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Correlation of MicroRNAs-122a Gene Expression with Diabetic for Iraqi Patients

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Abstract

This study was carried out to describe the gene expression of the micro RNA 122a gene with the development of diabetes in Iraq. The difference in gene expression between patients and healthy controls was properly considered. In this study, blood was isolated from 121 individuals divided into two groups as follows: 80 samples of diabetic patients and 41 samples from a healthy control. miRNA was isolated and transformed into cDNA, and the expression of mi122a was measured by qRT-PCR. The researchers looked at the relationship between age and gender and the occurrence of diabetes, as well as how they compared to controls. When comparing the mean gene expression level (Ct) of patient groups to the corresponding Ct means in the control group, the results revealed a discrepancy. Also the gene expression folding ($2^{-\Delta\Delta Ct}$) of the micro RNA 122a gene reflect differences in the expression, the level of micro RNA 122a was (20.504) in patients with diabetes compared to control groups with significant differences. On the other hand, gender and family history showed a significant difference between patients and health monitors. For age and type of diabetes, they showed a significant difference between patients and health monitors. Our results indicate that diabetes can affect all ages in both males and females. This study aims to correlate the expression of miRNA 122a with the occurrence of diabetes in the Iraqi population.

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1. Introduction

Diabetes Mellitus (DM) is a long-term condition in which the body's ability to produce or respond to the hormone insulin is impaired. Or when the body is unable to use the insulin it produces, resulting in high blood glucose levels (hyperglycemia) [1, 2], where insulin is a hormone produced by pancreatic beta cells that acts as a key to letting glucose from food to move from the bloodstream to the body's cells for energy production. High blood glucose levels have been related to long-term harm to the body and organ and tissue failure [2]. Generally, inactive lifestyle and being overweight are the biggest causes for diabetes. In addition to the family history of diabetes, older age, High cholesterol or/and high triglycerides, Women who have had gestational diabetes,

Smoking, and alcohol [3]. MicroRNAs (miRNAs) are short, non-coding RNA molecules that are only 18-25 nucleotides long. These tiny, abundant molecules have been demonstrated to serve key regulatory roles in a range of biological and pathological processes since their discovery. [4, 5]. In humans, about 2000 miRNAs have been found, and it is estimated that they influence one-third of the genes in the genome. MiRNAs have been connected to a number of human disorders, and they are being studied as clinical diagnostic and therapeutic targets [6]. Several studies in the biological fluids like serum and plasma have detected miRNAs [7, 8], cerebrospinal fluid [9], saliva [10], breast milk [11], tears, peritoneal fluid, urine, bronchial lavage, colostrum, seminal fluid [12], and ovarian follicular fluid [13]. MiRNAs are extremely stable, resistant to degradation for up to four days at room temperatures and under deleterious conditions, including boiling, multiple cycles of freezing, and high and low pH [7, 14]. In 2002, the systematic cloning and sequencing of small RNAs from various mouse tissues led to miR-122 being identified as abundant miRNA in the liver [15]. miR-122a was the most common isolated miRNA of the adult liver, reaching approximately 70 percent of all cloned miRNAs [16]. miR-122a is found in the human liver, mice, woodchuck, human primary hepatocytes, and cultured liver-derived cells, such as mouse Hepa 1-6 cells and human Huh7 cells [17]. miR-122 derived from a single genomic locus on chromosome 18 in humans, and expression of miR-122 has been found in 18 vertebrate species, including humans, frogs, and zebrafish [18]. miR-122 was shown to modulate systemic iron homeostasis through suppression of mRNAs, hemochromatosis (Hfe), and hemojuvelin (Hjv) [19]. Finally, miR-122 is a tumor suppressor, and its expression is frequently decreased or abolished in hepatocellular carcinoma (HCC). These mRNAs encode the hormonal hepcidin activators, which regulate iron accessibility [20].

2. Experimental Procedure

2.1. Study Groups and Blood Sampling

The total number of participants in this study was 121 individuals, study groups included eighty patient's samples of Iraqi men, women, and children diagnosed with different types of diabetes, aged between (6-80 years). Furthermore, forty-one samples of apparently healthy individuals of both sexes, aged between (19-70 years) were obtained for control. From each participant, 5 ml of whole blood was needed to be collected from the venous blood directly into an EDTA-containing tube, and this procedure was done under aseptic conditions.

