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The effects of gene polymorphisms in tumor necrosis factor-α on the susceptibility of type I diabetes mellitus in an Iraqi population

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***Abstract*---**Background: Type 1 diabetes mellitus (T1DM) is a chronic, immune-mediated disease characterized by the destruction of insulin-producing β cells in the pancreas. This study investigated the influence of single nucleotide polymorphisms (SNP) in tumor necrosis factor (*TNF-a)* –308 G/A (rs1800629,) on T1DM patients. Method: The study was included 75 T1DM patients and 25 healthy subjects. The SNP–308 G/A *TNF-a* gene was detected by using polymerase chain reaction restriction fragment length polymorphism (RFLP-PCR) technique in T1DM patients and controls. Results: The genotype distribution results of the -308G/A SNP of Tumor Necrosis Factor - α (*TNF- a*)-308 G/A gene showed significant difference ($p<0.05$) between controls (GG: n= 24, 96%; GA: n=1, 4%; AA: n=0, 0%) and T1DM patients (GG: n= 44, 58.67%; GA: n=21, 28%; AA: n=10, 13.33%).These results showed an increased in GA, AA genotype and G allele of the *TNF- a*(-308) G/A in T1DM patients than controls, and they were significantly more likely than controls to have the mutant allele (OR=18.431, 95%CI= 2.464-137.855, p=0.00001). These results indicate a possible role for the G genotype in T1DM disease . Conclusions: The present study suggested that the -308 G/A SNP in the *TNF -a* gene statistically association ($p<0.05$) with the risk of T1DM occurrence in Iraqi patients.

***Keywords*---**T1DM, (TNF-α) gene -308G/A, polymorphism, RFLP-PCR.

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic immune-mediated disease with a subclinical prodromal period, characterized by selective loss of insulin-producing-β cells in the pancreatic islets of genetically susceptible individuals (Knip & Siljander, 2016). The prevalence of diagnosed T1DM among US adults in 2016 and 2017 was 0.5% (xu *et al.*, 2016&2017). Other reported have confirmed that the incidence of T1DM continues to increase in many countries, including the US, Korea, Romania, Iraq, and Poland (Serban *et al.,* 2015; Kim *et al.*, 2016; ALMahfoodh *et al.*, 2017; Chobot *et al.*, 2017; Rogers *et al.*, 2017). Incidence of T1DM in children under 15 years of age in Ireland, Scandinavia, Japan and Western Australia (Berhan *et al.*, 2011; Haynes *et al.,* 2015; Roche *et al.,* 2016) The reasons for these differences to be elucidated.

Tumor necrosis factor - α (*TNF*-α) is a potent proinflammatory cytokine and immunomodulator produced by activated macrophages, monocytes, CD4+ lymphocytes, natural killer cells, neutrophils, mast cells, eosinophils, and neurons (Cekici *et al.,* 2014). It is involved in a variety of metabolic disorders such as T1DM, T2DM, and obesity. It blocks the action of insulin, causing insulin resistance. In humans, serum concentration of *TNF-a* is elevated in T2DM, being associated with impaired glucose tolerance, enhanced insulin resistance, islet dysfunction, and increased T2DM risk (Role of TNF-α in the immunopathogenesis of Behçet's disease (BD) and the effect of treatment with TNF-α blockers.

The *TNF-a* gene is located in the class III region of the MHC at chromosome 6p21.32, is 2,676 bp long and contains 4 exons and 3 introns (Al Naqbi *et al.,* 2021). This study explored the association of SNP of (*TNF*-α) –308 G/A (rs1800629,) by using the method of polymerase chain reaction restriction fragment length polymorphism (RFLP-PCR) with the risk of T1DM development in Iraqi population.

