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Changes of MicroRNA-377 in Diabetic Nephropathy

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Abstract

Background: MicroRNAs (miRNAs) play important roles in modulating gene expression. In cultured mesangial cells, MiR-377 exhibited the greatest change on exposure to high glucose and led to increased fibronectin production in *in-vitro* diabetic nephropathy. Our aim was to identify the pattern of microRNA-377 changes in human diabetic patients with different stages of diabetic nephropathy. **Methods:** The study included 45 patients divided into 5 groups; patients with stage 1 & 2 diabetic nephropathy (DN), stage 3 & 4 DN, diabetics without DN, stage 1 & 2 chronic kidney disease (CKD), and stage 3 & 4 CKD. The following tests were done to all patients; serum creatinine, estimated glomerular filtration rate (eGFR), albumin to creatinine ratio, blood sugar, urine analysis, glycated hemoglobin, fundus examination. The RNA was extracted from plasma samples, TaqMan microRNA (miRNA) assays from applied Bio systems were used for analysis of MicroRNA-377. **Results:** This study found that patients with the highest median of the interquartile range (IQR) of miR-377 were those of group 1 with significant difference between them and all other groups. MiR-377 was significantly correlated with glycated hemoglobin but not with eGFR. **Conclusion:** Plasma miR-377 is highly significantly increased in human diabetic patients with early rather than late diabetic nephropathy, diabetics without nephropathy, or those at any stage of other causes of CKD. Our findings confirm the role of miR-377 as a potentially novel target in the development of diabetic nephropathy in humans.

Keywords

MicroRNA, MiR-377, Diabetic Nephropathy, Chronic Kidney Disease

1. Introduction

The incidence of diabetic nephropathy is rising and is now the leading cause of

end-stage kidney disease in the US, Europe, and Japan. Hence diabetic nephropathy has become a disease with a heavy impact in the world [1].

The main pathological features of diabetic nephropathy include glomerular hypertrophy and mesangial expansion along with podocytopathy and accumulation of extracellular matrix proteins. Essential hyperglycemia, glycation end products, protein kinase c and oxidative stress may contribute to the pathogenesis of diabetic nephropathy [2].

Several typical cell signaling pathways have been proven to be involved in diabetic nephropathy. Transforming growth factor (TGF-B) [3], phosphoinositide3_kinase protein kinase B (PI3K_AKt) pathway [4], nuclear factor Kappa light chain enhancer of activated B cells (NF_kB) [5]. Although the complete pathogenesis has not yet been fully uncovered.

MicroRNAs (miRNAs) are ultrashort non coding RNAs of about 21 - 25 nucleotides that have recently proved to regulate modulating gene expression thus affecting almost every key cellular function. Almost 60% of the human protein-coding genes can be targeted by miRNAs was elucidated and thus research on miRNA has attracted a lot of interest. MicroRNAs were found to affect signaling pathways involved in the pathogenesis of diabetic nephropathy [6].

MicroRNAs stably bind the 3'-untranslated regions (UTRs) of their target mRNAs when there is identity in their first eight nucleotides. The degree of complementarity beyond those can determine whether the target mRNA is ultimately degraded or blocked from being translated or transcribed, with better matches undergo degradation [7].

Previous work in diabetic renal disease (performed in cell culture, animal models or formalin fixed human biopsy) has linked a number of microRNAs to the development of nephropathy.

Many studies on other microRNAs have proven their vital roles as MicroRNA-21 over activates PKB (Protein Kinase B) after targeting Phosphatase and Tensin homolog (PTEN) regulating colorectal cancer [8] while MicroRNA-451 decreases mesangial cells growth [9]. MicroRNA-93 decreases vascular endothelial growth factor (VEGF-A) secretion, which normally was over expressed with hyperglycemia and diabetic microvascular abnormalities [10]. MicroRNA-192 under expression due to diabetes promotes fibrogenesis in the kidney and liver [11]. Diabetic renal fibrosis was also found due to inhibition of microRNA-29 by TGF-B [12]. The miRNA-25 was reduced both in the kidney from diabetic rats and in high glucose-treated mesangial cells, accompanied by increase in NADPH Oxidase 4 (NOX4) [13].

