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MicroRNA-377 Expression Level as a Marker of Nephropathy in Type 2 Diabetes Mellitus

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ABSTRACT

Background: Diabetic nephropathy is one of the most dangerous complications of diabetes mellitus. To prevent these complications in diabetic patients monitoring of patients is a must. In last decades many authors were trying to investigate molecular biomarker to detect patients who are at risk. MicroRNA-377 is one of the promising biomarkers for prediction of diabetic nephropathy. **Objective:** This study aimed to investigate the role of miRNA-377 as early predictor of diabetic nephropathy in patients with type 2 diabetes mellitus.

Patients and methods: Seventy five patients with type 2 diabetes and 25 healthy control participants are enrolled in a case-control study. Clinical evaluation, and laboratory investigations including fasting plasma glucose, serum creatinine, fasting lipid profile, glycosylated hemoglobin, estimated glomerular filtration rate (eGFR) and albumin creatinine ratio, The expression of serum miRNA-377 was measured via quantitative real-time-polymerase chain reaction (qRT-PCR).

Results: Expression of miR-377 could differentiate diabetic patients from healthy control as the expression of miR-377 was significantly higher in overall T2DM patients than in the healthy control (2.5 fold change, P<0.001), and was progressively increased in the normoalbuminuric group and further increased in the microalbuminuric and macroalbuminuric groups (1.92, 2.76, 3.38 fold change respectively, P<0.001). MiR-377 expression levels were positively correlated with diabetes duration, fasting plasma glucose, $HbA1_C$, total cholesterol, LDL-C, triglycerides, creatinine and ACR, while miR-377 expression levels were significantly negatively correlated with HDL-C and eGFR.

Conclusions: MiR-377 might act as a promising biomarker for prediction of development of diabetic nephropathy in type 2 diabetes patients.

Keywords: Diabetic nephropathy, Micro RNA-377, Albumin/creatinine ratio

INTRODUCTION

Diabetes Mellitus (DM) is seen as one of the strongest enemies we must defeat. In line with the International Diabetes Federation (IDF), Egypt is ranked as the 8th country for the number of adults with diabetes (20–79 years) and for their healthcare expenditure. In accordance with WHO, the prevalence of type 2 diabetes mellitus (T2DM) in Egypt was roughly tripled over the last two decades. By 2030, it is estimated that the number of Egyptians with DM will increase to 6,726,000 [(1)]. Diabetic nephropathy (DN) is one of the most dangerous and prevalent complications that can lead to death in diabetic patients, in addition to being a principal causative factor to end-stage renal disease (ESRD) [(2)]. It is characterized by albuminuria (urine albumin/creatinine ratio (ACR) is >300 mg/g), and/or a glomerular filtration rate (GFR) less than 60 ml/min/1.73 m [(3)]. Current guidelines recommend that both parameters have to be measured, at least once a year, to diagnose and screen for or monitor DN [(4)].

Although microalbuminuria is considered as the gold standard for the diagnosis of DN, there are many reasons to consider ACR is not the perfect biomarker to be measured for the early detection of DN. Albuminuria is a non-specific biomarker as some other conditions may result in increased urinary excretion of albumin such as urinary tract infection, fever, exercise, hypertension, and congestive heart failure [(5)]. Alternatively up to 25% of patients with T2DM and diminished kidney function have little or no proteinuria, despite having biopsy-proven diabetic kidney disease (DKD) [(6)].

MicroRNAs are tiny molecules containing about 22 nucleotides created inside cells as short regulating noncoding RNA. They regulate several fundamental biological pathways and act on different cell functions to produce normal and pathological conditions in myriad biological systems. They act on RNA silencing and control post-transcriptional gene expression [(7)]. Various microRNAs are implicated in the pathogenesis of DKD, whereas others have a role in nephroprotection and so serve as promising therapeutic targets for DKD. Serum and urine microRNAs levels have additionally been considered in the early diagnosis and monitoring of patients with DKD. The detection of microRNA in biological materials is applicable in clinical research for the progress of diagnostic biomarkers for DKD, since early diagnosis may inhibit progression to kidney failure and cardiovascular events [(8)]. One of the miRNAs formerly reported to be implicated in the pathogenesis of DN is miRNA-377 that was found to enhance fibronectin expression in mesangial cells (MCs) through the reduction of manganese superoxide dismutase and p21-activated kinase [(9)].