Materials and Method

a) Study group: This study was included 75 patients. These samples were collected from laboratory of Najaf Center of Diabetes & Endocrine in Al-Sadr Teaching Hospital. All the patients selected for the present study were having T1DM and they diagnosed by specialist doctor. (Blood samples were obtained as part of the routine clinical protocol). Epidemiological information's about patients like age and gender was collected from patients Data sheets from hospital.
b) Control group: It consists from 25 healthy subject; all were without any inflammatory disorders or clinical manifestation of any disease.

Blood sample

The PCR test was performed on 2 ml of venous blood, which was collected tubes with anticoagulant Ethylenediaminetetraacetic acid (EDTA) from patients and controls.

DNA isolation and RFLP-PCR technique

Genomic DNA was isolated using protocol from Genomic DNA Mini Kit (Geneaid Biotech, Taiwan) protocol procedure, which specially was designed to purifying DNA from frozen blood. A sequence of SNP in region of promotor in *TNF- a* was amplified using the primer-pairs: forward primer 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and reverse primer 5′-TCCTCCCTGCTCCGATTCCG-3′. These primers have already been published previously (Ohtsuka *et al.,* 2003). Primer containers were first centrifuged at 13,000 rpm for 3 minutes, and then reconstituted with appropriate volume of TE buffer for each one (according to the manufacturer) in order to get 100 pmole/µl (stock solutions). Working solution with 10 pmole/µl, was prepared from stock solutions.

According to the manufacturer's instruction DNA quality extracts were analyzed by electrophoresis. The extracted genomic DNA concentration was estimated by using Nanodrop spectrophotometer (THERMO, USA), which measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at (260 /280 nm) according to Ausubel *et al.* (2003). The RFLP-PCR was performed to detect (*TNF*-α)-308G/A gene polymorphism. All DNA samples were amplified individually using primers and corresponding cycling condition (as described in Table 1) by using (THERMO, USA).

Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) is a method established by Grodzicker *et al.* in 1974, it had used to identify DNA polymorphisms among different individuals. The 107bp PCR product was digested with 10 unit of *Ncol* restriction enzyme (10 unit is sufficient, generally 1 µl Was used (Wilson *et al.*,1997), Source*: Nocardia Corallina* at 38^{o}C (synthesized by Promega, US, Cat. No R6515). After the *Ncol* digestion (it left to digest 4 hours), one of these results were yielded for each sample:

a) Two fragments for allele G (homozygous wild genotype patient, GG). The original PCR fragment had one *Ncol* cleavage site so the restriction enzyme found the cutting position to produce 87 and 20 bp fragments.
b) 107bp fragment for allele A. The PCR fragment had no *Ncol* enzyme cleavage site (homozygous mutant genotype patient, AA).
c) 87, 20 and 107bp fragment for both G and A allele (a heterozygous genotype patient, GA) (Aslebahar *et al.,* 2019). Finally, the gel electrophoresis method, which included preparing the gel loading and running the gel, was done according to Sambrook & Russell (2001) as the following:
d) A 2% agarose gel was made by mixing 2 g agarose with 100 ml 1X TBE buffer.

Table 1: Primers sequences for Tumor Necrosis Factor- α (*TNF-a)* gene -308 G/A polymorphism

Gene	Primers sequences		PCR product	Ref.
(TNF–α) gene-308 G/A	Forward	5′-AGGCAATAGGTTTTGAGGGCCAT-3′	107 bp	(Ohtsuka *et al.,* 2003).
	Reverse	5′-TCCTCCCTGCTCCGATTCCG-3′		

(rs18006 29)				

Statistical analysis

Statistical analyses of all results were carried out by the help of Statistical Package for the Social Sciences (SPSS) version 23 software statistical package using t-test and Chi-square test (with P value at level of significance less than 0.05) to compare value of results between groups. Result values were expressed as mean ± SE, number of patients, or percentages.

