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Evaluation of Mir 29c-3p and Mir 31-5p Serum Values in Predicting Nephropathy in Type 2 Diabetic Patients

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Abstract: Diabetic Nephropathy (DN) is a prime complication of diabetes with various pathological mechanisms. microRNAs (miRNAs) have emerged as an important factor in the pathogenesis of renal disorders. We aimed to analyze serum miR 29c-3p and miR 31-5p in diabetic patients concerning albuminuria and eGFR. Also, the correlations between these miRNAs and cystatin C were assessed. This study was conducted on 180 Type 2 Diabetes Mellitus (T2DM) patients and 60 age and gender-matched controls. The patients were divided into three groups: Normoalbuminuric (Group I), microalbuminuric (Group II), and macroalbuminuric (Group III). miRNA expression analysis was assessed via real-time PCR. The diabetic patients showed decreased miR 29c-3p and miR 31-5p serum values and increased cystatin C compared with the controls. miR31-5p had better sensitivity (92.31%), specificity (93.62%) and AUC (0.958) than miR 29c-3p (69.23% sensitivity, 85.11% specificity and AUC = 0.785) in the prediction of an eGFR <60 mL/min/1.73 m^2 in normoalbuminuric patients. Both miR 29c-3p and miR 31-5p could be used as early diagnostic and prognostic biomarkers for DN in T2DM. In particular, circulating miR 31-5p outperformed the other miRNAs in detecting normoalbuminuric diabetic patients with lower eGFRs.

Keywords: miR 29c-3p, miR 31-5p, Cystatin C, T2DM

Introduction

Diabetic Nephropathy (DN) is the main cause of the morbidity and mortality associated with Diabetes Mellitus (DM). Its prevalence is 25-40% even with the proper control over the blood glucose level (D'Addio *et al*., 2014). Hyperglycemia and renal hypertension lead to deterioration of renal function and a decrease in Glomerular Filtration Rate (GFR) over time, coinciding with the development of DN (Fineberg *et al*., 2013; Fowler, 2008). DN is considered to be a microvascular complication that is characterized by increased urinary albumin excretion caused by podocyte damage, triggering increased renal glomerular permeability and subsequent albuminuria (Persson and Rossing, 2018; Barutta *et al*., 2018). Although the urinary albumin levels of some diabetic patients are in the normal range, they are found to suffer from advanced renal pathological changes, hence why albuminuria is not the ideal biomarker for the early detection of DN. The detection of other biomarkers is important for the early detection of renal dysfunction (Moresco *et al*., 2013).

Inflammation acts as a vital factor in the progression of DN. Since the last decade, scientists have been focusing on different inflammatory and metabolic pathways in a trial to control the progress of nephropathy (Kim and Park, 2017). Cystatin C, a cysteine protease inhibitor, is continuously released by nucleated cells and poured into the bloodstream, with two hours as a half-life. It is freely filtered by renal glomeruli and metabolized by the proximal tubules, so its concentration depends totally on the GFR. A detected serum or urinary cystatin C is evaluated as a renal biomarker of early renal impairment in normoalbuminuric diabetic patients (Jeon *et al*., 2011). Studies in epigenetics, a recent field of research, have an important role in the pathophysiology of DN. Researchers have found microRNAs (miRNAs) that are capable of causing pathological changes in the kidney as well as those with reno-protective activity (Wu *et al*., 2014; Mukhadi *et al*., 2015). miRNAs are non-coding RNAs

involved in the modulation of gene expression via target mRNA degradation or subdued protein translation. Their lengths range from 20 to 30 nucleotides (Daugaard and Hansen, 2017). Circulating miRNAs represent promising candidate markers for the early monitoring of microvascular diseases (Guay *et al*., 2013). The miR-29s are known to be regulated in many tissues. They belong to a family of three members, divided into two clusters, that are transcribed polycistronically; the miR-29a/b-1 cluster is localized on human chromosome 7 and the miR 29c/b-2 cluster presents on chromosome 1. The miR-29 family is thought to participate in oxidative stress-mediated inflammatory response in DM (Kwon *et al*., 2019). Another type of microRNA is miR-31, which targets E-selectin, thus regulating the binding of neutrophils to endothelial cells. The increased expression of serum miR-31 has been reported in some patients with T2DM manifesting microvascular complications such as retinopathy, neuropathy, and nephropathy (Li *et al*., 2019).

In light of the significance of microRNAs and the evolving role of cystatin C as a high-value marker in DN, we aimed to evaluate the serum levels of miR-29 and miR-31 as molecular biomarkers in patients with DN concerning albuminuria and eGFR. Also, the correlations between these miRNAs and cystatin C were assessed.

