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Serum MiR-377 and MiR-29a in Type II Diabetic Patients with Diabetic Nephropathy

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Authors' contributions

Both authors contributed equally in conception, design, practical work, supervision, manuscript writing, editorial help, collection of data and data analysis throughout the whole study.

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Original Research Article

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ABSTRACT

Aim: The aim of our study is to evaluate the impact of serum miR-377 and miR-29a on the development and pathogenesis of diabetic nephropathy in type II diabetic patients.
Place and Duration of Study: The study was conducted in Faculty of Medicine, Fayoum University, Fayoum, Egypt, from July 2015 to December 2015.
Methodology: The present study was conducted on 110 subjects: 30 controls, 40 diabetic subjects with microalbuminuria and 40 diabetic subjects with macroalbuminuria. Blood and urine samples were taken from 110 subjects; Urine samples were collected for measurement of urine albumin. Serum was separated for detection of: Transforming growth factor beta 1 (TGF-β1) by Elisa and miR-377 and miR-29a by qRT-PCR.
Results: There is significant increase in the mean values of serum miR-377 [$P<0.001$ & $P<0.001$] and significant decrease in the mean values of serum miR-29a [$P<0.001$ & $P<0.001$] in diabetic subjects with macroalbuminuria compared with diabetic subjects with microalbuminuria and healthy control subjects.

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Conclusions: Serum MiR-377 is significantly increased and serum MiR-29a is significantly decreased in patients with diabetic nephropathy. Accordingly, these miRNAs have a strong potential to act as biomarkers to diagnose, treat and prognose diabetic nephropathy.

Keywords: Diabetic nephropathy; TGF-β1; miR-377; miR-29a.

1. INTRODUCTION

The diabetes mellitus incidence is increasing at alarming rates around the world and is estimated to rise to 552 million adults by 2030 [1,2]. Diabetic nephropathy (DN) is a serious complication of diabetes mellitus [3]. It is the major cause of progressive kidney disease and it participates in increased deaths among diabetic patients [4,5].

The pathogenesis of diabetic nephropathy is complex. The hyperglycemic medium in the diabetic patient can cause expression or activation of different proteins and signaling pathways, clarifying severe morphological changes in the kidney [6,7]. The earliest clinical sign for diagnosis of diabetic nephropathy is persistent microalbuminuria (30-300 mg/day) and change of the glomerular filtration rate (GFR) [8].

Transforming growth factor beta 1 (TGF-β1) is a crucial cytokine with major importance in diabetic nephropathy due to its contribution in scarring and fibrosis. It is an anti-inflammatory immune mediator which promotes hypertrophy of cells in diabetic kidney and cumulation of proteins of extracellular matrix [9]. TGF-β1 directly induces transcription of many genes of extracellular matrix in renal cells including tubular, endothelial and mesangial cells. It reduces production of collagenase and induces expression of tissue inhibitors of metalloproteinases, leading to inhibition of extracellular matrix turnover [10,11].

MicroRNAs (miRNAs) are a group of non-coding RNAs (18:24 nucleotides) that are expressed in all tissues and play important roles in human diseases, as diabetes [12,13,14]. These miRNAs can also circulate in the blood, thus acting as biomarkers for following up of disease onset and progression. Furthermore, the miRNAs in the circulation reflect those in the tissues [15,16].

There is an association between increased expression of miR-377 and increased production of fibronectin through repression of superoxide dismutase and p21-activated kinase. The cascade beyond regulation of miR-377 transcription is still unclear, though increased glucose is sufficient to stimulate expression of this miRNA [17].

MiR-377 targeted heme oxygenase 1 (HO-1), an essential antioxidant which contributed in oxidative redox signaling [18]. Also HO-1 prevents diabetic nephropathy through antioxidative impact; miR-377/HO-1 pathway might be a new pathway by which miR-377 stimulates diabetic nephropathy [19,20].

Three members of miR-29 family (miR-29a/ miR-29b/ miR-29c) were suppressed by TGF-β1 in proximal tubular cells (Rat renal proximal tubular cell line NRK-52E), primary mouse mesangial cells, and human podocytes. MiR-29 family repressed COL1 and COL4 expression in both mRNA and protein levels [21]. MiR-29a decreased in HK-2 cells (human proximal tubule cell line) under high glucose/TGF-β1 conditions. MiR-29a targeted directly 3′UTR of COL4α1 and COL4α2, leading to decreased expression of these two fibrotic genes [22].

The aim of this study is to evaluate the impact of serum transforming growth factor beta 1 (TGF-β1), miR-377 and miR-29a on the development and pathogenesis of diabetic nephropathy in type II diabetic patients.