2.2. miRNA Extraction from Blood Samples

The miRNA extraction from whole blood of both patient and healthy control by using the protocol in Easy Pure® miRNA Kit as the following:

1. Sample lysis: Add 1 ml of lysis buffer (LB10) to 200µl of blood, mix thoroughly by vortexing. Incubate for 5 minutes at room temperature.

2. For three phase's separation: added 200 µl of chloroform to the lysate. Incubate was all mixed for 2–3 minutes, then centrifuge for 15 minutes at 10,000 ×g. Then, the mixture was separated into a lower organic pink phase, interphase, and a colorless upper aquatic phase, containing the RNA. The RNA containing the aquatic phase was transferred into another clean tube.

3. For miRNA precipitation: Transferred to a fresh RNase-free tube, the colorless upper phase containing RNA. Add 96% -100% of ethanol to 1 volume of the transferred solution. We are inverting the tube to mixing gently. Then Add all the lysate to the RNA spin column, centrifuge at 12,000×g at room temperature for 30 seconds, and collect the flow-through. Add entire lysate into miRNA spin column, centrifuge at 12,000×g at room temperature for 30 seconds, and discard the flow-through.

4. For miRNA washing: Add 500µl of WB10 into the spin column centrifuge at 12000×g at room temperature for 30 seconds. Discard the flow-through.

5. For Store the isolated miRNA: Place the miRNA spin column into a clean 1.5 ml RNase free tube. Add 30-50 µl of RNase free water into the spin column matrix and incubate for 1 minute at room temperature, Centrifugation at 12000×g for 1 minute to elute miRNA, and then store miRNA at -80°C.

2.3. Primer Design

The primers were designed using the bioinformatics program NCBI (national center biotechnology information). Primers used in this study with their sequences are shown in Table 1.

Table 1: Primers and their sequences.

Primer	Sequence (5′→3′ direction)
MiRNA	
miR-122a	TGGAGTGTGACAATGGTGTTTGT
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

2.4. Gene Expression

cDNA Synthesis from mRNA

The cDNA synthesis was subjects by using protocol in EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix

Procedure

1. First strand cDNA synthesis
2. The reaction components and the volume needed shown in Table 2.

Table 2: Reaction components and volume.

Component	Volume
MiRNA	5
Anchored Oligo(dT)18 Primer(0.5µg/µl)	1µl
Random Primer(0.1µg/µl)	1µl
GSP	2 pmol
2×EX Reaction Mix	10µl
Easy Script®RT/RI Enzyme Mix	1µl
gDNA Remover	1µl
RNase-free Water	To 23µl

3. Incubation

The time and temperature needed shown in Table 3.

Table 3: Thermal cycler steps.

	Step1	Step2	Step3
Temperature	25°c	42°c	85°c
Time	10min	15min	5seconds

	Random Primer (N9)	Anchored Oligo(dT)18	Inactivate reverse transcriptase enzyme

2.5. Quantitative Real-Time PCR (qRT–PCR)

The levels of expression of the 122a gene were estimated by the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, which is a sensitive technique for quantifying steady-state mRNA levels. A quantitative real-time qRT-PCR SYBR Green test was used to confirm the expression of the target gene. Primer sequences for the 122a gene were designed in the current study, synthesized using NCBI's bioinformatics program (national center biotechnology information), and stored lyophilized at -23°C. In this study, Quantification RT PCR used green SYBR to recognize any double-stranded DNA, including cDNA [21]. Primer sequences are shown in Table 1.

2.6. Primer Preparation

The lyophilized primers were then prepared to produce a primer working solution for each stock solution at a concentration of 100 µM and stored (-23 °C) after dissolving in nuclease-free water. Diluting were done using 10µL in the 90 µL nuclease-free water of store solution of the primers and store at (-23°C) until use, producing a working solution with a concentration of 10 µM.

2.7. Quantitative Real-Time PCR (qRT–PCR) Run

Quantitative Real-Time PCR (qRT–PCR) was carried out using the Real-time PCR method [22]. The levels of gene expression and fold change were identified by measuring the threshold cycle (Ct) using the TransStart® Top Green qPCR SuperMix Kits components. The required volume of each component was calculated according to Table 4.

Table 4: Components of quantitative real-time PCR used in gene expression experiment.

Component	AQ131-01
2×TransStart® Top Green qPCR SuperMix	1 ml
Passive Reference Dye (50×)	40 µl
Nuclease-free Water	1 ml

Table 5: Thermal profile of gene expression.

