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The association of *TNF-a* Gene Polymorphims with the Incidence of Diabetes Mellitus in Adult Patients

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Abstract: Diabetes mellitus (DM) are defined as the most chronic metabolic disorder resulted from a complex interaction between environmental and heredity factors, Diabetes mellitus type 2 is Previously known as non-insulin dependent DM, the most common kind of diabetes is characterized by hyperglycemia, insulin resistance, and a relative lack of insulin. The *TNF- a* gene variant (rs 1800629) has been associated with an increased risk of developing diabetes. The aim of study is a relation between the polymorphisms in the *TNF* gene and the prevalence of type 2 diabetic mellitus (T2DM) in the Iraqis as a whole. Having 50 patients with diabetes and 50 subjects who appeared to be in good health as controls, The DNA was extracted and then quantification using Nanodrop,the integrity was exam through agarose gel electrophoresis were enrolled genotyping of *TNF- a* gene SNPs (rs1799724 and rs1800629) that determined by using High Resolution Melting (HRM) ,The results showed there were an increase in serum levels of Triglyceride and VLDL in DMT2 patients compared with apparently healthy subject .the result is found that no relation was found between rs1799724 SNP of *TNF-a* gene with the incidence of DMT2 in Iraqi patients .No effected of rs1799724 SNP of *TNF-a* gene on Lipid profile parameters . It was concluded that related with rs1800629 SNP of TNF-α ,GG genotype represent as a protective factor against DMT2 in incidence whereas ,GA genotype represent as a risk factor for DMT2 incidence .serum triglyceride and serum VLDL levels were affected by the polymorphism rs1800629 of *TNF-a.*

Keyword: Diabetic mellitus type 2, genetic polymorphism, TNF-α.

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Introduction

As a result of a lacking insulin, using insulin incorrectly, or both the varied illnesses that make up diabetes mellitus frequently appear with periods of hyperglycaemia and glucose intolerance. (1). In 2019, diabetes was the direct cause of 1.5 million deaths and 48% of all deaths due to diabetes.

By 2030, the number will be double and diabetes mellitus especially type 2 occurs all around the world, but in more developed nations, the rate is more prevalent. Ranging from 85-90% of all Diabetes mellitus cases. Increases in type 2 diabetes risk factors, particularly being overweight or obese, are substantially responsible for rising diabetes prevalence rates (2). With a frequency of 9.2%, the Middle East and North Africa (MENA) area possesses the second-highest diabetes prevalence.

The prevalence of diabetes is predicted to rise by 110% in the MENA region between 2017 and 2045, reaching 629 million people globally. Diabetes affects 1.4 million Iraqis. International Diabetes Federation (IDF) age-adjusted prevalence of T2DM in Iraq ranged from 8.5% to 13.9% (4). Researchers have used "Genome Scans" and "Association studies" in addition to

other techniques to look into the genetics of T2DM. Case-control studies that investigate associations include Genome Wide Association (GWA) studies, which investigate the relationship between a given disease status and a certain allele, Haplotype, genotype, or most frequently, a collection of single-nucleotide polymorphisms (SNPs). The GWAS, which were successfully carried out for the first time in 2005, are a significant tool for identifying various biological pathways, comprehending the pathophysiology of several complicated (multifactorial) disorders, and creating pharmacological therapies (5). Various cell types are affected in pleiotropic ways by the cytokine tumor necrosis factor alpha (TNF-α). It has been noted to be a significant regulator of inflammatory responses and to play a role in the pathophysiology of various inflammatory and autoimmune illnesses (6). Numerous SNPs have been identified in the human TNF- gene promoter region, with the potential to change the structural makeup of regulatory regions and impair the function and regulation of TNF synthesis. Given that the gene is located within the MHC region, it is now more likely than ever that SNPs at this locus will have a role in the emergence of a variety of illnesses, including T2DM (7) .In fact, there have been no studies on the association of -308 (rs1800629) of Iraqi people's TNF- gene and T2DM.

