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Correlation Between Random Blood Glucose, Glycated Hemoglobin Levels, and the Likelihood of Developing Type 2 Diabetes Mellitus in Connection to the Micro RNA-423 Rs6505162 C>A Gene Point Mutation

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ABSTRACT
Background: Diabetes Mellitus (DM) is a metabolic disease characterized by elevated blood sugar levels. Type 1 DM and Type 2 DM are the two categories based on the principles of causation. A short non-coding RNA molecule of between 21 and 25 nucleotides is called microRNA, or miRNA. By attaching itself to messenger RNA (mRNA) molecules and either preventing or accelerating their expression, it plays a critical part in the control of gene expression. **Material and Method**: Genomic DNA from 192 samples of whole blood obtained from the vein included (96 individuals diagnosed with diabetic type 2 and 96 healthy individuals as control). Tetra Amplification Refractory Mutation System (T-ARMS)-polymerase chain reaction (PCR) was used to amplifyMIR-423 rs6505162 C>A gene. Anti-Gad antibodies were detected by using of the Enzyme-linked Immunosorbent assay (ELISA) as indicator to distinguishing test between Type1 and Type IIdiabetes. Random Blood glucose levels (R.B.S) and the level of glycated hemoglobin (HbA1c) have been conducted.In the current study, the relationship between type 2 and MicroRNA-423(miRNA-23) gene variation at position rs6505162 C>A and R.B.S was studied.**Result:**Presentfindings revealed that there was a statistically significant difference in genotype distribution of the MIR-423 rs6505162 C>A gene and T2DM patients compered to controls (P<0.001).Concerning the MIR-423 genotypes and the biochemical markers HbA1c statistically significant link was found.Conversely, no statistically significant correlation was seen between MIR-423rs6505162 C>A genotypes distribution and R.B.S level.

I. INTRODUCTION

Diabetes is a severe chronic illness that develops over time as a result of either insufficient or non-useable insulin by the body. It is a metabolic ailment. An estimated 537 million adults globally, or 10.5% of all adults in this age range, suffer from diabetes. Globally, the number of persons with diabetes will rise from 643 million in 2030 to 783 million in 2045 (ArvindKumaret al., 2023).In individuals with diabetes mellitus, chronic hyperglycemia can exacerbate other metabolic abnormalities and harm multiple organ systems. This can result in life-threatening and incapacitating health complications, the most common being microvascular retinopathy, nephropathy, and macro-vascular (a 2- to 4-fold increased risk of cardiovascular diseases). Type 2 diabetes mellitus accounts for around 90% of all cases of diabetes. In DM type 2 the response to insulin is diminished, and this is defined as insulin resistance (Melanie et al., 2022 and Arvind Kumar et al., 2023). During this state, insulin is ineffective and is initially countered by an increase in insulin production to maintain glucose homeostasis, but over time, insulin production decreases, resulting in T2DM. Diabetes mellitus is most commonly seen in persons older than 45 years. Still, it is increasingly seen in children, adolescents, and younger adults due to rising levels of obesity, physical inactivity, and energy-dense diets (Choiet al., 2016). miRNA-423 is a type of microRNAs that has been studied in relation to type 2 diabetes mellitus. They have been implicated in various biological processes, including glucose and lipid metabolism, and are therefore of interest in T2DM research (Alex Caria et al., 2018). In 2019 reported that miR‑423 levels were significantly decreased in cases with proliferative diabetic retinopathy (Blum, et al. 2019). The inhibition and/or impairment of miRNA-423 production levels, decreases gluconeogenesis, reduce insulin resistance and decreases blood glucose The anti-GAD (glutamic acid decarboxylase) testis performed when it is necessary to differentiate between type I and type II diabetes, when gestational diabetes is detected in pregnant women, when the risk of congenital

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diabetes must be assessed(Aisha Al Senani et al., 2018).

II. MATERIALS & METHODS

2.1 Study Design

Current study conducted on 192 samples were divided into two equal groups. One of which included 96 samples that is represented the control group (healthy individuals, and the other group included persons with diabetes mellitus type 2. All samples were collected from AL_Diwaniyah Teaching Hospital. The recruitment period of the patients and controls was from October 2022 to January 2023Prior to the collection of samples from any patients or control individuals, informed permission was acquired. The Scientific Research Ethics Committee of the Diwaniyah Health Department, the Training and Human Development Department, and the Knowledge Management and Research Division granted ethical approval.

