of glucose uptake transporters (GLUT-4) hyperinsulinemia itself induce augmented production of TNF - in serum in obese type 2 DM patients. High glucose uptake results in apoptosis of beta cells in pancreas with increase expression of IL-1BB messenger ribonucleic acid and its levels. TNF-alpha also has pro-apoptotic effect by activation of transcription factor nuclear factor kappa B target genes in cultured beta cells of pancreas.

The TNF and LTA genes are arranged in tandem within the major histo-compatibility complex class III region on the short arm of chromosome 6. LT- $\alpha$  RNA was detected in both macrophage and non-macrophage populations in term deciduas. LT- $\alpha$  is a member of the tumor necrosis factor (TNF) super-family that was originally thought to be functionally redundant to TNF [4, 5].

The aim of the study was to investigate the frequency of alleles and polymorphic genotypes of the TNF- $\alpha$  gene, locus-308 (rs-1800629) and lymphotaxine alpha +1 252 A<G in diabetic patients compared to the control group. The TNF- $\alpha$  is a cytokine that arise in inflammation condition to regulate activation, differentiation, and proliferation of cell. The cytokine was regulating the continuity of the cell. The TNF- $\alpha$  structure in the form of 17 kilodaltons (kDa) protein was constructed from 185 amino acids [6, 7, 8]. Coding of TNF- $\alpha$  genes are on chromosome 6 (6p 21.3). TNF- $\alpha$  synthesis is regulated by specific TNF- $\alpha$  gene sequences. Single nucleotide polymorphism (SNPs) or substitution of one nucleotide base in the TNF- $\alpha$  gene sequences influence the synthesis or levels of TNF- $\alpha$ . The SNIPs of TNF- $\alpha$  gene was postulated as a key genetic factor in several diseases. In the gene polymorphism (-308G/A) shown that the substitution of a guanine (G) nucleotide by an adenine (A) nucleotide [9, 10].

## Study sampling and study design

The work was conducted from June 2021 to September 2021, and subjects were collected from diabetes outpatient clinics. Genetic analysis of this study was carried out at Wasit University / Department of Biology.

### Inclusion criteria

The study included two groups:

Group 1: included 100diabetic patients.

Group 2: included 50 apparently healthy unrelated volunteers. The diagnosis was according to ADA (2013) criteria.

All patients and healthy volunteers were subjected to the following:

History taking, laboratory investigations (fasting blood glucose, glucose tolerance test HA1C, lipid profile, and detection of both TNF-308G/A (rs-1800629) gene polymorphisms and LT- $\alpha$  gene, position +252 (rs909253).

### ARMS-PCR

ARMS-PCR approach was used for detecting polymorphisms at TGF- $\beta$ 1 gene positions. AccuPower PCR PreMix (Bionner, Korea) was prepared depending on [11, 12] with some modifications. A total 20 $\mu$ l of PCR reaction was used: 12.5  $\mu$ l master mix (1x), 1  $\mu$ l of each primer (1  $\mu$ M) and 2  $\mu$ l of DNA template (100 ng). Total volume of reaction was completed with nuclease-free water. 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- $\beta$ 1 gene position codon 25 +915\*G/C. The reaction mixers were placed in Thermo cycler (Esco, Singapore).

### PCR conditions

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). Most of patient have hb1c (45%) was equal to or greater than 9 mg have been explained carry aa and ga genotype, this indicate TNF considered one of predisposing factor to develop T2DM

and (34%) of patients 7-9 mg had poor control of their blood sugar the blood; While, the remaining 21% (12 males + 9 females) had good glucose control (<7 mg).

#### Statistical analysis

Our data were analyzed using of ANOVA test in GraphPad Prism (6.0.1) Software at a significant differences of  $P \leq 0.05$  [13, 14].

## Results and discussion

In our study we found that, in patients with T2DM (GG genotype: 12.50%, AA genotype: 41.67% and GA genotype: 45.83%). In the healthy volunteers (GG genotype: 100%, AA genotype 0% and GA genotype: 0%).

