Expression of microRNA-377 and microRNA-192 and their potential as blood-based biomarkers for early detection of type 2 diabetic nephropathy

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Abstract. The increased incidence of diabetic nephropathy (DN) in type 2 diabetes (T2D) requires novel markers for the early detection of DN. Previously, microRNAs (miRs) have been demonstrated to be promising disease biomarkers. The present study evaluated the biomarker potential of DN-associated miR-377 and miR-192 in the early stages of DN. The study included 85 participants: 55 patients with T2D (30 without DN and 25 with DN) and 30 healthy controls. The patients with T2D were classified according to albumin-to-creatinine ratio and were split into three groups: Normoalbuminuric group (n=30), microalbuminuric group (n=15) and macroalbuminuric group (n=10). Reverse transcription-quantitative polymerase chain reaction analysis was used to evaluate blood miR expression. It was observed that there was higher miR-377 expression and lower miR-192 expression in T2D patients with and without DN compared with healthy controls (P<0.05). miR-377 was higher in the normoalbuminuric group and gradually increased in the microalbuminuric and macroalbuminuric groups (P<0.05), whereas miR-192 was lower in the macroalbuminuric group compared with the normoalbuminuric group (P<0.05). Regression analysis revealed direct associations between the two miRs and albuminuria (P<0.05). miR-377 was independently associated with DN risk, even following multivariable adjustment, and albuminuria was the only predictor of miR-377 (P<0.001). In discriminating overall patients from healthy subjects, ROC analysis revealed areas under the curve (AUCs) of 0.851 for miR377 and 0.774 for miR-192 (P<0.001). In discriminating the normoalbuminuric group from the microalbuminuric/macroalbuminuric groups, the AUCs were 0.711 (P=0.008) and 0.70 (P=0.049) for miR-377 and miR-192, respectively. In patients with microalbuminuria and macroalbuminuria, miR-377 correlated positively with albuminuria and negatively with renal function, whereas miR-192 correlated negatively with albuminuria and positively with renal function (P=0.001), and the two miRs were correlated with known risk factors of DN (P<0.05). The results suggested that blood-based miR-377 and miR-192 may serve as potential biomarkers for early detection of DN. Further validation studies are required with larger sample sizes.

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Introduction

It has become increasingly apparent that the non-protein coding parts of the genome, including microRNAs (miRs), are crucial for normal development and function in humans (1). miRs regulate gene expression by mediating post-transcriptional gene silencing through degradation of target mRNAs or translation inhibition (2). Although the exact mechanism underlying miR targeting and activity is not completely understood, a single miR may regulate multiple target genes, and a single gene may be regulated by multiple miRs (3). Thus, miRs are predicted to control the expression of 50-60% of all coding genes within mammalian genomes (3). Consequently, miRs have been demonstrated to regulate a number of biological processes, including growth, metabolism and inflammatory responses (4), and alterations in their expression have been frequently observed in various diseases (5,6).

As miRs have additionally been identified extracellularly, packaged in microvesicles or bound to proteins, they are very stable and protected from RNase digestion (7,8). In addition, the expression levels of circulating miRs in the blood differed considerably between healthy people and those with diseases, suggesting that they may be ideal biomarkers for a number of diseases, including cancer (9,10), type 2 diabetes (T2D) and associated vascular complications (11-14).

Diabetic nephropathy (DN) is an important long-term microvascular complication of diabetes mellitus and is a notable cause of end-stage renal disease (ESRD) with a high mortality rate, primarily among patients with T2D who

frequently remain undiagnosed for a number of years (15) as hyperglycemia develops gradually and may not produce any symptoms (15,16). DN is characterized by glomerular basement membrane thickening, mesangial expansion and hypertrophy, and an accumulation of extracellular matrix (ECM) proteins (17). Although the pathogenesis of DN is not completely understood, numerous mechanisms have been proposed to be involved, including the overproduction of reactive oxygen species, formation of advanced glycation end products, activation of protein kinase C and upregulation of transforming growth factor (TGF)-β1, leading to the deposition of ECM proteins, including collagen and fibronectin in the mesangium, and renal tubulointerstitium of the glomerulus and basement membranes (18,19).

Clinically, DN is characterized by increased rates of urinary albumin excretion and decreased renal function and glomerular filtration rate. Diabetic patients at risk of developing DN go through the stages of normoalbuminuria, microalbuminuria and macroalbuminuria, and eventually ESRD (20). While microalbuminuria may be an indicator of early DN, macroalbuminuria represents DN progression (20). It has been reported that impaired renal function may present in diabetic patients in the absence of significant increases in microalbuminuria, or while remaining normoalbuminuric (21,22). Moreover, a number of DN-associated structural alterations have been demonstrated to be already manifested prior to the development of microalbuminuria (23,24). Therefore, microalbuminuria has been regarded as an indicator of kidney damage rather than a DN prognostic marker (25,26). Microalbuminuria is strongly and independently associated with increased cardiovascular risk among individuals with and without diabetes (27), and was associated with impaired arterial and venous endothelium-dependent vasodilation in patients with T2D (28). These studies highlight the requirement for more sensitive biomarkers for the early detection of DN (29,30), which is a key point in disease management. A previous study reported that numerous miRs regulate signaling pathways in the diabetic kidney, and their dysregulation contributes to DN pathogenesis and development (31). Among the earliest studied miRs that regulate pathological pathways induced in DN are miR-377 and miR-192.

miR-377 has been demonstrated to be overexpressed in *in vitro* and *in vivo* models of DN and its increase suppressed the translation of p21-activated kinase and manganese superoxide dismutase 1 and 2, and enhanced the production of fibronectin, a matrix protein that excessively accumulates in DN (32). By contrast, miR-192 has been reported to be implicated in the development of matrix accumulation by controlling TGF-β-induced collagen type 1 α-2 (COL1A2) expression by downregulating the E-box repressors zinc finger E-box-binding homeobox (ZEB)1 and ZEB2 (33), and thus serves a vital role in the development and progression of DN.