Materials and Methods

Study Design and Participants

This study was performed via a collaboration between the Medical Biochemistry and Molecular Biology and the Internal Medicine and Clinical Pathology Departments at the Faculty of Medicine, Menoufia University, Egypt, from February 2019 to March 2020. This study included 180 T2DM patients divided into three groups depending on Albumin–Creatinine Ratio (ACR) value: Group I (60 patients; normoalbuminuria [ACR <30 mg/g]), Group II (60 patients; micro-albuminuria [ACR more than 30 and less than 300 mg/g]) and Group III (60 patients; macro-albuminuria [ACR >300 mg/g]). The patients were compared with 60 healthy subjects. T2DM was diagnosed according to the directions of the American Diabetes Association: Fasting blood glucose >126 mg/dL or 2 h blood glucose after loading with 75 g glucose >200 mg/dL in an oral glucose tolerance test or glycated hemoglobin (HbA1c) >6.5% in patients with classic symptoms of hyperglycemia (ADA, 2015). DN cases were chosen from the nephrology unit of the Internal Medicine Department, Menoufia University Hospital, with a history of DN lasting for more than 3 months (ACR >30 mg/g). Subjects with other primary or secondary renal diseases, malignancy, hepatic or cardiac failure, and severe infections were excluded.

This study was conducted per the Declaration of Helsinki. Informed written consent was obtained from all the participants, approved by the Ethical Committee of Medical Research, Faculty of Medicine, Menoufia University.

Data Collection

Medical and demographic history, family history, duration of DM, Body Mass Index (BMI) [weight (kg)/height (m^2)], smoking history, and systolic and Diastolic Blood Pressure (SBP and DBP, respectively) were obtained for all the participants.

Biological Samples and Biochemical Procedures

Urine Samples

Fifteen milliliters of fresh morning voided urine was obtained from every subject in sterile containers to measure creatinine and albumin for ACR calculation. Urinary albumin was estimated via a solid-phase enzyme-linked immunosorbent assay (DRG International Inc., USA; cat. #EIA-2361). Urinary creatinine was measured using colorimetry (DIAMOND Diagnostics Kits, Germany).

Blood Samples

Ten milliliters of venous blood were withdrawn from each subject after overnight fasting. Two milliliters of blood were placed in a sodium fluoride-containing tube for the measurement of blood glucose profile (FBG and 2 h PPG) using the glucose oxidase method (Spinreact Diagnostics Kit, Girona, Spain) (Trinder, 1969) and HbA1c using quantitative colorimetric measurement kits (Techo Diagnostics, Anaheim, CA, USA) as a percentage of total hemoglobin (Gonen and Rubenstein, 1978). Four milliliters of blood were placed in plain tubes, left to clot at room temperature, and then centrifuged for 15 min at 4,000 rpm. The separated sera were used to measure the lipid panel (TC, HDL-C, TGs) via the standard enzymatic colorimetric kits (Spinreact Diagnostics Kit, Spain) (Siedel *et al*., 1983) and renal function tests (serum urea and creatinine) via the standard enzymatic colorimetric kits (DIAMOND Diagnostics Kits, Germany). The Friedewald formula was used to calculate LDL-C (Friedewald *et al*., 1972). The eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) formula from serum creatinine (NKF, 2002; Levey *et al*., 2006). The Human Cystatin C (Cys-C) ELISA Kit was used to assay cystatin C (SunRed, China; cat. #201-12-1105). Four milliliters of blood were placed in an EDTA tube for miRNA analysis.

miRNA Expression Profiling

Purification and cDNA Synthesis

The miRNeasy® Mini Kit (QIAGEN, Germany; cat. #217004) was used for the purification of miRNA from whole blood. Both the yield and the purity of RNA were assessed using a NanoDrop instrument (Thermo

Scientific, USA). Purified miRNA was stored at-80°C. Complementary DNA (cDNA) was obtained via reverse transcription using the miScript II RT Kit (QIAGEN, Germany; cat. #218161). Each reaction was performed on ice with a total volume of 20 µL; 4 µL of the miScript HiSpec RT buffer, 2 µL of miScript Nucleics Mix, 2 µL of the miScript™ reverse transcriptase, and 2 µL of nuclease-free water were pipetted into every well, followed by 10 µL of extracted miRNA. Analysis was performed in a 2720 Applied Biosystems thermal cycler (Singapore) for one cycle: 37°C for 60 min and 95°C for 5 min to inactivate the reverse transcriptase. The cDNA produced was stored at-20°C.

