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RESEARCH ARTICLE

The Role of miRNA- 126 in The Development of End Stage Renal Disease

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ABSTRACT

The prevalence of chronic kidney disease (CKD) is on the rise globally, mostly due to the high burden of diabetes and hypertension. Chronic kidney disease is described as a silent condition due to a lack of obvious clinical symptoms, particularly in its early stages, as a result, most affected individuals are unaware of their disease status and are often only detected at an advanced stage of the disease. The aim of this study was to reveal the role of microRNAs (miRNAs) in the development and prognosis of CKD. miRNAs, are a class of short, single-stranded, non-coding RNAs approximately around 22 nucleotides in length that down regulate gene expression through translational repression or degradation of messenger RNA (mRNA) by binding to the mRNA's 3'-untranslated region thereby inhibiting translation into protein. miRNAs are implicated as playing a role in chronic kidney disease due to their role in apoptosis, cell proliferation, differentiation and development. The current study focused on miRNA-126, which controls endothelial cell function and maintains vascular homeostasis. Patients with CKD have been found to have lower levels of miRNA-126. Dysregulation of this miRNA is associated with disease progression. This article summarizes the techniques used to isolate and quantify miRNA-126. The article provides a brief overview of the challenges faced by miRNA research and the potential use of miRNA molecules for the management of CKD, this suggests a research strategy focused on developing standardized and reliable biomarkers for therapeutic use.

INTRODUCTION

The occurrence of chronic kidney disease (CKD) is increasing worldwide, and by 2045, CKD is expected to be the fifth leading cause of mortality(Motshwari et al., 2023). The rise in the number of CKD cases is mainly due to the high prevalence of hypertension (HTN) and diabetes mellitus (DM), other causes including viral infection and advancing age(Bikbov et al., 2020). Chronic kidney disease is sometimes called a silent disease because there are no definite symptoms in its early stages. As a result most affected individual are unware of their disease status and are often only detected at an advanced stage of disease(Bağriaçik & Dikmen, 2024). In addition, there is a distinct association between CKD and an increased likelihood of cardiovascular diseases (CVD). Besides, people with CKD are more prone to the death caused by CVD than to the probability of advancing to end-stage renal disease (ESRD)(Cambray et al., 2024). The early detection of the CKD and the screening of the high-risk persons are the most important things to do in order to stop the disease progression and to reduce the negative health consequences that are associated with it (Li et al., 2020).

The human genome has approximately 22,000 genes that encode for proteins. On the other hand, other genes are transcribed into non-coding RNAs (ncRNAs), which are RNA molecules that do not

encode polypeptides. These ncRNAs comprise about 80% while the rest 20% of transcription is associated with making mRNA (López-Jiménez & Andrés-León, 2021).

miRNAs are non-coding RNA molecules about 18–25 nucleotides in length, the primary function of the miRNA is to regulate gene expression either by destroying the mRNA or by blocking the mRNA translation into functional proteins. They are produced from endogenous hairpin-shaped transcripts within the genome. The human genome encodes roughly one thousand miRNAs, which potentially regulate about 60% of all genes, as they bind to the coding mRNA due to partial complementarity(Dandare et al., 2023). miRNAs function as negative regulators of gene expression by either degrading or inhibiting the translation of their target mRNAs(Venneri & Passantino, 2023). These molecules are initially transcribed by RNA polymerase II as longer RNA products known as Pri-miRNA. This Pri-miRNA is then processed in the nucleus by RNase III enzyme Drosha and DiGeorge syndrome critical region 8 (DGCR8), a step critical for miRNA maturation. The resulting pre-miRNA hairpins, approximately 60–70 nucleotides long, are exported to the cytoplasm by exporting. There, Dicer, another RNase III enzyme, cleaves them into a double-stranded miRNA/miRNA duplex of about 22 base pairs. In the final maturation step, one strand from this duplex is integrated into the RNA-induced silencing complex (RISC). which directs it to its target mRNA to suppress gene expression. The other strand is either quickly degraded or serves distinct biological functions, potentially as a miRNA itself(Bofill-De Ros & Vang Ørom, 2024).

miRNAs are known to be responsible for the occurrence of many diseases in humans by controlling the vital biological processes that lead to the development of a disease, for example, apoptosis, development, and proliferation (Wang et al., 2024) . miRNAs are critical in the initiation and progression of CKD, with their levels varying according to the stage of the disease. These variations in miRNA levels are linked to the severity of the condition(Khan et al., 2020). Liang et al. have identified miRNAs as novel potential biomarkers capable of detecting early kidney damage, highlighting their promise in indicating the stage or severity of the disease. Therefore, they assayed the level of miRNA-126 in patients with end stage renal disease expecting to be able to provide new insights into the development of end stage renal disease. The expression level of miR-126 was considerably decreased in the CKD Moreover, those in the highest tertile of miR-126 had a significantly lower risk for CKD compared with the lowest tertile of the miR-126(Fujii et al., 2021). Previous investigations have showed that patients with end stage renal disease and hemodialysis exhibit a low expression of miR-126(Fourdinier et al., 2021).