2. MATERIALS AND METHODS

This study was conducted on 110 subjects with their ages ranging between 41-70 years. They were classified into 3 groups:

Group I: Included 30 healthy individuals without diabetes mellitus. They were 12 females (40%) and 18 males (60%) aged 54.80 ± 7.71 years. Healthy individuals were selected from outpatient's clinics at El Fayoum University Hospital.

Group II: 40 diabetic subjects with microalbuminuria, they were 13 females (32.5%) and 27 males (67.5%) aged 48.80 ± 4.965.

Group III: 40 diabetic subjects with macroalbuminuria, they were 17 females (42.5%) and 23 males (57.5%) aged 63.60 ± 4.189.

The diabetic subjects were selected from the internal medicine department, El Fayoum University Hospital, Faculty of Medicine, Fayoum University, Fayoum, Egypt. The study was carried out in the period from July 2015 to December 2015. The study design, objectives and methods were compatible with the world medical association (WMA) declaration of Helsinki 2013. The research ethical committee at Fayoum University approved the protocol of study. The protocol was discussed to all the study participants, and a written informed consent was obtained from each participant. All participants were volunteers.

Full history including age, sex, diabetic duration and treatment (oral hypoglycemic drugs and insulin) were recorded for all subjects. Medical history of chronic renal diseases other than diabetic nephropathy, chronic liver diseases, heart diseases, coronary artery diseases, autoimmune diseases, malignancy were important exclusion criteria. The eGFR was calculated for all subjects using Cockcroft–Gault equation, CG-GFR = [(140-age in years) X (actual weight in kg) X 0.85 (if female)] / [(72 X serum creatinine in mg/dl)] [23].

2.1 Samples Collection

Blood samples were drawn in the morning; portion of the blood was collected on EDTA for the determination of glycated hemoglobin, the other portion is incubated at 37℃ for 10-15 minutes then centrifuged at 3000 rpm to separate serum for the other determinations. Urine samples were collected in the morning, centrifuged at 1000 rpm, and the supernatant was used for detection of albumin.

2.2 Methods

Urine albumin was determined by Kits produced by BioSystem S.A. Costa Brava, Barcelona (Spain) [24]. Glycated hemoglobin (HBA1c) was determined by Kits produced by BioSystem S.A Costa Brava, Barcelona (Spain) [25]. Serum Creatinine was determined by Kits produced by BioSystem S.A Costa Brava, Barcelona (Spain) [26]. Serum TGF-β1 was measured by ELISA using Human TGF β1 PicoKine™ ELISA Kit, Boster Biological Technology, Pleasanton, USA [27].

2.2.1 Determination of serum miRNAs

2.2.1.1 RNA extraction

Total RNA including miRNAs was extracted from 200 µL(serum or peritoneal fluid) by mirVana PARIS kit (Ambion, Life Technologies, Texas, USA) by mixing with 200 µL of 2X Denaturing Solution at room temp. 400 µL of chloroform were added and vortexed for 30-60 sec, this was followed by centrifugation at ≥ 10000 rpm at room temperature for 5 min. The upper aqueous phase was carefully removed and transferred to a new tube and 1/3 volume 100% ethanol was added. For each sample, a Filter Cartridge was placed into one of the Collection Tubes, 300 µL of this mixture was applied to a Filter Cartridge and centrifuged at 10000 rpm at room temperature for 30 sec. After the mixture had passed through the filter, 2/3 volume 100% ethanol was added to filtrate and mix thoroughly. The mixture was passed through a second filter cartridge and the flow through was discarded. 700 µL of miRNA Wash Solution 1 was added to the filter cartridge and centrifuged at 10000 rpm at RT for 15 sec and the flow through was discarded. 500 µL of Wash Solution 2/3 was added to the filter cartridge and centrifuged at 10000 rpm at RT for 15 sec and the flow through was discarded. A second 500 µL of Wash Solution 2/3 was added and the flow through was discarded. The filter cartridge was replaced in the same Collection Tube and the assembly was centrifuged for 1 min. The filter cartridge was transferred into a new collection tube and 100 µL of nuclease-free water was added to the center of the filter, and the cap was closed then Centrifuged for 30 sec to recover the RNA. The elute was collected and stored it at -20 or colder.