2.2 Inclusion and Exclusion criteria

All of the 192 the subjects that included in current study were citizens. 96 of them (cases group) clinically confirmed with T2DM (both males and females), all of the cases group were have been random plasma glucose levels >150 and the level of glycated hemoglobin (HbA1c) was>9.2% on the day the blood sample is taken. All of the individuals who were classified as diabetes patients used either oral hypoglycemic medications or insulin injections in addition to having random blood glucose (R.B.S) readings more than 300 mg/dl.Patients with type 2 diabetes who also had other major chronic conditions such liver cirrhosis, renal failure, or cancer were not allowed to participate in the trial. Those with type 1 diabetes were not allowed to participate in the trial.

2.3 Patient's selection and blood sample collection

Total 192 study subjects were included in present study. Out of 192 study subjects, 96 were T2DM and 92 healthy subjects. Three mL of blood sample were drawn in EDTA vials only after the informed consent being obtained from patients and healthy subjects. All various variables like HbA1c, R.B.S, and Anti-Gad antibody test, age, weight, and sex, case history, and duration of T2DM were among the many characteristics that were evaluated from the T2DM patients and controls. The biochemical parameters were measured in accordance with accepted practices.

2.4 Anti-Gad analysis

All reagents, standard solutions and samples as instructed were prepared. All reagents werebrought to room temperature before use and the analysis performed as per the manufacturer's instructions. The assay was performed at room temperature. The mean of two or three readings was used to determine whether a sample is positive or negative for Human Anti-Glutamic Acid Decarboxylase. This value is calculated by comparing the sample to a control wells and using the formula: cutoff value.

- Cutoff Value = average Negative Control value + 0.15
- While ODsample < Cutoff Value: Negative
- While ODsample ≥Cutoff Value: Positive

2.5 DNA Extraction

Tow mL of blood samples were processed for all cases that detected as a diabetes mellitus type 2. By RBCs lysis buffer for lysing red blood cells and the DNeasy Blood K (FAVOURGEN) was used to extract genomic DNA in accordance with the manufacturer's instructions. Before being used, the isolated DNA was dissolved in nuclease-free water and kept at 4˚C.

2.6 Genotyping of MIR423 (Micro RNA-423)

Extracted DNA was used to study theMIR423 genepolymorphism at rs6505162C>Tand it's correlation with type2 diabetes byusing of T-ARMS-PCR primers method. Primers for MIR423gene atrs6505162 C>Awere designed by (Mohammad Muzaffar et al., 2022). All primers were depicted in (Table 1). After protocol standardization, the programmer was set for amplification at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycle, final extension for 5 minutes at 72°C respectively.

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Table 1: Set of ARMS primers used for MIR423 rs6505162C>Aamplification (Mohammad Muzaffar et al., 2022)

Gene	Primers	Amplicon size	Tem C^0
MIR423 rs6505162 C>A	**OF**: 5'-TTTTCCCGGATGGAAGCCCGAAGTTTGA-3'	**336**	**62 C^0**
	OR: 5'-TTTTGCGGCAACGTATACCCCAATTTCC-3'		
	Allele C: 5'-TGAGGCCCCTCAGTCTTGCTTCCCAA-3'	**160**	
	Allele T:5'-CAAGCGGGGAGAAACTCAAGCGCGAGG-3'	**228**	

2.7 Statistical analysis

Statistical analysis was performed using IBM SPSS 23.0 (NY, USA). Genotype and allele frequency were analyzed by PopGen32, version 1.31 (Yeh & Yang, 1999). In this study, the Chi-square or Fisher's exact tests were used to determine whether there were significant differences in frequencies between this sample of the Iraqi population and other populations of other studies. P-value ≤ 0.05 was considered statistically significant. To evaluate the genotype distributions consistency with Hardy–Weinberg equilibrium, the chi-squared test (χ^2) was used (Rodriguez et al., 2009).