Amplification by Real-Time PCR (miScript® SYBR® Green PCR Kit [QIAGEN, Germany; cat. #218073])

Before amplification, the cDNA samples were diluted with nuclease-free water at a ratio of 1:5. A total volume of 25 µL was used (12.5 µL of SYBR Green Master Mix, 3.5 µL of nuclease-free water, 4 µL of diluted cDNA, 2.5 µL of the miScript universal primer, and 2.5 µL of the miScript primer). The miRNA RNU6 was used as the reference miRNA. The miScript primer assay containing miRNA-specific forward primers was used to detect mature miRNA; hsa-miRNA-29c-3p, UAGCACCAUUUGAAAUCGGUUA and hsa-miRNA-31-5p, AGGCAAGAUGCUGGCAUAGCU (miScript Primer Assay Kit, QIAGEN, Germany; cat. #MS00003269 and #MS00003290, respectively). The data were analyzed via an ABI 7500 real-time PCR instrument with software version 2.0.1, with the following cycling conditions: Initial activation step at 95°C for 15 min and then 40 cycles (94°C for 15 sec 55°C for 30 sec, and 70°C for 30 sec). The expression levels of miRNA 29 c-3 p and miRNA 31-5p were normalized to those of RNU6 and calculated via the comparative $2^{-\Delta\Delta Ct}$ method to achieve the relative quantification of each miRNA.

Statistical Analysis

The data was analyzed using SPSS version 20 (SPSS Inc., released 2011; IBM SPSS Statistics for Windows, version 20.0, Armonk

Results

The demographic, clinical, and laboratory data of the diabetic patients (n = 180) and controls (n = 60) is listed in Table 1. They matched in terms of age and gender (p = 0.775 and p = 0.709, respectively). Statistically significant differences (p<0.001) were found between the diabetic patients and the controls in terms of BMI, SBP, DBP, FBG, 2 h PPG, HbA1c, TG, TC, HDL-C, LDL-C, urea, creatinine, ACR and eGFR as well as cystatin C. The expression levels of miR 29c-3p and miR 31-5p were significantly decreased in the patients than in the controls (p = 0.048 and p = 0.002, respectively).

Stratification of Diabetic Patients According to ACR

Among the three studied subgroups, gender, age, duration of DM, BMI, FBG, 2 h PPG, TG, and LDL-C did not differ significantly. Group III, compared with Groups I and II, had significantly (p<0.001) elevated SBP and DBP as well as higher levels of urea, creatinine, cystatin C and ACR, while HDL-C levels and the eGFR were significantly decreased. Additionally, Group II reported a significant increase in TC (p = 0.008), cystatin C (p<0.001), and ACR (p<0.001) compared with Group I as well as elevated blood levels of HbA1c (p<0.001) compared with Groups I and III. Regarding the expression of miR 29 c-3 p, Group I showed significantly decreased levels compared with patients with albuminuria in either Group II or Group III (p_1 = 0.013 and p_2 = 0.046, respectively), no significant differences were found between Groups II and III (p_3 = 0.623). The miR 31-5p results demonstrated lower levels in Group III compared with Groups I and II (p_2<0.001 and p_3 = 0.019, respectively) and in Group II compared with Group I (p_1 = 0.013) (Table 2).

Stratification of Diabetic Patients According to eGFR

Diabetic patients with an eGFR <60 mL/min/1.73 m^2 (n = 71) exhibited significantly (p<0.001) higher values of urea, creatinine, ACR and cystatin C as well as significantly (p<0.001) lower levels of miR 29c-3p and miR 31-5p compared with patients with an eGFR ≥60 mL/min/1.73 m^2 (Table 3).

Stratification of Patients According to ACR and eGFR

In Group I, patients with an eGFR <60 revealed significantly higher levels of urea (p = 0.004), creatinine (p<0.001) and cystatin C (p<0.001). The levels of miR 29c-3p and miR 31-5p were significantly lower in these patients (p = 0.03 and p<0.001, respectively). In Group II, patients with an eGFR <60 showed significantly higher values of creatinine (p = 0.005) and significantly lower values of cystatin C and miR 29c-3p (p = 0.005 and p<0.001, respectively). However, urea, ACR, and miR 31-5 p did not show any significant differences in this group. In Group III, we found significantly higher values of urea,

creatinine, ACR, and cystatin C ($p<0.001$) and lower values of miR 29c-3p and miR 31-5p in patients with eGFR <60 versus patients with eGFR ≥60 (Table 4).

The correlation results of miR 29c-3p with the clinicopathological parameters (Table 5) are as follows: In Group I, the serum miR 29c-3p level was positively correlated with TC and was negatively correlated with urea; in Group II, it was positively correlated with BMI, HbA1c, HDL-C, and eGFR and was negatively correlated with SBP, urea, creatinine, and ACR; and in Group III, it was positively correlated with eGFR and negatively correlated with DBP, TC, LDL-C, urea, creatinine, ACR and cystatin C.

Regarding miR 31-5p, in Group It was found to be positively correlated with FBG and eGFR and negatively correlated with urea, creatinine, and cystatin C. In Group II, it was positively correlated with TC, HDL-C, LDL-C, and eGFR and negatively correlated with HbA1c, creatinine, ACR, and cystatin C. In Group III, it was positively correlated with eGFR and was negatively correlated with DBP, TC, LDL-C, urea, creatinine, ACR, and cystatin C (Table 5).