Step	Temperature	Duration	Cycles
Enzyme activation	94 ℃	30 Sec	Hold
Denature	94 ℃	5 Sec	37
Anneal/extend	65 ℃	15 Sec	
Dissociation	55 ℃ - 95 ℃		

The Ct threshold cycle was calculated using real-time cycler software for each sample. All samples were run in duplicate, and mean values were calculated.-Expression data of selected genes were normalized against housekeeping. The ΔΔCt method was used as was recommended for data analysis, and results were expressed as folding change in gene expression as follow: For each sample, the difference between the Ct values (ΔCt) for each gene of target and the housekeeping gene was calculated

ΔCt (control) = Ct (gene) – Ct (HKG)

ΔCt (patient) = Ct (gene) – Ct (HKG)

The difference in ΔCt values represented as ΔΔCt for the genes of interest was calculated as follow:

$\Delta\Delta Ct = \Delta Ct$ (patient) – ΔCt (control)

The fold-change in gene expression was calculated as follow:

Fold change=$2^{-\Delta\Delta Ct}$

Statistical Analysis
The Statistical Analysis System- SAS (2012) application was used to detect the effect of various factors on study parameters. To compare means, the T-test and the Least Significant Difference –LSD test were used. Chi-square test was used to compare between percentage (0.05 and 0.01 probability) significantly. Estimate of the Correlation coefficient of the variables in this study [23], [24].

3. Results and Discussion

3.1. Quantitative Expression of 122a

Total miRNA was extracted from all samples.

3.2. cDNA Reverse Transcription

On the second day of miRNA extraction, complementary DNA reverse transcription was conducted. A common primer reaction was applied since it was needed to have cDNA for the 122a gene and housekeeping gene. The efficiency of cDNA concentration was determined through the efficiency of qPCR conducted later on. All steps were associated with perfect yield reflecting efficient reverse transcription. According to the Tm of each primer supplied in the manufacturers, instructions the annealing of the optimal primers temperature was calculated depending on the following equation:

- (Tm) Melting Temperature =2 (A+T) + 4 (G+C).
- (Ta) Annealing Temperature = Tm – (2-5) ℃.

The melting temperature for both reverse and forward primer were also calculated according to the above equation. The lowest temperature (℃) was chosen by comparing the annealing temperature for forward and reverse primers. Quantitative expression of the 122a gene and housekeeping gene miRNAU6 were assessed by Real-Time Polymerase Chain Reaction, in which the relative quantitation method was used. The gene expression level was normalized to the level of a housekeeping gene and quantified by the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) method as shown in Figures 1 & 2, respectively.

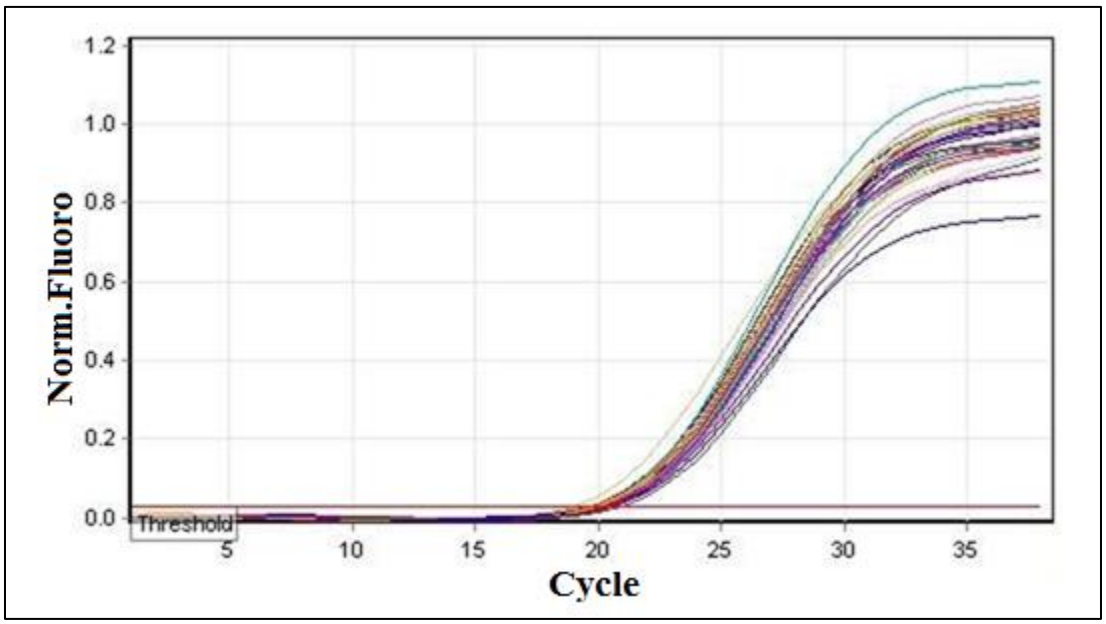

Figure 1: Amplification plots of a miR-122a gene by RT-qPCR.

