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Assessment of insulin receptor substrate-1 (G972R) SNP and correlated with total antioxidant capacity in T2DM patients

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Abstract
Background and Aim: The prevalence and incidence of Type 2 Diabetes Mellitus (T2DM) have significantly increased globally in the past few years, around two to three times each. A lower age of onset is caused by a number of risk factors, such as female sex, maternal diabetes or gestational diabetes, ethnicity, family history, and extreme obesity. The aim of the present research to assess levels of Malondialdehyde (MDA) and Total Antioxidant Capacity (TAOC) as a markers of the oxidative stress in Iraqi patients diagnosed with Type 2 Diabetes Mellitus (T2DM) and correlate with G972R SNP of ISR-1 gene.
Methods: The Babylon province was the site of this research in the period from September 1, 2023, to March 30, 2024. There were 90 subjects in all for the study: Relatives of hospital patients and hospital staff without type 2 diabetes were used to enroll 50 patients with the disease and 40 healthy controls. The two research cohorts were matched for age. The following parameters were ascertained by analyzing blood samples from both groups: Glycated Hemoglobin (HbA1c), Total Antioxidant Capacity (TAOC), and malondialdehyde (MDA) as a measure of oxidative stress. PCR-RFLP used for genotyping analysis of G972R SNP.
Results: Compared to the control group, the T2DM group's mean serum TAOC was considerably lower (32.21±3.12 U/ml vs. 52.22±4.76 U/ml). This suggests that there is a very substantial difference between the groups. Furthermore, TAOC and HbA1c showed statistically significant inverse relationships in the T2DM group. The RR genotype creates 212, 31 bp bands (Which did not appear on our gel), the GG genotype produces just 243 bp bands, and the GR genotype produces 243, 212, 31 bp bands, according to the results of the G972R SNP analysis using the restriction enzyme *MvaI*.
Conclusion: This research shown strong correlation between increase oxidative stress and lowering of TAOC with the incidence of T2DM in Iraqi population through the significant alteration in G972R SNP of ISR-1 gene.

Keywords: T2DM, G972R SNP, ISR-1, antioxidant, oxidative stress

Introduction
The pathophysiology of Type 2 Diabetes Mellitus (T2DM), the most common metabolic disease, is influenced by insulin resistance and reduced pancreatic β cell insulin secretory capacity.When genetic predisposition and environmental factors like age, food, and physical activity come together, diabetes develops [1]. A worldwide epidemic of diabetes mellitus has been announced [2]. According to data from 2005, According to estimates from the World Health Organisation (WHO), more than 180 million people globally have diabetes [3]. Without action, this number is probably going to more than double by 2030. In 1995, the WHO estimated that 2.4% of Myanmar's population had diabetes mellitus, and by 2025, that number is expected to rise to 3.2% [4]. An important role in the insulin signaling pathway is played by insulin receptor substrate-1 (IRS-1) [5]. An insulin signalling absence could result from a failure in IRS-1 function because it is the first substrate in this cascade [6]. Therefore, genetic alterations in IRS-1, the most prevalent of which is a glycine to arginine mutation at codon 972 (G972R), may potentially aid in the development of insulin resistance [7]. People with Type 2 Diabetes Mellitus (T2DM) were more likely to have the IRS-1 (G972R) polymorphism, particularly if they were obese [8]. Throughout studies, the polymorphism's prevalence fluctuates, most likely due to genetic, racial, and ethnic differences [9]. Additionally, there are contradictory results regarding the IRS-1 (G972R) polymorphisms with blood glucose control, fasting plasma insulin, and insulin resistance. In non-obese patients, there was no difference in insulin sensitivity or pancreatic β-cell activity between

carriers and wild type individuals; however, in obese patients, these differences were substantial [10]. Since genetic data on the frequency of IRS-1 (G972R) polymorphism in Iraq may vary from different areas, this is a preliminary examination. The current study looked at the incidence of polymorphism and its association with total antioxidant capacity.

Materials and Methods

Study design

This investigation was carried out at Babylon University's College of Medicine's Biochemistry Department laboratory. The timing of the sample collection was from September 1, 2023, to March 30, 2024.The purpose of the questionnaires was to gather data from the control and patient groups.

Fifty patients with Type 2 Diabetes (T2D) (20F and 30 M) whom were not using any anti-diabetic medications participated in the study. The mean ± Standard Deviation (SD) was 51±4.23 years, while the patient's ages ranged from 41 to 59 years.

All of these groups' samples had medical diagnoses. They were gathered from Hilla City's Merjan Teaching Hospital. Forty subjects (17 F and 23M) were taken as control groups (CON) without any diabetic history and the mean ±SD of age was (49±3.77) years and the range of (37-52) years.

FBG, HbA1c, and TAOC analysis

Both control and patient individuals had their venous blood samples taken while they were seated using a disposable syringe (5 ml). Subjects who are fasting will be asked to come for a blood sample. Each individual had a vein punctured to get seven milliliters of blood; 2.5 milliliters were placed in EDTA tubes, and the other 4.5 milliliters were gradually poured into disposable tubes filled with deciding gel. The blood in the EDTA tubes was utilized to calculate the FBG, HbA1c, and TAOC, and the rest was deep-frozen at -40 °C to be used in the study's genetic component later.