In Wang *et al.* study [1], *in vitro* as well as in diabetic mouse models *in vivo*, microRNA-377 was consistently up-regulated relative to controls which decreased expression of P21-activated kinase (PAK_1) and superoxide dismutase 1 and 2 (SoD_1, SoD_2) with increased fibronectin production; a major matrix protein accumulated in excess in diabetic nephropathy. Hence, micro RNA-377 is positioned to have a critical role in the mesangial cell response to the diabetic community.

Nevertheless, there have been no comprehensive studies examining microRNA changes in human bio-fluids in diabetic nephropathy in relation to the level of urinary albumin and clinical outcome.

Aim of the Work

To identify the pattern of microRNA-377 changes in human diabetic patients in different stages of diabetic nephropathy and in comparison to patients with other causes of chronic kidney disease. To our knowledge, this is the first research to study the changes of miR377 in human subjects with diabetic nephropathy.

2. Materials and Methods

The study was conducted from 2016 to 2018, at Ain shams university Hospital in Cairo, and Quessena Hospital El Monfia governorate in Egypt, including 45 patients divided into 5 groups:

Group 1: 10 diabetic patients with stage 1 & 2 diabetic nephropathy (DN),

Group 2: 9 patients with stage 3 & 4 DN,

Group 3: 9 diabetics without DN,

Group 4: 9 patients with other causes of chronic kidney disease (CKD) stage 1 & 2,

Group 5: 8 patients with CKD stage 3 & 4.

Inclusion Criteria:

1) Diabetes Mellitus defined as increased glycated HbA1c with or without elevated creatinine levels (±Diabetic Nephropathy).

2) CKD due to other causes defined as increased creatinine levels with normal HbA1c levels.

3) Elevated albumin/creatinine ratio due to hyperfiltration of early DN or other causes.

Exclusion Criteria:

1) Diabetes Mellitus Type I.

2) Age below 20 years.

3) Hemodialysis End Stage Renal Disease Patients.

4) Patients with unstable comorbidities eg: ischemic heart disease with reduced ejection fraction.

Full history was taken and clinical examination was made to all patients. The following tests were done to all patients; serum creatinine, estimated glomerular filtration rate (eGFR), albumin to creatinine ratio, blood sugar, urine analysis, glycated hemoglobin, fundus examination and pelvic abdominal ultrasound. The RNA was extracted from plasma samples, TaqMan microRNA assays from Applied Biosystems were used for analysis of MicroRNA-377 and its control gene U18. The miRNA-U18 was analyzed for normalization of correction ratio.

A simple, two-step protocol required only reverse transcription with a miRNA-specific primer, followed by real-time PCR with TaqMan probes using the protocol of TaqMan™ Small RNA Assays user guide (Publication Number

4364031).

1) Reverse transcription complementary DNA (cDNA) synthesis of miRNA:

The TaqMan microRNA reverse transcription kite (Applied Biosystem) was used according to the manufacture instructions, each 15 μL Reverse transcription (RT) reaction consists of 7 μL master mix, 3 μL of 5× Rt primer and 5 μL RNA sample.

The master mix (total volume 7 μL) components are 0.15 μL deoxyribose nucleotide triphosphate (dNTP), 1 μL (50 U/μL) Multiscribe RT enzyme, 1.5 μL 10× RT Buffer, 0.19 μL (20U/μL) RNAase inhibitor and 4.16 μL nuclease free water.

The tube was sealed and mixed thoroughly by inverting the solution, then centrifugation to bring the solution to the bottom of the tube. The tube was incubated on ice for 5 minutes and kept on ice until to be loaded to thermal cycler.

The c DNA synthesis was performed in a thermal block cycler with heated lid (Bio Metra) with following steps: priming at 16˚C for 30 min, transcription at 42˚C for 30 min and enzyme inactivation at 85˚C for 5 min.

2) Real time PCR (quantification of miRNA):

For the detection of mature microRNAs, the TaqMan MiRNA assay (Applied bio system) was used. The PCR reaction mixture of 10 uL contained: 1 uL microRNA specific c DNA, 5 uL TaqMan 2× universal PCR Master Mix No Amp Erase UNG, 0.5 uL gene specific (mir-377-U_{18}) TaqMan real–time PCR–Assay solution (20×) and 3.5 uL nuclease free water.