Given that circulating miRs reflect a tissue-specific injury or expression (34), and as a number of these circulating miRs may represent a potential novel source of non-invasive biomarkers for kidney diseases (35,36), the present study aimed to evaluate the expression of miR-192 and miR-377 and to establish their potential as blood-based biomarkers in the early stage of DN in patients with T2D.

Materials and methods

Patients and healthy controls. In the period between January 2013 and October 2014, 55 patients diagnosed with T2D with and without DN at King Abdullah University Medical Centre (Arabian Gulf University, Manama, Kingdom of Bahrain) and 30 healthy control individuals without any history of T2D, were recruited. Patient characteristics are displayed in Table I. The following parameters were studied in the participants: Age, sex, body mass index (BMI), mean blood pressure, fasting blood glucose (FG) levels, glycated hemoglobin (HbA1c), diabetes duration, urinary albumin excretion rate (AER), albumin-to-creatinine ratio (ACR), serum creatinine, estimated glomerular filtration rate (eGFR), total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglyceride. The diabetic patients were divided into three groups according to ACR as follows: 30 patients with normoalbuminuria (ACR, <3.5 mg/mmol), 15 patients with microalbuminuria (ACR, 2.5-25 mg/mmol) and 10 patients with macroalbuminuria (ACR, >25 mg/mmol). The diabetic patients fulfilled the World Health Organization criteria for T2D (37). Renal function was established based on eGFR according to the Modification of Diet in Renal Disease formula (38). Informed consent was provided by the patients and procedures were approved by the Medical Research and Ethics Committee at the College of Medicine and Medical Sciences, Arabian Gulf University.

Blood collection and miR extraction. Peripheral blood samples (5 ml) were collected from the participants in EDTA-coated tubes (BD Biosciences, Franklin Lakes, NJ, USA). Aliquots of 0.5 ml EDTA-blood were mixed with 1.3 ml RNA later, an RNA stabilizing agent (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The samples were stored at -80˚C until processing. Total RNA, including miRs, was extracted from whole blood using a blood miR kit (Qiagen GmbH, Hilden, Germany), as previously described (11,14,36). RNA quality and concentration were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA purity was determined by measuring the absorbance ratios 260/280 nm and 260/230 nm. All RNA samples were diluted to final identical concentrations of 20 ng/μl.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was reverse transcribed for the target miRs (miR-377 and miR-192) and the housekeeping miR (RNU6B) using a TaqMan microRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). For cDNA synthesis, RNA (20 ng) was mixed with specific stem-loop RT primers (3 μl), 100 mM dNTPs (0.15 μl), 10X RT buffer (1.5 μl), 20 U/μl RNase inhibitor (0.19 μl) and 50 U/ml MultiScribe™ Reverse Transcriptase (1 μl). Nuclease-free water was added to a final volume of 15 μl. The reactions were incubated in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 96-well plate at 16˚C for 30 min, followed by 42˚C for 30 min, 85˚C for 5 min and then 4˚C.

Subsequently, the expression levels of miR-377 and miR-192 were determined via qPCR using RNU6B as a control. qPCR was performed using a TaqMan microRNA assay on a Real-Time

Table I. Baseline characteristics of patients.

Characteristic	T2D	DN		Healthy
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	
No. of subjects	30	15	10	30
Age, years	60.3±12.2	61.2±6.1	67.3±6.0[a,b]	56.4±5.1
Sex, male/female	12/18	11/4	4/6	14/16
BMI, kg/m^2	25.7±5.2	29.1±5.7[a,b]	27.1±5.5[a,b]	24.2±4.6
FG, mmol/l	8.6±13.6[a]	9.5±1.8[a,b]	9.8±1.7[a,b]	4.3±0.6
HbA1c, %	8.7±2.6[a]	9.7±1.1[a,b]	11.2±1.6[a,b]	5.0±0.7
Diabetes duration, years	15.0±4.4	16.2±4.8	18.0±2.9[b]	-
Mean blood pressure, mmHg	87.5±5.3	95.1±11.6[a,b]	105.1±17.4[a,b]	86.9±4.0
Albuminuria, mg/day	6.0±3.5	100.3±41.9[a,b]	325.4±24.3[a,b]	4.9±1.7
ACR, mg/mmol	1.0±0.7	13.9±7.3[a,b]	30.4±8.7[a,b]	0.78±0.3
Serum creatinine, μm/l	68.1±16.0[a]	100.7±38.8[a,b]	141.1±33.0[a,b]	55.4±10.0
eGFR, ml/min/1.73 m^2	97.0±10.6	76.9±6.6[a,b]	55.4±8.3[a,b]	106.3±13.7
LDL, mmol/l	2.4±1.1[a]	3.5±1.6[a,b]	4.2±1.1[a,b]	2.1±0.8
HDL, mmol/l	1.3±0.2	1.2±0.2	1.4±0.3	1.3±0.3
Triglyceride, mmol/l	1.6±0.5	2.0±1.1[a,b]	3.1±1.1[a,b]	1.6±0.6
Total cholesterol, mmol/l	4.6±1.1	4.6±1.3	4.9±0.3[a,b]	4.3±0.6

The values are presented as the mean ± standard deviation, as a number or percentage. [a]P<0.05 vs. healthy group, [b]P<0.05 vs. normoalbuminuric group. FG, fasting glucose; HbA1c, glycated hemoglobin; BMI, body mass index; ACR, albumin/creatinine ratio; eGFR, estimated glomerular filtration rate; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DN, diabetic nephropathy; T2D, type 2 diabetes.

PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.), as previously described (10,11,14,36), with the cycling conditions of 95˚C for 10 min, 95˚C for 15 sec and 60˚C for 60 sec for 40 cycles. The primers used were: miR-377 forward, 5'-ACAAAAGTTGCCTTTGTGTGAT-3' and reverse, GGCTAGTCTCGTGATCGA-3'; miR-192 forward, 5'-CTG ACCTATGAATTGACAGCCA-3' and reverse, 5'-GCTGTC AACGATACGCTACGT-3'; and RNU6B forward, 5'-GCTTCG GCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTC ACGAATTTGCGTGTCAT-3'.

Each sample was run in duplicate, and the quantity of miRs in each sample was calculated as the quantification cycle (Cq) and normalized to that of endogenous control RNU6B. For each sample, the difference in the Cq of the target and the Cq of the reference was calculated as ΔCq, and the fold change of relative expression (ΔΔCq) was determined by calculating the difference in ΔCq of the case and ΔCq of the control. The relative quantification was calculated using the $2^{-\Delta\Delta Cq}$ method as previously described (10,11,14,36). The data were analyzed using Sequence Detection software, version 1.7 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Statistical analysis. Differences in the expression of miRs and other clinical parameters between cases (T2D or DN) and controls were obtained using the Student's t-test. Differences in the expression of miRs and other clinical parameters among the three subject groups were completed using one-way analysis of variance and Tukey post hoc tests. All quantitative data were presented as the mean ± standard deviation.

Multivariate tests were performed using the regression models for association, with unadjusted odds ratios ([a]Model 1 and [b]Model 1) or adjusted odds ratios ([a]Model 2, [b]Model 2) for different variables such as age, sex, BMI, FG, HbA1c, diabetes duration, mean blood presser, LDL, triglyceride and total cholesterol.

Linear regression stepwise analyses were performed to identify independent predictors. Receiver operating characteristic (ROC) analyses were performed and the areas under ROC curves (AUCs) were used as the accuracy indexes for evaluating the diagnostic ability of the miRs. For correlation analyses, Pearson's correlation coefficient was used. All statistical analyses were performed using SPSS software version 21 (IBM Corps., Armonk, NY, USA) and a 2-sided P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics of participants. A total of 85 patients were recruited in this study: 55 patients with T2D with and without DN, and 30 healthy control subjects. The diabetic patients were classified according to ACR into three groups: Normoalbuminuric group (n=30), microalbuminuric group (n=15) and macroalbuminuric group (n=10). The baseline characteristics of participants are presented in Table I.

Among the patients, there were 12 males and 18 females in the normoalbuminuric group, 11 males and 4 females in the microalbuminuric group, and 4 males and 6 females in the macroalbuminuric group. In the control group, 14 were male and 16 were female. The mean age was 60±12 years in the normoalbuminuric group, 61±6.1 in the microalbuminuric group, 67±6.0 in the macroalbuminuric group and 56±5.1

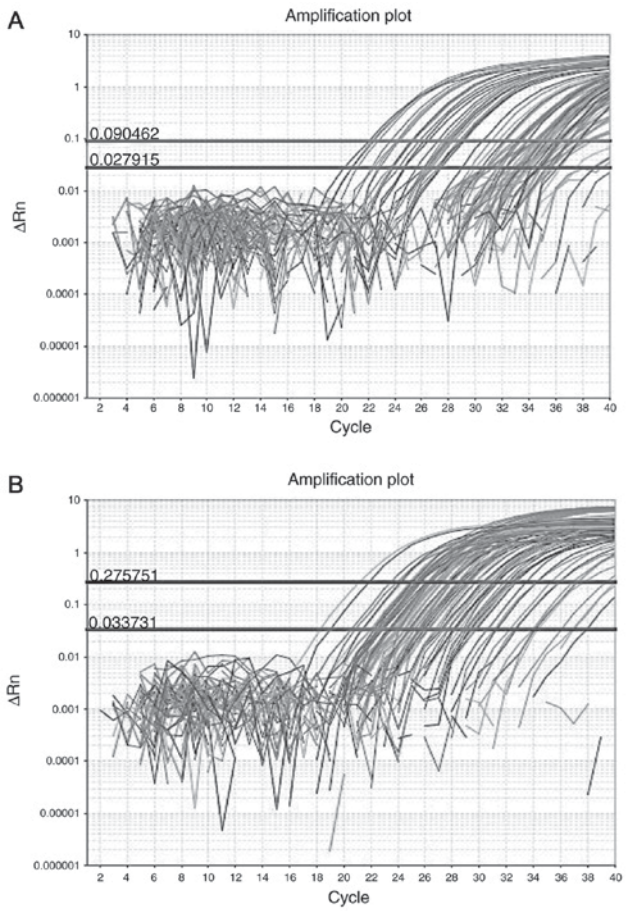

Figure 1. Amplification curves obtained by reverse transcription-quantitative PCR. (A) Amplification curve of miR-377. (B) Amplification curve of miR-192. Each amplification curve represents the quantification cycle of the PCR reaction, which is the level of signal that reflects a significant increase over the baseline signal. PCR, polymerase chain reaction; miR, microRNA; ΔRn, delta Rn value.

