

The Role of Some Urinary MicroRNAs in the Incidence of Renal Failure of Patients With Type 2 Diabetes In Iraq

Omar A.Al-Hashimi¹ ; Zubaidah A. Khudhair Aljashaami²

1,2 . Depart. of Biology , College of Education for Pure science , Tikrit University , Iraq

omarbio11382@gmail.com

Abstract: The current study includes the detection of the role of microRNA in the development of kidney failure in patients with type 2 diabetes, by measuring the levels of gene expression of two genes (MiR-21 and MiR-16) where 100 hundred blood samples and morning urine were collected for patients. People with kidney failure by (35) samples, and patients with type 2 diabetes by (35) samples and compared them with the control group of healthy subjects (30) samples. Their ages ranged between (35-65) years. Gene expression levels were measured by extracting microRNAs from the urine, using the Real time qPCR technique, performing statistical analyzes including extracting the value of the Fold expression by $2^{(-\Delta\Delta Ct)}$ and testing the F value of the samples with some measurements. Vital variables in blood and urine, where the results showed an estimate of the amount of MiR-21 convergence in the level of gene expression between the two groups of patients with diabetes and the group of kidney failure means there were no significant differences between them at the probability level ($P < 0.05$), while a significant moral difference was observed in The level of gene expression of the MiR-21 gene between patients and healthy controls at a probability level ($P < 0.05$). As for the levels of MiR-16 expression, it showed a convergence between the two groups of diabetics and the group failure of kidneys for the value of Fold expression with no significant differences at the probability level ($P < 0.05$) for the F test. On the contrary, the level of the gene expression of the miR-16 for healthy people exceeded the expression of patients Broad range and significant difference for F value (0.30) for probability level ($P < 0.05$).

This study clarified the role that MiR-21 and MiR-16 play in the possibility of developing kidney failure through the disorder in the amount of genetic expression about them, which indicates the possibility of benefiting from them as two vital indicators in the urine that can be inferred from the development of the disease in people with diabetes .

Introduction

Kidney disease is one of the diseases that has spread recently in a striking way, some of which are genetically and some of which are acquired and others are unknown reasons so far, and miRNAs in the kidneys play a large role in the formation of organs and in causing many diseases, including Kidney cancer, diabetic nephropathy and glomerulopathy. The parietal cells from Bowman's portfolio edit or secrete miRNAs related to the development of renal fibrosis (Sydwell, et al, 2015). These experimental evidence lines also found an analog in patients with diabetes, which opens up the possibility of being used as biomarkers for kidney diseases associated with diabetes, which often lead to kidney failure as a result of the persistently high levels of blood sugar, which is accompanied by frequent infection of the urinary tract or a certain disorder as a result The use of chemical or herbal drugs affecting the kidney function (Smith, 2017). Failure Renal kidney failure, known as the last stage of kidney disease, is the most important stage, and it is the two types of acute kidney failure that occur during short periods ranging between hours and days and the kidneys may regain their

function The main thing after treating the cause, as for the other type, which the kidneys lose permanently, is called chronic kidney failure (Al-Suwayda, 2010). It is described as a gradual and irreversible deterioration of kidney function, where the body cannot get rid of metabolic process residues, and is unable to maintain the balance of water and ions in the normal range, and thus the level of urea and creatinine in the blood increases. There are several reasons that lead to chronic kidney failure, the most important of which is disease Diabetes Mellitus, as Diabetic nephropathy is a major cause of kidney failure with 45.3% of cases (Al-Juburi, 2015). Diabetes is referred to as a group of metabolic disorders characterized by high blood sugar and physiological changes in the metabolism of energy resulting from a lack of relative or complete insulin secretion or insulin activity or both, and it also includes disorders of metabolism of carbohydrates, proteins, fats, water and electrolytes that can lead Thus to death (Whitney et al, 2002). Numerous studies have found that miRNA, or miRNA, or miRs, are small pieces of RNAs that are approximately 19-25 nucleotides involved in gene blocking after transcription in all eukaryotes. It was discovered in 1993 in *Caenorhabditis elegans*, where it was found that it plays crucial roles in gene regulation networks and is related to the incidence of various kidney diseases, including chronic kidney disease (CKD) Chronic Kidney Diseases. Regulating basic cellular activities such as growth, differentiation, programmed cell death, immune regulation, and organic formation (Chandrasekaran *et al*, 2012). The importance of miRNA remained unknown due to its unusual properties and function unknown at the time, after which approximately 17,000 miRNA were identified so far with more than 1900 in humans, the discovery of RNAi revolutionized understanding of gene regulation and led to Identification of multiple classes of small RNA involved in gene regulation (Reinhart et al, 2000).