TAOC were assessed by using of spectrophotometric assay depending on the manufacture protocol provided with kit.

Genetic analysis for IRS-1 (G972R) SNP

Whole blood, frozen blood, and buffy coat are just a few of the sources from which complete DNA can be easily and quickly extracted with a genomic DNA extraction kit. Procedure of DNA extraction and purification was done depending on steps that mention in the protocol of DNA extraction kit. The final stage in any DNA extraction process is to use a nanodrop to measure the concentration of DNA and assess its quality. As illustrated in figure (1), absorbance measurements are taken at 260 nm.

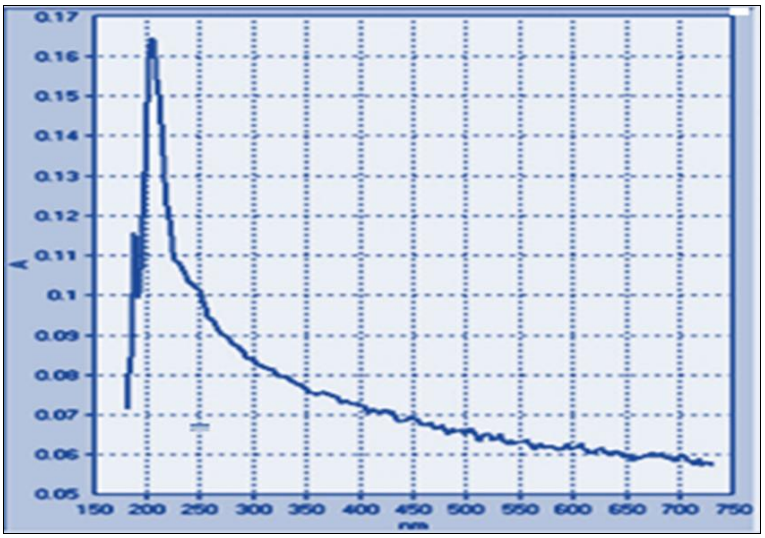

Fig 1: DNA purity measurement using a scan drop device

Using the agarose gel electrophoresis technique, the presence of DNA isolated by the earlier process was found. DNA fragments are separated, identified, and purified using this conventional technique. In accordance with primer sequences that amplify the polymorphism area of the insulin substrate-1 gene primers were employed a collection of primers that includes reverse primer was 5´-AGGACGGTGCGGTGAGAGTG-3´, and the forward primer was 5'-TTTGGGAGTGATCAGTCTGGCTAC-3'.

For visualize agarose gel, the UV transilluminator with a gel documentation unit was used as seen in figure (2), the gel was exposed to UV light with a wavelength of 260 nm and a canon digital camera was used to take pictures.

Fig 2: The genomic DNA pattern that extracted from whole blood

Insulin Receptor Substrate-1 (IRS-1) genotyping (G972R) determination

In the first step, PCR optimization was carried out using a gradient temperature that ranged from 50 to 64 degrees Celsius with 0.3 step changes in PCR wells. Finding the ideal annealing temperature required careful consideration of this. 20 µl of the overall reaction volume contained a PCR reaction mixture that contained 5-50 ng of template DNA, 250 mM of each dNTP, 5 µl of buffer, 1 U Taq DNA polymerase enzyme (Bioneer), 10 pmol of each primer, and 1.5 mM MgCl2. Following the determination of the optimal annealing temperature of 53Co, this was carried out.

Once the optimal annealing temperature was determined, the thermocycler was set up to amplify the target DNA fragments as shown in Table 1.

Table 1: The amplification protocol of IRS-1 genotyping analysis

Stages	Temperature (C°)	Time	Function	Cycles
1	95	5 min	Initial denaturation	
2	95	45 sec	DNA denaturation	35
	60.8	1 min	Primer annealing	
		45 sec	Template elongation	
3	72	7 min	Final elongation	
4	4	-	Incubation	Hold

For genotyping based on restriction endonuclase cleavage, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is utilized. Introduce the method of single nucleotide polymorphism. The region around the restriction enzyme site is amplified by PCR, and the result is then digested by the appropriate enzyme for genotyping. This procedure entailed preparing 5 units of the restriction enzyme MvaI and 10 µl of each PCR result (Containing 243 fragments) in the buffer recommended by the manufacturer for three hours at 25 °C. Digested amplified DNA fragments were electrophoresed on 1.5% agarose, stained with EtBr, and then exposed to UV light to view the bands.

Results

Table (4) shows the attributes of T2DM group and CON group. The mean age difference between the patients in both groups and the female control is not statistically significant (p>0.05). In comparison to control subjects, there was a significant increase in BMI (p<0.05) T2DM group.