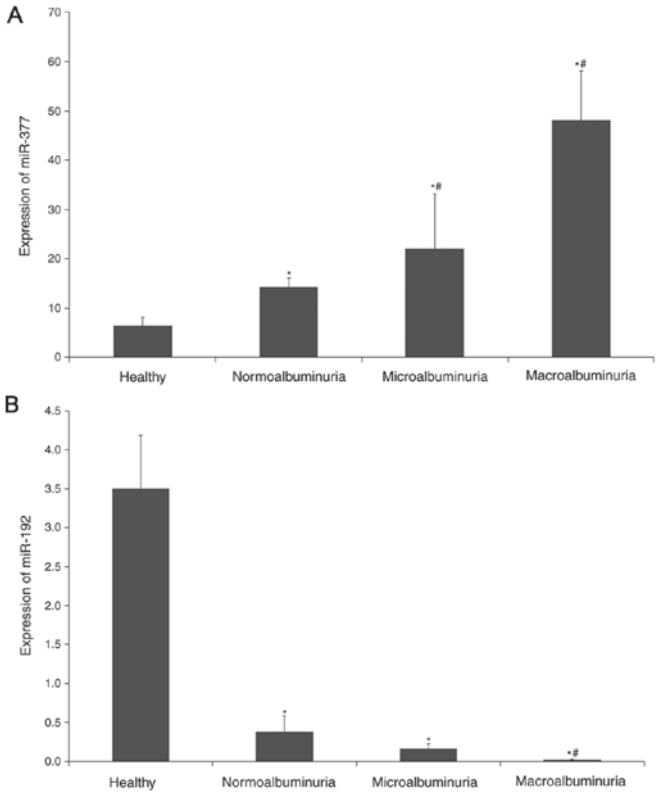

Figure 2. Expression of miR-377 and miR-377 in association with albuminuria. (A) miR-377 and (B) miR-192 expression levels. The relative expression levels of miR-377 and miR-192 were quantified by reverse transcription-quantitative polymerase chain reaction in the peripheral whole blood of patients with type 2 diabetes with normoalbuminuria, microalbuminuria and macroalbuminuria, and in healthy control subjects. Data are presented as the mean fold change ± standard deviation. *P<0.005 vs. healthy and #P<0.005 vs. normoalbuminuria. miR, microRNA.

in the control group. The mean age was thus significantly higher in the diabetic patients compared with the healthy controls (P<0.05). The values of FG and HbA1c differed among the subject groups and were significantly higher in the diabetic patients compared with the healthy controls (P<0.05). The values of BMI, mean blood pressure, ACR, serum creatinine, total cholesterol, LDL and triglyceride were significantly higher in patients with microalbuminuria and macroalbuminuria, compared with patients with normoalbuminuria and controls (P<0.05). There were no significant differences in HDL values among the subject groups (P>0.05). Furthermore, eGFR values were significantly lower in patients with macroalbuminuria compared with patients with microalbuminuria and normoalbuminuria.

Detection of miR-377 and miR-192 in whole blood samples. Whole blood samples were collected from subject groups. miRs were extracted from peripheral blood and converted to cDNA. Validation experiments were performed to evaluate the reliability of miR-377 and miR-192 assays by RT-qPCR. The results demonstrated that miR-377 and miR-195 were successfully amplified in whole blood samples from subject groups with high PCR efficiency. The amplification curves for miR-377 and miR-192, plotted as the increase in fluorescence emissions vs. cycle numbers, are presented in Fig. 1. The amplified RT-qPCR products were analyzed by 1.5% agarose gel electrophoresis in 1X Tris-Borate-EDTA (TBE) buffer containing 89 mM of Tris Base and 89 mM of Boric Acid. Electrophoresis was performed at 100 V for 60 min. Gel was stained with ethidium bromide for 10 min prior to visualization using a UV transilluminator (data are not shown).

Expression of miR-377 and miR-192 and their association with albuminuria. Having successfully validated the reliability of miR-377 and miR-192 assays from whole blood samples, the present study compared their expression levels in the groups of patients with normoalbuminuria, microalbuminuria and macroalbuminuria, and the healthy control subjects by RT-qPCR. The mean expression of miR-377 and miR-192 normalized to the mean expression of RNU6B was calculated to determine the fold change in miR expression using the comparative $2^{-\Delta\Delta Cq}$ method.

Fig. 2 illustrates the expression of miR-377 and miR-192 in the subject groups. The expression of miR-377 was significantly higher by 3.5-fold in overall T2D patients with and without DN compared with the healthy controls (P<0.005) and was progressively increased in the normoalbuminuric group and further increased in the microalbuminuric and macroalbuminuric groups (P<0.005). miR-377 expression was 3.4-fold higher in the macroalbuminuric group and was

Table II. Multivariate logistic regression analysis of miR-377 and miR-192.

	miR-377			miR-192		
	OR	95% CI	P-value	OR	95% CI	P-value
[a]Model 1						
Normoalbuminuria	1.12	1.031-1.215	0.02	0.621	0.386-0.99	0.049
Microalbumiuria/Macroalbuminuria	1.2	1.085-1.289	0.001	0.153	0.03-0.92	0.040
[b]Model 1						
Normoalbuminuria	1.32	1.03-1.244	0.011	0.821	0.63-1.43	0.25
Microalbumiuria/Macroalbuminuria	1.234	1.06-1.44	0.007	0.472	0.09-1.32	0.41
[a]Model 2						
Microalbumiuria/Macroalbuminuria	1.067	1.022-1.115	0.04	0.421	0.109-0.132	0.13
[b]Model 2						
Microalbumiuria/Macroalbuminuria	1.119	0.98-1.217	0.018	0.505	0.123-0.634	0.223

[a]Model 1: The reference category was the healthy control group, unadjusted. [b]Model 1: The reference category was the healthy control group, adjusted for age, sex, BMI, HbA1c, mean blood presser, LDL, triglyceride and total cholesterol. [a]Model 2: The reference category was the normoalbuminuric group, unadjusted. [b]Model 2: The reference category was the normoalbuminuric group, adjusted for age, sex, BMI, fasting glucose, HbA1c, diabetes duration, mean blood pressure, LDL, triglyceride and total cholesterol. miR, microRNA; OR, odds ratio; CI, confidence interval; LDL, low density lipoprotein; BMI, body mass index; HbA1C, glycated hemoglobin.