Materials and methods

Blood samples were taken from 50 T2DM patients and 50 seemingly healthy controls. The T2DM patients were selected from National center of teaching laboriteres (NCTL) in medical city. Patients who had been diagnosed with T2DM by doctors, WHO recommendations served as the basis for the diagnostic standards for diabetes. In addition to showing signs of diabetes, an individual was deemed to have diabetes if their fasting blood sugar level was above 126 mg/dl. (8).Blood samples were taken from T2DM patients and seemingly healthy controls by vein puncture and placed in EDTA anticoagulated tubes for peripheral blood samples of T2DM and control groups. EDTA anticoagulant tubes containing 2 ml of blood were then freezing in order to be used as the source for DNA extraction. For biochemical investigation, 3 ml were kept. The HbA1c done by using full automated TOSH G8, the lipid profile and fasting blood sugar were done by Aboot C4000. Total genomic DNA was obtained using *EasyPure*®Genomic DNA Kit (TransGen, biotech EE121**)** whole fresh blood that was taken for the procedure. Nanodrop (200C apparatus, Thermo Scientific, USA) was used to assess the purity and concentration of DNA samples. DNA purity levels should be between 1.7 and 1.9..the sequence of primers used for SNPs sequence were from the bioinformatic programs (NCBI) show in (Table 1).

Table (1): The study's designed primers

Primer	Sequence(5′→3′ direction)
	TNF-α (SNP Genotyping) rs 1799724
Forward	TGTCCAGGGCTATGGAAGTC
Reverse	CTGGAGGCTCTTTCACTCC
	TNF-α (SNP Genotyping rs) rs 1800629
Forward	ACCTGGTCCCCAAAAGAAAT
Reverse	TTTGTGTGTAGGACCCTGGAG

A rotor gene Q Real-time PCR System (QIAGEN) was used to perform qPCR-HRM, an HRM analysis with a 0.2 °C scale from 55 to 95 °C follows. *2xTransStart*®Tip Green qPCR Super Mix Synthetic SNP sequences were evaluated using duplicates. To identify allelic differences, qPCR-HRM was used on triplicate synthetic. Controls, and normalized melting curves (NMC) and differential curves (DC) were constructed using the HRM Tool included in the integrated program (Rotor gene 4.4). (Table 2) The HRM SNPs experiment uses quantitative real-time PCR components.

Table (2): The HRM SNPs experiment uses quantitative real-time PCR components

Components	20 µl rxn
***2xTransStart*®Tip Green qPCR Super Mix**	10
Nuclease free water	5
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
DNA	3

The Statistical Analysis System-SAS (2018) program was utilized. To significantly compare between means, the T-test was utilized. Chi-square test was used to compare percentages (0.05 and 0.01 likelihood) in a significant way. The study's estimated odds ratio and confidence interval.The means of the groups under study were compared using an independent samples t-test. Utilizing Hardy-Weinberg equilibrium (HWE), allele and genotype frequencies were studied (9).

Results and discussion

Subject data

A total of 100 blood samples were taken from type 2 diabetic patients (n = 50) and 50 seemingly healthy volunteers as controls, according to data on various parameters gleaned from the questionnaires that patients filled out .The percentages of male to female were (46%:54%), (48%:52%) for apparently healthy subjects (control), patients with T2DM respectively summarized in (Table 3).

Table (3): The distribution of the basic characteristics of subjects in both study groups

Criteria		Control	T2DM	p-value
Age (years)	<40	17 (34%)	10 (20%	NS
	50-40	25 (50%)	19 (38%	NS
	>50	8 (16%)	21(42%)	0.0326 *
Gender	Male	23(46%)	24 (48%	NS
	Female	27 (54)	26(52%)	NS
Family history	Yes	8 (16%)	40(90%)	0.007 **
	No	42 (84%)	5 (10%)	0.003 **
Body mass index	18.5 – 25	15 (30%)	12(24%)	NS
	>30	35 (70%)	38(76%)	NS

***P<0.05 , **P<0.01**

Biochemical characteristics

The biochemical characteristics of the T2DM patients and apparently healthy subjects (control) are summarized in (Table 4). Serum cholesterol levels were unaffected by diabetes mellitus type 2 in the present study ,cholesterol level in blood is based on other risk factors such as age, gender, family history, smoking, high blood pressure physical inactivity obesity (10). The serum levels of triglyceride and VLDL were significantly increased in DMT2

patients versus apparently healthy subjects (191.26 ±13.09 versus 126.48 ±11.85; 38.25 ±2.62 versus 25.29 ±2.37 and this result is agreement with (11).

