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Original Article

Gene Expression of miRNAs Let-7aAssociated with Diabetes in Iraqi Population

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Abstract

miRNAs regulate protein abundance and control diverse aspects of cellular processes and biological functions in metabolic diseases, such as obesity and diabetes. Lethal-7(Let-7) miRNAs specifically target genes associated with diabetes and have a role in the regulation of peripheral glucose metabolism. The present study aimed to describe the gene expressions of the let-7a gene with the development of diabetes in Iraq and the difference in the expression of this gene in patients with diabetes and healthy individuals. The association between age and gender with the development of diabetes was studied in this study and the results were compared with those of healthy individuals in the group of control. Based on the obtained results, there was a lack in the mean of gene expression level (ΔCt) in patients, compared to controls. Moreover, the gene expression folding (2-ΔΔCt) of the let-7a reflects significant differences in terms of gene expression between groups of patients and controls, and the level of let-7a expression was reported to be 12.97 in patients with diabetes. On the other hand, significant difference was observed in terms of age and gender between diabetic patients and controls. The findings suggest that diabetes can affect individuals in all age groups and occur regardless of gender in both males and females. Based on the obtained results in this study, the gene expression level of miRNA let-7a was lower in diabetic patients compared to healthy individuals in the group of control. This also reflects differences in the gene expression fold (2-ΔΔCt) of gene let-7a between both groups of patients and controls.

Keywords: Gene expression, Diabetes, Micrornalet-7a

1. Introduction

Diabetes Mellitus (DM) is a chronic disease that impairs the body's ability to produce / or respond to the insulin hormone. This disorder leads to a significant increase in the levels of glucose in the blood (i.e. hyperglycemia) (1), and high glucose levels have been linked to long-term damage to the body and the failure of various organs and tissues (1). Overweight, inactive lifestyle, a family history of diabetes, older age, and high cholesterol or/and high triglycerides are among major causes of diabetes. On the other hand, smoking and alcohol consumption in women with gestational diabetes may lead to the development of diabetes as well (2).

miRNAs are short, non-coding, endogenous RNA molecules that are only 18-25 nucleotides long. Since their discovery, these small and abundant molecules have played important regulatory roles in a variety of biological and pathological processes (3, 4). In 1993, Lee et al. discovered miRNAs in the nematode Caenorhabditis elegans. Over 2000 miRNAs have been discovered in humans, and it is thought that they collectively regulate one-third of the genes in the human genome. miRNAs have been linked to a variety

of human diseases and are being investigated for use as clinical diagnostics and therapeutic targets (5).

miRNAs have been detected in several studies performed on biological fluids, such as serum and plasma (6, 7) as well as in cerebrospinal fluids (8), such as saliva (9), breast milk (10), tears, peritoneal fluid, urine, bronchial lavage, colostrum, seminal fluid (11), and ovarian follicular fluid (12). miRNAs are extremely stable and 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, 13). Lethal-7 (Let-7) was one of the first discovered miRNAs. It was first identified as a developmental timing regulator for the nematodes and Caenorhabditis elegans, and was considered to be a hetero chronic gene (14).

The let-7 family is one of the well-studied miRNA clusters and is well conserved in various animal species (15-17).Let-7iswidelybeing used and the potential use of let-7 as therapeutics for metabolic and immune-related diseases has been discussed in different studies (15-17). miRNAs have recently been shown to support insulin signaling and glucose homeostasis and highlight the potential pathological roles for miRNA expression in metabolic disorders, such as insulin resistance(IR), obesity, and T2D (18). Proper metabolic homeostasis control is essential for human health maintenance. Analysis of global expression showed that changes in the miRNA levels are linked to a variety of metabolic diseases (19). It has been shown that miRNAs regulate central metabolic pathways and therefore play a vital role in maintaining metabolic homeostasis and the energy balance of organisms (20). The present study was designed to investigate the gene expression of the let-7a gene with the development of diabetes in Iraq.

2. Material and Methods

2.1. Study Groups

The study participants included 121 individuals who were assigned to two groups. Group 1 included 80 Iraqi male and female patients diagnosed with different types of diabetes in the age range of 6-80 years, and Group 2 (control group) included 41 apparently healthy individuals of both genders in the age range of 19-70 years.

2.2. Blood Sampling

In total, 5 ml of venous blood was collected from each participant and transferred directly into an EDTA-containing tube under aseptic conditions.

2.3. miRNA Extraction from Blood Samples

The miRNA was extracted from the blood of participants (both patients and healthy controls) using Easy Pure® miRNA Kits (TransGen Biotech Co., LTD, China), based on the following procedures:

2.3.1. Sample Lysis

Initially, 1 ml of lysis buffer (LB10) was added to 200µl of blood and mix thoroughly by vortexing. Subsequently, incubation was performed for 5 min at room temperature.