III. RESULTS

3.1 Molecular study

Polymerase chain reaction (PCR) products were seen on an Analytic Jena, Inc. UV trans illuminator after being separated by using of a 2% agarose gel and stained with 2 µl of RED Safe dye (Thermo Fisher Scientific, Inc.). The thermos-cycling conditions procedure that used in present experiment wereaccording to Mohammad and his team in 2022. Primers FO and RO flank the exon of the, 423 rs6505162 C>A gene resulting in primers FO and RO band of 336 bp, and primers IR 160 amplify a wild-type allele (C allele), generating and Forward mutant amplify (A allele) generate a band of 228 bp. The results are depicted in Fig. 1.

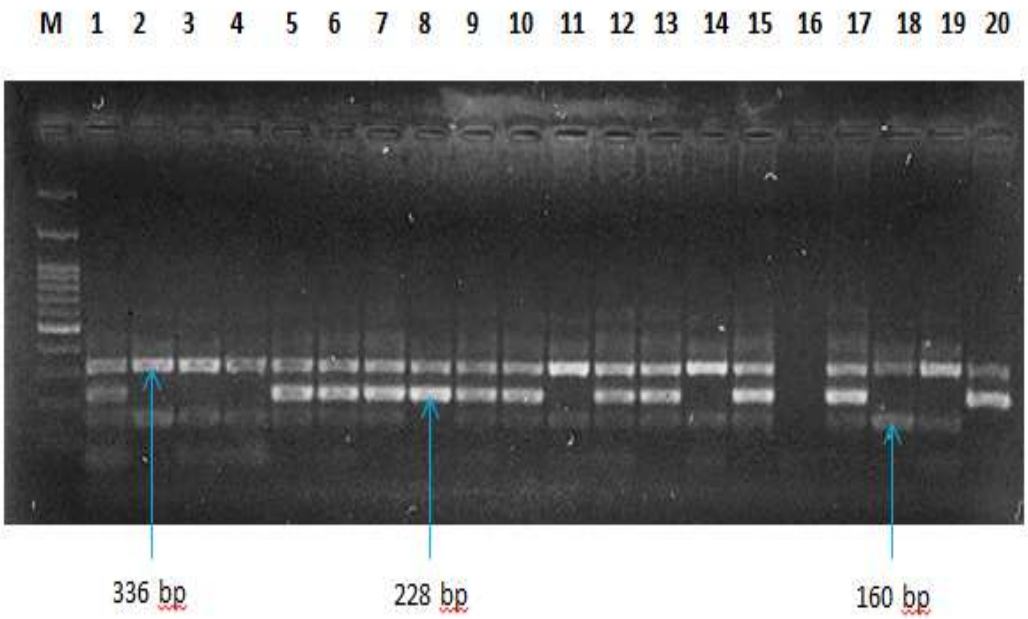

Figure .1 Detection of MIR--423 rs6505162 C>A gene polymorphism by T-ARMS-PCR in T2DM patients. Lane M, marker 100-bp DNA ladder, lanes 8, and 20 represented wild type genotypes 228 bp (C/C) lanes, 2,3,4,11,14,18 and 19 represented mutant.

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3.2 MIR-423 rs6505162 C>A gene variation with type2 diabetes mellitus

The findings show that the MIR-423 rs6505162 C>A genotype distribution differed significantly (P<0.001) between T2DM patients and controls. In patients, the frequency of CC, CA, and AA genotypes was 30, 46.7, and 23.3%, whereas in controls, it was 27.8, 55.5, and 16.7%. Compared to diabetes patients, healthy controls had a greater prevalence of the C allele (55.72%) Table (2).

According to data in Table 3, in the codominant model, the CA genotype was linked to T2DM with OR=1.715 and (P=0.248). The MIR-423 rs6505162 C>A genotypes in dominant and recessive alleles did not differ significantly, according to the data.

Table 2: Association of MIR-423 rs6505162 C>A gene variation in T2DM patients and controls.

Gene	Groups	Total NO.	Genotypes %			X2	P value
			CC	CA	AA		
MIR-423 rs6505162	**Cases**	**96**	29(30%)	45(46.7%)	22(23.3%)	13.03	**<0.001***
	Control	**96**	27(27.8%)	53(55.5%)	16(16.7%)	33.84	**<0.001***
Allele Frequency							
MIR-423 rs6505162	**Cases**	**C**	103(53.64)	**A**	89(46.35)	2.04	0.153
	Control	**C**	107(55.72)	**A**	85(44.27)	5.04	**0.025***

MIR: microRNA**; X^2: chai**-square test**; *:** significant at P<0.05.

