RESEARCH ARTICLE

Role of Micro-RNA 146a in Iraqi Patients with Type 1 Diabetes Mellitus

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is a progressive autoimmune disease. According to growing data, microRNAs (miRNAs) appear to have a crucial impact in processes associated with T1DM Mellitus pathogenesis, involving immune system activities, beta-cell metabolism, and apoptosis.[1] So, this study aims to look into the amount of the expression of miRNA146a as a possible diagnosis biomarker in T1DM patients. The study population comprised 60 T1DM patients of both genders, 38 females and 22 males. In addition to 30 normal, healthy individuals with ages ranging between ≤16 years old and >16 years old. A biochemical test was performed to determine the concentration of random blood sugar (RBS). An enzymatic colorimetric assay measured blood glucose using kits supplied by Spinreact S, A. Spain. The sandwich immunodetection method was used to determine the percentage of glycated hemoglobin (HbA1c%) in the blood of the study's subjects. The miRNA extraction from the serum of all study subjects via a protocol of *EasyPure*® miRNA kit, relative quantification (RQ) of miRNA146a expression, in serum sample was estimated using reverse transcriptase quantitatively real-time polymerase chain reaction. Results showed no significant difference among age groups and study parameters, as well as non-significant differences between both genders and study parameters. There was a significant difference between the duration of diabetes and RBS ($p = 0.020$) and non-significant differences between duration and HbA1c ($p = 0.067$). The findings clarified a significant ($p = 0.0001$) increase in expression of miRNA146a in the serum patients group depending on 2-ΔCt and 2-ΔΔCt methods (2.732 folding changes ± 0.11), compared with a control group (1.0 folding changes ± 0.00), and the non-significant difference between patients and control ($p = 0.328$) in fold expression of miRU6. (1.03 folding changes ± 0.07), (1.00 folding changes ± 0.00) respectively. However, the correlation coefficient between CT microRNA146a and parameters study showed that highly significant difference between CT microRNA146a and RBS ($p = 0.0003$), HbA1c($p = 0.0082$). And non-significant difference between CT microRNA U6 and RBS, HbA1c ($p = 0.392$), ($p = 0.911$), respectively. The application of miRNA146a as a possible diagnostic biomarker of T1DM by using multiple techniques to get a more precise prediction of T1DM.

Keywords: miRNA146a, Reverse transcriptase, T1DM.

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INTRODUCTION

Type 1 and type 2 diabetes are the two most frequent types of diabetes.[2] Type 1 diabetes mellitus (T1DM), also known as insulin-dependent diabetes mellitus (IDDM), is an autoimmune disease in which T-cells invade and destroy pancreatic beta cells, with genetic and environmental factors that play a major role.[3,4]

Diabetes is becoming more common all over the world at an unprecedented pace. Over the last three decades, the status of diabetes has changed. It was previously thought to be a minor condition affecting the elderly. It is now a leading cause of morbidity and mortality in the young and middle-aged.[5]

The presence of sugar or HbA1c in the blood is used to identify diabetes. Measurement of HbA1c is considered essential to the management of diabetes patients.[6]

In several eukaryotic lineages, microRNAs are 22 nucleotides RNA that guide post-transcriptional suppression of mRNA targets. These short RNAs help form the expression of most mRNAs in man and animals, and microRNAs, a family of endogenous short non-coding RNA have been identified as critical regulators in genomic systems.[7,8] MiRNAs are highly persistent in the bloodstream and thus might be used as disease indicators. MiR-146a is a slightly well-known miRNA in inflammatory autoimmune diseases and linked to the development of numerous autoimmune illnesses, including type 1 diabetes mellitus, systemic lupus erythematosus, and multiple sclerosis.[9,10] Multiple genes are targeted by MiR-146a in the Toll-like receptor 4 (TLR4) nuclear factor-κB (NF-κB) pathway, an important immune and the inflammatory

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response pathway that regulates the inflammatory response.[11] In addition, vascular endothelial cells, smooth muscle cells, and monocytes/macrophages all expressed miRNA-146a.[12-14]

The miR-146 family includes other miRNAs found in most innate and adaptive immune cells. The miR-146 family of microRNAs regulates inflammation.[15,16]

MATERIALS AND METHODS

Subjects

A total of 60 patients of both genders 38 females and 22 males from Baghdad province, were enrolled in this study. Also, 30 normal, apparently healthy individuals aged between ≤16 years old and >16 years old were performed from February 2020 to January 2021 at Al-Sadr Hospital and Ibn-Albalady Hospitals.