Table (4): Comparison between control and patients groups according to the selected Lipid profile Mean ± SE

Group	Serum lipid profile				
	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	VLDL (mg/dl)	LDL (mg/dl)
Control	173.52 ±5.96	126.48 ±11.85	38.78 ±0.94	25.29 ±2.37	109.44 ±5.74
Patients	189.12 ±6.24	191.26 ±13.09	36.08 ±0.99	38.25 ±2.62	115.09 ±5.81
T-test	17.144 NS	35.056 **	2.718 *	7.011 **	16.22 NS
P-value	0.074	0.0004	0.050	0.0004	0.491

*** (P≤0.05), ** (P≤0.01).**

The blood sugar and HbA1c significantly increased in patien when compared with healthy individual (Table 5) this result agreement with(12).

Table (5): Serum FBS and HbA1c in patients with Type 2 Diabetes Mellitus and control subject (mean ± SE)

Group	Mean ± SE	
	HbA1c (%)	F.B.S.
Control	5.23 ±0.07	93.08 ±1.47
Patients	9.82 ±0.29	254.28 ±11.54
T-test	0.589 **	23.091 **
P-value	0.0001	0.0001

**** (P≤0.01).**

DNA concentration and purity

The lysis of red blood cells, removal of undesired impurities including protein and RNA, and destruction within the white blood cell membrane are all steps in the DNA extraction process. The quantification of DNA by Nanodrop These results revealed that the frozen blood samples yielded enough DNA concentration (45-58 ng/µl) , while the DNA purity (1.7-2). The DNA integrity was exame through agarose gel electrophoresis. DNA it appeared as a single sharp band when visualized under UV light next ethidium bromide stain (Figure 1).

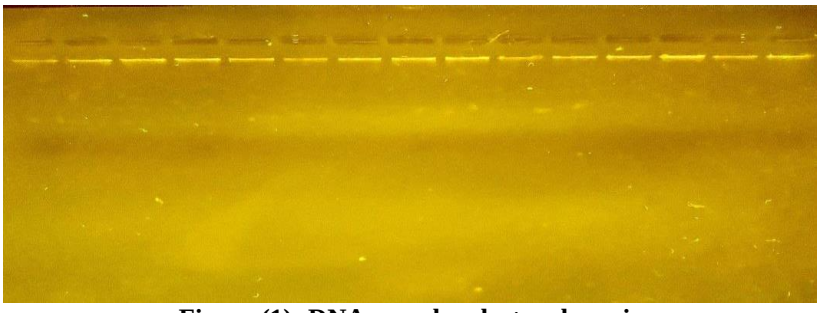

Figure (1): DNA samples electrophoresis

The TNF-α Gene polymorphism rs 1799724

The genotype and allele frequency distributions of -857 (1799724) are shown in (Table 6), (Figure 2). No relation was found between rs1799724 SNP of *TNF-a* gene with the incidence of DMT2 in Iraqi patients. No effect of rs1799724 SNP of *TNF-a* on lipid profile parameters (Table 7).