2.2.1.2 Reverse transcription (RT) and quantitative Real Time-PCR (qRT-PCR)

20 µL of eluted RNA was reverse transcribed by incubation for 1 H at 42℃, 3 min at 93℃, and then maintained at 4℃ using the miRNeasy serum Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Expression of miR-377 and miR-29a was evaluated by qRT-PCR analysis according to the manufacturer's protocol. U6RNA was used as an endogenous control. For real time PCR, we

mixed 10 µL of diluted RT products (cDNA), 12.5 µL SYBR Green Master Mix (Qiagen, Valencia, CA, USA), 0.5 µM of each specific primer, and RNase-free water to a total volume of 25 µL. Real-time PCR reactions were performed using Applied Biosystems 7500 Real Time PCR System (Foster city, CA, USA) with the following thermal cycling parameters: 95℃ for 5 min followed by 35 cycles of 95℃, 57℃, and 75℃ for 10, 30, and 30 sec, respectively. The cycle threshold (CT) is defined as the cycles' number which is required for the fluorescent signal to cross the threshold in real-time PCR. Expression of miRNAs was accounted as ΔCt value which was calculated by subtracting the U6RNA CT values from the CT values of the studied miRNAs. The normalized ΔCt values resulting were used in the calculation of relative expression values by using 2- Δ(Ct), these values are related directly to levels of the miRNA expression.

2.3 Statistical Analysis

Data was collected and coded to facilitate data manipulation and data analysis was performed using SPSS software version 18 under windows 7. Descriptive analysis in the form of numbers and percentages for qualitative data, and arithmetic means as central tendency measurement, standard deviations as measure of dispersion for quantitative parametric data, and inferential statistic test:

2.3.1 For quantitative parametric data

- In-depended student t-Test used to compare measures of two independent groups of quantitative data
- One way ANOVA test in comparing more than two independent groups of quantitative data.

- Bivariate pearson correlation test to test association between variables
- Sensitivity and specificity test for testing a new test with ROC curve "Receiver Operating Characteristic".

The level P ≤ 0.05 was considered the cut-off value for significance.

3. RESULTS AND DISCUSSION

There is statistically significance difference with *P*-value <0.05 between all study groups as regards to age, eGFR, level of HBA1c, Albumin in urine, and TGF-β1. Also there is statistically significance difference with *P*-value <0.05 between diabetic subjects with macroalbuminuria and diabetic subjects with microalbuminuria as regards to duration of DM with high mean among diabetic subjects with macroalbuminuria**.** On the other hand there is no statistically significance difference with *P*-value >0.05 as regards to sex and S.creatinine level between study groups (Table 1).

Table 1. Demographic and biochemical data of healthy control and diabetic subjects

Variables	**Control**	**Diabetic subjects with microalbuminuria**	**Diabetic subjects with macroalbuminuria**	***P-value***
	Mean ±SD	**Mean ±SD**	**Mean ±SD**	
Age (years)	54.8 ± 7.7	48.8±4.9	63.6±4.2	<0.001 [a,b,c]
Sex Females %	12 (40%)	13 (32.5%)	17 (42.5%)	0.6
Males %	18 (60%)	27 (67.5%)	23 (57.5%)	
Duration of DM (years)	-------	5.3±1.8	16±3.2	<0.001 [c]
Retinopathy (%)	-------	8 (20%)	14 (35%)	
Neuropathy (%)	-------	16 (40%)	29 (72.5%)	
Ischemic heart disease (%).	-------	9 (22.5%)	33 (82.5%)	
eGFR (ml/min)	108.8±8.5	99.4±11.6	85.4±5.4	<0.001 [a,b,c]
HBA1c (%)	5.1±0.47	7.9±0.41	7.5±0.42	<0.001 [a,b,c]
S.Creatinine(mg/dl)	0.89±0.14	0.91±0.18	0.90±0.14	0.8
Albumin in urine (mg/L)	5.1±2.8	140.1±74.6	851.6±114.4	<0.001 [a,b,c]
TGF-β1(pg/ml)	12.4±6.5	215.1±87.5	1798.5±399.7	<0.001 [a,b,c]

***a**: significance between G1, and G2, **b**: significance between G1, and G3, **c**: significance between G2, and G3*

There is statistically significance difference in serum MiR-377 and MiR-29a with P-value <0.05 in diabetic subjects with macroalbuminuria in comparison with diabetic subjects with microalbuminuria and control group with high mean of MiR-377 and low mean of MiR-29a among diabetic subjects with macroalbuminuria. There is no statistically significance difference in serum MiR-377 and MiR-29a with P-value >0.05 between diabetic subjects with microalbuminuria and control group (Table 2).

There is statistically significance positive correlation in diabetic subjects between MiR-377 and each of age(r=0.72), duration of DM(r=0.82), level of albumin in urine(r=0.87), and TGF-β1(r=0.84). Also there is statistically significance negative correlation between MiR-377 and each of eGFR(r=-0.59), HBA1c(r=-0.36) and **MiR-29a(r=-0.37**). On the other hand there is no statistically significance correlation between MiR-377 and S.creatinine (Table 3).