To evaluate the validity of miR 29c-3p and miR 31-5p to predict patients with an eGFR <60 among diabetic patients, a ROC curve was constructed and the cutoff values for each of the two miRNAs were reported; miR 31-5p had a better performance with a cutoff value ≤3.9, providing a sensitivity of 85.92% and a specificity of 73.39%, while miR 29c-3p, at a cutoff value ≤1.6, provided a sensitivity of 60.56% and a specificity of 87%. The combinations of both miRNAs improve the sensitivity of miR 29c-3p (Table 6). Additionally, in patients with normoalbuminuria, miR 31-5p reported very good performance in predicting patients with an eGFR <60. miR 31-5p, with a cutoff value ≤2.54, had a sensitivity of 92.31% and a specificity of 93.62%, while miR 29c-3p, at a cutoff value ≤0.98, had a sensitivity of 69.23% and a specificity of 85.11% in predicting patients with an eGFR <60. The combinations of both miRNAs improve the performance of miR 29c-3p (Table 6).

Table 1: Demographic, clinical and laboratory data of the studied groups

Patients (n = 180)	Control (n = 60)	Test of sig	p	
Gender Male	91(50.6%)	32(53.3%)	$\chi^2 = 0.139$	0.709
Female	89(49.4%)	28(46.7%)		
Age (years)				
Mean ± SD.	53.9±8.4	53.5±8.2	t = 0.286	0.775
Duration of diabetes (years)				
Mean ± SD.	9±2.9	–	-	-
BMI (kg/m^2)				
Mean ± SD.	32±5.8	21.9±1.7	t = 20.942*	<0.001*
SBP (mmHg)				
Mean ± SD.	128.3±11.6	113.5±12.2	t = 8.457*	<0.001*
DBP (mmHg)				
Mean ± SD.	78.7±8.3	73.4±5.3	t = 5.717*	<0.001*
FBG (mg/dl)				
Median (IQR)	150(121 – 187.5)	84(80-94)	U = 785.5	<0.001*
2hr PPG (mg/dl)				
Median (IQR)	216.5(158.5 – 301.5)	95(87-101.5)	U = 785.5	<0.001*
HbA1c (%)				
Median (IQR)	8(7.1 – 8.7)	5.3(4.4-5.8)	U = 28.0	<0.001*
TG (mg/dl)				
Mean ± SD.	150.3±48.2	90.3±9.6	t = 15.830*	<0.001*
TC (mg/dl)				
Mean ± SD.	198.2±29.5	159.7±12.1	t = 14.259	<0.001*
HDL-C (mg/dl)				
Mean ± SD.	44.5±10.1	52.2±7.4	t = 6.318*	<0.001*
LDL-C (mg/dl)				
Mean ± SD.	130.6±25.1	90.3±7.1	t = 19.340*	<0.001*
Creatinine (mg/dl)				
Median (IQR)	1(0.8-2.3)	0.5(0.4-0.7)	U = 1001.5	<0.001*
Urea (mg/dl)				
Median (IQR)	33(26-76.5)	23(13-33)	U = 2438.0	<0.001*
ACR (mg/g)				
Median (IQR)	175(17.1-1075)	15(9-18.5)	U = 1962.0	<0.001*
eGFR (ml/min/1.73m^2)				
Median (IQR)	69.1(33-97)	89(79-94)	U = 3549.0	<0.001*
Cystatin C (mg/dl)				
Median (IQR)	1.4(0.9-3.2)	0.7(0.7-0.8)	U = 1128.5	<0.001*
miR 29c-3p				
Median (IQR)	3.8(1.5-19.3)	10.7 (2.5-19.1)	U = 6321.0	0.048*
miR 31-5p				
Median (IQR)	4 (0.3-10.6)	18.5(0.7-62)	U = 3929.5	0.002*

BMI: Body Mass Index, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, FBG: Fasting Blood Glucose, 2 hr PPG: Two-hour Postprandial Glucose, HbA1c: Glycated Hemoglobin, TG: Triglycerides, TC: Total Cholesterol, HDL-C: High-Density Lipoprotein cholesterol, LDL-C: Low-Density Lipoprotein Cholesterol, ACR: Albumin Creatinine Ratio, eGFR: Estimated Glomerular Filtration Rate, χ^2: Chi-square, t: Student t-test, U: Mann Whitney test, *: Statistically significant at p-value ≤0.05

Table 2: Comparison between the diabetic patients' subgroups according to demographic, clinical, and laboratory data