Results and Discussion

a) Controls: Among the 25 healthy subjects; 24 (96%) had found as homozygous GG alleles, 1(4%) found as heterozygous genotype (with the G and A alleles (GA), and no healthy subjects had found as homozygous genotype AA alleles; (GG: n= 24, 96%; GA: n=1, 4%; AA: n=0, 0%) (Table 2 & figure 1).

Table 2: The results of genotypic frequencies of -308 (G/A) polymorphism in the *TNF*-α gene in patients and controls

Genotypes	Healthy controls (N=25)	Diabetes mellitus type 1patients (N=75)
GG	24(96%)	44 (58.67%)
GA	1 (4%)	21 (28%)
AA	0(0%)	10 (13.33%)
P-value	0.005**	
Alleles frequency	N(%)	N(%)
G allele	49 (98%)	109 (72.67%)
A allele	1 (2%)	41 (27.33%)
X^2 P-value OR (95%CI)	14.507 0.00001* 18.431(2.464-137.855)	

Data were expressed as number and a percentage (N%). *p <0.05 significant. Abbreviations: X^2= chi-square, OR= odds ratio, CI= confidence interval.

b) Patients: Among the 75 T1DM patients; 44(58.67%) had found as homozygous GG alleles, 21 (28%) found as heterozygous genotype (with the G and A alleles (GA), and 10 (13.33%) had found as homozygous genotype AA alleles; (GG: n= 44, 58.67%; GA: n=21, 28%; AA: n=10, 13.33%).

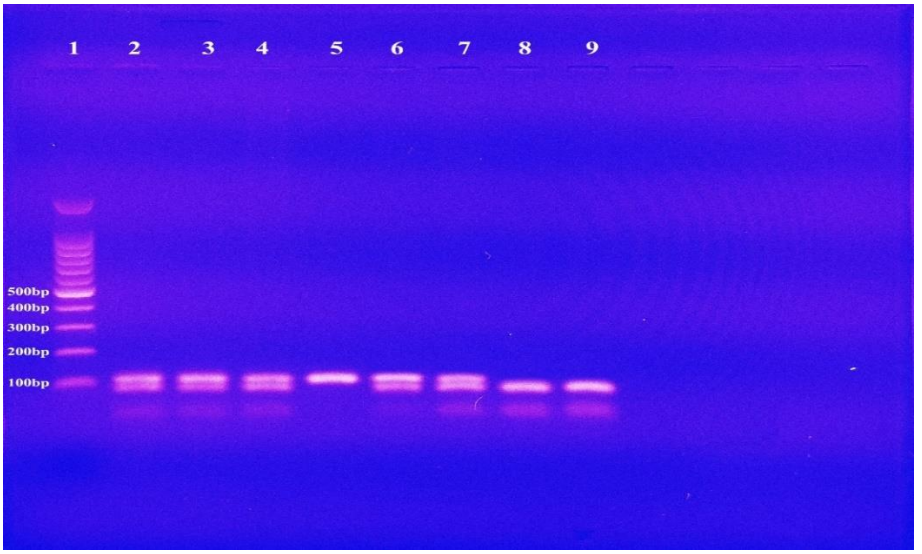

Figure 1: The electrophoresis image of RFLP-PCR analysis of -308 G/A SNP in the *TNF-a* gene. Lane 1: 100 bp DNA Ladder; Lane 8, and 9: homozygous genotype GG (87 and 20 bp which undetectable on gel because of small size); Lane 2,3,4,6 and 7: heterozygous genotype GA (107, 87 and undetectable 20 bp bands); Lane 5: mutant homozygous genotype AA (107 bp uncut bands).

That means the frequencies of -308 G/A SNP (rs1800629) in the *TNF -a* gene in the 75 Iraqi of T1DM patients in Al-Najaf province were with significant differences with that of the 25 healthy controls group ($p<0.05$). The result showed an increased in GA, AA genotype and G allele of the - 308 G/A SNP in *TNF- a* gene in T1DM patients than controls, and they were significantly more likely than controls to have the mutant allele (OR=18.431, 95%CI= 2.464-137.855, p=0.00001). These results indicate a possible role for the G genotype in T1DM disease.