Diabetic Nephropathy

Diabetic nephropathy occurs as a complication of diabetes, as high blood sugar for a long time causes damage to the kidney units Nephrons and glomeruli responsible for blood purification and removal of excreta, as it may lead to scarring of these glomeruli and impaired kidney function, and the disease may develop into a disease Chronic kidneys or kidney failure in some cases, due to complications caused by diabetes, which is characterized by the accumulation of extracellular proteins, glomerular basal membrane thickness, expansion, enlargement and division of intermediate cells (Kato et al, 2007).

Renal failure

A pathological condition in which the kidneys become unable to get rid of the final metabolic products from the blood, regulate fluids, electrolytes, acid and base balance in extracellular fluids (Ricci *et al.*, 2016). Renal failure was previously known as Uremic poisoning, depending on the symptoms associated with kidney failure, which include high urea and creatinine in the blood. Beginning in 1847, the term uremia was replaced by the term uremia to indicate blood contamination in the urine, until contemporary medical literature settled on the term kidney deficiency (Shafi *et al.*, 2012).

Biogenesis of MicroRNA

The miR is constructed from the deoxyribonucleic acid, where many miRs are encoded by the genetic regions that fall into the introns and the un encoded genetic areas of the RNA (Shi *et al*,2007). The miR in the nucleus begins with two paths, the canonical pathway and the second path, which is unconventional or non-canonical pathway.

Canonical pathway

The miR RNA is transcribed by the RNA polymerase II or III to produce a pri-miRNA, which is processed by two RNA III enzymes (Dicer and Drosha) where they are called the microprocessor complex. After treatment, pre-miRNA precursors that are transported outside the nucleus to the cytoplasm by Exportin5 / RanGTP are then produced to be processed again in the cytoplasm by Dicer, resulting in a mature miRNA strand of 22bp. The mature thread is combined with the Argonaute (AGO) proteins present in the cytoplasm to form an induced-silencing complex (miRisc) that in turn targets the mRNAs to induce inhibition of translation. The miRisc complex may bind to the GW182 family proteins, which in turn cut the target mRNA thread, where the cut mRNA is then subjected to decomposition by Exoribonuclease (XRN1).

Non-canonical pathway:

In this path, the small hairpin RNA (shRNA) is synthesized by the microprocessor complex and exported to the cytoplasm by Exportin5 / RanGTP, where it is also processed by Argonaut 2 (AGO2) independently of the Dicer enzyme to form mature RNA. miRNA then the latter merges into the MiRisc functional complex to start functioning.

As for the Mirtron molecule resulting from binding (mRNA with spliceosome) and m7G pre-miRNA resulting from the binding (7-methyl guanine with pre-miRNA), they depend on the Dicer enzyme to complete their maturation process, but they differ in the mechanism of their export outside the nucleus, where Mirtron is exported from Exportin5 / RanGTP. The export of the m7G pre-miRNA molecule to the cytoplasm is done by Exportin1. All paths eventually lead to the functional MiRisc complex, which in turn either induces translation inhibition or mRNA is interrupted by the family proteins GW182 and then mRNA is degraded by the enzyme XRN1 (Wei *et al.*, 2014).