Sample Collection

Under strict aseptic conditions, ten milliliters of venous blood were drawn from each patient. Blood samples were separated into two groups. Two milliliters were dispensed in a sterile EDTA tube for glycated hemoglobin determination. The second half was put into sterile gel tubes and left to coagulate for around 15 minutes before being centrifuged at 1000 rpm for five minutes at room temperature to separate it. The serum was poured into sterile plain tubes, which were firmly sealed and maintained at 20°C until the test was performed.

METHODOLOGY

Biochemical Test

In this study, the study's subjects determined RBS Level and HbA1c%. Blood glucose was measured by enzymatic colorimetric assay.[17] Using kits supplied by Spinreact S, A. Spain. Measurement of the percentage of HbA1c in the blood of patients and control groups by sandwich immunodetection method ichroma™ HbA1c (Boditech, Korea).[18]

Relative Quantification of the miR-146a Expression

Real-time PCR was used to measure the expression of miRNA 146a (RTq-PCR (miRNA extraction from the serum of both patient and healthy control by using a procedure of *EasyPure*® miRNA Kit (Transgenbiotech, China).[19] Applying the manufacturer's methodology, which is as follows: Briefly, after melting, the sample lysate buffer10 (LB10) was added into a microcentrifuge tube, mixed thoroughly by inverting 4 to 6 times, and incubated at room temperature for 5 minutes. To separate the aqueous phase, chloroform was added to the lysate. Vortexed samples were incubated at room temperature for 3 minutes before being centrifuged at 4°C for 15 minutes at 14,000 rounds per minute. To create adequate binding conditions for all RNA molecules, ethanol was added to the separated aqueous phase. After that, specimens were then eluted in the RNase-free water and kept at −20°C till further processing. According to the manufacturer's instructions, reverse transcription of miRNA-146a was done for cDNA synthesis using the EasyScript One-Step gDNA removal and complementary DNA (cDNA) Synthesis Super Mix kit. The expression miRNA 146a by RTq-PCR analysis according to the manufacturer's procedure, the forward primer: 5′- CAGTGCGTGTCGTGGAGT -3′ while the revers 5′- GGGTGAGAACTGAATTCC-3′. miRNA 146a reverse Transcriptase primer 5′-GTCGTATCCAGTGCGTGTCGTGTCGGC-3′. Reference gene was miRU6 small nuclear, the forward primer: 5′-AGAGAAGATTAGCATGGCCCCT-3′ and the reverse primer 5′- GCGAGCACAGAATTAATACGAC-3′. For the RT-PCR, The SYBER Green master mix was combined with cDNA. TransStart® Green qPCR SuperMix kit.[20] A non-template control (NTC), non-amplification control (NAC), and non-primer control (NPC) were used as negative controls in each reaction. PCR tube enriched with forward and reverse miRNA 146a specific primers. The Real-time PCR reaction were done using a smart cycler Real-time PCR System (Corbett research PCR, ANDbio, USA). The cycle threshold (CT) is described as the number of cycles and required the fluorescent signal to cross the threshold in the real-time PCR, the quantitative gene expression was reported as the ΔCT value, and obtained via subtracting the CT values of reference gene from the CT values of target gene of miRNA-146a, and the comparative expression as measured by: (ΔΔCt) = (Ct of target gene normal – Ct of housekeeping gene normal)-(Ct of target gene case–Ct of housekeeping gene case). The relative expression in the target gene was calculated on the estimated mean difference ($2^{-\Delta\Delta Ct}$).[21]

Statistical Analysis

The Statistical analysis and graphing were performed with the statistical package for social sciences (SPSS) 24.0 to determine the impact of various variables on research parameters. To make a meaningful comparison between means, the T-test was performed. To make a meaningful comparison between percentages, the Chi-square test was performed (0.05 and 0.01) probability. In this study, the correlation coefficient between variables was estimated.

RESULTS AND DISCUSSION

A questionnaire filled out by direct interview with the control and case groups was used to examine some of the demographic features listed in Table 1. The study revealed that the rate of females (63.33%) with type 1 diabetes (T1D) is more than males (36.67%). Statistical analysis showed significant differences in the mean value between males and females (p = 0.029). As well

Table 1: Some of factors studied in patients and control groups

		Mean ± SE		
Factor		*Patient*	*Control*	*p-value*
Gender No. (%)	Male	22 (36.67%)	12 (40.00%)	0.029 *
	Female	38 (63.33%)	18 (60.00%)	
Age (year)	Mean ± SE	14.06 ± 0.98	14.73 ± 1.26	0.685 NS
Weight (kg)	Mean ± SE	38.96 ± 2.04	46.83 ± 2.97	0.030 *
Duration (year)	Mean ± SE	5.20 ± 1.08	-	-
Ketone bodies No. (%)	Positive	59 (98.33%)	0 (0.00%)	0.0001 **
	Negative	1 (1.67%)	30 (100%)	