Table 3: Comparisons between T2DM patients and controls with MIR-423 rs6505162 C>A genotypes

Mode Inheritance	Cases =96	Control=96	OR	X^2	P-value
Co-Dominate					
CC	29(30)	27(27.8)	1.106	0.101	0.751
CA	45(46.7)	53(55.5)	1.715	1.33	0.248
AA	22(23.3)	16(16.7)	1.48	1.18	0.277
Dominate					
CC	29(30)	27(27.8)	1.106	0.101	0.751
CA+AA	67(69.79)	69(71.87)	0.904	0.101	0.751
Recessive					
CC+CA	74(77.08)	80(83.33)	0.672	1.18	0.277
AA	22(22.91)	16(16.7)	1.48	1.18	0.277
Allele frequency					
C	103(53.64)	107(55.72)	0.919	0.168	0.682
A	89(46.35)	85(44.27)	1.087	0.168	0.682

X^2: Chai-square test**; OR:** odds ratio**; *:** significant at p<0.05.

3.3 Comparisons of age and sex with MIR-423 rs65 05162 C>A genotypes and

Multivariate analysis based on logistic regression (OD) was used to compare the MIR-423 rs6505162 C>A genotypes with comorbid conditions and T2DM severity statistically. The MIR-423 rs6505162 C>A genotypes (CC, CA, and AA) were shown (Table 4) to significantly correlate with the individuals' age groups (48-58 and 37-47), as well as their sex (P. 0.008, 0.028, and 0.006), respectively.

Table 4: age and sex of the type 2 diabetes patients with MIR-423 rs6505162 C>A genotypes among diabetes patients.

Subject Characteristic	No.	Genotypes %			X2	DF	P-value
		CC	CA	AA			
Association with sex							
Male	35	13(37.14)	14(40)	8(22.85)	2.65	2	0.265
Female	61	16(26.22)	30(49.18)	15(24.59)	10.37	2	**0.006***
Association with age							

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26-36 year	12	4(33.33)	4(33.33)	4(33.33)	0	2	1
37-47 year	16	2(12.5)	10(62.5)	4(25)	9.75	2	***0.008**
48-58 year	24	5(20.83)	13(54.16)	6(25)	7.12	2	***0.028**
59-69 year	29	13(44.82)	10(34.48)	6(20.68)	3.82	2	0.148
70-79 year	15	5(33.33)	7(46.66)	3(20)	2.4	2	0.301

X^2: chai-square test; DF: degree of freedom; *: significant at P<0.05.

3.4 Comparisons the RBS and HbA1c of the type 2 diabetes patients with MIR-423 rs6505162 C>A gene.

Table (5) displays the statistically significant link found between the MIR-423 rs6505162 C>A genotypes (CC, CA, and AA) and the biochemical markers HbA1c. Conversely, no statistically significant correlation was seen between the MIR-423 genotypes and the random blood sugar level in the case and control groups.

Table 5 : RBS and HbA1c of the type 2 diabetes patients with MIR-423 rs65051.62 C>A genotypes

Parameters	**Genotypes%**			**F test**	**P-Value**
	CC	**CA**	**AA**	**†**	
RBS mg/dl	264.2±15.86	238.4±16.21	207.04±15.34	2.37	0.098
HbA1c %	10.48±0.32	8.90±0.34	9.42±0.39	5.22	**0.007***

***: mean± standard error; †: one way ANOVA test; *: significant at P<0.05.**

3.5 Association of anti- glutamic acid decarboxylase (Anti- GAD) with type 2 diabetes.

Titer of Anti-GAD in patients and control:

Among 96 patients with type 2 diabetes mellitus, only 4 cases (4.17%) had positive for autoantibodies (Anti-GAD). Whereas control group had 3 (3.13%) positive for anti-GAD with no significant variances between patient and control. The mean of Anti-GAD titer between both groups showed highly significant (P<0.001) differences between patients and control groups with control predominance as showed in table (6). Concerning level of Anti-GAD, RBS and HbA1c in patients according to the age groupsshowed in (Table 7).