The current study aimed to investigate the role of miRNA-377 as early predictor of diabetic nephropathy in patients with type 2 diabetes mellitus and to correlate it with other parameters of disease progression.

PATIENTS AND METHODS

This case-control study comprised 75 patients with T2DM and 25 healthy age- and sex-matched

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participants, which were included as a control group. Patients were recruited from the Outpatient Clinics of Internal Medicine Department, Zagazig University Hospitals in the period from February 2021 to June 2021. Patients were divided according to their ACR into three groups, 25 patients with normoalbuminuria (ACR <30 mg/g), 25 patients with microalbuminuria (ACR 30-299 mg/g) and 25 patients with macroalbuminuria (ACR ≥ 300 mg/g).

All patients included were diagnosed with T2DM for greater than or equal to 5 years excluding patients with type1 diabetes mellitus, renal stones, urinary tract infection, or history of nephrotoxic drug usage in the last 3 months. Pregnant females, patients with nephropathy caused by acute inflammation, tuberculosis, cancer, autoimmune diseases or cardiovascular disease were excluded from the study.

Ethical approval:

Written informed consent was obtained from every participant included in the study. Approval for the study was obtained from The Research Ethics Committee, Faculty of Medicine, Zagazig University. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

All participants were subjected to thorough history taking, clinical evaluation, height and weight measurement, body mass index (BMI) calculation and laboratory investigations including fasting plasma glucose, serum creatinine, fasting lipid profile (Total cholesterol, HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and serum triglycerides)(Cobas 8000, Roche Diagnostics), glycosylated hemoglobin (HbA1c)(Cobas 6000, Roche Diagnostics),and estimated glomerular filtration rate (eGFR) according to the Modification of Diet in Renal Disease formula [(10)]. Early morning urine samples were taken for assessing for ACR. Precautions were taken to exclude exercise, marked hyperglycemia, or menstruation in females at the time of sampling. Two of three urine samples were used as evidence of albuminuria.

Measurement of miRNA -377 gene expression:

The expression of serum miRNA-377 was measured via quantitative real-time-polymerase chain reaction (qRT-PCR). Five milliliters of venous blood was collected and placed in a serum separator tube gel. The blood was centrifuged and serum was transferred into 2 ml Eppendorf tubes, then another centrifugation step was done at high speed 12,000 rpm for 15 min to remove cell debris completely, leaving only circulating RNA. Serum was separated within 4 h after withdrawn from patient and controls and stored at -80°C until RNA extraction.

RNA extraction:

RNA extraction was done according to the manufacturer's instructions by using the miRNeasy serum/plasma kit (QIAGEN GmbH, Hilden, Germany, cat.no.217184) that combines phenol/guanidine-based lysis of samples and silica-membrane-based purification of total RNA. This step is followed immediately by RNA conversion to complementary DNA (cDNA).

RNA conversion to complementary DNA (cDNA):

Purified RNA was used for one-step reverse transcription using miScript II RT kit (Qiagen, Hilden, Germany, cat. no. 218161) following the manufacturer's instruction. Reactions included 15-µl RNA, 4-µl 5x miScriptHiSpec Buffer containing nucleics mix and 1 µl miScript Reverse Transcriptase. Former reactions were incubated for 60 min at 37°C then for 5 min at 95°C. The temperature sequence was performed by Gene Amp PCR System 9700 thermocycler (Perkin Elmer, Singapore). Lastly, cDNA samples were Stored at –80°C till analysis.