1.4-fold higher in the microalbuminuric group compared with the normoalbuminuric group (P<0.005).

Conversely, miR-192 expression was decreased by 14-fold in in overall T2D patients with and without DN compared with the healthy control group (P<0.005). miR-192 expression was 2.4-fold lower in the microalbuminuric group compared with the normoalbuminuric group, although it did not reach statistical significance (P>0.05); however, it was significantly lower by 19-fold in the macroalbuminuric group compared with the normoalbuminuric group (P<0.005).

Regression analysis. To further evaluate the association of miR-377 and miR-192 with albuminuria and DN risk, regression analysis was performed. Table II summarizes the results of the multivariate logistic regression analysis for miR-377 and miR-192.

Using the healthy control group as the reference category, miR-377 was directly associated with albuminuria and exhibited an odds ratio (OR) of 1.12 [95% confidence interval (CI), 1.031-1.215; P=0.02] for normoalbuminuria and an OR of 1.2 (95% CI, 1.085-1.289; P=0.001) for microalbuminuria/macroalbuminuria. This association remained statistically significant following multivariable adjustment including age, sex, BMI, HbA1c, mean blood pressure, LDL, triglyceride and total cholesterol (OR, 1.32; 95% CI, 1.03-1.244; P=0.011 for normoalbuminuria; and OR, 1.234; 95% CI, 1.06-1.44; P=0.007 for microalbuminuria/macroalbuminuria).

miR-192 was directly associated with albuminuria and exhibited an OR of 0.621 (95% CI, 0.386-0.99; P=0.049) for normoalbuminuria and an OR of 0.153 (95% CI, 0.03-0.92; P=0.040) for microalbuminuria/macroalbuminuria. However, this association was not significant following adjustment for different parameters (P>0.05).

When the present study further evaluated the associations of these miRs with DN risk using the normoalbuminuric group as the reference category (Table II), multivariate logistic regression analysis revealed that miR-377 was significantly associated with the severity of albuminuria and DN risk (OR, 1.067; 95% CI, 1.022-1.115; P=0.04). In addition, miR-377 was demonstrated to be independently associated with DN risk with an OR of 1.119 (95% CI; 0.98-1.217; P=0.018) adjusted for age, sex, BMI, FG, HbA1c, diabetes duration, mean blood pressure, LDL, triglyceride and total cholesterol. By comparison, miR-192 was not an independent risk factor of DN (OR, 0.421; 95% CI, 0.109-0.132; P=0.13).

In addition, on applying a linear stepwise regression model using miR-377 as a dependent variable, only albuminuria was identified as a significant predictor of miR-377, among a number of other variables included in the analysis, including age, sex, BMI, FG, HbA1c, diabetes duration, mean blood pressure, total cholesterol, triglyceride and LDL (P<0.001).

Evaluation of the biomarker potential of miR-377 and miR-192 for early detection of DN. To further explore whether miR-377 and miR-192 may be used potential diagnostic biomarkers of DN, ROC analyses were performed on the subject groups. The ROC curves were constructed for the two miRs and AUCs were calculated.

The ROC curves for miR-377 yielded an AUC of 0.851 (95% CI, 0.745-0.957; P<0.001) in distinguishing overall diabetic patients from healthy subjects (Fig. 3A), and an AUC of 0.711 (95% CI, 0.565-0.857; P=0.008) in discriminating the normoalbuminuric group from the microalbuminuric/macroalbuminuric groups (Fig. 3B).

For miR-192, the ROC curves yielded an AUC of 0.774 (95% CI, 0.645-0.903; P<0.001) in distinguishing overall diabetic patients from healthy subjects (Fig. 4A), and an AUC of 0.70 (95% CI, 0.542-0.854; P=0.049) in discriminating the normoalbuminuric group from the microalbuminuric/macroalbuminuric groups (Fig. 4B).