MICRORNAS IN URINE

MicroRNAs have been detected in urine.(Hanke *et al.*,2009) Theoretically, these urinary miRNAs may be filtered and excreted by, or directly from, the kidney and urinary tract. Scientists detected 22 different urinary miRNAs, but none was kidney-specific.(Melkonyan *et al.*2008) Analysis of miRNA expression in single urine samples revealed the miRNA ratios miR-126 : miR-152 and miR-182 : miR-152 were significantly elevated in urine of urothelial bladder cancer patients compared with urine of healthy donors and patients with urinary tract infections,enabling a separation of tumour patients from the control groups (Hanke *et al.*,2009) The ratio miR-126 : miR-152 showed an average 9.9-fold increase in urine samples from patients with bladder cancer in comparison with healthy donors. These studies have revealed a new possibility in the development of noninvasive investigation of kidney diseases by using specific urinary miRNAs as biomarkers for disease diagnosis or progression. Exosomes have also been detected in urine(Gonzales *et al.*,2008) Urinary exosomes are a rich source of intracellular kidney injury biomarkers because they are released from every segment of the nephron, including podocytes. Urinary exosomal transcription factors have already been proposed as renal tubular cell biomarkers for acute kidney injury.(Zhou *et al.*2008) demonstrated that levels of miR-27b and miR-192 in urinary exosomes could differentiate lupus patients with or without nephritis.(2008) It is expected that miRNA containing exosomes .

Material & methods

Sample collection

The study included collecting 100 morning Urine samples distributed between (35) samples for patients with type 2 diabetes and (35) samples for patients with Renal failure and who suffer from diabetes in the long time in addition to (30) samples for healthy people as a

control group, which included The study, the age group between 35 - 65 years, for the subject areas. Samples were obtained from different regions of the governorates of Iraq, Keeping the samples in sterile tubes and marking them according to the categories under consideration in the following way:

- The samples were collected in sterile tubes, marked by categories, and transferred directly to the laboratory
- Ten ml of each Urine sample was placed in sterile and labeled plastic tubes and placed in a Centrifuge
- The device was set to a power of (3000) r / min for 15 minutes
- Nine ml was removed from the upper filtrate of the sample quietly by pipette and disposed of directly
- The precipitate was mixed with the remainder of the liquid in the tube, then 70 line of Urine Conditioning Buffer, prepared by ZYMO RESEARCH / USA, was added.
- The samples were kept at a temperature of 25 ° C until the start of the extraction process

RNA extraction

RNA was extracted using RNA extraction kit (Direct-zol™ RNA MiniPrep,R2051, ZYMO RESEARCH / USA). The Direct-zol™ RNA MiniPrepprovides a streamlined method for the purification of up to 50 µg (per prep) of high-quality RNA directly from samples in TRI Reagent. Total RNA, including small RNAs (17-200 nt) including miRNA, is effectively isolated from a variety of sample sources (urine). the contents of this kit were listed in table 1.

Table 1: RNA extraction kit contents (Direct-zol™ RNA MiniPrep,R2051, ZYMO RESEARCH / USA).

Direct-zol™ RNA MiniPrep Kit Size (Preps)	R2051 (50)
TRI Reagent®	50 ml
Direct-zol™ RNA PreWash1 (concentrate)	40 ml
RNA Wash Buffer2 (concentrate)	12 ml
DNase I3 (lyophilized)	1
DNA Digestion Buffer	4 ml
DNase/RNase-Free Water	6 ml
Zymo-Spin™ IIC Columns	50
Collection Tubes	100

Isolation of RNA by conventional phase separation was shown to selectively enrich for some species of miRNA, leading to bias in downstream analysis. The Direct-zol™ method assures unbiased recovery of small RNAs including miRNA.

Conversion of RNA to cDNA

In this study, RNA was covered to cDNA according to used kit (PrimeScript™ RT reagent Kit). The additions and conditions of the conversion step were obtained in table 2.

Table 2: contents conversion RNA to cDNA

Reagents	Volumes
1- 5 × PrimeScript™ mix	2 µl
2- total RNA	3
3- RT-stem-loop primer*	0.5 µl
4- RNase Free dH ₂ O	up to 10 µl
	Total 10 µl

* sequence of RT-stem-loop primer was expected in table 3.

Performing RT-PCR

RT-qPCR technology was used to amplify miR-16, miR-21 and U6 genes depending on the working method of the diagnostic kit (KAPA SYBR® FAST qPCR Master Mix (2X) Kit, Instrument: Sacace, Origin: Italy) which is supplied as a 2X master mix with integrated antibody-mediated hot start, SYBR Green I fluorescent dye, MgCl₂, dNTPs, and stabilizers. Primers were designed by NCBI and supplied by Integrated DNA Technologies / USA, as shown in table 3. The conditions and additions of the reaction were obtained in table 4. Cycling Program was described in table 5.