	Group I (n = 60)	Group II (n = 60)	Group III (n = 60)	Test of sig.	p
Gender	35(58.3%)	27(45%)	29(48.3%)	$\chi^2 = 2.33$	0.315
Male	25(41.7%)	33(55%)	31(51.7%)		
Female					
Age (years)					
Mean ± SD.	54.7±8	52.7 ± 8.6	54.2 ± 8.5	F = 0.89	0.103
Duration of diabetes (years)					
Median (IQR)	9(7 – 12)	8.5(6.5 – 10)	9(7 – 11)	H = 9.9	0.735
BMI (kg/m^2)					
Mean ± SD.	31.9±6.2	33.2±5.5	31±5.5	F = 2.31	0.103
SBP (mmHg)					
Mean ± SD.	124.5±9.7	124.3±12.6	136.2±7.8	F = 26.79*	<0.001*
Sig.bet.Grps	$p_1 = 0.989$, $p_2<0.001^*$, $p_3<0.001^*$				
DBP (mmHg)					
Mean ± SD.	75.8±7.3	78.1±8.6	82.2±7.6	F = 9.97*	<0.001*
Sig.bet.Grps	$p_1 = 0.264$, $p_2<0.001^*$, $p_3=0.014^*$				
FBG (mg/dl)					
Median (IQR)	144.5(132 – 208.5)	165(120 – 184.5)	144(99.5-187.5)	H = 150.0	0.502
2hr PPG (mg/dl)					
Median (IQR)	211.5(166 – 305.5)	225(194 – 306)	215(146-259.5)	H = 216.5	0.936
HbA1c (%)					
Median (IQR)	7.5(7 – 8.4)	8.5(7.7 – 9.4)	7.8(7.2-8.5)	H = 7.950	0.002*
Sig.bet.Grps	$p_1 = 0.001^*$, $p_2 = 0.583$, $p_3 = 0.001^*$				
TG (mg/dl)					
Mean ± SD.	139.9 ± 52.6	151.6±51	159.5±38.4	F = 2.57	0.080
TC (mg/dl)					
Mean ± SD.	189.1±27.1	205.1±31.9	200.3±27.5	F = 4.85*	0.009*
Sig.bet.Grps	$p_1 = 0.008^*$, $p_2 = 0.088$, $p_3 = 0.635$				
HDL-C (mg/dl)					
Mean ± SD.	47±11.2	47.1±8.6	39.4±8.2	F = 12.97*	<0.001*
Sig.bet.Grps	$p_1 = 1.000$, $p_2<0.001^*$, $p_3<0.001^*$				
LDL-C (mg/dl)					
Mean ± SD.	129.8±25	128.5 ± 29.5	133.5±20.1	F = 0.64	0.530
Creatinine (mg/dl)					
Median (IQR).	0.9(0.8 – 1)	0.8(0.7-1.1)	2.7(2.2-6.4)	H = 103.1	<0.001*
Sig.bet.Grps	$p_1 = 0.378$, $p_2<0.001^*$, $p_3<0.001^*$				
Urea (mg/dl)					
Median (IQR)	30(25.5 – 34)	31.5(26-43.5)	93.5(27.5-138.5)	H = 29.44	<0.001*
Sig.bet.Grps	$p_1 = 0.237$, $p_2<0.001^*$, $p_3<0.001^*$				
ACR (mg/g)					
Median (IQR)	13.2(9 – 17.1)	175(130.5-185.5)	2855(1075-3600)	H = 159.1	<0.001*
Sig.bet.Grps	$p_1<0.001^*$, $p_2<0.001^*$, $p_3<0.001^*$				
eGFR (ml/min/1.73m^2)					
Median (IQR)	76.9(61.4-94.9)	80(60.1-97.5)	24(10-97.2)	H=27.97	<0.001*
Sig.bet.Grps	$p_1 = 0.781$, $p_2<0.001^*$, $p_3<0.001^*$				
Cystatin C (mg/dl)					
Median (IQR)	0.8(0.7 – 0.9)	1.3(1.2-1.4)	4(3.2-4.6)	H = 143.1	<0.001*
Sig.bet.Grps	$p_1<0.001^*$, $p_2<0.001^*$, $p_3<0.001^*$				
miRNA 29c-3p					
Median (IQR)	2.1 (1–14)	12.8 (2.1-17.1)	6 (0.6-24.3)	H = 6.95	0.031*
Sig.bet.Grps	$p_1= 0.013^*$, $p_2 = 0.046^*$, $p_3 = 0.623$				
miRNA 31-5p					
Median (IQR)	10(2.6 – 12.6)	4.4(0-15.1)	2.8(0.3-3.7)	H=23.360.001*	
Sig.bet.Grps	p1 = 0.013*, p2<0.001*, p3= 0.019*				