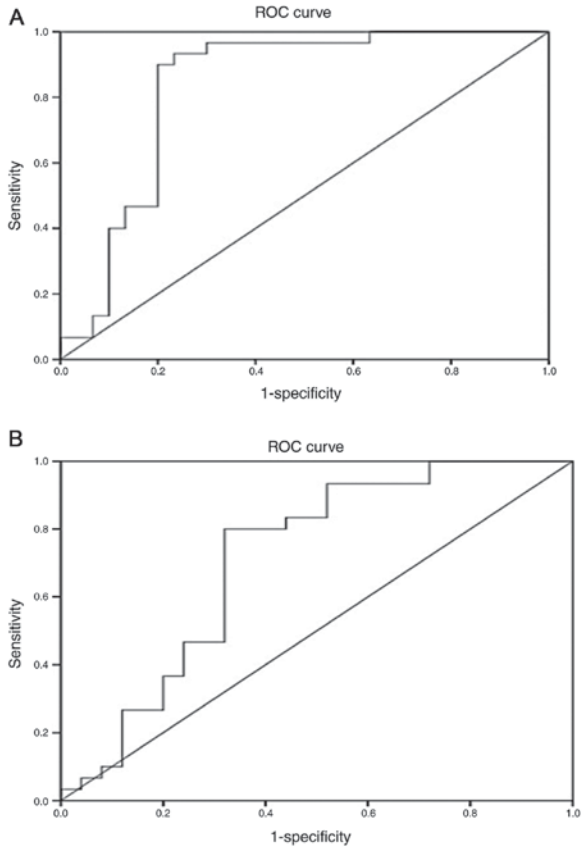

Figure 3. ROC curves of blood miR-375. (A) miR-375 distinguished overall patients from healthy subjects (AUC, 0.851; 95% CI, 0.745-0.957; P<0.001). (B) miR-375 distinguished the normoalbuminuric group from the microalbuminuric/macroalbuminuric groups (AUC, 0.711; 95% CI, 0.565-0.857; P=0.008). ROC, receiver operating characteristic; miR, microRNA; AUC, area under curve; CI, confidence interval.

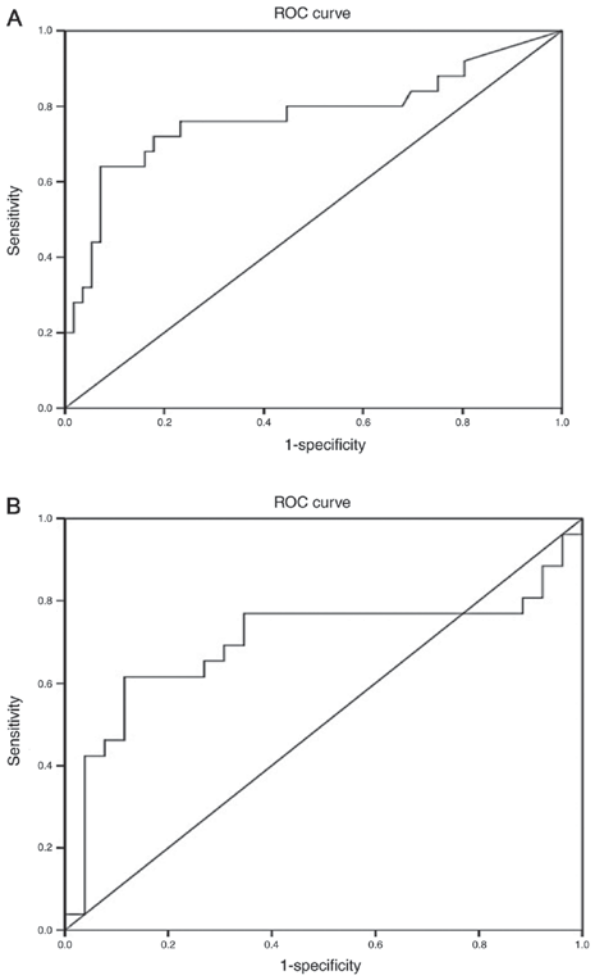

Figure 4. ROC curves of blood miR-192. (A) miR-192 distinguished overall patients from healthy subjects (AUC, 0.774; 95% CI, 0.645-0.903; P<0.001). (B) miR-192 distinguished the normoalbuminuric group from the microalbuminuric/macroalbuminuric groups (AUC, 0.70; 95% CI, 0.542-0.854; P=0.049). ROC, receiver operating characteristic; miR, microRNA; AUC, area under curve; CI, confidence interval.

Correlation between miRs and albuminuria, renal function and other risk factors of DN. To determine the correlation of miR-377 and miR-192 with albuminuria and renal function, Pearson's correlation coefficient analysis was undertaken in patients with microalbuminuria and macroalbuminuria.

As presented in Fig. 5, increased miR-377 expression was positively correlated with albuminuria (r=0.78; P=0.001) and negatively correlated with renal function (r=-0.74; P=0.001).

By comparison, a decreased miR-192 expression exhibited a positive correlation with albuminuria (r=-0.32; P=0.001) and a negative correlation with renal function (r=0.3; P=0.001) (Fig. 6).

In addition, increased miR-377 and decreased miR-192 expression correlated significantly with certain known risk factors of DN, including age, hyperglycemia, hypertension and lipid abnormalities (P<0.05; Table III).

Discussion

Among long-term diabetes-associated microvascular complications, DN accounts for a notable cause of ESRD with a high mortality rate in patients with T2D (15,16). A key pathological feature of DN is mesangial expansion as a result of ECM accumulation and glomerular basement membrane thickening, which consequently lead to increased extracellular depositions in the glomerulus (17). The exact molecular mechanism responsible for these pathological alterations remains uncertain. However, a number of reports have demonstrated that TGF-β1 is a key player and its upregulation promotes pathogenic collagen synthesis and cellular hypertrophy (18,19). The risk of developing DN begins with albuminuria, progressing from microalbuminuria towards macroalbuminuria, which indicates more serious kidney disease, and the progression from macroalbuminuria to renal failure is irreversible (39). Although microalbuminuria is regarded as the early sign of DN, it is more a diagnostic marker than a tool to predict DN, and a number of factors have called into question its ability to precisely detect disease progression. For example, a significant proportion of diabetic patients with microalbuminuria may revert to normoalbuminuria (40) and only ~30% of microalbuminuric patients progress to overt nephropathy subsequent to 10 years of follow up (41). Microalbuminuria is additionally an indicator of risk of cardiovascular disease among individuals with and without diabetes (27), and correlates with endothelial dysfunction in patients with T2D (28). Therefore, there is a requirement for improved biomarkers for early detection of DN, which may enable earlier diagnosis and facilitate more efficient intervention. Previous studies have defined the regulatory role