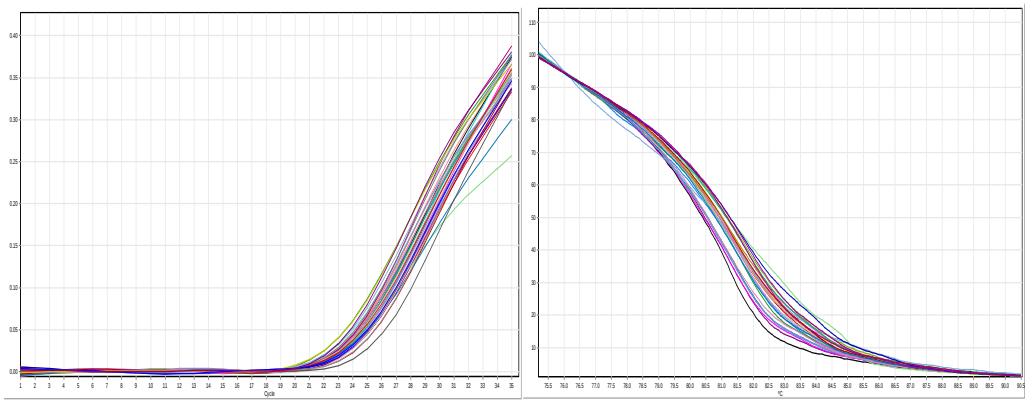

Figure (2): The result output of HRM for the three genotype in *TNF-a* SNP (rs 1799724).

Table (6): Distribution of sample study according to genotype of SNP1 (rs 1799724) in control and patients groups

Genotype	Control	Patients	$\chi 2$	P-value	O.R. (C.I.)
CC	28 (56.00%)	26 (52.00%)	0.074 NS	0.785	Ref. =1
CT	15(30.00%)	17 (34.00)	0.125 NS	0.723	0.31 (0.22-0.71)
TT	7 (14.00%)	7 (14.00)	0.00 NS	1.00 NS	0.05 (0.02-0.19)
Total	50	50	--	--	--
C	0.71	0.69	P-value =0.833 NS		
T	0.29	0.31`	P-value =0.902 NS		

NS: Non-Significant.

Table (7): Effect of gene polymorphism rs 1799724 in study parameters of patients

Parameters	Genotype			p-value
	CC	CT	TT	
Cholesterol (mg/dl)	189.7 ±11.55	187.7 ±12.03	194.5 ±12.64	0.764 NS
Triglyceride(mg/dl)	192.7 ±12.40	184.8 ±13.45	190.4 ±12.04	0.673 NS
HDL(mg/dl)	36.0 ±2.04	36.3 ±1.97	35.6 ±1.85	0.861 NS
VLDL(mg/dl)	38.5 ±2.85	36.9 ±1.98	38.0 ±2.06	0.704 NS
LDL(mg/dl)	115.5 ±6.02	114.4 ±4.38	120.8 ±7.02	0.658 NS

*** NS:Non-significant**

TNF-a Gene polymorphism (rs 1800629)

Tumor nerosis factor alpha (rs 1800629) single nucleotide polymorphism (SNP) G/A located on the upstream of the gene promoter at -308. Genotype and allele frequency percentage of rs1800629 (G/A) in *TNF alpha* in versus apparently healthy subject are presented in (Table 8), (Figure 3). As shown GG (wild) genotype frequency in control significantly ((P≤0.05), higher than in appartently patients (72% versus 36%, $\chi 2$ =6, O.R =1, respectively).Also the frequency of GA (heterozygous) genotype was significantly (P<0.01) higher in T2DM patients when compared with healthy control (46 versus 16 %, respectively: $\chi 2$ =7.258, OR=1.63).there was no significant difference were noted between T2DM patients and control as related with AA (mutant) genotype .according to these study GG genotype represent as a protective factor against DMT2 incidence whereas, heterozygous GA genotype represent to be a risk factor for diabetes mellitus type 2 incidence (46 versus 16 % ,$\chi 2$ =7.258,OR=1.63,

for T2DM and control, respectively). The results of present study support the association between genetic variation of *TNF-a* SNP rs1800629 polymorphism and increased risk of T2DM in Iraq population .genotype GA significantly associated as their wild allele (G) and risk variant allele A. The wild genotype GG act as protective factor from diabetic type 2. serum triglyceride and serum VLDL levels were affected by the polymorphism rs1800629 of *TNF-a* (Table 9).