The sequence variation in the regulatory region of *TNF-a* gene has been correlated with various autoimmune diseases. Several biallelic single nucleotide polymorphisms (SNPs) have been noted in the *TNF-a* gene. Among them one G >A transversion substitution polymorphism is located upstream of the gene at -308 and is known to influence TNF-α levels. In comparison with the *TNF-a* -308G allele, A allele has higher transcriptional activity and often connected to autoimmune diseases. The location of the gene within the major histocompatibility complex and the putative role of -308 G > A polymorphism on the promoter activity of *TNF-a* gene has raised the possibility that this polymorphism may influence immunologic homeostasis and contribute to the pathogenesis of many autoimmune diseases like Crohn's disease (CD), T1DM, and systemic lupus erythematosus (Sandhya *et al.,* 2013)

Genetic variations in *TNF* gene promoter region may regulate the production of TNF. Contrasting with the limitation of studies concerning other mutants, *TNF*-α promoter variants (-308 G/A SNP) being the most extensively studied. Individuals homozygous for the less common *TNF*-α-308 A allele have also been shown to have higher circulating TNF-α levels than those homozygous for the G allele and

have worse outcomes in response to infectious disease (Holmes *et al.*, 2003) The SNP located in the promoter regions of the *TNF*-α gene -308G/A were found to differentially affect binding of nuclear transcription factors, transcriptional activity, and protein production (Elahi *et al.,* 2009), and highlights on the potential role of -308 G/A SNP in regulation of *TNF*- α production occurs at the transcriptional and posttranscriptional levels (Wilson *et al.*, 1993). Hajeer *et al.* study (2001) confirmed that the *TNF- a* gene SNP at positions -308 G/A, relative to the transcription start site, have been shown to influence gene expression.

Present results were similar to the study by Das *et al.* (2006) which recorded that 80(61.5%) in T1DM patients had GG genotype, while 114(85.7%) in controls, 46(35.5%) patients had GA genotype and 19(14.3%) in controls, while 4(3%) patients had AA genotype. As mentioned above, they explained their results as follow: " The production of *TNF*-α is regulated at many levels, and there was clear evidence for transcriptional regulation, post-transcriptional control of mRNA abundance and for the effect of translational efficacy'. These results were consistent with the results of Feng *et al.* (2009) which found an association between -308 (G/A) polymorphism in the *TNF -a* gene and the risk for T1DM. They also found that the -308 (G/A) SNP in the *TNF -a* gene in the promoter region of *TNF* gene increased this cytokine level (TNF protein) in culture cells.

These results agreed with study by Szabo *et al.* (2014) which recorded that 53 (73.61%) T1DM patients had GG genotype versus 60 (66.66) among controls, while 17(23.61%) patients had GA genotype versus 30(33.33%) among controls while 2(2.77%) patients had AA genotype. The frequency of G allele was 123 (70.83%) in patients versus 150 (83.33%) among control group, while A allele frequency was 21 (29.16%) in patients versus 30 (16.66%) among control group. These results agreed with study by Lio *et al.* (2006) which clarified the relationship between Alzheimer's disease (AD) patients and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that 163 (73.4%) patients had GG genotype, 54 (24.3) patients had GA genotype, while 5 (2.3%) patients had AA genotype. The frequency of G allele was 380 (85.6%) in patients, while A allele was 64 (14.4%) in patients.

These results were in accordance with those of Kamali-Sarvestani *et al.* (2007) which found an association between Asthma and -308 (G/A) SNP in the *TNF -a* gene, where the results showed that 86 (81.9%) patients had GG genotype versus 89 (90.8%) among controls, 19 (18.1%) patients had GA genotype versus 9 (9.2%) among controls. The frequency of G allele was187 (95.4%) in patients, while A allele was 19 (9.05%) in patients. As well as another study by Zhang *et al.* (2011) which indicated that the variant A allele carriers had a 38% increased risk of asthma, when compared with the homozygote GG.