MicroRNA quantification by real-time quantitative RT-PCR (qPCR):

qRT-PCR was carried out by Stratagene Mx3005P" platform (Agilent Technologies, USA). The primers used were: miR-377 forward, 5′-ACAAAAGTTGCCTTTGTGTGAT-3′ and reverse, 5′-GGCTAGTCTCGTGATCGA-3′; and RNU6B forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′ and reverse, 5′-CGCTTCACGAATTTGCGTGTCAT-3′. SYBR Green Master Mix (Qiagen/ SABiosciences Corporation, USA) was used in the (RT-PCR) reaction according to the manufacturer's suggested protocol. The transcription levels of target miRs were normalized to those of RNU6B, which were used as internal control. The relative gene expression (fold change) of serum microRNAs expression levels were analyzed using the comparative threshold cycles method. For each sample, the difference in the cycle threshold (CT) of the target and the CT of the reference (RNU6B) were calculated as ΔCT sample. The same calculation was performed with controls (ΔCT control). Then, the relative expression (ΔΔCT) was determined by calculating the difference in ΔCT sample and ΔCT control. The relative quantification was calculated using the 2−ΔΔCT method as previously described [(11)].

Statistical analysis

Data analysis was performed using the software SPSS (Statistical Package for the Social Sciences) version 20 (SPSS INC. Chicago, IL, USA). Categorical variables were described using their relative percentages and chi square test (χ^2) was used to compare frequencies of these data. Quantitative variables were described using their means and standard deviations. To compare groups, analysis of variance ANOVA test (F test) was used, followed by the least significant difference (LSD) test for multiple comparisons between groups. Correlations between two quantitative parameters were done by Pearson correlation. Regression analysis was used to identify independent predictor variables for miR-377 expression.

Receiver operating characteristic (ROC) curve analysis was used to assess the best cutoff of studied parameters. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated. The level of statistical significance was set at 5% ($P < 0.05$). Highly significant difference was present if $p \leq 0.001$.

RESULTS

Demographic data and laboratory findings of the studied groups are presented in (Table 1). The relative expression of miR-377 could differentiate diabetic patients from healthy control as the expression of miR-377 was significantly higher in overall T2DM patients than in the healthy control (2.5 fold change, P<0.001), and was progressively increased in the normoalbuminuric group and further increased in the microalbuminuric and macroalbuminuric groups (1.92, 2.76, 3.38 fold change respectively, P<0.001) (Figure 1).

Table 1: Demographic data and laboratory findings of the studied groups

Laboratory finding	Control n=25	DM patients with normoalbuminuria n=25	DM patients with microalbuminuria n=25	DM patients with macroalbuminuria n=25	P
Age (years)	51.88 ± 6.77	55.20 ± 10.00	52.64 ± 7.34	56.16 ± 9.96	0.251
Gender*					
Male	12 (48)	13 (52)	14 (56)	12 (48)	0.932
Female	13 (52)	12 (48)	11 (44)	13 (52)	
Diabetes duration (years)		10.28 ± 1.969[a]	12.64 ± 2.797	13.00 ± 2.415	<0.001
BMI (kg/m^2)	16.2 ± 1.7	27.0 ± 3.2	28.0 ± 3.3	28.5 ± 4.3	<0.001
Fasting glucose (mg/dl)	87.24±9.73	115.24±17.8[b]	118±15.82[b]	128.92±15.80[a]	<0.001
HbA1c (%)	4.29±0.66	6.77±0.58[a]	7.52±0.40[b]	7.87±1.11[b]	<0.001
Total cholesterol (mg/dL)	151.52±13.99	166.92±10.05[a]	189.32±17.54[b]	192±12.54[b]	<0.001
HDL-C (mg/dL)	49.79±5.78	43.60±8.77[a]	39±7.45[b]	36.92±6.59[b]	<0.001
LDL-C (mg/dL)	88.72±13.52	95.44±14.11[a]	113.98±3.77[b]	116.89±10.67[b]	<0.001
Triglycerides (mg/dL)	112.22±23.54[a]	156.88±27.28	170.4±7.33	196.32±36.85[a]	<0.001
Creatinine (mg/dL)	0.77±0.14	0.96±0.21[b]	0.99±0.19[b]	1.11±0.20[a]	<0.001
ACR (mg/g)	11.47±.29	14.43±3.79	215.06±39.79[a]	344.24±20.91[a]	<0.001
eGFR (ml/min/1.73m$^{2)}$	106.76±7.28	99.16±6.07	82.08±17.48	67.51±6.58	<0.001
miR-377 expression	0.95±0.05	1.96±0.30	2.67±0.33	3.36±0.20	<0.001