MATERIALS AND METHODS

The study population

A case-control study included forty patients with chronic renal failure admitted to Kirkuk Teaching Hospital, Hemodialysis Center, and twenty apparently healthy individuals as a control group. Inclusion Criteria end stage renal disease patients on regular haemodialysis diagnosed by estimated Glomerular Filtration Rate (eGFR) less than 10 ml/min .

Ethical Approval

Ethical approval for this study was obtained from the Iraqi Ministry of Health|Kirkuk Health Department,with an assigned approval letter,No.9,dated 31 Februrary 2023.

Sample collection

Serum specimens (250 µl) were extracted from some chronic renal failure patients as well as from the control group and mixed with 500µl trizol solution for miRNA isolation.

Total RNA Extraction

RNA was extracted from serum samples of both patients and control groups using the TRIzol™ RNA isolation kit (Invitrogen, USA). The procedure for RNA isolation was described as following: For each 0.25 mL serum sample 0.50 mLTRIzol was added, and the lysate pipette was up and down several times to homogenize. Fifteen minutes' incubation was used to permit complete dissociation of the nucleoproteins complex. For each tube 0.15 mL of chloroform as of TRIzol™ Reagent was added for lysis. Incubation was done for fifteen minutes at -20°C and centrifuged the mixture at 12,000 ×g for 15 minutes. The mixture underwent an initial separation into a lower phase containing red phenol-chloroform, an intermediate phase, and an upper phase containing colourless water. The aqueous phase containing RNA was moved to a fresh tube. 0.250 mL of isopropanol was introduced into this phase in order to cause the RNA to precipitate. Subsequently, the mixture was subjected to incubation at a temperature of -20°C for a duration of 25 minutes, followed by centrifugation at a force of 12,000 x g for 10 minutes. As a result, a white, gelatinous precipitate of RNA settled at the bottom of the tube. The liquid portion was removed using a micropipette. After being vortexed to dissolve, the supernatant was re-suspended in 0.50 mL of 75% ethanol and centrifuged at 7500 × g for 5 minutes. Subsequently, a micropipette was used to pick out the liquid part. The RNA was dried by leaving the tube open for 15 minutes. Besides, the thermomixer was used to incubate the pellet at 60°C for 15 minutes after it had been suspended in 20 µL of RNase-Free water.

miRNA quantitation by Qubit 4.0

The Qubit™ miRNA High Sensitivity(HS) assay Kit was employed to quantify miRNA in the samples. The assay was very specific for miRNA over other types of RNA. The RNA assay is capable of quantifying the initial sample concentrations within the range of 10 ng/µL to 100 ng/µL. The test is conducted at room temperature and the signal remains consistent for a duration of up to 3 hours.

Primers design for miRNA-126 gene expression

The complementary DNA (cDNA) sequences of the housekeeping gene (U6), and miRNA-126 gene were acquired from the Gen Bank database of the National Center for Biotechnology Information (NCBI). Tables 1 below list the primer sequences that were utilized in this study for convenience.

Synthesis of cDNA from miRNA

The first-strand cDNA synthesis kit ProtoScript® was used. The expression levels of miRNA-126 were assessed using cDNA synthesis. This kit includes the (M-MuLV, MMLV) Reverse Transcriptase Mix and M-MuLV Reaction Mix, two optimized mixes. dNTPs and an optimized buffer are included in the M-MuLV Reaction Mix, whereas M-MuLV Enzyme Mix incorporates M-MuLV Reverse Transcriptase and Murine RNase Inhibitor, to do cDNA reverse transcription.

Quantitative Real-time PCR (qRT-PCR) for miRNAs under study

This step was divided into two phases, the first was done through the synthesis of cDNA from miRNA through specific primers for miRNA-126 and the cDNA synthesis kit. The procedure was performed through the steps below: Three microliters of total RNA from each sample extraction were distributed into separate PCR tubes. Ten microliters for each sample were added the Protoscript reaction mix, comprising dNTPs, buffer, and other vital components, was used. Subsequently, MuLV Enzyme was introduced into the reaction at a volume of 2µl per sample. The volume of particular primer was adjusted to 20µl by adding 3µl of nuclease-free water, resulting in a final volume of 2µl. The combination was incubated for 1 hour at 42 ºC using a thermocycler, and then the enzyme was inactivated at 80 ºC. The resulting cDNA product was quantified using Qubit 4.0 and stored for subsequent steps, including the second section of this protocol.The process entailed selecting the cDNA sample from both the patient and control groups to be used in the same experimental run. Each sample was accompanied by three PCR tubes: one for the target miRNA-126 and another tube for U6,

which serves as a housekeeping gene in this investigation. The quantification was determined by measuring the fluorescence intensity of syber green.