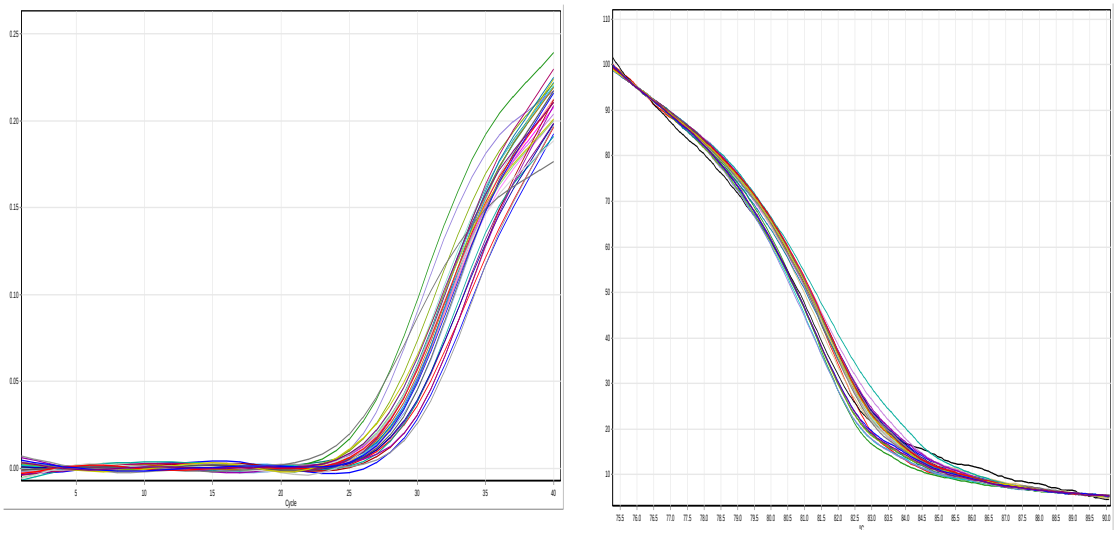

Figure (3): The result output of HRM for the three genotype in *TNF-a* SNP (rs1800629)

Table (8): Distribution of sample study according to genotype of SNP2 (rs 1800629) in control and patients groups

Genotype	Control	Patients	$\chi 2$	P-value	O.R	(C.I.)
GG	36 (72.00%)	18 (36.00%)	6.00 *	0.0143	-	-
GA	8 (16.00%)	23 (46.00)	7.258 **	0.0071	1.63	(0.91-3.27)
AA	6 (12.00%)	9 (18.00)	0.60 NS	0.438	0.501	(0.32-1.06)
Total	50	50	--	--	--	--
G	0.80	0.59				
A	0.20	0.41				

*** (P≤0.05).** (P≤0.01).**

Result of the present study agreement with Chinese study reported the polymorphism of *TNF-a* gene. The findings of the meta-analysis indicated that the *TNF-a* 308G>A polymorphism was strongly related with the likelihood of developing type 2 diabetes, and that the A allele at this locus may be a susceptibility allele in the Han Chinese population(13).

Table (9): Effect of gene polymorphism rs 1800629 in study parameters of patients

Parameters	Genotype			p-value
	GG	GA	AA	
Cholesterol (mg/dl)	179.0 ±14.82	193.1 ±11.66	194.8 ±13.07	0.184 NS
Triglyceride (mg/dl)	167.8 ±11.45b	218.5 ±16.75a	173.8 ±12.92b	0.047 *
HDL (mg/dl)	37.2 ±2.08	34.3 ±2.36	35.9 ±2.51	0.551 NS
VLDL (mg/dl)	33.5 ±2.44b	43.2 ±2.61a	34.7 ±2.56b	0.045 *
LDL (mg/dl)	111.9 ±7.51	113.3 ±8.64	125.8 ±8.13	0.094 NS

Conclusion

The carriers of GA genotype of rs 1800629 SNP of *TNF-a* are more exposure to DMT2 in Iraqi patients.

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