n: number of subjects. Data are represented as mean ± SD, or number (percentage)*
a: significant with other group. b:significant with control. p≤0.001 is highly significant

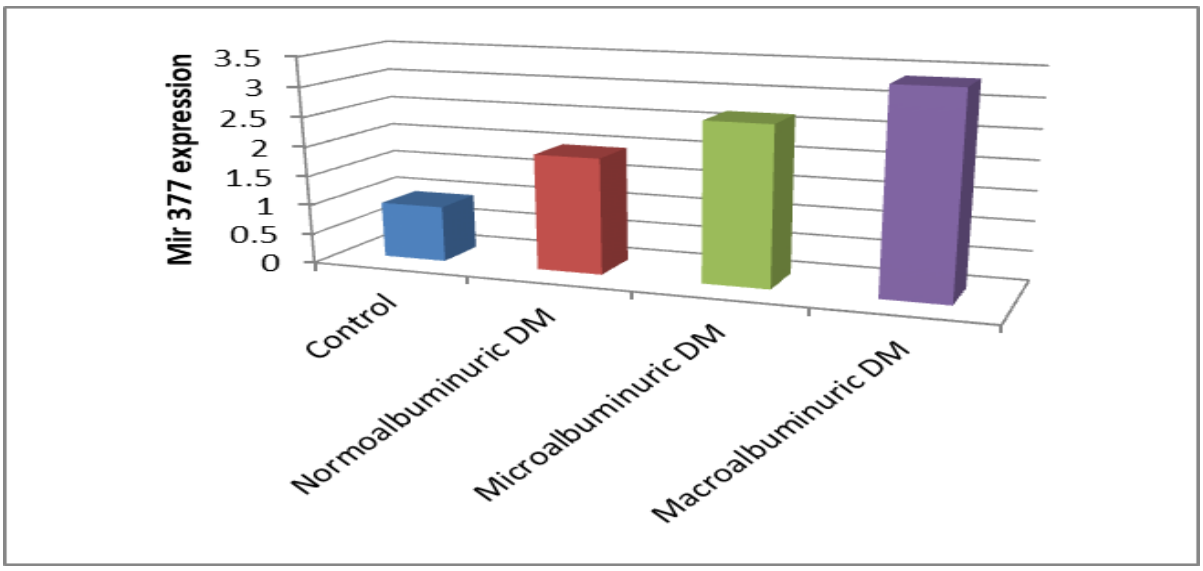

Figure (1): Relative expression of miR-377 in studied groups

The current results demonstrated a significantly positive correlation of miR-377 expression levels with diabetes duration, fasting plasma glucose, $HbA1_C$, total cholesterol, LDL-C, triglycerides, creatinine and ACR. While, miR-377 expression levels were significantly negatively correlated with HDL-C and eGFR (Table 2).

Table (2): Pearson correlation between miR-377 expression levels with some studied parameters in diabetic patients

Parameter	DM patients	
	r	p
Age (years)	0.035	0.766
Diabetes duration (years)	0.410	<0.001**
Fasting glucose (mg/dl)	0.340	0.003*
HbA1c (%)	0.421	<0.001**
Total cholesterol (mg/dl)	0.538	<0.001**
HDL-C (mg/dl)	- 0.338	0.003*
LDL-C (mg/dl)	0.536	<0.001**
Triglycerides (mg/dl)	0.236	0.041*
Creatinine (mg/dl)	0.337	0.003*
ACR (mg/g)	0.912	<0.001**
eGFR (ml/min/1.73m^2)	- 0.585	<0.001**

*****p<0.05 is significant. **p≤0.001 is highly significant.

As summarized in table (3), a linear regression analysis test revealed that miR-377 expression levels were independently correlated with ACR.