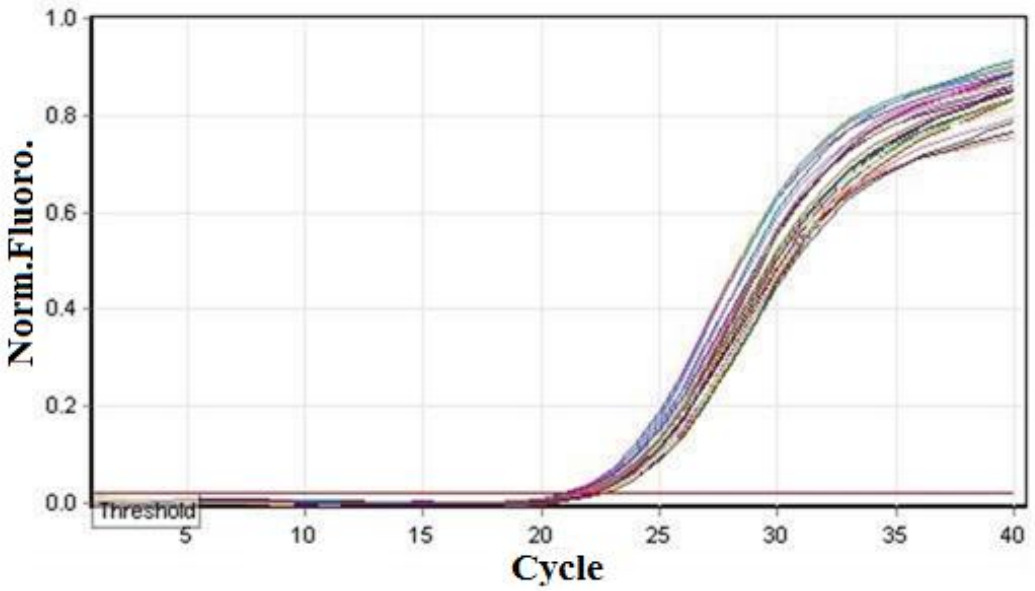

Figure 2: Amplification plots of miRNA U6 gene by RT-qPCR.

A representative melt curve 122a gene for samples analyzed by RT-PCR is shown in Figure 3.

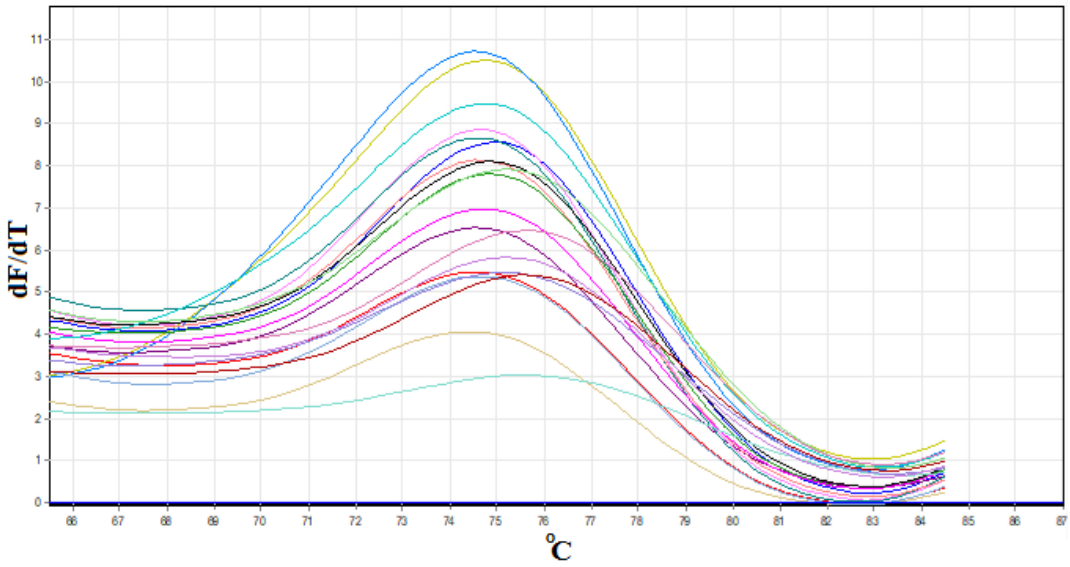

Figure 3: Melt curve of 122a gene after RT-qPCR analysis showing single peaks for different patients.