Table 3: Kidney functional parameters and miRNAs levels according to eGFR in diabetic patients (n= 180)

	eGFR <60 (n = 71)	Mann whitney test ≥60 (n = 109)	p	
Creatinine (mg/dl)				
Median (IQR)	2.6(1.3-6.1)	0.9(0.8-1)	836.0	<0.001*
Urea (mg/dl)				
Median (IQR)	91(39.5-124.5)	28(25-33)	767.0	<0.001*
ACR (mg/g)				
Median (IQR)	1100(171.5-3515)	43.2(14-185)	1643.0	<0.001*
Cystatin C (mg/dl)				
Median (IQR)	3.4(1.3-4.3)	1.2(0.8-1.5)	1361.5	<0.001*
miRNA 29c-3p				
Median (IQR)	1.5(0.6-6.1)	12.6(2.3-21.1)	2101.0	<0.001*
miRNA 31-5p				
Median (IQR)	1.2(0.1-3.2)	6.9(3.1-13.1)	1661.5	<0.001*

Table 4: Kidney functional parameters and miRNAs levels according to eGFR in diabetic patients' subgroups

	eGFR in group I <60 (n = 13)	eGFR in group II ≥60 (n = 47)	eGFR in Group III <60 (n = 14)	≥60 (n = 46)	<60 (n = 44)	≥60 (n = 16)
Creatinine (mg/dl)						
Median (IQR)	1.2 (1.1– 1.3)	0.9 (0.8– 1)	1.3 (0.7–2.8)	0.8 (0.6–1)	5.8(2.4–6.7)	2.1(1.6–2.4)
	U = 35.50*	p<0.001*	U = 163.50*	p = 0.005*	U=85.50*	p<0.001*
Urea (mg/dl)						
Median (IQR)	34 (32 –35)	29 (25– 32)	38 (26–78)	29.5 (26–42)	121.5(92.5–160)	17.1(14.6–21.5)
	U = 147.0*	p=0.004*	U = 222.50	P = 0.082	U = 0.0	p<0.001*
ACR (mg/g)						
Median (IQR)	17 (9.2– 18.8)	13 (9– 15)	177.5(170–187)	155.5 (129–185)	2899.5(1200–3705)	624.3(621.7–3000)
	U = 213.50	p = 0.099	U = 249.50	p = 0.205	U = 150*	p = 0.001*
Cystatin C (mg/dl)						
Median (IQR)	1.3 (1.2– 1.4)	0.8 (0.7 – 0.8)	1.2 (1.2–1.3)	1.4 (1.2–1.5)	4.1(3.5– 4.7)	2.6(2.6– 4)
	U = 0.0*	p<0.001*	U = 160.50*0.005*	U = 122.0*	p<0.001*	miR 29c-3p
Median (IQR)	1.6 (0.6 –2.3)	2.3 (1.7– 16.8)	1.2 (0.9–1.5)	14.3 (11.9–21)	5.9(0.5– 20.8)	79.2(3.8– 80.6)
	U = 184.50*	p = 0.030*	U = 0.0*	p<0.001*	U =150*	p = 0.001*
miR 31-5p						
Median (IQR)	0.6 (0.5– 1.3)	11 (7.1– 13)	0(0–7.8)	4.4 (0.1–19)	2.7(0.2– 3.2)	6.5(0.5– 6.7)
	U = 25.50*	p<0.001*	U = 237.50	0.140	U =150*	p = 0.001*

Table 5: Correlation between miR 29c-3p, miR 31-5p and clinicopathological parameters in each diabetic patients' subgroup

		miR 29c-3p	miR 31-5p				
		Group I	Group II	Group III	Group I	Group II	Group III
Age	r_s	0.237	-0.015	0.144	0.154	-0.140	0.124
	P	0.068	0.912	0.272	0.240	0.285	0.346
Duration of diabetes	r_s	-0.058	-0.176	-0.069	0.123	-0.093	-0.117
	P	0.658	0.178	0.599	0.348	0.480	0.372
BMI	r_s	-0.073	0.723	0.203	-0.100	0.119	0.237
	P	0.579	<0.001*	0.119	0.445	0.365	0.069
SBP	r_s	0.193	-0.279	-0.077	0.120	0.202	-0.061
	P	0.140	0.031*	0.560	0.362	0.121	0.643
DBP	r_s	0.145	-0.242	-0.391	0.030	0.039	-0.397
	P	0.271	0.063	0.002*	0.820	0.766	0.002*
FBG	r_s	-0.145	0.124	0.098	0.323	-0.010	0.112
	P	0.270	0.345	0.455	0.012*	0.938	0.394
2hr PPG	r_s	-0.224	-0.084	0.049	0.177	-0.043	0.092
	P	0.086	0.523	0.711	0.176	0.746	0.485
HbA1c	r_s	-0.159	0.262	0.056	0.242	-0.590	0.084
	P	0.226	0.043*	0.670	0.062	<0.001*	0.524
TG	r_s	0.060	0.206	-0.131	0.099	-0.086	-0.063
	P	0.651	0.115	0.317	0.450	0.512	0.631
TC	r_s	0.301	-0.052	-0.368	0.162	0.540	-0.256
	P	0.019*	0.692	0.004*	0.216	<0.001*	0.048*
HDL-C	r_s	-0.050	0.326	0.047	-0.144	0.493	0.053
	P	0.706	0.011*	0.719	0.274	<0.001*	0.688
LDL-C	r_s	0.114	-0.155	-0.443	0.032	0.265	-0.440
	P	0.387	0.237	<0.001*	0.810	0.041*	<0.001*
Creatinine	r_s	-0.074	-0.297	-0.647	-0.463	-0.450	-0.541
	P	0.574	0.021*	<0.001*	<0.001*	<0.001*	<0.001*
Urea	r_s	-0.261	-0.296	-0.651	-0.360	0.068	-0.567
	P	0.044*	0.021*	<0.001*	0.005*	0.606	<0.001*
ACR	r_s	-0.193	-0.318	-0.956	-0.198	-0.833	-0.952
	P	0.139	0.013*	<0.001*	0.129	<0.001*	<0.001*
eGFR	r_s	0.184	0.569	0.713	0.638	0.642	0.574
	P	0.159	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Cystatin C	r_s	-0.221	0.248	-0.731	-0.689	-0.544	-0.647
	P	0.090	0.056	<0.001*	<0.001*	<0.001*	<0.001*