The cycling condition were set as follows: initial activation of Taq polymerase at 95˚C for 10 min then amplification steps: denaturation at 95˚C for 15 secs, annealing/elongation at 60˚C for 1 min with fluorescence acquisition and final cooling step at 40˚C for 1 min. All non-template controls were negative.

Quantification of miRNA was estimated based on measured cycle thresholf (C_T) values defined as the number of cycles required for the fluorescent signal to cross threshold (*i.e.* exceeds background level).

Data from real time PCR are Cycle threshold value at which detectable signal is achieved where lower cycle threshold means larger amount of starting material (template). To compare levels or changes in gene expression, relative quantification is used. You need a calibrator sample to perform a relative quantification.

In normalization against a reference gene, the same amount of mass for each sample was used by measuring the starting amount of RNA that is added to the reaction.

Normalization ratio = $2\Delta C_t$ of target gene* − ΔC_t of calibrator gene** = $2\Delta\Delta C_t$, to determine corrected ratio of target gene.

If >1 this means good gene expression.

If <1 this means low gene expression.

*Delta C_t target = target gene − mean control.

**Delta C_t calibrator = reference (control) gene − mean control.

Where 4 healthy subjects were taken as controls for calculating the mean control value of both target gene (miR-377) and reference gene (control gene U_{18}).

Statistical Analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 23. The quantitative data were presented as mean, standard deviations and ranges when their distribution was found parametric and median inter quartile range (IQR) when their distribution was found non parametric. Also qualitative variables were presented as number and percentages.

3. Results

Chi-Square Test showed no significant difference in number of male and female (Table 1 & Table 2).

There was no significant difference in age in different groups (Table 3).

The total percentage of patients with high glycated hemoglobin (HA_{1C} > 7.0% = 53 mmol/mol) was 63% of the studied groups. All patients (100%) with stage 1 & 2, 50% of patients with stage 3 & 4 diabetic nephropathy and 33.3% of diabetic patients without nephropathy had high glycated hemoglobin (Table 4 & Figure 1).

Table 1. Descriptive table showing the gender in different groups.

			Group				
			Diabetic nephropathy stage 1, 2	Diabetic nephropathy stage 3, 4	Diabetic patients only	Chronic kidney disease stage 1, 2	Chronic kidney disease stage 3, 4
sex	Male	Count	6	5	2	5	1
		% within group	60.0%	55.6%	22.2%	55.6%	12.5%
	Female	Count	4	4	7	4	7
		% within group	40.0%	44.4%	77.8%	44.4%	87.5%
Total		Count	10	9	9	9	8
		% within group	100.0%	100.0%	100.0%	100.0%	100.0%

Table 2. Comparison between different groups according to the gender.

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.980[a]	4	0.137
Likelihood Ratio	7.536	4	0.110
Linear-by-Linear Association	3.088	1	0.079
N of Valid Cases	45		

[a]6 cells (60.0%) have expected count less than 5. The minimum expected count is 3.38.

Table 3. Comparison table showing mean age and standard deviation in each group.

Groups	Age	
	Mean ± SD	Range
Diabetic nephropathy stage 1, 2	52.00 ± 15.18	23 - 70
Diabetic nephropathy stage 3, 4	58.22 ± 11.52	35 - 74
Diabetic patients only	40.44 ± 18.90	0 - 60
Chronic kidney disease stage 1, 2	42.88 ± 13.23	23 - 60
Chronic kidney disease stage 3, 4	47.33 ± 15.94	27 - 75
One Way ANOVA test	F = 1.955 P-value = 0.121	

Table 4. Descriptive table showing HA_{1C} in diabetic groups.

			Group			Total
			Diabetic nephropathy stage 1, 2	Diabetic nephropathy stage 3, 4	Diabetic patients without nephropathy	
HA_{1C}	High glycated hemoglobin HA_{1c} (HA_{1C} > 7.0% = 53 mmol/mol)	Count	10	4	3	17
		% within group	100.0%	50.0%	33.3%	63.0%
	Controlled glycated hemoglobin (HA_{1C} > 7.0% = 53 mmol/mol)	Count	0	4	6	10
		% within group	0.0%	50.0%	66.7%	37.0%
Total		Count	10	8	9	27
		% within group	100.0%	100.0%	100.0%	100.0%

X^2 = 9.847; p-value = 0.007.