3.3. Fold of miRNA 122a Expression Depending on $2^{-\Delta Ct}$ Method

In the patients, means Ct of miRNA 122a was (20.504), means Ct of miRU6 was (17.66), ΔCt (Means Ct of miRNA 122a - Means Ct of miRU6) was (2.844), $2^{-\Delta Ct}$ was 0.1392742, experimental group/ Control group was 0.1392/0.0688, Fold of gene expression was 2.0237 ±0.489. In control healthy means Ct of miRNA 122a was 21.491, means Ct of miRU6 was 17.63, ΔCt (Means Ct of miRNA 122a Means Ct of miRU6) was 3.861, $2^{-\Delta ct}$ was 0.0688, experimental group/ Control group was 0.0688/0.0688. Fold of gene expression was 1.00 ±0.00. These show highly significant between patients and healthy control. These, due to the dysregulation of miR-122 (the most abundant miRNA in the liver, implicated in several hepatic functions), diabetic livers further

strengthens their implications in hepatic abnormalities and hyperglycemia [25]. These results are shown in Table 6.

Table 6: Fold of miRNA 122a expression Depending on $2^{-\Delta Ct}$ Method.

Groups	Means ct of *miRNA122*	Means ct of miRU6	ΔCt (Means ct of *miRNA122* - Means Ct of miRU6)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group 2 Patient	20.504	17.66	2.844	0.1392742	0.1392/0.0688	2.0237 ±0.489
Group 1 control healthy	21.491	17.63	3.861	0.0688	0.0688/0.0688	1.00 ±0.00
T-test (P-value)	--	--	--	--	--	0.519 ** (0.0001)
** (P≤0.01).						

3.4. Correlation of Factors with Diabetes and Control Groups

Of the 80 patients, 44 (55.00%) had a family history of diabetes, and 36 (45.00%) patients had no family history, and the mean of HbA1c of patients were (9.93 ±0.32).

3.5. Sex Distribution

Among the 80 diabetic patients, the distribution was as follows: 26 (32.50%) were males, and 54 (67.50%) were females. While from the forty-one healthy individuals, 18 (43.90%) were males, and 23 (56.10%) were females.

Individuals in all study groups were randomly selected so that the difference in sex distribution does not reflect any significance. 121 samples' frequency only represents their availability at the time of collection, which was not fixed in intervals, for gender and family history, which showed a significant difference between patients and the health control. For age and Diabetes type, it showed a highly significant difference between patients and the health control. The Frequency distribution of study groups is shown in Table 7.

Table 7: Results of factors in sample study.

Factors		Patients	Control	P-value
Gender No (%)	Male	26 (32.50%)	18 (43.90%)	0.0392 *
	Female	54 (67.50%)	23 (56.10%)	
Diabetes type No (%)	Type 1	10 (12.50%)	-	0.0001 **
	Type 2	70 (87.50%)	-	

Family history	Yes	44 (55.00%)	-	0.0477 *
No (%)	No	36 (45.00%)	-	
Age (year)	Mean ±SE	51.48 ±1.95	36.36 ±1.65	0.0001 **
BMI (kg/m^2)	Mean ±SE	27.89 ±0.58	-	---
Patient's age at the time of diabetes (year)	Mean ±SE	41.50 ±1.65	-	---
HbA1c	Mean ±SE	9.93 ±0.32	-	---
* (P≤0.05), ** (P≤0.01).				

4. Conclusions

This research was one of the first researches at the university to study microRNA and its effect on the genetic transmission of diseases due to its close relationship with the disease and preventing the transmission of the disease to offspring through anti-allergic synthesis. MicroRNA inhibits translation of the disease-carrying gene.
Our results concluded that the gene expression level of miR 122a was lower in diabetic patients than in healthy control. This also reflects differences in the gene expression fold ($2^{-\Delta\Delta Ct}$) of gene 122a.
Thus, lower levels of expression of miR 122a are associated with the occurrence and progression of diabetes in Iraq.
As for gender and family history, it showed a significant difference between patients and health monitors. For age and type of diabetes, they showed a significant difference between patients and health monitors. Our results indicate that diabetes can affect all ages in both males and females.

Conflict of Interest

The authors declare that they have no conflict of interest.

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