Table 4: The clinical parameters of study groups

Parameter	Patients			p-value
		Mean ± SD	Rang	
NO.	T2DM	50	-	-
	CON	40		
Age (year)	T2DM	51 ± 4.23	41-59	> 0.05ss
	CON	49±3.77	37-52	> 0.05
BMI (kg/m^2)	T2DM	27.44±2.09	20- 34	< 0.05*
	CON	24.38±2.85	19-38	< 0.05*
MDA (ng/ml)	T2DM	33.8 ± 4.29	24-40	< 0.001**
	CON	12.3 ± 3.23	9-15	< 0.001**
TAOC(U/ml)	T2DM	32.21±3.12	22- 40	< 0.001**
	CON	52.22±4.76	40-66	< 0.001**
HbA1c (%)	T2DM	9.01±1.22	6.5- 11.7	< 0.001**
	CON	5.21±0.99	4.6-6.1	< 0.001**

In order to find polymorphisms in the IRS-1 G972R (rs 1801278) gene, restriction endonuclease enzymes, such as *MvaI*, target a particular sequence of the amplified DNA product. Previous research using the restriction enzyme *MvaI* has shown that the RR genotype produces 212, 31 bp bands (Which did not show up on our gel), the GG genotype produces just 243 bp bands, and the GR genotype produces 243, 212, 31 bp bands (Table 5).

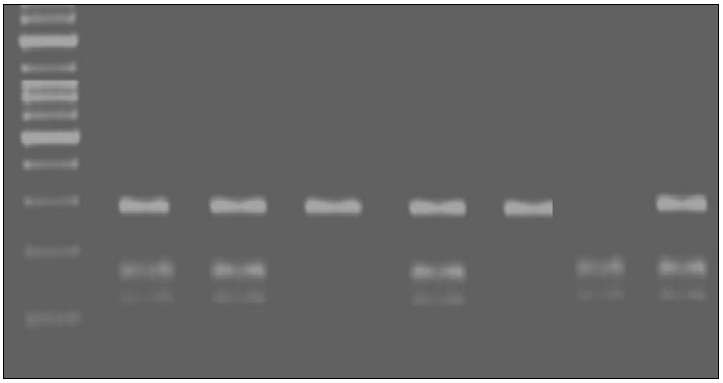

Fig 3: PCR-RFLP gel electrophoresis analysis of IRS-1 SNP. Lane M: Ladder (DNA marker), Lane 1,4 represented of GR genotype, lane 2,3,6,7 represented of GG genotype, and RR genotypes

Table 5: IRS-1 SNP with allele frequency

Group	No.	Genotype			Allele frequency	
		GG (%)	GR (%)	RR (%)	G	R
T2DM	50	31 (62)	12(24)	7(14)	82%	18%
CON	40	27(67.5)	10(25)	3(7.5)	57%	43%
Total	90	58	22	10		

Discussion

T2DM accounts for about 90% of the total number of cases. In T2DM, there is a blunted response to insulin, and it is called insulin resistance. The insulin becomes inert in this condition, which, as discussed above, first leads to compensatory production of insulin that succeeds in maintaining the glucose levels, but then over a time period production diminishes to yield T2DM. This condition predominantly results in T2DM as seen commonly above the age of 45 years [12]. The current study's findings that T2DM patients had higher oxidative stress and lower TAOC than controls supported previous research showing that people with T2DM had lower total antioxidant capacity and higher oxidative stress [13-15]. In Iraq, the prevalence of diabetes has significantly increased over the last ten years, rising from 19.6 cases per 1000 people in 2000 to 42.3 cases per 1000 people in 2015, a 115% increase (16 Insulin receptors substrate-1 (IRS-1) is essential for insulin signalling. Because it hinders insulin production and results in insulin resistance, the glycine to arginine replacement (GGG ↔ AGG substitutions) at codon 972 (G972R) (rs1801278), a frequent polymorphism of the IRS-1 gene, may be harmful in the progression of type-2 diabetic mellitus (T2 DM) [17]. Numerous risk factors, both changeable and unchangeable, are known to contribute to the early-phenotype presentation of illness. The increasing number of children and adolescents who are overweight or obese appears to be the most important of these. Furthermore, younger T2DM patients are far more likely than older ones to have ectopic adiposity, especially in the liver, which appears to be an important contributor to insulin resistance in this population [18]. The results of this research highlight how challenging it is to determine how much 'low-frequency-low-risk' variations contribute to the risk of type 2 diabetes. Approximately 200,000 study participants would be required to have 80% power to detect a 9% increase in diabetes risk at a genome-wide significance level in the particular context of the R972 mutation. In these situations, a technique that aims to improve the definition of the outcome and reduce its variability may significantly improve our capacity to identify genetic influences, which would reduce the size of the sample that is needed. This findings imply that one component of such a strategy would be concentrating on early-onset diabetes, which is distinguished by a deeper genetic foundation.

Conclusion

The association between oxidative stress, lifestyle, and diabetes mellitus is highlighted by low TAOC, which may be a potential predictor of an increased incidence of T2DM patients. The study found that compared to healthy controls, patients with T2DM had decreased levels of TAOC.

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