Table III. Correlation between miRNAs and risk factors of DN.

	miR-377		miR-192	
Parameters	r	P-value	R	P-value
Age	0.270	0.031	-0.439	0.041
FG	0.085	0.001	-0.392	0.001
HbA1c	0.625	0.001	-0.2	0.001
Diabetes duration	-0.103	0.001	0.392	0.001
BMI	-0.064	0.01	0.107	0.03
Mean blood pressure	0.171	0.001	-0.20	0.001
Total cholesterol	0.137	0.001	-0.07	0.001
Triglyceride	0.353	0.001	0.20	0.001
LDL	0.001	0.031	-0.316	0.001

FG, fasting glucose; BMI, body mass index; LDL, low-density lipoprotein; miRNA, microRNA; miR, microRNA; DN, diabetic nephropathy; HbA1c, glycated hemoglobin.

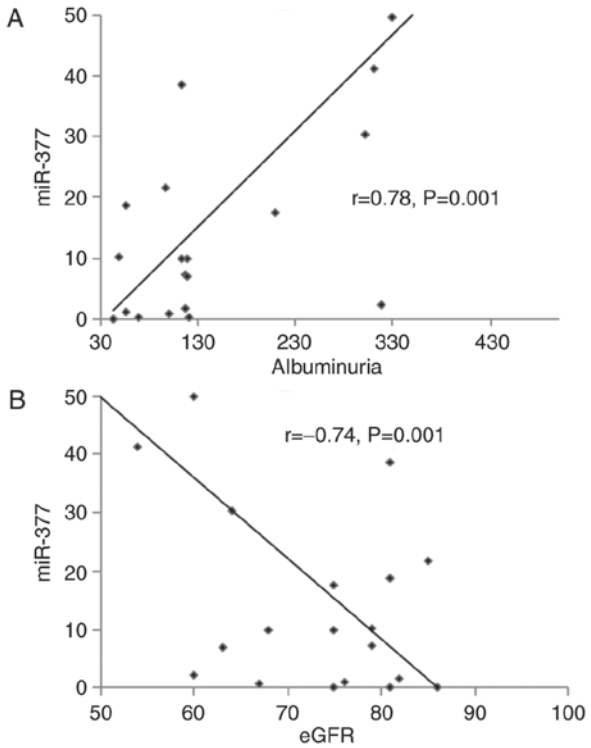

Figure 5. Correlation between miR-377, albuminuria and renal function. (A) Correlation between miR-377 and albuminuria. (B) Correlation between miR-377 and renal function. miR, microRNA; eGFR, estimated glomerular filtration rate.

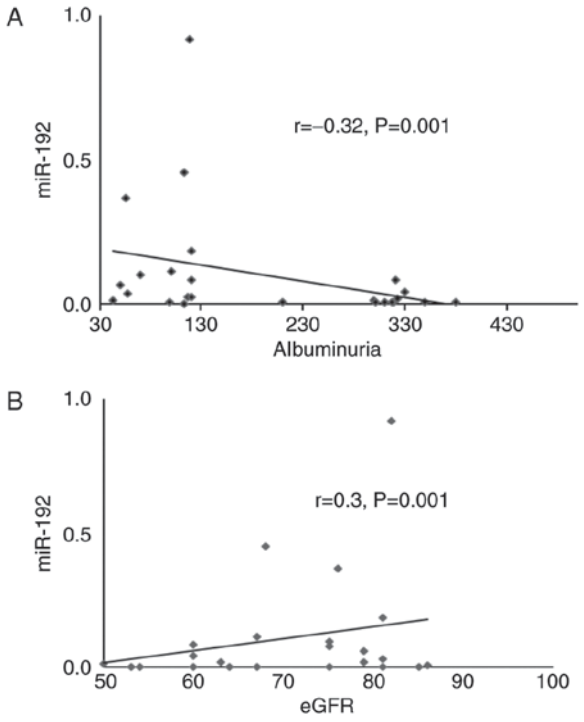

Figure 6. Correlation between miR-192, albuminuria and renal function. (A) Correlation between miR-192 and albuminuria. (B) Correlation between miR-192 and renal function. miR, microRNA; eGFR, estimated glomerular filtration rate.

of miRs in renal development, physiology and pathology (31), and dysregulation of a number of these miRs has been demonstrated to contribute to the pathogenesis and development of DN (31). Findings that miRs are stably present extracellularly in the blood circulation (7) and other body fluids, including the urine and saliva (42), have suggested that circulating miRs act as signaling molecules outside the cell and may be developed as non-invasive biomarkers for a variety of diseases.

Previous results from the authors' laboratory (10,11,14,36) and numerous other reports (9,13,43) have demonstrated that miRs in peripheral whole blood may serve as disease biomarkers.

The present study evaluated the expression of two DN-related miRs (miR-377 and miR-192) in the peripheral blood of patients with T2D with different levels of albuminuria (normoalbuminuria, microalbuminuria and macroalbuminuria) and healthy controls to establish their potential as biomarkers in the early stage of DN.

It was observed that the expression of miR-377 was significantly increased in overall diabetic patients compared with healthy controls. miR-377 expression was gradually increased

in patients with normoalbuminuria and further increased in patients with microalbuminuria and microalbuminuria. This significant increase in miR-377 expression with the severity of albuminuria may suggest a potential role of miR-377 in DN progression.