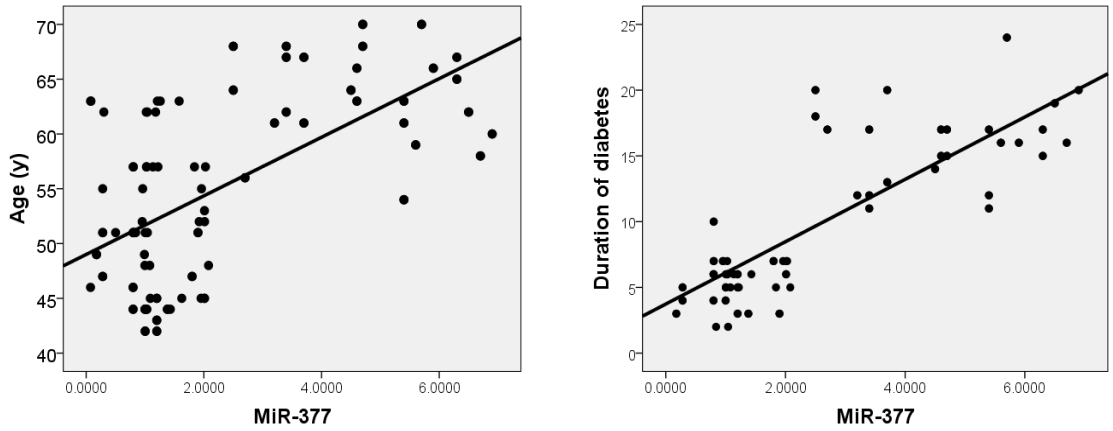
Table 2. Serum MiR-377 and MiR-29a in healthy control and diabetic subjects

Variables	Control	Diabetic subjects with microalbuminuria	Diabetic subjects with macroalbuminuria	*P-value*
	Mean ±SD	Mean ±SD	Mean ±SD	
MiR -377	1.01±0.63	1.13±0.52	4.7±1.4	<0.001 [b,c]
MiR- 29a	1.18±0.42	1.01±0.37	0.69±0.32	<0.001 [b,c]

***a**: significance between G1, and G2, **b**: significance between G1, and G3, **c**: significance between G2, and G3.*

Table 3. Correlation between serum MiR-377 and study variables in both diabetic subjects

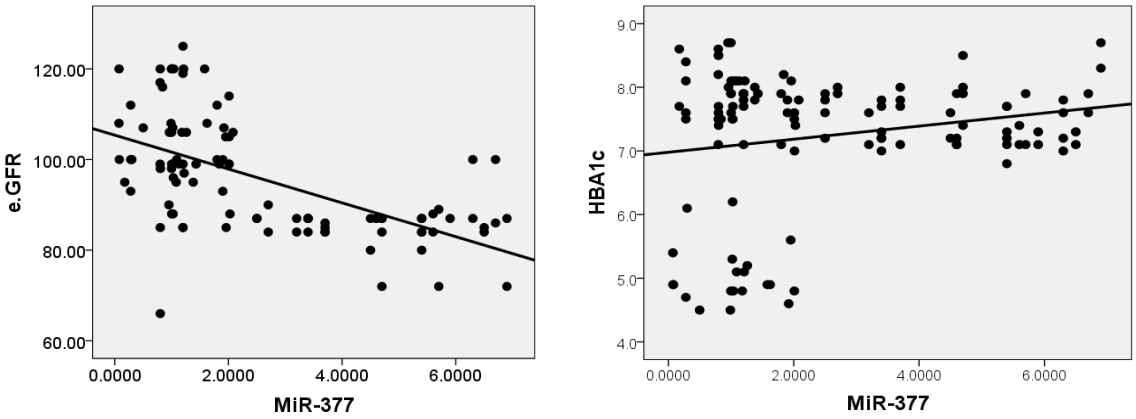
Variables	MiR -377		
	r	*P-value*	Sig.
Age (years)	0.72	<0.001	HS
Duration of DM (years)	0.82	<0.001	HS
eGFR	-0.59	<0.001	HS
HBA1c	-0.36	<0.001	HS
S. Creatinine	-0.02	0.8	NS
Albumin in urine	0.87	<0.001	HS
TGF-β1	0.84	<0.001	HS
MiR-29a	-0.37	<0.001	HS