2.3.2. Three Phases Separation

In total, 200 µl of chloroform was added to the lysate. All mixtures were incubated for 2-3 min and then centrifuged for 15 min at 10,000 g. Subsequently, 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.

2.3.3. miRNA Precipitation

The colorless upper phase containing RNA was transferred to a fresh RNase-free tube, and 96%-100% of ethanol was added to 1 volume of the transferred solution. The tube was inverted to mix gently. Subsequently, the lysate was added to the RNA spin column, centrifuged at 12,000g at room temperature for 30 sec, and the flow-through was collected. The entire lysate was added into the miRNA spin column, centrifuged at 12,000g at room temperature for 30 sec, and the flow-through was discarded.

2.3.4. miRNA Washing

Initially, 500µl of WB10 was added into the spin column and centrifuged at 12,000g at room temperature for 30 sec. The flow-through was discarded.

2.3.5. Store the Isolated miRNA

The miRNA spin column was placed into a clean 1.5 ml RNase-free tube. Subsequently, 30-50 µl of RNase-

free water was added into the spin column matrix and incubated for 1 min at room temperature. Centrifugation was conducted at 12000g for 1 min to elute miRNA, and then miRNA was stored at -80°C.

2.3.6. Primer Design

The primers were designed using National Center for Bioinformatic Information (NCBI) handbook. Table 1 presents primers sequences used in this study.

2.3.7. Quantitative Expression of let-7a

Total miRNA was extracted from all samples.

2.3.7.1. cDNA Reverse Transcription

The complementary DNA reverse transcription was conducted on the second day of miRNA extraction. A common primer reaction was applied since it was needed to have cDNA for the let-7a 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 by the manufacturers, the annealing temperature of the optimal primers was calculated according to the following equations:

• Melting Temperature(Tm) =2 (A+T) + 4 (G+C).

• Annealing Temperature (Ta)= Tm - (2-5) ℃.

The temperatures of melting for reverse and forward primer were also calculated according to the above equation. The annealing temperatures for forward and reverse primers were compared to choose the lowest temperature (℃).

2.4. Gene Expression

2.4.1. cDNA Synthesis from mRNA

The cDNA was synthesized using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix protocol.

2.4.2. Procedure

1. First-strand cDNA synthesis

2. The reaction components and the required volume are presented in table 2.

3. Incubation

The required time and temperature are presented in table 3.

Table 1. Primers sequences

Primer	Sequence (5′→3′ direction)
MiRNA	
let-7a	TGAGGTAGTAGGTTGTATAGTT
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

Table 2. Reaction components and volumes

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

Table 3. Thermal cycler steps

	Step1	Step2	Step3
Temperature	25°c	42°c	85°c
Time	10min	15min	5sec
	Random Primer (N9)	Anchored Oligo (dT)18	Inactivation of reverse transcriptase enzyme

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

The expression levels of the let-7a gene were estimated using the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, which is a sensitive technique for the quantification of steady-state mRNA levels. A quantitative real-time qRT-PCR test was performed to confirm the expression of the target gene. Primer sequences for the let-7a gene were designed and synthesized using the NCBI handbook and stored lyophilized at -23°C. In this study, quantification of RT-PCR products was performed based on SYBR Green to recognize any double-stranded DNA, including cDNA (21). Primer sequences are presented in table 1.

2.6. Primer Preparation

The lyophilized primers were then used to produce a stock solution at a concentration of 100 µMto be used each time as a primer working solution. This prepared stock was then stored at -23 °C after dissolving in nuclease-free water. Subsequently, 10µL of the prepared stock was diluted in in the 90 µL nuclease-free water of lyophilized primers and stored at -23°C to be used later. This yielded a working solution with a concentration of 10 µM.

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

The qRT–PCR was carried out using the Real-time PCR method (22).The levels of gene expression and fold change were determined by measuring the threshold cycle (Ct) value using the TransStart® Top Green qPCR SuperMix Kit (Transgen Biotech Co., LTD, China). The required volume of each component was calculated afterward (table 4).

Table 5 tabulates the thermal profile of gene expression.

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. Data were analyzed using the ΔΔCt method and the obtained results were expressed as folding change in gene expression. For each sample, the difference between the CT values (ΔCt) for each target and the housekeeping gene was calculated using the following equations:

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 based on the following formula:

ΔΔCt=ΔCt (patient) – ΔCt (control)

The fold-change in gene expression was calculated as:

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

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 ℃		

2.8. Statistical Analysis

The Statistical Analysis System (SAS; 2012) application was used to detect the effect of various factors on study parameters. The T-test and the Least Significant Difference test were used to compare means. The Chi-square test was used to compare percentages (0.05 and 0.01 probability). The correlation of coefficient was estimated for each variable in this study (23, 24).