Significantly * (p ≤0.05), Highly Significant ** (p ≤0.01), NS: Non-Significantly.

as, the results revealed that a non-significant difference was found in the mean value of age between T1DM patients (14.06 ± 0.98) and healthy subjects (14.73 ± 1.26) (p = 0.685). However, statistical analysis showed that the mean diabetic patients' weight (38.96 ± 2.04) is less than that of healthy subjects (46.83 ± 2.97). There is a highly significant difference (p = 0.030). Furthermore, there is a highly significant difference found in the mean value of ketone bodies between T1DM patients (98.33%) and non-diabetic healthy control (0.00%) (p = 0.0001).

The study showed that RBS concentration in patients (329.68 ± 17.41) mg/dL and control (92.57 ± 1.65) mg/dL there is highly significant differences between patients and control (p < 0.001) as shown in Table 2. Figure 1. The study showed highly significant differences (p < 0.001) between patients (9.91 ± 0.32)% and control (4.43 ± 0.08) % in HbA1c, as shown in Table 2 and Figure 2. Clinicians can gain an overall view of the typical blood sugar levels over weeks/months by monitoring HbA1c.

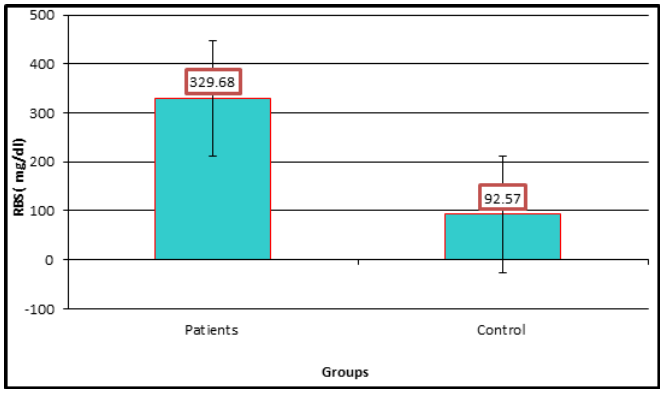


Figure 1: Comparison Between Patients and control in RBS.

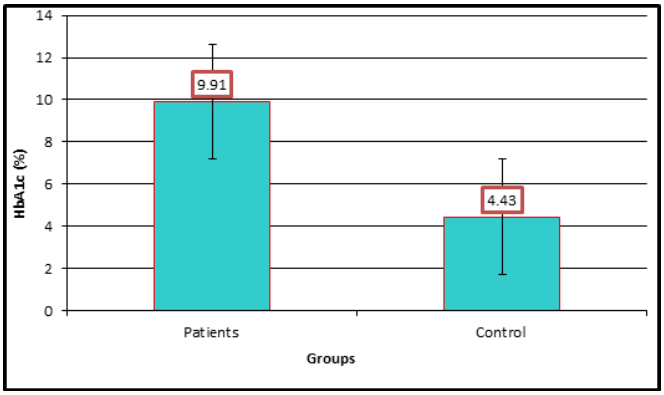


Figure 2: Comparison Between Patients and control in HbA1c.

Table 2: Comparison between patients and control in RBS (mg/dL) and HbA1c (%).

	Mean ± SE	
Group	*RBS (mg/dL)*	*HbA1c (%)*
Patients	329.68 ± 17.41	9.91 ± 0.32
Control	92.57 ± 1.65	4.43 ± 0.08
t-test	49.147 **	0.934 **
p-value	< 0.001	< 0.001

Highly Significant ** (P ≤0.01).

In this study, the effect of age groups on the study's parameters of patients is summarized in Table 3. The statistical analysis pointed that non-significant differences between age groups and RBS (p = 0.079), HbA1c (p = 0.252).

Table 4 summarizes the impact of genders on the study's parameters of patients. Statistical analysis revealed that non-significant differences between gender groups and RBS (p = 0.871), HbA1c (p = 0.669).

The effect of duration of diabetes on the study's parameters of patients is summarized in Table 5. The statistical analysis suggested significant differences between duration and RBS (p = 0.020). And non-significant differences between duration and HbA1c (p = 0. 067).