(a) **(b)**

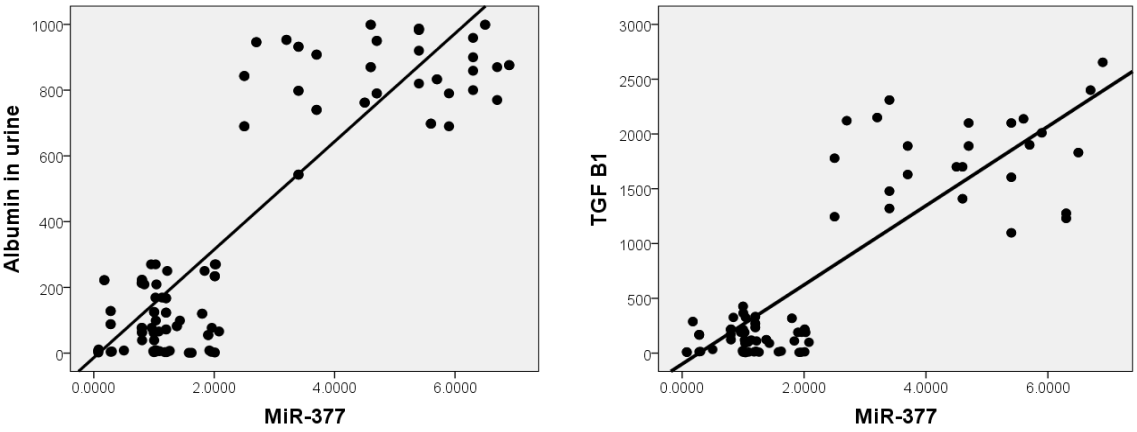
Fig. 1. Correlation between serum MiR-377 and age (a) and duration of diabetes (b) in both diabetic subjects

There is statistically significance negative correlation in diabetic subjects between MiR-29a and each of age(r=-0.36), duration of DM(r=-0.36), level of albumin in urine(r=-0.42), and TGF-β1(r=-0.42). Also there is statistically significance positive correlation between MiR-29a each of eGFR(r=0.34) and level of HBA1c(r=0.26). There is no statistically significance correlation between MiR-29a and S. creatinine (Table 4).



(a) **(b)**

Fig. 2. Correlation between serum MiR-377 and eGFR (a) and HBA1c (b) in both diabetic subjects



(a) **(b)**

Fig. 3. Correlation between serum MiR-377 and Albumin in urine (a) and TGF-β1 (b) in both diabetic subjects

Table 4. Correlation between serum MiR-29a and study variables in both diabetic subjects

Variables	MiR-29a		
	r	P-value	Sig.
Age (years)	-0.36	<0.001	HS
Duration of DM (years)	-0.36	<0.001	HS
eGFR	0.34	<0.001	HS
HBA1c	0.26	0.01	S
Creatinine	-0.02	0.8	NS
Albumin in urine	-0.42	<0.001	HS
TGF-β1	-0.42	<0.001	HS

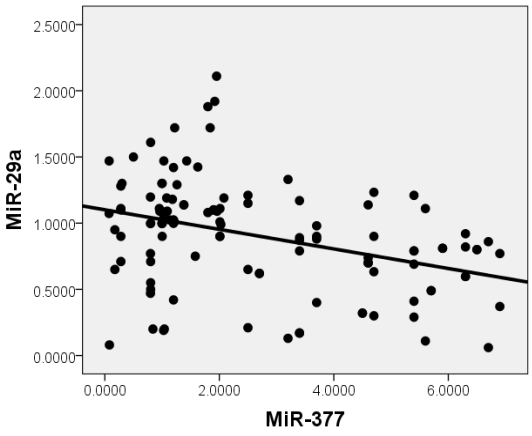
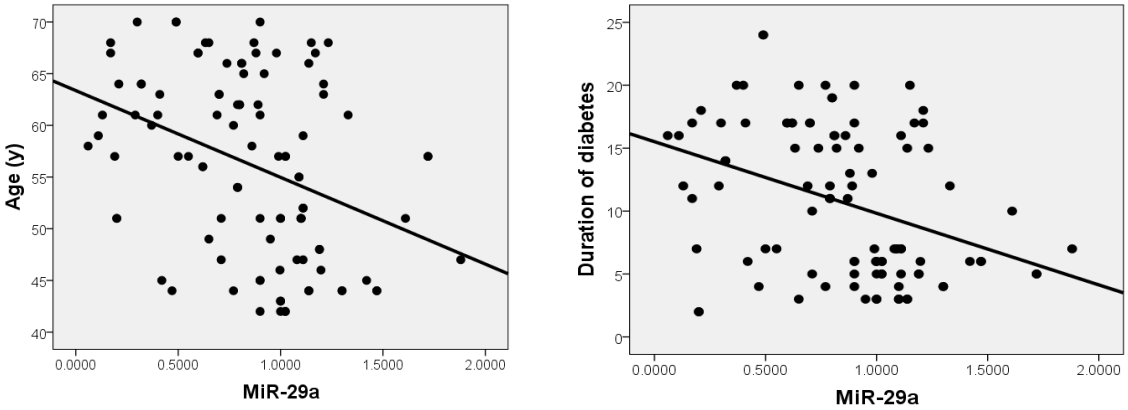