There have been no previous reports investigating circulating miR-377 in DN, to the best of our knowledge, and the present study may be the first to demonstrate increased blood miR-377 in patients with DN throughout disease progression.

miR-377 is a key regulator and serves a critical role in the pathophysiology of DN. Elevated levels of miR-377 in high glucose culture or TGF-β-treated human and mouse mesangial cells repressed the expression of p21-activated kinase and superoxide dismutase, and enhanced the production of fibronectin protein, a matrix protein that excessively accumulates in DN (32). Thus, miR-377 was suggested to be a target for therapy (32).

The present study additionally revealed that the expression of miR-192 was significantly lower in overall diabetic patients compared with healthy controls, and was significantly lower in patients with microalbuminuria compared with patients with normoalbuminuria. However, the decrease in miR-192 expression was not significant between microalbuminuria and normoalbuminuria. The results of decreased miR-192 expression in patients with macroalbuminuria and microalbuminuria compared with patients with normoalbuminuria observed in the present study are consistent with a recent report by Ma *et al* (44) conducted on a large cohort, which demonstrated that miR-192 was significantly decreased in patients with macroalbuminuria (n=148) compared with patients with normoalbuminuria (n=159). However, the results of the present study differ from a previous study by Chien *et al* (45) who reported increased expression of miR-192 in overt proteinuria patients compared with microalbuminuria patients.

miR-192 was initially identified to be enriched in the normal renal cortex (46) and serves an important role in normal kidney function (47). miR-192 regulates TGF-β-induced COL1A2 expression by targeting and downregulating E-box repressors ZEB1 and ZEB2; thus, it is involved in matrix accumulation in DN (33). Low expression of miR-192 was observed in renal tubular cells cultured in a high glucose concentration and TGF-β1, and in renal biopsy samples from patients with established DN in association with tubulointerstitial fibrosis and a reduction in eGFR (48). TGF-β1 additionally decreases the expression of miR-192 in rat proximal tubular cells, mesangial cells and human podocytes (49). In contrast to these observations, Putta *et al* (50) demonstrated that miR-192 levels were increased by TGF-β1 in cultured glomerular mesangial cells and in glomeruli from diabetic mice, and that decreased renal miR-192 resulted in a reduction in renal fibrosis and improves proteinuria.

A number of factors have been demonstrated to have an important impact on the development of DN, including age (>45 years), long duration of diabetes mellitus, poor glycemic control (high HbA1c), hypertension and hyperlipidemia (51,52).

The present study evaluated the association of miR-377 and miR-192 with albuminuria and DN risk using different models of regression analysis. A direct, significant association between increased miR-377 or decreased miR-192 and albuminuria was observed. Of note, miR-377 expression was significantly associated with the progression of albuminuria, supporting the authors earlier suggestion of the association between miR-377 DN progression. In addition, the association of miR-377 with the degree of albuminuria remained significantly independent of known risk factors of DN, including age, sex, BMI, blood pressure, total cholesterol, triglyceride and LDL. In addition, in linear stepwise regression analysis, the present study demonstrated that albuminuria was the only significant predictor of miR-377 among other variables, including age, sex, BMI, FG, HbA1c, diabetes duration, mean blood pressure, total cholesterol, triglyceride and LDL.

When the present study evaluated the possibility of using blood miR-377 and blood miR-192 as biomarkers for DN, ROC analysis revealed that the two miRs were significantly able to discriminate overall patients from healthy subjects. The two miRs exhibited a significant ability to discriminate patients with normoalbuminuric from patients with microalbuminuria/macroalbuminuria. The present study may be the first to demonstrate the potential of blood-based miR-377 and miR-192 biomarkers for early detection of DN. Conversely, previous studies of miR biomarkers of renal disease revealed that miR-192 in urine extracellular vesicles is a useful biomarker for the early stage of DN (53).

In the present study, Pearson's correlation coefficient analysis undertaken on patients with microalbuminuria and macroalbuminuria demonstrated that increased miR-377 was positively correlated with albuminuria and negatively with renal function, while decreased miR-192 was negatively correlated with albuminuria and positively with renal function. Furthermore, miR-377 and miR-192 were significantly correlated with age, hyperglycemia, diabetes duration, hypertension and lipid abnormalities. These results may indicate that higher expression of miR-377 and lower expression of miR-192 in the blood may be used as useful biomarkers to evaluate renal damage and the risk of DN.

Although the results of the present study demonstrated that miR-377 and miR-192 in peripheral blood may be utilized as biomarkers for early detection of DN, it should be noted that the evaluation of the biomarker potential of these miRs was conducted in a relatively small sample size, and larger studies are recommended for validation of the present results.

In summary, increased miR-377 expression and decreased miR-192 expression was observed in the blood of patients with T2D with and without DN compared with healthy controls, and blood miR-377 expression was increased with the severity of albuminuria. miR-377 and miR-192 were directly associated with albuminuria, miR-377 was independently associated with DN risk, and albuminuria was observed to be the only predictor of miR-377. The two miRs were correlated with albuminuria, renal function and other risk factors of DN. It was additionally revealed that miR-377 and miR-192 may be utilized as blood-based biomarkers for early DN prediction in patients with T2D. The present study may provide the first evidence of the association between miR-377 and the risk of DN, and its potential usefulness as a biomarker in the early stage of DN.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author, on reasonable request.

Authors' contributions

GAK: Project development, data management, data analysis, manuscript writing, manuscript editing. HAAM: Project development, data collection, data analysis, manuscript writing.

Ethics approval and consent to participate

Ethical approval to conduct the present study was obtained from the Medical Research and Ethics Committee of the College of Medicine and Medical Sciences, Arabian Gulf University (Manama, Bahrain). All participants provided informed consent for the use of their blood samples and data.

Consent for publication

All participants provided informed consent for the use of their blood samples and data.

Competing interests

The authors declare that they have no competing interests.

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