Significant differences were seen in the distribution of three different genotypes across the groups under study ( $P = 0.0001$ ). In contrast to the GA, AA and A alleles, which were more prevalent in the patient group, our study showed that individuals with T2DM had a lower frequency of the GG genotype and the G allele than healthy controls. But all of the controls had the G allele and none had the A allele. It was in accordance with previous studies that discovered strong correlations between the risk of type 2 diabetes mellitus and the TNF- gene polymorphism (-308G/A) (15-17). A study from Egypt also revealed that G/A genotype patients had greater rates of type 2 diabetes than G/G genotype patients [18]. In contrast to previous research, which lacked statistical differences [19-21]. Most patients (45%) with AA, GA genotype had hb1c level equal to or greater than 9 mg, and this indicates that TNF is considered as one of the predisposing factors for the development of T2DM.

Table (1): Distribution of TNF- $\alpha$  G/A SNPS among the study groups

Genotypes and alleles	Group1 Diabetics No =100	Group 2 Control No =40	p -value between each 2 groups
<b>Homozygous AA</b>	40 (41.67%)	0 (0.00%)	0.0001
<b>Wild type GG</b>	12 (12.50%)	50 (100%)	0.0001
<b>Heterozygous GA</b>	44 (45.83%)	0 (0.00%)	0.0001
<b>A allele</b>	0.65	1.00	0.0001
<b>G allele</b>	0.35	1.00	0.0001

Our study clarified that none of the controls had the A allele and all had the G allele. On the other hand, the present study couldn't find any homozygous AA genotype in the healthy controls studied population, contrary to patients group which carry the same genotype in 41.67% frequency. It may be thus thought that the G allele is a protective factor from the development of T2DM in some Wasit province population and speculated that individuals with the A allele were more susceptible to T2DM than those with the G allele. Also, homozygosity for A-allele conferred a more than a twofold increased risk of T2DM. However, the current study cannot propose strictly that rs1800629 (G/A) polymorphism of the TN F- $\alpha$ -308 gene contribute to the prevalence of T2DM in selected area population. There are many studies concerning the investigation of TNF- $\alpha$  G-308A genotype in patients with T2DM, which suggested that the association between TNF promoter genotypes and this disease is quite controversial. Ethnic and other disparities in their study designs may show a role in these unpredictable results [21].

Regarding the other gene under study, the results recorded three genotypes (GG, GA, AA) in the diabetic group with consecutive proportions of 28.57%, 51.02% and 20.41% versus 93.62%, 1.03, 4.26 in the other group. The results showed a very significant difference ( $P \leq 0.01$ ) in GA and AA between the studied group, but GG did not show any significant difference (p value = 0.0593).

Meanwhile, it was found that the recurrence rate of the G allele in both diabetic and non-diabetic patients was 54%, 95% while the A allele was 46%, 5%, respectively (Table 2)

Table (2): Genotype and allele frequency of (+252A) SNP in TNF-  $\beta$  gene in studied subjects

GenotypeTNF- $\beta$	Patient No (%)	Control No (%)	Chi-Square: $\chi^2$	P-value	O.R. (C.I)
GG	28 (28.57%)	44 (93.62%)	3.356 NS	0.0593	Ref.
GA	50 (51.02%)	4 (1.03%)	47.078 **	0.0001	2.07 (1.16-4.37)
AA	22 (22.41%)	2 (4.26%)	14.727 **	0.0001	1.46 (0.85-2.71)
Allele	Frequency				
G	0.54	0.95	--	--	--
A	0.46	0.05	--	--	--
** (P $\leq$ 0.01), NS: Non-Significant.					

According to several reports, association between this polymorphism and other variables such as fasting glucose and triglycerides had been shown in patients under controlled diet [19]. On the contrary, this interpretation of the data is consistent with the results of a study demonstrating that the LTA variants were not related to the risk of coronary artery disease or myocardial infarction in a German population [22]. Walton et al. [23] also failed to demonstrate a direct association between variations in LTA and myocardial infarction in a large population-based study involving 15,800 subjects.

## Conclusions

Genetic variable in 308- position of TNF-alpha gene has direct association with glucose level and lipid parameters so it As risk factor for predisposing to insulin resistance in population with 308-A alleles (TNF-alpha hyper producer), so this is expected to cause insulin resistance as recorded in a previous study. Variations in LTA are associated with an increased risk of type 2 diabetes (OR=1.24) and other features of the metabolic syndrome. The diabetogenic effect of LTA was even more pronounced in patients with early-onset type 2 diabetes.

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