We investigated the potential value of miR-377 expression in diabetic nephropathy by ROC analyses. The ROC curves for miR-377 yielded an AUC of 0.886 (95% CI, 0.804-0.969; P<0.001) in distinguishing overall diabetic patients from healthy subjects. The cutoff value was (1.725) (additionally, the sensitivities and the specificities were 88% and 84%. PPV, NPV and accuracy were 94.2%, 70% and 82.25% respectively (Figure 2).

We further investigated the potential diagnostic value of miR-377 expression by ROC tests in T2DM patients. When we discriminated normoalbuminuric patients from the microalbuminuric/ macroalbuminuric patients, we found the cutoff values of 2.540 and the AUC was 0.860 (95% CI =0.745-0.97; P<0.001). Additionally, the sensitivities and the specificities were 86 % and 84%. PPV, NPV and accuracy were 91%, 75% and 85% respectively (Figure 3).

Table (3): Multiple linear regression analysis showing variables independently associated with miR-377 expression in DM patients

	Unstandardized coefficient		Standardized coefficient	t	p
	Beta	Standard error	Beta		
HbA1c (%)	0.014	0.044	0.019	0.321	0.749
Total cholesterol (mg/dL)	-0.001	0.002	-0.040	-0.653	0.516
HDL-C (mg/dL)	-0.004	0.004	-0.056	-1.013	0.315
LDL-C (mg/dL)	-0.004	0.003	-0.091	-1.417	0.161
Triglycerides (mg/dL)	-0.009	0.064	-0.113	-1.542	0.174
Creatinine (mg/dL)	0.248	0.158	0.080	1.565	0.122
ACR (mg/g)	0.005	0.000	0.996	12.315	**<0.001****
eGFR (ml/min/1.73m$^{2)}$	0.000	0.002	0.100	0.147	0.884

**p≤0.001 is highly significant

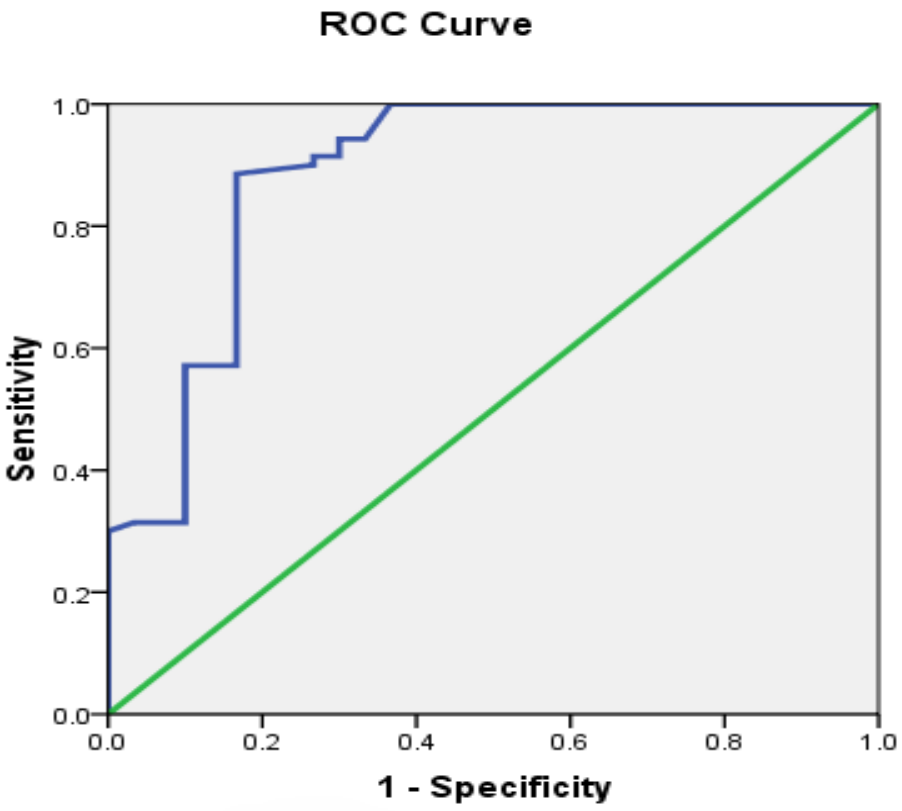

Figure (2): ROC curve of miR-377 expression levels for discriminating DM patients from controls.