Table 6: Agreement (sensitivity, specificity) for miR 29c-3p and miR 31-5p to predict patients with eGFR <60 in the diabetic patients (n = 180) and in patients with normo-albuminuria (n = 60)

patients with eGFR <60 in the diabetic patients (n = 180)	AUC PPV	p NPV	95% C.I LL	Cut off[#] UL	Sensitivity	Specificity
miR 31-5p	0.785 73.39	<0.001[*] 67.8	0.718 88.9	0.852	≤3.9	85.92
miR 29c-3p	0.735 87.16	<0.001[*] 75.4	0.654 77.2	0.815	≤1.6	60.56
miR 31-5p + miR 29c-3p	0.833 74.31	<0.001[*] 68.5	0.776 89.0	0.890		85.92
patients with eGFR <60 with normo-albuminuria (n = 60)	AUC PPV	p NPV	95% C.I LL	Cut off[#] UL	Sensitivity	Specificity
miR 31-5p	0.958 93.62	<0.001[*] 80.0	0.903 97.8	1.013	≤2.54	92.31
miR 29c-3p	0.785 85.11	0.002[*] 56.2	0.625 90.9	0.944	≤0.98	69.23
miR 31-5p + miR 29c-3p	0.953 95.74	<0.001[*] 85.7	0.897 97.8	1.010		92.31

Discussion

DN is a prime complication of diabetes with various pathological mechanisms. The aberration of glucose and lipid metabolism, the dysregulation of inflammatory factors, and podocyte injury are the main pathological processes associated with DN progression (Chen *et al*., 2018). Such progression is usually manifested by increased albumin excretion from normoalbuminuria to microalbuminuria and then macroalbuminuria (Hostetter, 2003). Despite a decreased eGFR, some patients with DN still reported normal levels of urinary albumin and even microalbuminuria manifested in renal impairment following a prolonged silent stage of the disease, which explains the need for more accurate biomarkers for DN (Suarez *et al*., 2013). Recently, miRNAs have emerged as an important factor in the pathogenesis of renal disorders, and the abnormal expression of miRNAs was reported to be associated with DN, suggesting miRNAs as potential biomarkers for DN (Chen *et al*., 2018; Tayel *et al*., 2020).

In this analysis, circulating expression levels of miR 31-5p and miR 29c-3p were evaluated in T2DM patients categorized according to ACR into normo-, micro-and macroalbuminuric groups. Furthermore, the potential correlations of these miRNAs with kidney functional parameters, including cystatin C, were investigated to evaluate their significance as early indicators of DN development.

This study revealed significantly elevated levels of the serum cystatin C and a stepwise reduction of the serum miR 31-5p in T2DM patients progressing from normoalbuminuria to macroalbuminuria. Moreover, a significant reduction of miR 31-5p was observed in patients with an eGFR <60 mL/min/1.73 m^2 among normoalbuminuric and macroalbuminuric patients concerning patients with an eGFR >60 mL/min/1.73 m^2, while ACR was significant only in patients with macroalbuminuria. miR 31-5p showed negative correlations with creatinine and cystatin C and a positive correlation with eGFR in patients with normoalbuminuria, microalbuminuria, and macroalbuminuria.