Briefly centrifuge the PCR tubes to eliminate bubbles and gather the liquid (1 minute at 2000g), then configure the Real-Time PCR program according to the specified thermocycling protocol outlined in Table 3.

Statistical Analysis

The statistical analysis was performed with Graph Pad Prism analytical software and comparison were made where required via Qi squire and T test. T-Test were applicable and interpreted as P value > 0.01 : Highly Significant (HS). • 0.1 ≤ (P value) ≤ 0.5: Significant (S) • P valus > 0.05 : Non –signifiant (NS)

RESULTS AND DISCUSSION

The present data demonstrated that most of patients involved in the study were males 22 (75%), while 8 (25%) were females as depicted in Table 4.

The results from the current study revealed that the majority of patients had a BMI between 20-25, accounting for 44.67% of the sample. Patients with a BMI between 26-30 had the second highest rate, while the lowest proportion of patients had a BMI below 29, as indicated in Table 5.

Regarding comorbidity diseases, the present results showed that chronic renal failure patients were most likely complaining of hypertension 30% followed by diabetes 60% and 10 % were diagnosed with cardiovascular disease as demonstrated in Table 6.

Regarding the gene expression of miRNA-126, the RT PCR data illustrated significant downregulation of miRNA-126 in patients (P<0.0001) compared to a control group with mean fold change 0.65 and 3.01 respectively when applying Mann Whitney tTest as depicted in Table 7 and Figure 1. The amplification curves in Figure 1 display the relative gene expression of miRNA-126 in both study groups.

The current investigation revealed a statistically significant reduction in miRNA-126 levels among the patients, consistent with findings from previous studies. Recent research underscores the pivotal role of miRNAs in CKD pathophysiology, highlighting the potential role of circulating miRNAs as alternative markers for early CKD detection, progression monitoring, and treatment response assessment.

Circulating and urinary miRNAs are the most promising biomarkers that can be used for the diagnosis of pathological states due to their stability in the body fluids and their detectability using validated quantification techniques (1).In the general population and people with comorbidities like DM and HTN, serum and whole blood samples of miRNA-126 were found to be reduced and this was inversely related to the CKD prevalence and positively related to the eGFR(Carmona et al., 2020).

A prospective study showed that lower levels of miRNA-126 were associated with an increased risk of developing CKD and the fast worsening of kidney function over five years (12). Moreover, a previous study proved that miR-126 has an atheroprotective effect and it influences the vascular smooth muscle cell turnover and the regulation of their contractile phenotype(Nguyen et al., 2021). Therefore, the decrease in miRNA-126 expression could potentially be the underlying reason for the impaired blood vessel function commonly observed in individuals with CKD. Additionally, miR-126 downregulation may have a role in both the detection and the management of vascular problems associated with CKD(Motshwari et al., 2023).

The present study is inconsistent with some studies which reported an increase in miRNA-126 expression in persons with diabetic kidney disease, particularly in urine, plasma, and serum samples. This upregulation could potentially represent a compensatory mechanism triggered by microvascular endothelial cells under stressful conditions(Motshwari et al., 2022). The previous study was noticed the serum levels of miRNA-126 reduced as the renal function decreased. However, there are insufficient and even conflicting data available on the dependency between kidney function and miRNA concentrations in plasma and urine. Some investigators have examined the possibility of the aggregation of RNases that are present in renal failure in plasma and which may facilitate the further degradation of circulating miRNAs. However, the miRNAs are not free but bound to exosomes and Argonaute proteins which provide protection to them(Scullion et al., 2021).

On the other hand, in the last stages of CKD, there is a reduction in the plasma concentration of miRNA-126. Additionally, findings from studies indicate that miRNA-126 may confer protective effects on kidney tissue(14). Endothelial miRNA-126 is recognized as a significant regulator of angiogenesis and is involved in endothelial repair and homeostasis. Therefore, it appear that the regulation of miRNA-126 expression could become a possible therapeutic method that will be used to restore the endothelial regeneration in blood vessels and protect the kidney tissue, thus, reducing the atherosclerosis and the CKD-related damage(Nammian et al., 2020).Other study also found that the amount of miRNA-126 was less in CKD and hemodialysis subjects than in kidney transplant patients. The present result are in line with previous result who have demonstrated that circulating miRNA-126 is reduced in ESRD and hemodialysis patients(Franczyk et al., 2022).

CONCLUSION

The current study demonstrated the essential function of miRNAs in CKD and consequently, their possible use in the diagnosis and treatment of CKD. Also emphasized the critical importance of miRNA-126 both as a biomarker for early detection of kidney injury and as a possible focus for treatment strategies. The marked decrease in miRNA-126 levels found in patients with CKD compared to the control group further substantiates its significant involvement in the diseases progression.