Quantitative expression of the let-7a gene and housekeeping gene miRNAU6 were assessed using Real-Time PCR through the relative quantitation method. The gene expression level was normalized to the level of a housekeeping gene and quantified by the ΔCt value and $2^{-\Delta\Delta Ct}$ method as shown in figures 1 and 2, respectively.

A representative melting curve of the let-7a gene for samples analyzed by RT-PCR is shown in figure 3.

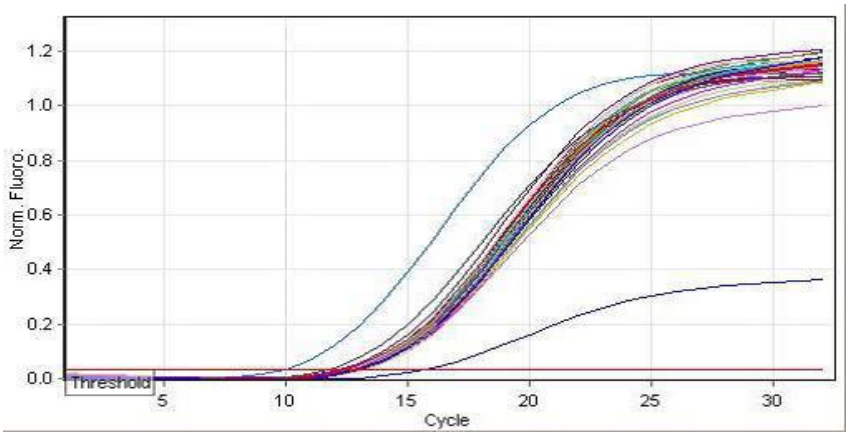

Figure 1. Amplification plots of let-7a gene by RT-qPCR

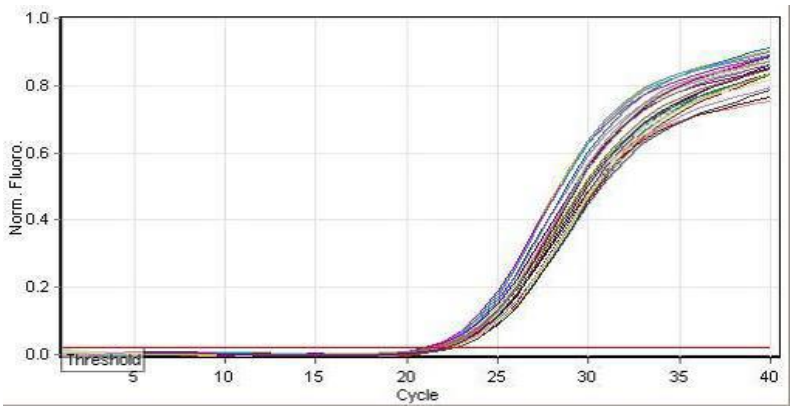

Figure 2. Amplification plots of miRNA U6 gene by RT-qPCR.

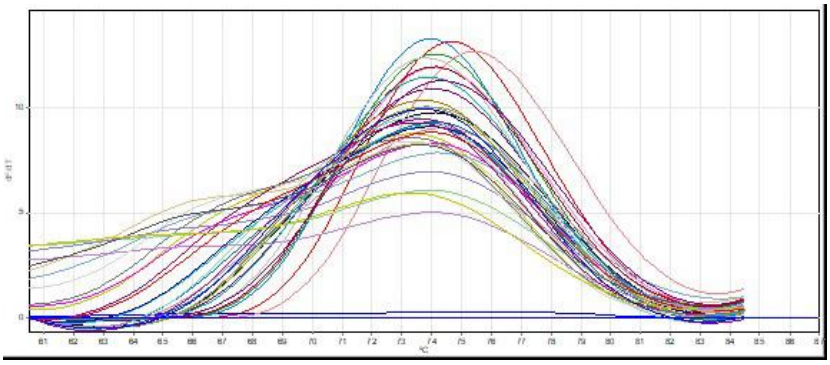

Figure 3. Melting curve of let-7a gene after RT-qPCR analysis showing single peaks