These results agreed with study by Hounie *et al.* (2008) which clarified the relationship between obsessive-compulsive disorder (OCD) patients and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that GG, GA and AA genotype frequencies were 69.4, 27.9% and 2.7%, respectively, among the cases and 83.2%, 15.2% and 1.6%, respectively, among the controls. Present results were similar to Utriainen *et al.* (2010) which found the relationship between Premature adrenarche (PA) patients and -308 (G/A) polymorphism in the

TNF -a gene, where the results showed that 53(75%) patients had GG genotype and 74(76%) in controls, 19(26%) patients had GA genotype and 20(21%) in controls, while 1 patient (1.4%) had AA genotype and 3(3%) in controls.

present results agreed with study by Fan *et al.* (2010) which recorded relationship between Tuberculosis (TB) patients and -308 (G/A) SNP in the *TNF -a* gene, where the results showed that 60 (53.1%) patients had GG genotype versus 77 (68.2%) among controls, 46 (40.7%) patients had GA genotype versus 32 (28.3%) among controls, while 7 (6.2%) patients had AA genotype versus 4 (3.5%) among controls. At the same time, this study in accordance with those Prasad *et al.* (2010) study which found a correlation between Guillain–Barre syndrome (GBS) patients and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that 97 (69.3%) patients had GG genotype and 177 (85.9%) in controls, 36 (25.7%) patients had GA genotype and 26 (12.6%) in controls, while 7 (5%) patients had AA genotype and 3 (1.5%) in controls. The frequency of G allele was 230 (82.1%) in patients and 380 (92.2) in controls, while A allele was 50 (17.9%) in patients and32 (7.8%) in controls.

These results agreed with study by Mosaad *et al.* (2011) which clarified the relationship between Rheumatoid arthritis (RA) patients and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that 97 (79.5%) patients had GG genotype and 17 (14.2%) in controls, 21 (17.2%) patients had GA genotype and 92 (76.7%) in controls, while 4(3.3%) patients had AA genotype and 11(9.2%) in control. The frequency of G allele was 215 (88.1%) in patients, while A allele was 29 (11.9) in patients.

These results were similar with Ardebili *et al.* (2011) study which found 32.5% of Alzheimer patients had GG genotype and 87.73% in controls, while 45.62% patients had GA genotype and 12.27% in control, while 21.87% patients had AA genotype. They concluded that *TNF-a* as an important pro inflammatory cytokine is unregulated in Alzheimer's patients. This cytokine plays an important role in pro inflammatory responses of immune system including regulation and catabolism. The expression of *TNF-a* is regulated at transcriptional and post transcriptional levels. As well as another study Flex *et al.* (2014) which reported an association between Alzheimer's disease and -308 (G/A) SNP in the *TNF -a* gene, where the results showed that 310 (58.2%) patients had GG genotype versus 458 (64.2%) among controls, 196 (36.8%) patients had GA genotype versus 225 (31.6%) among controls, while 27 (5.1%) patients had AA genotype versus 30 (4.2%) among controls.

present results were similar with study by Bozkurt *et al.* (2012) which found that GG genotype frequncywas 66 (76.7%) in primary open-angle glaucoma (POAG) patients and 171 (88.6%) in controls, 19 (22.1%) patients had GA genotype patients and 21 (10.9%) in controls, while 1 patient (1.2%) had AA genotype and 1 (0.5%) in controls. These results were similar with study by Al-Shobaili *et al.* (2012) which recorded that acne vulgaris cases had significantly higher frequency of both the GG and AA homozygous forms than controls (73.8% and 63.6%, respectively).