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Table 1: The miRNA-126 primers utilized in this study (the primers were designed in this study)

Primers of miRNA-126	**Sequence 5' 3' ⟶**
miRNA-126 forward primer	GGGCATTATTACTTTTGG
miRNA-126 reverse primer	TGCGTGTCGTGGAGTC
miRNA-126 Reverse Transcription Primer	GTCGTATCCAGTGCGTGTCGT GGAGTCGGCAATTGCACTGGA TACGACCGCATT
U6 Forward primer	GAGAAGATTAGCATGGCCCCT
U6 Reverse Primer	ATATGGAACGCTTCACGAATTTGC

U6: housekeeping gene

Table 2: The component of reaction mix with their quantity

Component	**Reaction (20 µL)**
Luna Universal qPCR Master Mix	10
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Template DNA	5
Nuclease-free Water	4

Table 3: Program for real-time PCR with indicated thermo cycling.

Cycle No.	**Stage**	**Temperature**	**Time**
1	Initial Denaturation	95°C	60 seconds
40 for U6, miRNA-126	Denaturation	95°C	15 seconds
	Annealing and Extension	60°C for (U6, miRNA34a and 126)	30 seconds
1	Melt Curve	60-95°C	40 minute

Table 4: Distribution of the study groups according to sex.

Sex	**Patients**		**Control group**	
	No.	%	No.	%
Male	22	75	21	70.00
Female	8	25	9	30.00
Total	30	100.00	30	100.00

Table 6:Distribution of the study groups according to BMI.

BMI	Patients		Control group	
	No.	%	No.	%
< 20	16	10.67	4	8.00
20-25	67	44.67	33	66.00
26-30	49	32.67	11	22.00
> 30	18	12.00	2	4.00
Total	150	100.00	50	100.00

Table 4: Comorbidity diseases in the study groups.

Comorbidity factors	Patients		Control	
	No.	%	No.	%
Diabetes	9	30	0	0.00
Hypertension	18	60	0	0.00
Cardiovascular diseases	3	10	0	0.00
Total	30	100.00	20	0.00

Table 5: *miRNA-126* gene expression in study groups.

Gene expression of miRNA-126 (Mean±SD)	Patients	Control		*P. value*
	0.65 ± 0.2517	3.01	0.732	<0.0001

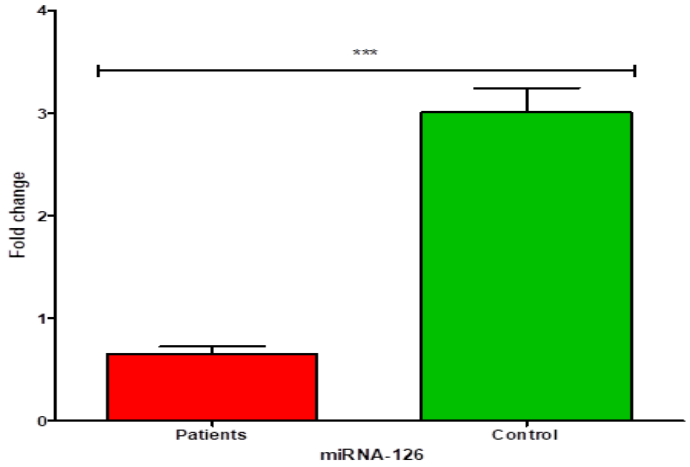

Fig. 1. Gene expression of miRNA-126 in the study group (*P<0.0001*).

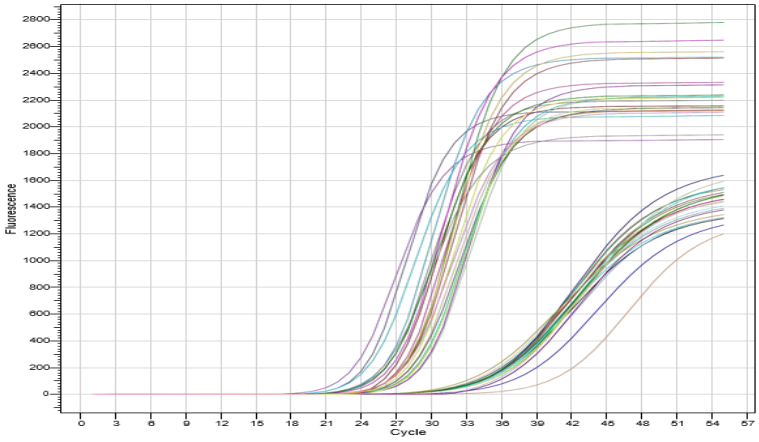

Fig. 2: RT PCR Amplification curve of FAM channel of miRNA-126 in the patients (right) and the control group (left).