3. Results and Discussion

3.1. Fold of miRNA let-7a Expression Depending on 2-ΔCtMethod

In the group of patients, means Ct of miRNA let-7a, means Ct of miRU6 and ΔCt (Means Ct of miRNA let 7a - Means Ct of miRU6) were reported at 12.97, 17.66, and -4.69, respectively. Moreover, 2-ΔCt was obtained at 25.812536 and experimental group/ Control group was estimated at 25.812/9.986. In the group of healthy controls, the fold of gene expression, means Ct of miRNA let 7,means Ct of miRU6 ΔCt (Means Ct of miRNA let-7a Means Ct of miRU6) and 2-ΔCt were measured to be2.584 ±0.562,14.31, 17.63, -3.32, and 9.9866444, respectively. In addition, experimental group/Control group and the fold of gene expression were obtained at 9.986/9.986 and1.00 ±0.00, respectively. These results were in line with those obtained by Santovito, De Nardis (25), which showed that based on quantitative RT-PCR analysis, let-7a levels significantly increased following 12 months of antidiabetic treatment. Similar findings had been reported previously by Abed and Al-Khafaji (22).Table 6 presents the mentioned results.

Table 6. Fold of miRNA let-7a expression depending on 2-ΔCtMethod

Groups	Means Ct of *miRNA let-7a*	Means Ct of miRU6	ΔCt (Means Ct of*miRNA let-7a* - Means Ct of miRU6)	$2^{-\Delta Ct}$	Experimental group/ Control group	Fold of gene expression
Group 2 (Patients)	12.97	17.66	-4.69	25.812536	25.812/9.986	2.584 ±0.562
Group 1 (Healthy controls)	14.31	17.63	-3.32	9.9866444	9.986/9.986	1.00 ±0.00
T-test (P-value)	-	-	--	--	--	0.493 (0.0001)
(P≤0.01)						

3.2. Correlation of Factors in Patients with Diabetes and Healthy Controls

In total, 44 (55.00%) out of the 80 patients had a family history of diabetes, while 36 (45.00%) patients had no family history of the disease. The mean±SD of HbA1c inpatients was obtained at 9.93±0.32.

3.3. Gender Distribution

Among diabetic patients (n=80), 26 (32.50%) and 54 (67.50%) patients were males and females, respectively. The number of males and females among healthy individuals (n=41) were18 (43.90%) and 23 (56.10%), respectively. These findings were in agreement with those in previously published studies (21-26).

Due to the fact that individuals in both study groups were randomly selected, the difference in gender distribution does not reflect any significance and only represents their availability at the time of collection. The frequency distribution of study groups is presented in table 7.

The ΔCt means of miRlet-7a was reported at 12.97in the blood samples of patients with diabetes, while in the group of control, ΔCt means was 14.31, indicating a significant differences between patients and the healthy controls. These results agree with those previously published in the study conducted by Santovito, De Nardis (25) in which the quantitative RT-PCR analysis showed that let-7a levels were significantly increased following 12 months of antidiabetic treatment. This result can be explained by biological function of the let-7 family which promotes glucose intolerance (26).

This was one of the first researches conducted on Iraqi population to study miRNA and its effect on the genetic transmission of diseases through the prevention of the disease transmission to offspring by synthesis of anti-allergic agents. In the other words, miRNA inhibits translation of the disease-carrying gene.

Based on the obtained results, the gene expression level of miRNA let-7a was lower in diabetic patients, compared to healthy individuals. This also indicates differences in the gene expression fold (2-ΔΔCt) of gene let-7a.

Therefore, lower expression levels of miR let-7a was shown to be associated with the occurrence and progression of diabetes in Iraq population.

Moreover, a significant difference was observed between patients and healthy controls in terms of gender and family history of the disease. In addition, there was a significant difference between patients and healthy controls in terms of age and type of diabetes. The obtained results indicate that diabetes can affect both males and females in all age ranges.

Table 7. Results of factors in sample study

Factors		Patients	Control	P-value
Gender No (%)	Male Female	26 (32.50%) 54 (67.50%)	18 (43.90%) 23 (56.10%)	0.0392 *
Diabetes type No (%)	Type 1 Type 2	10 (12.50%) 70 (87.50%)	- -	0.0001 **
Family history No (%)	Yes No	44 (55.00%) 36 (45.00%)	- -	0.0477 *
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 when diagnosed with diabetes (year)	Mean ±SE	41.50 ±1.65	-	---
HbA1c	Mean ±SE	9.93 ±0.32	-	---

* (P≤0.05), ** (P≤0.01).

Authors' Contribution

Study concept and design: N. S. Y.
Acquisition of data: Z. A. S
Analysis and interpretation of data: M. F. A.
Drafting of the manuscript: Z. A. S
Critical revision of the manuscript for important intellectual content: N. S. Y.
Statistical analysis: Z. A. S
Administrative, technical, and material support: N. S. Y.

Ethics

All investigations were conducted in accordance with the Ethics Committee of University of Technology, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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