These results were consistent with the results of Bhayal *et al.* (2013) which clarified the relationship between Gastric cancer (GC) and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that 28.07% patients had GG genotype and 33.19% in controls, 66.67% patients had GA genotype and 10.92% in controls, while 5.26% patients had AA genotype and 10.92% in controls. These results agreed with study by Vázquez-Huerta *et al.* (2014) which indicated that the GG genotype frequency was 130% in Chronic kidneydisease (CKD) patients and 165% in controls, GA genotype frequency was 19% in patients and 27% in control while AA genotype frequency was 1% in patients.

Also, these results agreed with study by Gheita *et al.* (2015) which showed relationship between Rheumatoid arthritis (RA) patients and -308 (G/A) SNP in the *TNF -a* gene. They recorded that 62.8% patients had GG genotype, 23.2% patients had GA genotype and 14% patients had AA genotype. These results were consistent with the results of Piotrowski *et al.* (2015) which reported a relationship between Systemic lupus erythematosus (SLE) patients and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that 162 (0.62%) patients had GG genotype versus 369(0.70%) among controls, 89(0.34%) patients had GA genotype versus 142 (0.27%) among controls, while 11(0.04%) patients had AA genotype versus 17(0.03%) among controls.

In same time, this study agreed with a study done by Sudhir *et al.* (2016) which recorded that 66% of women with Recurrent miscarriage (RM) had homozygous wild type genotype GG and 4% of women had heterozygous GA and homozygous mutant genotype AA, respectively. Among control groups, 79%, 16%, and 5% of women showed GG, GA, and AA genotypes, respectively. The results were in accordance with those of Furquim *et al.* (2016) which recorded an association between Temporomandibular disorder (TMD) patients and -308 (G/A) SNP in the *TNF -a* gene. They recorded that 79 (86.81%) patients had GG genotype and 111 (73.02%) in controls, 8 (8.79%) patients had GA genotype and 32 (21.05%) in controls ,while 4 (4.4%) patients had AA genotype and 9 (5.93%) in controls.

These results were consistent with Sobhan *et al.* (2018) which observed increase in GG genotype frequency. They found that 79 (71.8) Osteoarthritis (OA) patients had GG genotype and 85 (70.8%) in controls, 30(27.2%) patients had GA genotype and 33 (27.5%) in controls ,while 1 (0.9%) patients had AA genotype and 2 (1.6%) in controls. These results were in accordance with those of Stavros *et al.* (2021) which found an association between Recurrent pregnancy loss (RPL) and -308 (G/A) SNP in the *TNF -a* gene. They recorded that 27% patients had GG genoptype and 25% in control group, while 28% patients had GA genotype and 18% in control group, 7% patients had AA genotype and 6% in control group.

Study by Razeghinejad *et al.* (2009) which clarified the relationship between Glaucoma patients and -308 (G/A) polymorphism in the *TNF -a* gene, where the results showed that 109 (84.5%) patients had GG genotype and 63 (86.3%) in controls, 20 (15.5%) patients had GA genotype and 9 (12.3%) in control, while no patients had AA genotype and no healthy subject. The frequency of G allele 238 (92.3%%) patients and 135 (92.5) in controls, while A allele was 20 (7.7%) in patients and 5 (2.2%) in controls.

These results disagreed with study by Abutorabi *et al.* (2015) about the role of -308 (G/A) polymorphism in the *TNF -a* gene in Endometriosis patients and where the results showed that 65 (100%) patients had GG genotype, no patients had GA genotype, while no patients had AA genotype. These results were different with study by Luleyap *et al.* (2013) which about role of -308 (G/A) SNP in the *TNF -a* gene in obsessive-compulsive disorder (OCD), where the results showed that no patients had GG genotype, 13.5% patients had GA genotype, while 86.5% patients had AA genotype.

Conclusions

The present study suggested that the -308 G/A SNP in the *TNF -a* gene statistically association ($p<0.05$) with the risk of T1DM occurrence in Iraqi patients.

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