Jeon *et al*. (2011) agreed that the significance of detecting the level of cystatin C in normoalbuminuric patients may be due to the tubular insult that started before glomerular manifestation and added that cystatin C levels are related to subclinical tubular injury and can be used to assess renal involvement before the occurrence of albuminuria. Also, (Gupta *et al*., 2017) stated that cystatin C values may be considered an early biomarker compared with microalbuminuria and creatinine levels, the most common previously used markers for nephropathy (declining renal function) in diabetic patients.

Rovira-Llopisa *et al*. (2018) reported similar results of the decreased expression of miR31-5p in DN and added that the protective role of miR31-5p in inflammation-derived leukocyte-endothelial interplay may explain the reduction in miR31-5p levels observed in their study. (Barutta *et al*., 2018) believed that low-grade chronic inflammation played an important role in the pathogenesis of microvascular complications of diabetes. Moreover, (Suarez *et al*., 2010) recognized miR-31 to be a negative regulator of TNFα- cells. The adhesion molecule ICAM-1 showed a negative correlation with miR-31 levels, supporting the association of miR-31 with the provocative and adhesive particulates. Also, miR-31-5p was reported to be down-regulated and to have a negative correlation with prognosis in patients with renal cell carcinoma (Li *et al*., 2019).

Currently, miR 29c-3p values are reduced in diabetic patients with normoalbuminuria compared with patients with albuminuria (the micro-and macroalbuminuric groups). This reduction is also related to an eGFR <60 mL/min/1.73 m^2 in all groups. miR 29c-3p is negatively correlated with cystatin C only in diabetic patients with macroalbuminuria while positively correlated with eGFR in the microalbuminuria and macroalbuminuria groups.

The results of miR-29 in DN are controversial. In

line with our results, (Gondaliya *et al*., 2020) reported the downregulation of miR-29b and the consequent upregulation of HDAC4 expression in both the in vivo and in vitro models of DN. They added that miR-29b mimics the inhibition of podocyte inflammation and reduces HDAC4 expression and glomerular damage and fibrosis.

Lv *et al*. (2013) confirmed these findings and reported the downregulation of miR-29 in the urinary exosomes of patients with chronic kidney disease than in the controls. They also demonstrated a positive correlation between miR-29c and eGFR as well as a negative association between miR-29c and the extent of tubulointerstitial fibrosis. (Chung *et al*., 2015) identified significant miRNAs for diabetic kidney disease progression, including miR-29c, which was found to regulate the anti-inflammatory protein, tristetraprolin and control the inflammatory process (Guo *et al*., 2017).

Additionally, (Pezzolesi *et al*., 2015), T1DM, found that miR-29 is a regulator of TGF-β and prompt development of End-Stage Renal Disease (ESRD) versus those with albuminuria with conserved

Members of the miR-29 family are known to control the genes of the extracellular matrix and regulate fibrosis (Hsu *et al*., 2016). The upregulation of miR-29 was reported to hinder the stimulation of Akt, a vital factor in insulin signaling. Moreover, along these lines, this increased expression may assume a defensive function against vascular complications of diabetes (Peng *et al*., 2013). On the other hand, **(**Chien *et al*., 2016) discovered that DN patients with a rapid decline of eGFR reported elevated expression levels of the miR-29 family compared with stable patients.

To validate the performance of miR 31-5p and miR 29c-3p to detect patients with an eGFR <60 mL/min/1.73 m^2, a ROC curve was constructed. It revealed that the evaluation of miR 31-5p, either alone or combined with miR 29c-3p, outperformed miR 29c-3p in the differentiation of patients with eGFR above and below 60 mL/min/1.73m^2 among the total diabetic patients and specifically among the normoalbuminuric group. Therefore, we can assume the use of miR 31-5 p in combination with miR 29c-3p as potential markers in predicting patients with early renal impairment even before the appearance of albuminuria, which may be represented after a long silent stage of the disease.

Conclusion

Overall, we can emphasize the importance of circulating miR 31-5p and miR 29c-3p as potential biomarkers for DN and their role, specifically miR 31-5p, as early indicators and prognostic biomarkers of renal impairment in T2DM patients even before albuminuria becomes apparent.

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Author's Contributions

Mona Salah Habieb: Performed the laboratory investigations and the molecular analysis besides selecting the study design, participated in writing and final manuscript.

Nesreen Gamal-Eldin Elhelbawy: Participated in writing and revising the paper and approved the final manuscript.

Khaled Mohmed Elzorkany and Mohamed Z. Nooh: Collected the samples and analyzed and interpreted the results. Participated in writing and revising the paper and approved the final manuscript.

Mohammad G. Elhelbawy: Performed laboratory investigations and molecular analysis. Participated in writing and revising the paper and approved the final manuscript.

Heba F. Khader: The major contributor in writing the manuscript and approved the final manuscript.

Ethical Statement

This study was conducted per the Declaration of Helsinki. Informed written consent was obtained from all the participants, approved by the Ethical Committee of Medical Research, Faculty of Medicine, Menoufia University.

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