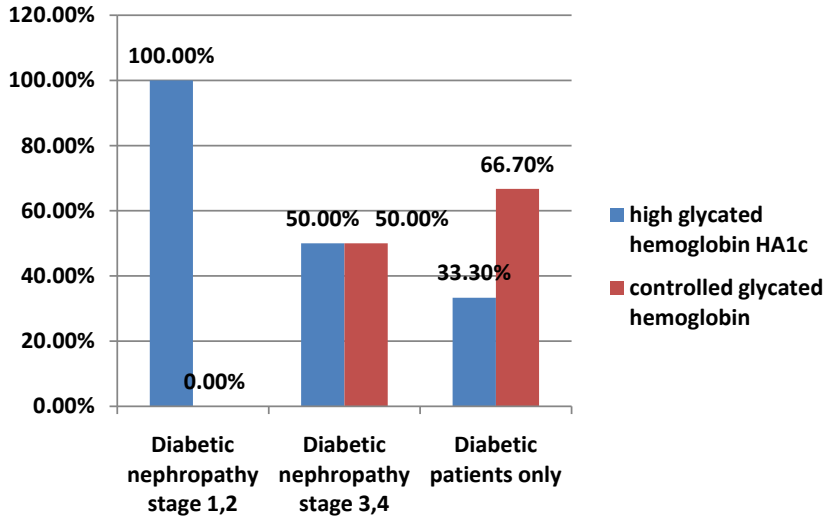

Figure 1. Percentage of controlled and high glycated hemoglobin.

Table 5 showed highly significant difference between patients with high HbA_{1c} and those with controlled HbA_{1c} in diabetic groups.

Table 6 shows significant difference between group 1 (DN stage 1 & 2) versus group 2 (DN stage 3 & 4), group 3 (DM without nephropathy) and all stages of CKD.

Figure 2 showed that the highest median IQR of miR-377 was present in DN stage 1 & 2 and the lowest median IQR was present in CKD stage 1 & 2.

Table 7 and Figure 3 showed that patients with uncontrolled diabetes mellitus (with higher HA1C) had significantly higher miR377 than those patients with controlled diabetes mellitus.

There was no significant correlation between GFR and miR-377 (Table 8).

Table 5. Difference in HA1C between patients with high and controlled glycated hemoglobin.

Chi-Square Tests			
	Value	df	Asymptomatic. Sig. (2-sided)
Pearson Chi-Square	9.847[a]	2	0.007
Likelihood Ratio	13.047	2	0.001
Linear-by-Linear Association	8.837	1	0.003
N of Valid Cases	27		

[a]3 cells (50.0%) have expected count less than 5. The minimum expected count is 2.96.

Table 6. Comparing miR-377 between all groups by Kruskal-Wallis test.

	$2^{-\Delta\Delta C_t}$ of miR377		Test value	P-value	Sig.
	Median (IQR)	Range			
Diabetic nephropathy stage 1, 2 (group I)	55.87 (13.31 - 576.53)	3.05 - 102,658			
Diabetic nephropathy stage 3, 4 (group II)	1.59 (0.15 - 8.17)	0.04 - 20.97			
Diabetic patients only (group III)	1.98 (0.97 - 5.25)	0.1 - 1176.27	10.774	0.029	S
Chronic kidney disease stage 1, 2 (group IV)	1.27 (0.11 - 2.47)	0 - 648.07			
Chronic kidney disease stage 3, 4 (group V)	2.89 (0.23 - 5.46)	0.01 - 49.18			

Post hoc analysis									
Group I vs group II	Group I vs group III	Group I vs group IV	Group I vs group V	Group II vs group III	Group II vs group IV	Group II vs group V	Group III vs group IV	Group III vs group V	Group IV vs group V
0.005	0.016	0.021	0.008	0.847	0.691	1.000	0.336	0.929	0.624

Kruskal-Wallis test.

Table 7. Comparison between patients with high HA_{1C} and those with controlled HA_{1C} as regard miR377.