The results of this study revealed a highly significantly (p = 0.0001) rise in expression of miRNA146a in the patient's serum group depending on $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods was (2.732 folding changes ± 0.11), compared with apparently healthy control group (1.0 folding changes ± 0.00) as shown clearly in Tables 6 and 7; Figure 3. In comparison to the patient, the ΔCt mean of miRNA146a was considerably greater in the control group. The expression of the miRNA146a gene was shown to be upregulated in T1DM patients. These results agree with Kamali *et al.* (2016), who noticed that the expression of miR-146a is upregulated under hyperglycemic condition.[22]

There is a non-significant difference between patients and control (p = 0.328) in the fold expression of miRU6. (1.03 folding changes ±0.07), (1.00 folding changes ±0.00) respectively, as shown in Table 8 and Figure 4.

The correlation coefficient between CT MicroRNA146a and parameters study showed that highly significant difference

Table 3: Effect of Age groups on study's parameters of patients

	Parameters mean ± SE	
Age groups	*RBS (mg/dL)*	*HbA1c (%)*
(≤ 16) yrs.	337.17 ± 20.75	10.08 ± 0.39
(> 16) yrs.	269.75 ± 14.69	9.24 ± 0.39
t-test (p-value)	84.86(0.079) NS	1.634(0.252) NS

NS: Non-significant

Table 4: Effect of gender on study parameters of patients.

	Parameters mean ± SE	
Gender groups	*RBS (mg/dL)*	*HbA1c (%)*
Male	323.73 ± 26.92	10.37 ± 0.57
Female	333.13 ± 22.90	9.64 ± 0.40
t-test (p-value)	72.93 (0.871) NS	1.36 (0.669) NS

NS: Non-significant

Table 5: Effect of Duration of Diabetes in parameters study of patients.

	Parameters Mean ± SE	
Duration (years)	*RBS (mg/dL)*	*HbA1c (%)*
0–1 yr.	260.27 ± 32.17	8.90 ± 0.62
1–5 yr.	298.43 ± 24.26	9.41 ± 0.59
>5 yr.	386.69 ± 27.79	10.78 ± 0.44
T-test (P-value)	0.0201 *	0.0676 NS

Significantly * (P ≤0.05), NS: Non-Significantly.

Table 6: Fold of miR146a expression depending on $2^{-\Delta Ct}$ Method

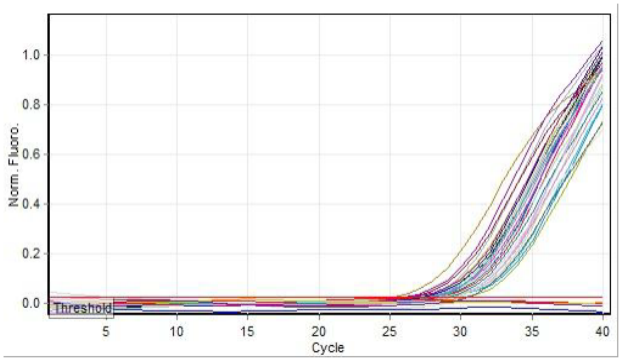
Groups	*Means Ct of miR146a*	*Means Ct of miRU6*	*ΔCt (Means Ct of miR146a - Means Ct of miRU6)*	$2^{-\Delta Ct}$	*Experimental group/ Control group*	*Fold of gene expression*
Patient	27.68 ± 1.83	17.49 ± 1.76	10.19 ± 0.64	0.0008560	0.0008560/0.0003133	2.732 ± 0.11
Healthy	29.18 ± 1.52	17.54 ± 0.96	11.64 ± 0.71	0.0003133	0.0003133/0.0003133	1.0 ± 0.00
p-value	-	-	-	-	-	0.0001 **

Highly Significant ** ($p \leq 0.01$).

Table 7: Fold of miR146a expression depending on the 2-ΔΔCt method

Groups	*Means Ct of miR146a*	*Means Ct of miRU6*	*ΔCt (Means Ct of miR146a - Means Ct of miRU6)*	*Mean ΔCt calibrator (ct miR146a -ct miRU6*	*ΔΔCt*	*2-ΔΔCt*	*Experimental group/ Control group*	*Fold of gene expression*
Patient	27.68 ± 1.83	17.49 ± 1.76	10.19 ± 0.64	16.2 ± 0.72	-6.01	64.445	64.445/32.990	2.732 ± 0.11
Healthy	29.18 ± 1.52	17.54 ± 0.96	11.64 ± 0.71	16.2 ± 0.72	-4.56	32.990	32.990/32.990	1.0 ±0.00
p-value	-	-	-	-	-	-	-	0.0001 **

Highly Significant -** ($P \leq 0.01$).