Fig. 4. Correlation between serum MiR-377 and MiR-29a in both diabetic subjects

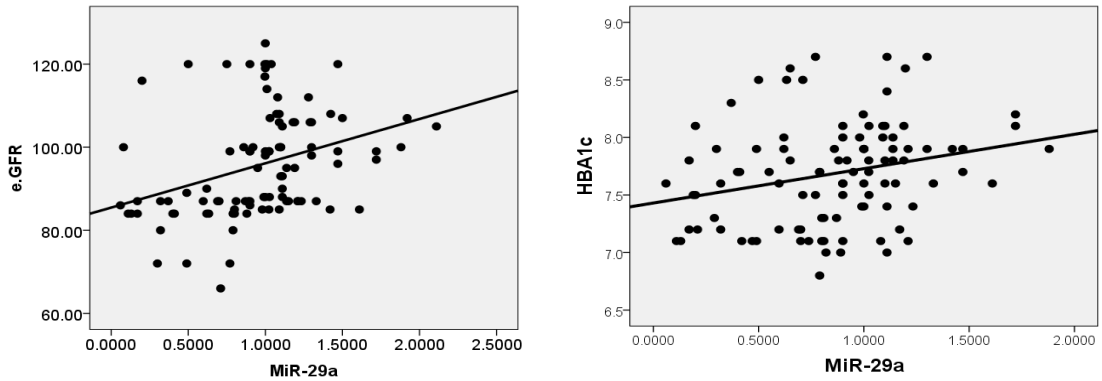
The multivariate linear regression model analysis was conducted to explore the explanatory power of different risk factors in prediction of MiR-377 level, it illustrates that there was statistical significance predictors with p-value <0.05 to **duration of DM** and **TGF- B1** in both diabetic subjects(with p-value 0.03 and 0.02 respectively).

The multivariate linear regression model analysis was conducted to explore the explanatory power of different risk factors in prediction of MiR-29a level, it illustrates that there was no statistical significance predictors with p-value >0.05 to age, duration of DM, eGFR, albumin in urine, HBA1c and creatinine in both diabetic subjects. On the other hand there is statistical significance predictors with p-value <0.05 to **TGF-B1** in both diabetic subjects.



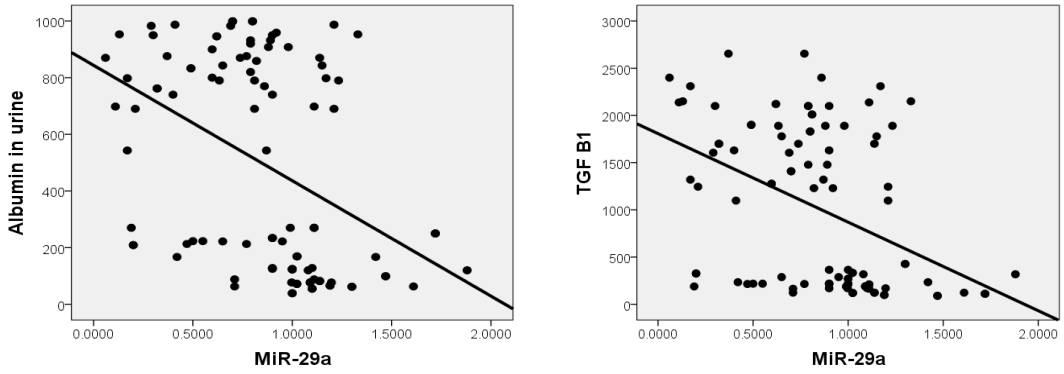
(a) **(b)**

Fig. 5. Correlation between serum MiR-29a and age (a) and duration of diabetes (b) in both diabetic subjects



(a) **(b)**

Fig. 6. Correlation between serum MiR-29a and eGFR (a) and HBA1c (b) in both diabetic subjects



(a) (b)

Fig. 7. Correlation between serum MiR-29a and Albumin in urine (a) and TGF-B1 (b) in both diabetic subjects