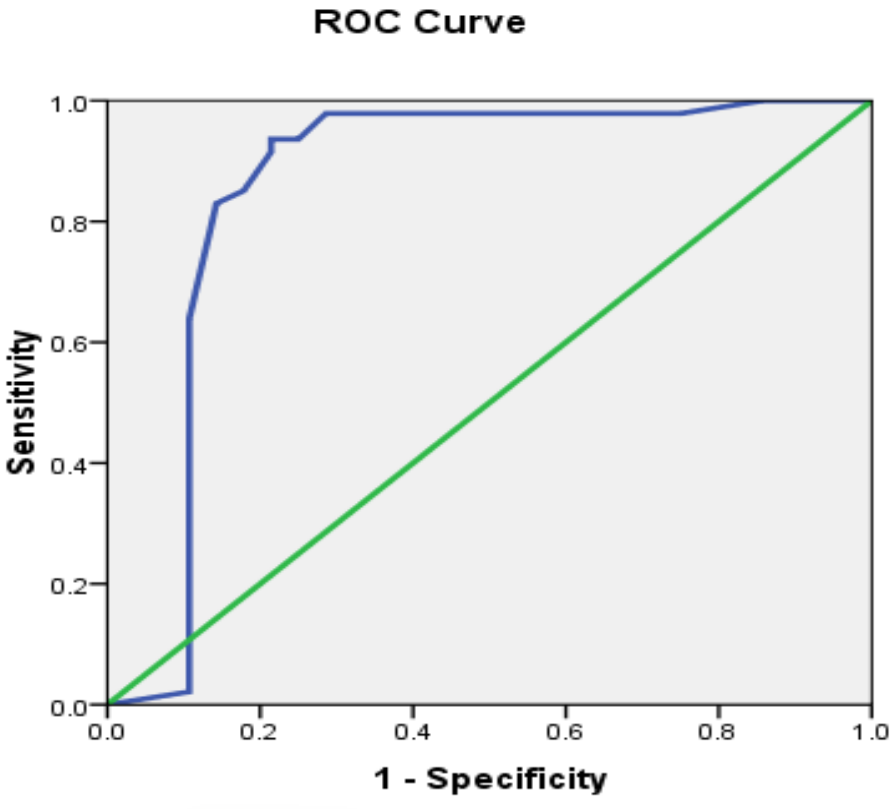

Figure (3): ROC curve of miR-377expression levels for discriminating patients with DN from patients without DN.

DISCUSSION

Diabetic nephropathy is the principal cause of renal failure that leads to renal replacement therapy all over the world [12]. Early detection of diabetic nephropathy is still a challenge. The diagnosis is based on determining the level of urinary albumin and on estimating the GFR [4]. On the other hand, these markers have many limitations. It was reported that there are some phenotypes of DN with neither microalbuminuria nor decreased eGFR[13], due to the tubular affection in DM, that may precede glomerular dysfunction resulting in normal serum creatinine and normal level of albumin in urine [14]. In some patients, the progression of DKD to ESRD may happen without a transition from microalbuminuria to overt proteinuria because many of these patients, by time, became normoalbuminuric once more [15,16]. All these findings may suggest a nonalbuminuric pathway for the progression of DKD. Hence, there is a need for new, easily accessible and noninvasive biomarkers for early recognition of diabetic nephropathy. Among these biomarkers is the microRNA expression, which are affected by the pathological conditions involving kidneys [17].