Table 3: Primers sequence used in this study.

Primer		direction	Sequence (5'→3')
Reference gene	U6	Forward	AGC GAA GTC CCT TCG GGG
miR-21		Forward	TAG CTT ATC AGA CTG ATG TTG A
miR16		Forward	TAG CAG CAC GTA AAT ATT GGC G
Stem loop			GA AGA AGG CGA GGA GCA GAT CGA GGA AGA AGA CGG AAG AAT GTG CGT CTC GC TTC TTT CNN NN
Reverse universal			GTG CAG GGT CCG AGG T

Table 4: RT-qPCR reaction's conditions and additions.

Component	20 µL (Final volume)	Final concentration
KAPA SYBR FAST qPCR Master Mix (2X) Universal	10 µL	2x
Forward primer	0.4 µL	0.2µM
Universal Reverse primer	0.4 µL	0.2µM
Nuclease-free water	Up to 10 µL	
Template DNA Sample Volume	-	1pg-100ng

Table 5: RT-PCR Cycling Program

Step	Temp. (°C)	Time	Cycle	Scanning
Enzyme activation	95 °C	05:00 min	Hold	
Denaturation	95.0 °C	:00:20	40	
Annealing	60.0 °C	:00:20 sec		
Extension	72.0 °C	:00:20 sec		

Statistical analysis:

The Levac method was used to measure $2^{(-\Delta\Delta Ct)}$ to estimate the level of gene expression for the miR-21 and miR-16 genes based on the value of the Cycle Threshold (CT) value for each patient and healthy sample plus a reference gene with extracting the ΔCt value for each of the samples (Denman and McSweeney, 2005) according to the equations below:

$$\Delta Ct (\text{patients}) = Ct (\text{patients}) - Ct (\text{reference})$$

$$\Delta Ct (\text{Controls}) = Ct (\text{controls}) - Ct (\text{reference})$$

$$\Delta\Delta Ct = \Delta Ct (\text{patients}) - \Delta Ct (\text{controls})$$

$$\text{Normalized target gene expression level} = 2^{(-\Delta\Delta Ct)}$$

Results and Discussion

The current study dealt with measuring the difference levels in the microRNA gene expression in morning urine samples of three groups of patients and healthy people. The samples included the group of patients with type II diabetes, and the group of patients with diabetic nephropathy who reached the End Stage of Kidney Diseases (ESRD) In addition to the control group who are healthy, the study dealt with measuring the levels of miR-21 and miR-16 nucleic acid and comparing them with the U6 (Reference gene) using the real time qPCR, as shown in the figures.(1,2,3).