Table 5. Linear regression analysis to determine the risk factors of affecting level of MiR-377 in both diabetic subjects

Variables	B	SE	Sig.	CI
Constant	3.5	2.9	0.2	-2.3:9.2
Age (years)	-0.006	0.02	0.8	-0.05:0.04
Duration of disease (years)	0.1	0.04	**0.03**	0.01:0.2
eGFR	0.004	0.01	0.7	-0.02:0.03
Albumin in urine	0.002	0.001	0.07	0.00:0.003
HBA1c	-0.38	0.27	0.2	-0.93:0.2
Creatinine	-0.36	0.7	0.6	-1.7:0.98
TGF B1	0.001	0.00	**0.02**	0.00:0.001

Table 6. Linear regression analysis to determine the risk factors of affecting level of MiR-29a in both diabetic subjects

Variables	B	SE	Sig.	CI
Constant	1.1	0.97	0.3	-0.85:3
Age (years)	-0.006	0.008	0.5	-0.02:0.01
Duration of disease (years)	0.002	0.02	0.9	-0.03:0.03
eGFR	0.004	0.004	0.5	-0.01:0.006
Albumin in urine	0.00	0.00	0.5	0.00:0.001
HBA1c	0.09	0.09	0.3	-0.09:0.28
Creatinine	-0.19	0.23	0.4	-0.6:0.4
TGF-B1	0.00	0.00	0.01	-0.001:0.00

Table 7. Sensitivity and specificity of serum MiR-377 and MiR-29a in diagnosis of diabetic nephropathy in diabetic patients

Variable	Sensitivity	Specificity	Accuracy (AUC)	Cut off point
MiR-377	100%	100%	100%	2.29
MiR-29a	98%	71.4%	84.7%	2.05

As regards the ROC curve: The best cut off value for diagnosis of diabetic nephropathy using **MiR- 377** is 2.29 with sensitivity 100% and specificity 100% and the best cut off value for diagnosis of diabetic nephropathy using **MiR-29a** is 2.05 with sensitivity 98% and specificity 71.4%. So **MiR- 377** and **MiR- 29a** can be used as biomarkers for diagnosis of diabetic nephropathy.

(a) serum MiR-377 in diabetic nephropathy **(b) serum MiR-29a in diabetic nephropathy**

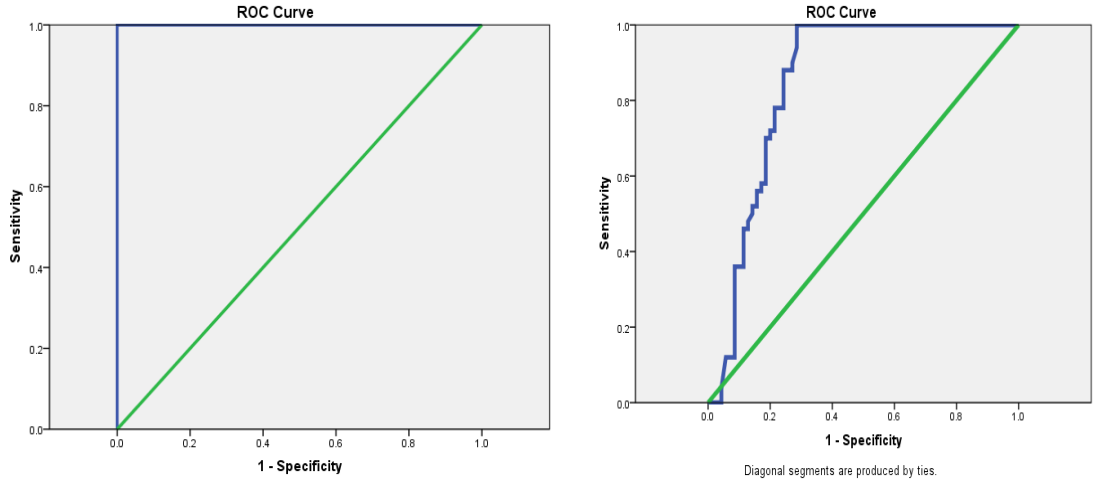


Fig. 8. ROC curve analysis of serum MiR-377(a) and MiR-29a (b) as diagnostic markers of diabetic nephropathy in diabetic patients

3.1 Discussion

Diabetic nephropathy occurs in almost 50% of type II diabetic patients, resulting in chronic renal disease and renal failure [28,29]. It is the main cause of end-stage renal disease and high mortality in diabetic patients [30,31].