MicroRNAs have gained its strength as renal biomarkers and offered good perspectives for the future management of DKD added to GFR and albuminuria testing in disease diagnosis and monitoring [8]. The role of miRNAs in DN progression is through the development of renal fibrosis [18]. MiRNA-377 was found to enhance fibronectin expression in mesangial cells (MCs) through the down regulation of manganese superoxide dismutase and p21-activated kinase [9].

In the present study, expression of miR-377 was significantly higher in overall patients than in the healthy control, and was progressively increased in the normoalbuminuric group and further increased in the microalbuminuric and macroalbuminuric groups. These findings are in agreement with **Al-Kafaji and Al-Muhtaresh** [19] who observed that miR-377 expression was increased in diabetic patients and gradually increased in patients with normoalbuminuria and further increased in patients with microalbuminuria and macroalbuminuria. Also, **Abdelghaffar and his colleagues** [20] found that a higher percentage of miRNA-377 upregulation was present in diabetic children and adolescents with DN compared to the group without nephropathy. Moreover, another study done by **Elamir and Ibrahim** [21] showed that miR-377 is significantly increased in diabetic nephropathy patients.

The glomerulus is primarily implicated in diabetic nephropathy. Principal features involve mesangial cell hypertrophy, apoptosis, and excessive production of the normal extracellular ingredient, fibronectin [22]**.** MiR-377 is increased in DN, and overexpression of miR-377 can target the inhibition of the synthesis of some vital mesangial cell proteins such as, PAK1, superoxide dismutase1 (SOD1), and superoxide dismutase2 (SOD2). This cause fibronectin accumulation. Also, an upregulation in miR-377 expression enhances oxidative stress in mesangial cells. Thus, inhibition of miR-377 was suggested to be a target for treatment of DN [9].

The current results demonstrated a positive correlation of miR-377 expression levels with diabetes duration, fasting plasma glucose, $HbA1_C$, total cholesterol, LDL-C, triglycerides, creatinine and ACR. While, miR-377 expression levels were significantly negatively correlated with HDL-C and eGFR. This is concordant with previous research by **Al-Kafaji and Al-Muhtaresh** [19] who reported that increased miR-377 expression was positively correlated with albuminuria, hyperglycemia, diabetes duration, and lipid abnormalities, while negatively correlated with renal function. These results indicate that upregulation of miR-377 is a useful biomarkers to evaluate renal damage and the risk of DN. Moreover, **Elamir and Ibrahim** [21] found a significant positive correlation between MiR-377 and duration of DM, level of albumin in urine and significant negative correlation with eGFR in diabetic subjects

This study revealed that miR-377 expression levels were independently correlated with albumin creatinine ratio. These findings are in agreement with **Al-Kafaji and Al-Muhtaresh** [19] who demonstrated that albuminuria was the only significant predictor of miR-377 among other variables, including fasting glucose, HbA1c, diabetes duration, total cholesterol, triglycerides and LDL

In the present study, miR-377 yielded ROC-AUC of 0.886 for overall diabetic patients versus from healthy subjects. At 1.725 cutoff, the sensitivity was 88%%, the specificity was 84% and accuracy was 82.25%. So, miR-377 was significantly able to discriminate overall diabetic patients from healthy subjects. When we discriminated normoalbuminuric patients from the microalbuminuric/macroalbuminuric patients, we found that at cutoff value of 2.540, the AUC was 0.860, the sensitivity was 86 %, the specificity was 84% and accuracy was 85%.

Limitations of this study included the small sample size, and lack of prospective validation to understand the association of the miR-377 to the degree of progression or prevention of DN.

CONCLUSION

MiR-377 was significantly higher in overall patients than in the healthy control, and was significantly increased with the severity of albuminuria. This gradual increase in different stages may indicate its potential role in the pathogenesis of diabetic nephropathy and its usefulness as an early biomarker for diagnosis even in the absence of albuminuria. In short, the emergence of miRNAs provides new ideas for the diagnosis and treatment of DN. Aiming to help finally in choosing appropriately personalized medications for each one of these patients.

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