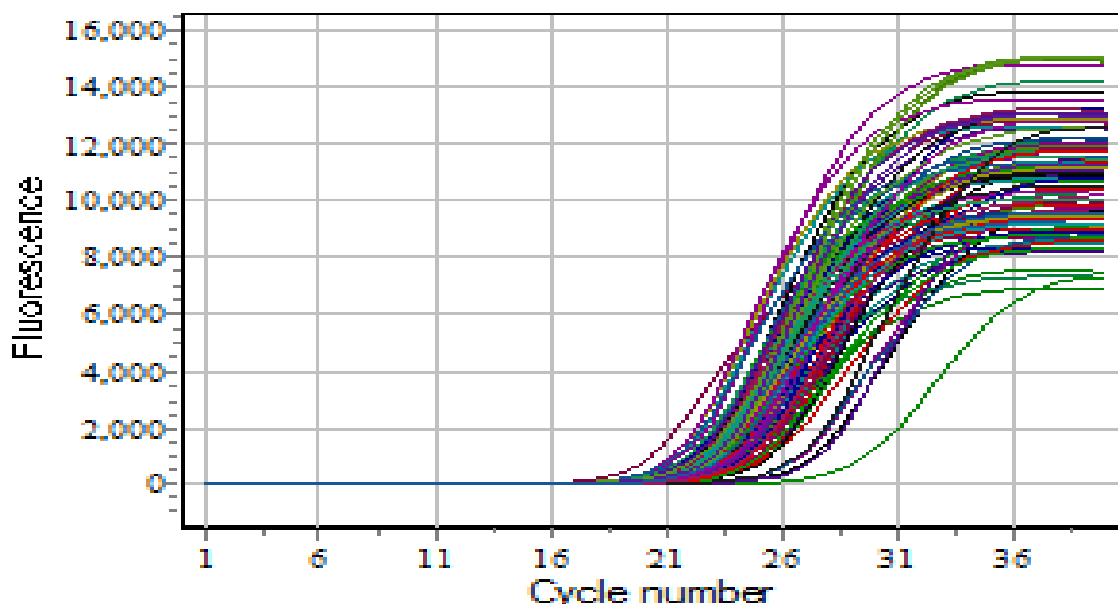


Figure (1): Diagram of the amount of gene expression of the U6 gene (Reference gene) in both patients and healthy .

miR-21 : A vital sign of good stability in extracellular body fluids is that they are present and stable and can be easily measured in tissues and body fluids, including urine and have great importance as kidney regulators (Ortiz-Quintero, 2016.). The level of folding expression

between the two groups of patients with diabetes and the group of kidney failure without significant differences for the F test, while both groups showed a significant difference when compared to the standard sample for the probability level $P < 0.05$. The decrease in gene expression is evident whenever the patient progresses and his condition deteriorates towards the final stage of kidney failure, as shown in figure (4). In the kidney, or released into the cavity by cells located on different areas of the kidney. Anne of large-scale studies, to test the ability of specific RNA to predict risk and respond to treatment (Wonnacott et al, 2017). These results were consistent with a 2016 study by (Gaede and his group 2017), and (Song and her group ,2018), by (Song et al, 2018) .

The approximate levels of expression between the group of patients with diabetes and the group of patients with diabetic nephropathy did not show any significant differences between them, except that the levels of expression decreased relatively, and this indicates that the expression of miR-21 decreased in both groups at the same frequency and thus can be considered a decrease in gene expression through Estimating its amount in the release is a biological sign of the possibility of developing the condition of people with diabetes towards the worst, which is the nephropathy that leads to the final stage of kidney failure. tensin, and excessive expression of B-protein kinase (PTEN) may alter or activate diabetic nephropathy, therefore targeting by miR-21 may inhibit the occurrence of diabetic impairment (Li et al, 2010), and one study confirmed that increasing expression of miR-21 It resulted in inhibition of the proliferation of mesangial cells in high glucose states, as well as the deposition of the extracellular substance, which is the main cause of kidney failure (Sydwell et al. , 2015).