HbA_{1C}	miR377		Test value	P-value	Sig.
	Median (IQR)	Range			
High glycated hemoglobin	9.82 (1.93 - 121.94)	0.04 - 102658	−1.970	0.049	S
Controlled glycated hemoglobin	1.66 (0.27 - 6.44)	0.1 - 20.97			

Table 8. Correlation between miR-377 and eGFR in each group.

	miR377									
	Diabetic nephropathy stage 1, 2		Diabetic nephropathy stage 3, 4		Diabetic patients only		Chronic kidney disease stage 1, 2		Chronic kidney disease stage 3, 4	
	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value
eGFR (ml/min)	0.167	0.693	−0.336	0.376	0.347	0.399	0.435	0.242	0.238	0.570

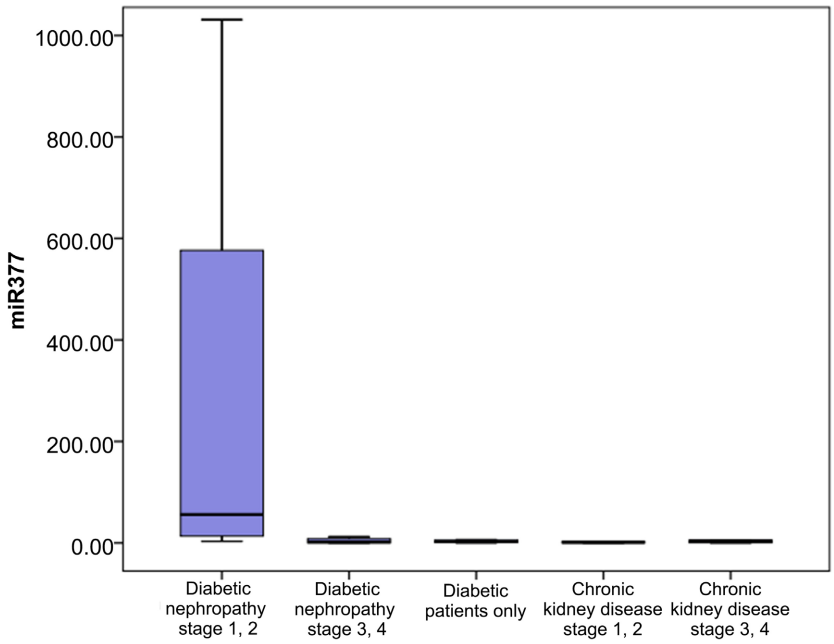

Figure 2. Median IQR of miR-377 in each group.

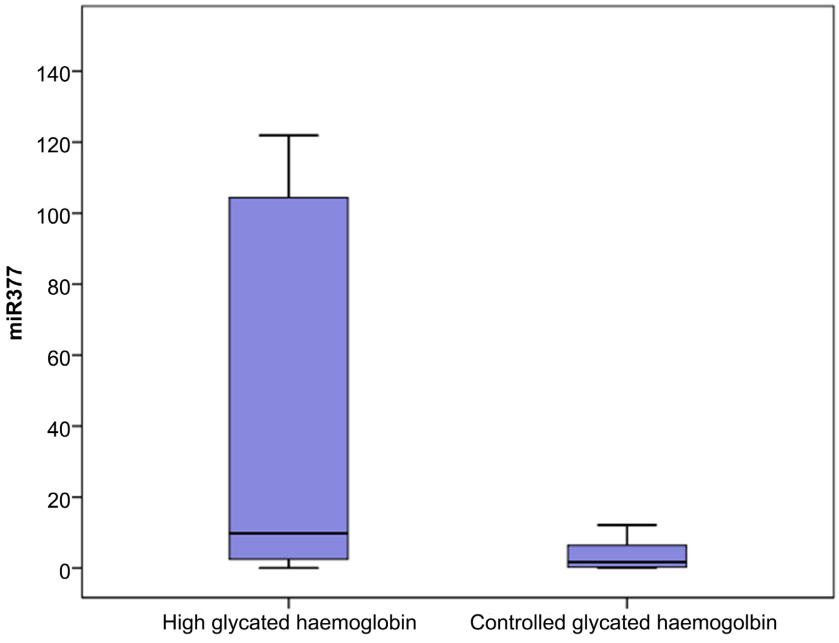

Figure 3. Comparison between patients with high HA1C and those with controlled HA1C as regard miR377.

Table 9 & Figure 4 showed that, with a cut off value of 6.44 IQR, miR377 had a sensitivity of 87.50% and specificity of 87.50% in detecting early diabetic nephropathy in diabetic patients.

Table 10 & Figure 5 showed that, with a cut off value of 2.47 IQR, miR377 had a sensitivity of 100.00% and specificity of 77.78% in distinguishing early diabetic nephropathy from other causes of early CKD.