Table 6:level of anti-GAD among type 2 diabetes mellitus in comparison with control group**.**

Groups	**Results of ELISA**	**Frequency %**	**Titer (mean ±SE*)**
Control	**Positive**	3(3.13)	0.306 ± 0.062
	Negative	93(96.87)	
Cases	**Positive**	4(4.17)	0.113 ± 0.019
	Negative	92(95.83)	
P- Value = <0.001			

Table 7 : level of Anti-GAD, RBS and HbA1c in patients according to the age groups**.**

Age Groups	Anti-Gad (Mean ±SE)	RBS mg/dl (mean ±SE)	HbA1c % (mean± SE)
26-36	0.102±0.004	254.2±33.1	10.57±1.11
37-47	0.106±0.02	266.2±26.04	10.2±1.11
48-58	0.167±0.082	192.3±16.95	8.78±8.78
59-69	0.089±0.013	243.2±22.6	9.32±9.32
70-79	0.090±0.013	236.7±31.4	9.79±9.79
P. value †	**0.671**	**0.292**	**0.191**

***:** Standard error**; †:** t-independent T-test.

IV. DISCUSSION

In recent studies by Yang et al.,(2021) has been reported that expression of the mature miRNA-423 influenced by the presence or absence of A allele at rs6505162 position.Current findings in present study is quite with Yang Zhenstudy, they discovered that suppressing liver miR-423-5p in diabetic mice improves insulin resistance, suppresses gluconeogenesis, and increases blood sugar and fatty liver. Additionally, they observed that through the inhibition of the hepatic FAM3A/ATP/Akt pathway, the overexpression of

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miR-423-5p boosted elevated blood glucose, and gluconeogenesislevels, as well as obesity in healthy mice (Yang et al., 2021). On the other hand, data in present study disagreement with Zeng's results that conducted in Pakistani population (Zeng et al.,2023).

The pre-miRNA sequence of hsa-mir-423, which codes for two mature miRNAs (hsa-miR-423-3p and hsa-miR-423-5p), contains the SNP rs6505162 C/A. It is theoretically possible for the SNP in the pre-miRNA sequence to change how miRNA is processed and expressed. Important cellular pathways may malfunction as a result of this pre-miRNA modification (Wang et al., 2020). According to Yuan et al. (2017), the majority of recent research has also suggested that miR-423is important in controlling cell growth and death. According to recent studies, miR-423targets SUFA protein to prevent myoblast differentiation and proliferation (Ge et al., 2018). Another study revealed that in renal proximal tubular epithelial cells, miRNA-423 promotes apoptosis driven by hypoxia and deoxygenation. (Yuan et al., 2021).According to earlier research, patients with proliferative diabetic retinopathy had considerably lower blood levels of miR-423 (Zhao et al., 2022). According to Weili Yang et al. (2017), suppression of miR-423-5p lowers gluconeogenesis, lowers insulin resistance, and raises blood glucose. nevertheless, overexpression of miR-423 in the liver. However, there was no meaningful correlation found between the amount of random blood sugar and the MIR-423 genotypes.Mature miR-423 that was produced from a precursor with the same sequences in humans and mice. According to a number of recent clinical investigations, people with type 2 diabetes, poor glucose tolerance, or morbid obesity had lower levels of miR-423 in their blood (Ortega et al., 2014). Specifically, in individuals with type 2 diabetes, there is a correlation between the level of miR-423 in the blood and variations in fasting blood glucose (Ortega et al., 2014). In contrast, the circulating miR-423-5p level is increased in obese children (Prats-Puig et al., 2013). So far, the role and mechanism of miR-423-5p in regulating glucose and lipid metabolism remain unknown. Bioinformatics prediction revealed that human, mouse, and rat FAM3A mRNAs are the potential targets of miR-423-5p suggesting that miR-423-5p may regulate glucose and lipid metabolism by repressing FAM3A expression and its downstream signaling transduction. Li et al., (2015), found that miR-4513 rs2168518 was associated with blood pressure, lipids, and blood glucose levels, and, as expected, risk for DM. miR-499 rs3746444 and miR-423 rs6505162 were associated with blood pressure and high-density lipoprotein (HDL) levels.

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