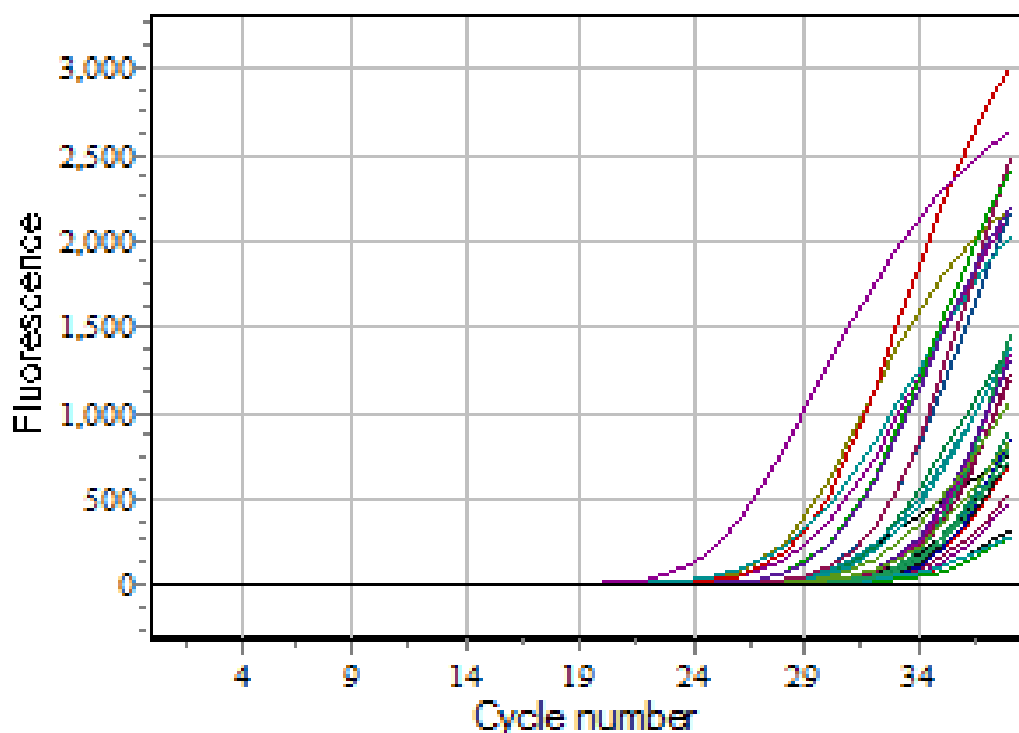


Figure (2): Diagram of gene expression (miR-21) for both patients and healthy.

miR-16 : Results of estimating the level of miR-16 gene showed a convergence between the group of diabetics and renal failure of the value of folding expression with no significant differences at the probability level $P < 0.05$) for the F test. On the contrary, the level of the gene expression of the mir-16 DNA for healthy people exceeded the expression of the patient groups With a wide range and significant difference for the value of the F test (0.30) and the probability level $P < 0.05$) as shown in Figure (4). The studies that were conducted on the

relationship of miR-16 with diseases and the results that were reached proved most studies related to the development of diseases, especially cancerous ones, where studies indicated that miR-16 was largely organized in RCC kidney cells. Decreased miR-16 regulation reduced cell proliferation, migration and increased programmed cell death levels, while overexpression of miR-16 accelerated cellular proliferation and migration, indicating that miR-16 may act as a tumor gene in RCC. The additional study of miR-16 in RCC may clarify the molecular mechanisms of RCC carcinogenesis and assist in the development of new biomarkers and treatment options (Chen et al., 2015), on the other hand, studies have indicated a low level of expression of miR-16 with type 2 diabetes being It aims to regulate the work of a number of cellular signals that regulate the level of insulin, which aggravates the pleural effects of the disease dangerously leading to the patient's kidney failure and this explains the low level of expression in the samples of the patients under study (Bork-Jensen et.al., 2015). To indicate that the miR-16 can be considered a reference gene for chronic kidney disease as the most stable internal reference gene in the data set obtained (Lange et al, 2017) the current study agreed with this study through the stability and stability of gene expression in all samples The study thus enables, for the first time, to our knowledge, to consider that the miR-16 gene is a reference gene for renal failure resulting from diabetic nephropathy.