Many studies have been allocated into trying to understand how miRNAs regulate and are regulated by factors that participate in kidney disease [32,33,34]. MiRNA dysregulations have been identified in many research areas including diabetic nephropathy [33]. MiRNAs are pivotal regulators of cellular and molecular pathways, so identifying the targets of diabetic nephropathy - associated miRNAs can provide further insight into the pathogenesis of DN [34].

In diabetes mellitus, many miRNAs are upregulated in diabetic kidney. These miRNAs bind to the 3′UTR of renoprotective genes resulting in their diminished expression. Accordingly, these upregulated miRNAs participate in the pathogenesis of DN. On the contrary, downregulated miRNAs showed renal-protective impacts [35].

As many miRNAs are now identified in the pathology of diabetic nephropathy, these small molecules provide new potential for therapeutic intervention. Many trials to decrease or increase expression of miRNAs using one of many delivery approaches in animal models of diabetic nephropathy in vivo are reported. Recent trends to dominate the expression of miRNA levels like using nuclease-resistant, chemically-modified, oligonucleotides (miRNA mimics and inhibitors), that might be developed for human use in the future [36].

There was increased expression of miR-377 in high glucose cultured or TGF-β treated human and mouse mesangial cells. Increased miR-377 leading to repression of p21-activated kinase and superoxide dismutase, which promoted fibronectin expression. Calculated analysis indicated that there was decrease in the activity of some target genes as PAK 1 and SOD1/2 by miR-377. This leads to increased vulnerability to oxidant stress and cumulation of fibronectin in extracellular matrix. Thus, this miRNA could have a pivotal role in the response of mesangial cells to the diabetic medium and could be assumed a target miRNA for therapy [17].

The members of MiR-29 family (miR-29a, miR-29b and miR-29c) are responsible for anti-fibrotic effects in diabetic nephropathy. MiR-29a targeted directly 3′UTR of COL4α1 and COL4α2, leading to decreased expression of these two fibrotic genes [37].

The miR-29 family targets a group of mRNAs which encode proteins implicated in fibrosis, as multiple collagens, elastin, and fibrillin. Thus,

decreased expression of miR-29 would be predicted to activate the expression of these mRNAs and promote the fibrotic response [21].

In cultured human proximal tubular epithelial cells, increased glucose and TGF-β1 decrease miR-29a expression. Collagen IV was reported as a target of miR-29a. Thus, miR-29a regulates collagen expression. Decreased miR-29a levels in diabetes may enhance more deposition of collagen, thus mediating the pathogenesis of diabetic nephropathy [22].

Our aim in this study is to evaluate the impact of serum miR-377 and miR-29a on the development and pathogenesis of diabetic nephropathy in type II diabetic patients.

According to our study we found significant increase in the mean values of serum miR-377 in diabetic subjects with macroalbuminuria compared with diabetic subjects with microalbuminuria and healthy control subjects [P <0.001 & P <0.001]. Also, we found significant decrease in the mean values of serum miR-29a in diabetic subjects with macroalbuminuria compared with diabetic subjects with microalbuminuria and healthy control subjects [P <0.001 & P <0.001].

Similar results were obtained by Kantharidis et al who demonstrated that TGF-β1, which is increased in diabetic nephropathy, is able to upregulate miR-21, miR-93, miR-192, miR-216a and miR-377, but downregulates the miR-29 family [33].

Our results are consistent with Hao et al who showed that several miRNAs were found to participate in the pathogenesis of diabetic nephropathy, so their levels are increased as miR-192, miR-217, miR-200b/c, miR-21, miR-195 and miR-377. While others showed renal protective effects, so their levels are decreased as miR-451 and miR-29 family [38].

As regards miR-377, our results confirmed the findings of Wang et al who found overexpression of miR-377 in human mesangial cells exposed to increased glucose levels. Also, miR-377 had been associated with elevated expression of the matrix protein, fibronectin which is accumulated in excess in diabetic nephropathy [17].

As regards miR-29a, our results are coincided with Du et al. [22] who showed that miR-29a repressed expression of fine-tune collagen and so the decrease of miR-29a caused by elevated glucose may increase the risk of more collagen deposition in proximal tubule cells (PTCs) that plays a significant role during diabetic nephropathy.

4. CONCLUSION

Several miRNAs are concerned with diabetic nephropathy. Some of them participate in the development and pathogenesis of the disease but other miRNAs act as preventers of the disease. To restore the expression of miRNAs to normal level may be a therapeutic potential to halt or attenuate disease progression. We have shown that miR-377 is significantly increased and miR-29a is significantly decreased in patients with diabetic nephropathy. Accordingly, these miRNAs have a strong potential to act as biomarkers to diagnose, treat and prognose diabetic nephropathy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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