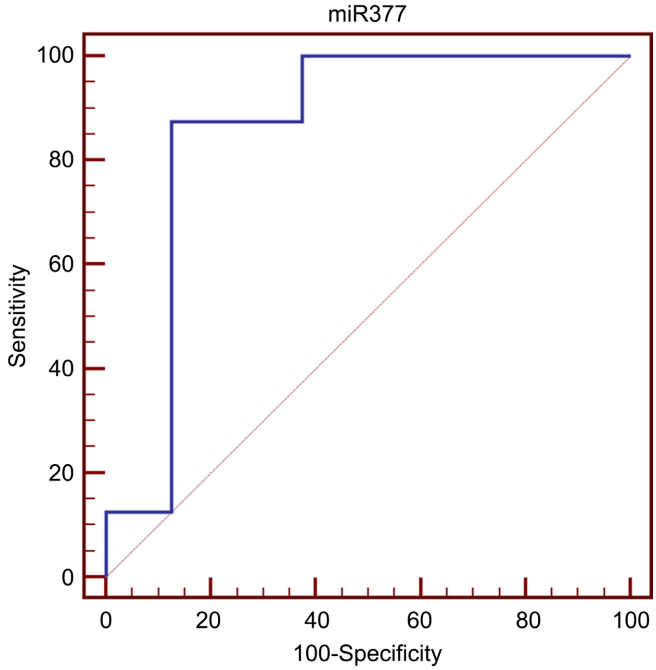

Figure 4. Roc curve analysis of miR-377 in diabetics with early diabetic nephropathy.

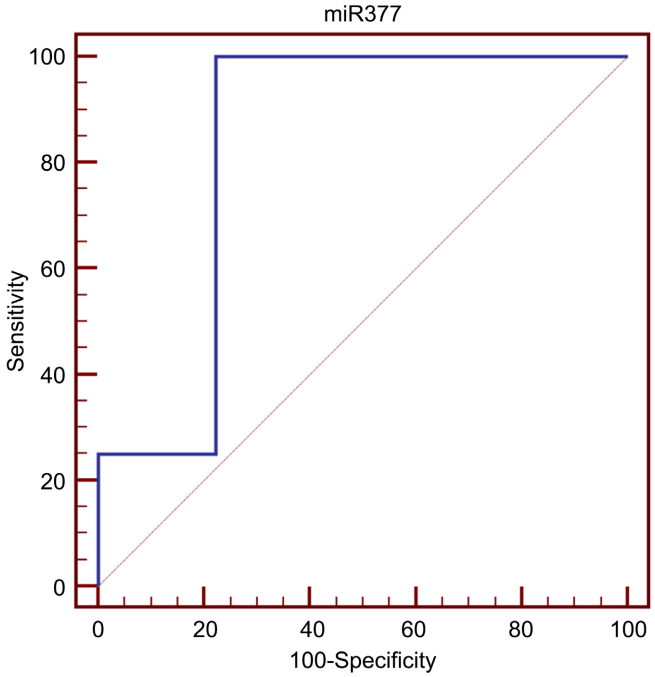

Figure 5. Roc curve analysis of miR-377 in CKD stage 1 & 2.

Table 9. Role of miR377 in prediction of early diabetic nephropathy.

Cut off of miR377 value	AUC	Sensitivity	Specificity	+PV	−PV
>6.44	0.859	87.50	87.50	87.50	87.50

Table 10. Role of miR377 in prediction of diabetic nephropathy vs. other causes of early chronic kidney disease (stage 1 & 2).

Cut off of miR377 value	AUC	Sensitivity	Specificity	+PV	−PV
>2.47	0.833	100.00	77.78	80.0	100.0

4. Discussion

Previous work in DN (performed in cell culture, animal models or formalin fixed human biopsy) has linked a number of microRNAs to the development of nephropathy.

Studies on the changes of miRNA in induced DN mice, human cultured mesangial cell revealed that miR-377 was the microRNA with the highest relevant changes to high glucose in cultured mesangial cells [1]. To our knowledge this is the first research to study the changes of miR377 in human subjects with DN as well as in patients with CKD secondary to other causes.