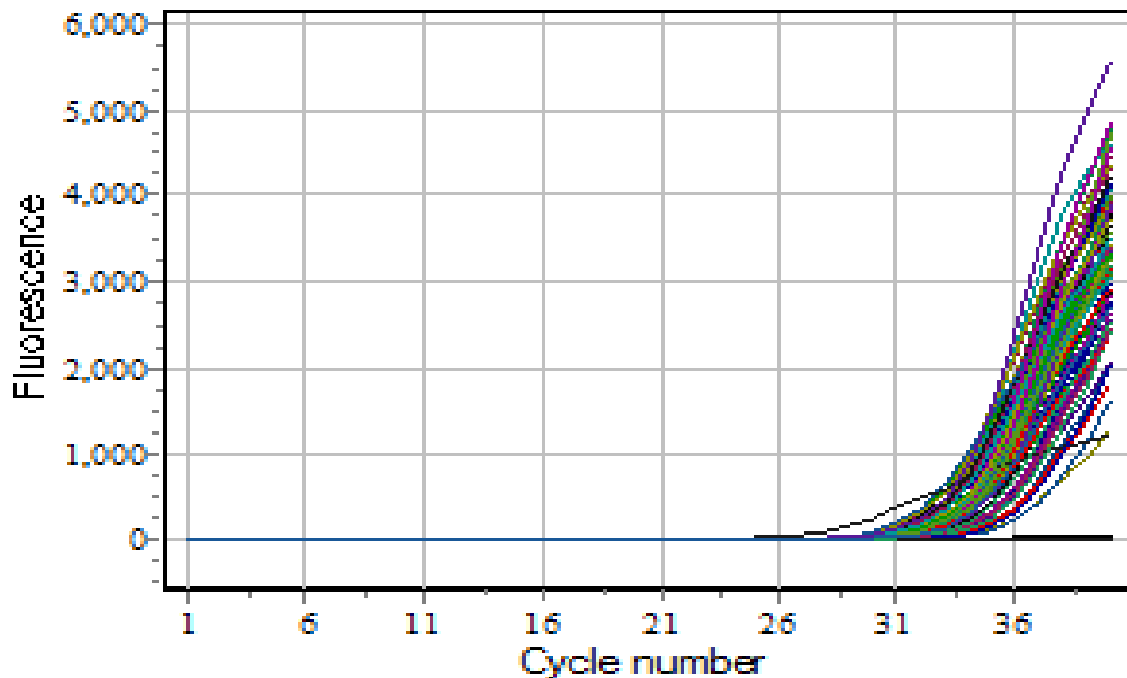


Figure (3): Diagram of the amount of gene expression (miR-16) for both patients and healthy.

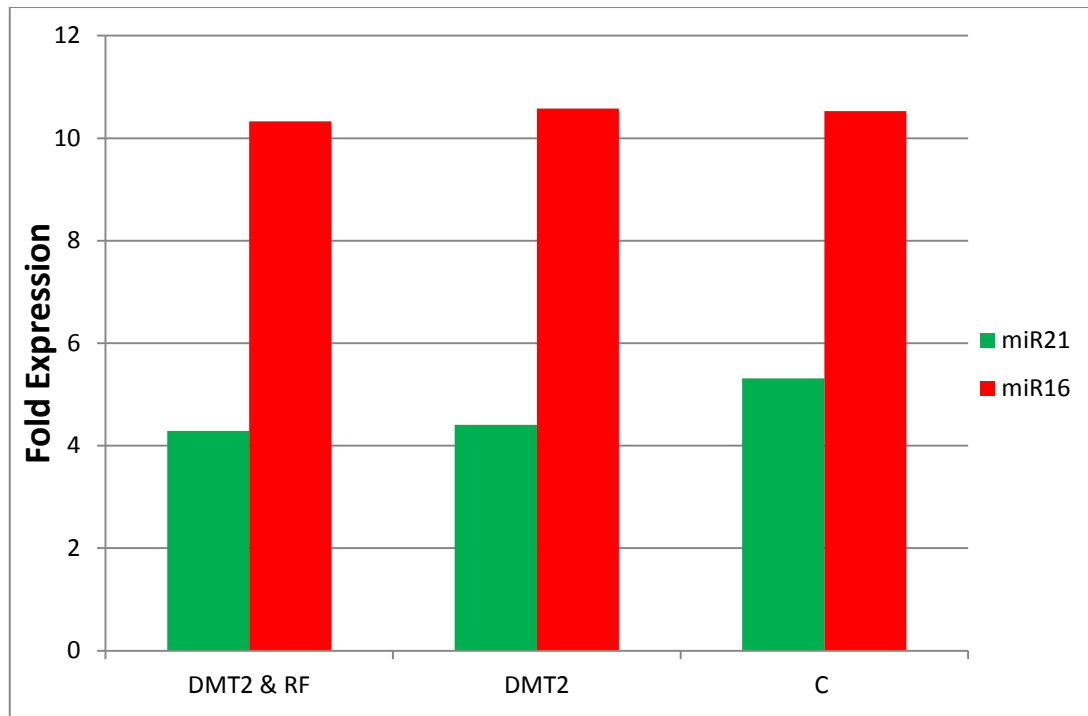


Figure (4) the level of gene expression for both (miR-21) and (miR-16) in patient and healthy samples.

Conclusion:

This study clarified the role that MiR-21 and MiR-16 play in the possibility of developing kidney failure through the disorder in the amount of genetic expression about them, which indicates the possibility of benefiting from them as two vital indicators in the urine that can be inferred from the development of the disease in people with diabetes .

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