Standard Curve

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		92	Unknown	29.41			
2		88	Unknown	27.54			
3		97	Unknown	29.45			
4		74	Unknown	28.66			
5		98	Unknown	30.45			
6		83	Unknown	30.94			
7		70	Unknown	29.41			
8		73	Unknown	28.48			
9		68	Unknown	28.72			
10		63	Unknown	29.16			
11		71	Unknown	28.41			
12		100	Unknown	30.43			
13		60	Unknown	30.56			
14		101	Unknown	28.56			
15		57	Unknown	29.35			

Figure 3: MiR-146a amplification plots by qPCR. Samples included a healthy study group. The photograph was taken directly from the Qiagen Rotor gene qPCR machine.

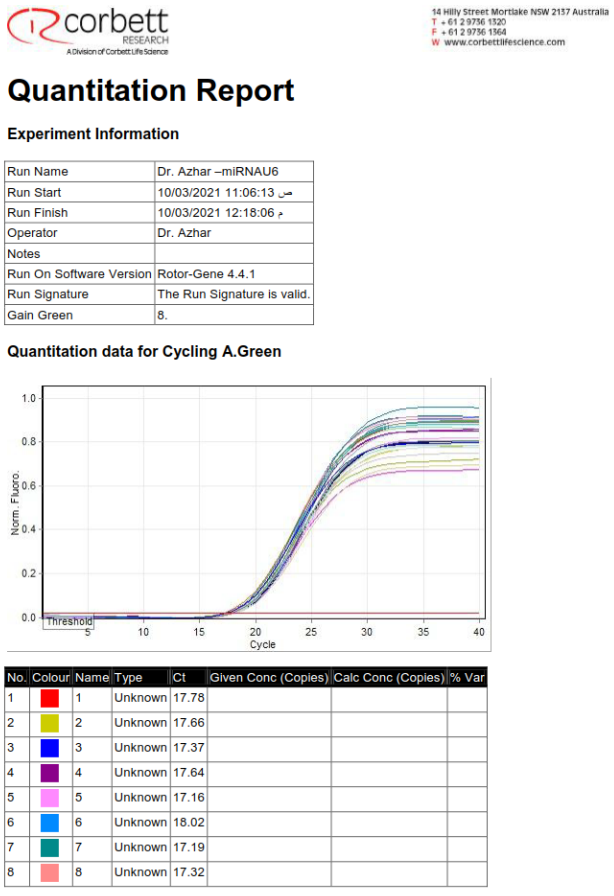


Figure 4: miRU6 amplification plots by qPCR Samples included all study groups. The photograph was taken directly from the Qiagen Rotor gene qPCR machine.

between CT microRNA146a and RBS (p = 0.0003), HbA1c(p = 0.0082), as shown in Table 9.

Table 10 showed non-significant difference between CT microRNA U6 and RBS, HbA1c (p = 0.392), (p = 0.911) respectively.

Table 8: Comparison of miRU6 fold expression between study groups.

Group	*Means Ct of miRU6*	2^{-Ct}	*Experimental group/Control group*	*Fold of gene expression*
Patient	17.49 ±1.76	5.43E-06	5.43E-06/5.23E-06	1.03 ± 0.07
Healthy	17.54 ± 0.96	5.23E-06	5.23E-06/5.23E-06	1.00 ± 0.00
p-value	-	-	-	0.328 NS

NS: Non-significant.

Table 9: Correlation coefficient between CT MicroRNA146a and studied parameters

Parameters	*Correlation coefficient-r with CT MicroRNA146a*	*p-value*
RBS (mg/dL)	-0.37 **	0.0003
HbA1c (%)	-0.28 **	0.0082

Highly Significant ** ($p \leq 0.01$).

Table 10: Correlation coefficient between CT MicroRNA U6 and studied parameters

Parameters	*Correlation coefficient-r with CT miRNA U6*	*p-value*
RBS (mg/dL)	-0.09 NS	0.392
HbA1c (%)	0.01 NS	0.911

NS: Non-significant.

CONCLUSION

Considering the regulatory roles of miRNAs in immune cells and β-cells. It is thought that miRNAs might be employed as a therapy for the treatment of T1DM. Although studies on the roles of miRNAs in T1DM are in their infancy, advancement in our understanding of the roles of miRNA in this illness may assist enhance therapeutic care in the future.[23] The expression of miRNA 146a gene was shown to be a useful and helpful marker in the prediction of T1DM.

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