In the present study, 45 gender and age matched patients were examined to assess plasma miR377 value. It revealed higher serum miR377 in stage 1 & 2 DN more than in diabetics without nephropathy, patients with DN stage 3 & 4 and all stages of CKD due to other causes (**Table 6**).

In the present study, the high miR-377 in stage 1 and 2 DN, go with the results of Wang *et al.* [1] who followed up experiments in two separate mouse models of DN in which the expression of miR-377 was consistently upregulated. This confirms the relevant elevation of miR-377 levels in human patients with DN.

Also, the present study showed significant correlation between HA_{1C} and value of $2^{\Delta\Delta C_t}$ miR377, Wang *et al.* [1] found that miR-377 was significantly overexpressed in human mesangial cells exposed to high glucose levels rather than TGF-B as better approximating the condition that glomerular cells are exposed to *in vivo*.

Superoxide dismutases catalyze the reduction of reactive oxygen species (ROS) to less toxic forms [14]. Excessive glucose levels result in generation of ROS through the mitochondrial electron-transport chain [15], which is further complicated in diabetes by coexistent SOD deficiencies [16]. Wang *et al.* [1] showed that miR-377 targets and suppresses translation of important mesangial cell proteins including SoD_1 and SoD_2 and PAK_1, resulting in their deficiencies, leading to acceleration of diabetic renal injury [17].

Hydrogen peroxide (H_2O_2) continuously generated by glucose oxidase up regulates TGF-B1 and fibronectin expression in mesangial cells. Thus playing an important role in hyperglycemic induced renal injury [18].

Elamir and Ibrahim (2016) studied the impact of DN on miR-377 and found

that macroalbuminuria type 2 diabetics showed higher mean and significant difference in miR-377 [19].

Although urinary albumin excretion remains an essential tool for risk stratification and monitoring disease progression, a number of factors put into question its sensitivity and specificity: Microalbuminuria (MA) has originally been thought to be predictive of future overt DN in 80% of patients, but recent evidence indicated that only 30% progress to overt DN after 10 years [20]. Despite advanced structural alteration in the glomerular basement membrane may already have occurred by the time MA becomes clinically evident [21], a significant proportion of patients with MA can revert to normo-albuminuric [22]. Taken together, these results suggest that MA is perhaps more a diagnostic marker than a tool to predict DN and therefore the need to identify alternative biomarkers for the earlier prediction of DN.

In our research, the quantity real time PCR of miR-377 can distinguish DN stage 1 & 2 from diabetics without nephropathy by a sensitivity of 87.5% and specificity 87.5% and the ability of the test to distinguish DN stage 1 & 2 from CKD stage 1 & 2 by sensitivity 100% and specificity 77.78% (**Table 9**, **Table 10**, **Figure 4**, **Figure 5**).

Table 4 shows that 63% of diabetics had high glycated hemoglobin. Higher levels of HA1C were associated with increased risk for development of DN, and all DN stage 1 & 2 patients (100%) while 50% only of stage 3 and 4 DN had high HA1C. However *William and Garge* [23] and *Chen et al.* [24] found substantial increase in HA1C from stage 1 to 5 DN. Taking into consideration that HA1C becomes controlled as ESRD progresses.

Elamir A and Ibrahim T (2016) [19] found a negative correlation between miR-377 and eGFR. But in our research there was no correlation between GFR and miR-377 in all groups (**Table 8**). This indicates that miR-377 could be a good marker for detection of early diabetic nephropathy that is not affected by the glomerular filtration and kidney function tests.

The stability of miRNAs in body fluids, together with the fact that miRNAs testing is non-invasive and has a high accuracy and specificity, renders miRNAs potentially better biomarkers than proteins and mRNAs. However, it is actually time consuming and expensive. Further optimization of these procedures is required before they can be used in routine clinical practice.

5. Conclusion

Plasma miR-377 is highly significantly increased in human diabetic patients with early rather than late DN, diabetics without nephropathy, or in those with CKD due to other causes at any stage. Our findings confirm the role of miR-377 as a potentially novel target in the development and management of diabetic nephropathy in humans.

Ethical Approval

All procedures performed in studies involving human participants were ap-

proved by the Ain Shams University Hospitals ethics committee at which the studies were conducted with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed oral consent was obtained from all individual participants included in the study.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

There is no conflict of interest between the authors. This research was solely funded by the authors themselves.

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