Original Article

MicroRNA-21 as an Early Marker of Nephropathy in Patients with Type 1 Diabetes

Abstract

Introduction: Diabetic nephropathy (DN) is the most common cause of chronic kidney disease worldwide. A major challenge is to identify early diabetic nephropathy. microRNAs (miRNAs) are short noncoding RNA sequences and regulate a wide range of biological processes as cell differentiation, proliferation, cell metabolism and apoptosis. miRNAs may have a role in molecular mechanisms linked to cellular pathways of DN. The aim of this study was to investigate the level of microRNA-21 as a potential marker of early nephropathy in type 1 diabetes mellitus (T1DM). **Methods:** A total number of 340 participants were included and classified into 3 groups; Group I included 100 healthy participants, Group II included 120 patients with T1DM with <5 years duration, and Group III included 120 patients with T1DM with >5 years duration. All participants were submitted to detail clinical examination, laboratory investigations, urinary albumin/creatinine ratio (ACR), estimated glomerular filtration rate (eGFR) as well as plasma microRNA-21 assays. **Results:** Blood pressure and ACR were significantly higher in group III than groups I and II. Further, microRNA-21 was significantly higher in group III than groups I and II, and more in group II than group I. microRNA-21 starts to rise in group II before microalbuminuria. miRNA-21 at a level of 0.01 had a greater sensitivity 94.1% and specificity 100% for identifying DN than ACR at level 45 mg/gm with sensitivity 88.2% and specificity 89%. **Conclusion:** Plasma microRNA-21 can serve as an early marker for diagnosis and identifying diabetic nephropathy in T1DM.

Keywords: *Diabetic nephropathy, microalbuminuria, microRNA-21, type 1 diabetes mellitus*

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Introduction

Diabetes mellitus, a global public health issue, results from insufficient production of the insulin (T1DM) or from ineffective insulin action (T2DM). T1DM can be classified as immune-mediated or idiopathic and majority of T1DM is of immune-mediated.[1] Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD), which has a major impact on the morbidity and has high mortality rate[2] Urinary albumin excretion (UAE) increased and called microalbuminuria (MA), when the UAE rate reached 30-300 mg/24hr, while macroalbuminuria when the UAE rate exceeds 300 mg/24 hr.[3] Multicenter studies have shown that approximately 20-30% of T1DM individuals have MA after an average of 5-10 years duration of diabetes.[4]

Several pathologic alterations are implicated in pathogenesis of DN, including increased accumulation and deposition of extracellular matrix, which results in expansion of the mesangial matrix, thickening of the glomerular basement membrane (GBM) and tubulointerstitial fibrosis. Hyperglycemia was suggested as the main pathogenic factor that triggers and sustains the activity of several molecular signaling pathways, such as those involving increased oxidative stress, high pro-inflammatory cytokine production, up regulation of renal transforming growth factor beta-1 (TGF-β1) expression, and activation of local fibroblasts and the renin-angiotensin-aldosterone system (RAAS).[5]

MicroRNAs (miRNAs) are a large family of short noncoding RNA sequences, approximately 20-22 nucleotides, which were synthesized in the cell nucleus through a complex multi-step biosynthetic process, starting from RNA polymerase II[6] and modulate gene expression of specific mRNA targets and regulate a wide range of biological cell processes, including cell growth and proliferation, differentiation, organogenesis, metabolism, stress response,

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and tissue remodeling.[7] It has been estimated that the human genome contains more than 2500 mature miRNAs[6] and they could regulate 74-92% of all protein-encoding mRNAs in a tissue- and/or cell-specific manner.[8]

MicroRNAs are detectable in plasma, urine, cerebrospinal and other extracellular fluids.[9] The presence of circulating endogenous miRNAs may be identified, measured and used as potential disease biomarkers.[10] There is evidence that the levels of several miRNAs were found deregulated in plasma and urine from patients with T1DM compared to age matched controls.[11] miRNA-21 is known to induce fibrosis in many organs, including the heart[12] and kidney[13] and promotes TGF-β mediated effects on endothelial dysfunction,[14] therefore, miRNA-21 levels in plasma and urine of young T1DM patients might serve as a potential noninvasive biomarker to identify patients with a high risk for ongoing endothelial dysfunction and therefore future kidney and cardiovascular injuries.[15]

Microalbuminuria is considered to be a standard marker of risk factor for DN and progressive renal insufficiency.[16] However, MA may not be as sensitive and specific a predictor of the DN.[17] There is evidence that miRNAs may have a role in molecular mechanisms linked to cellular pathways of DN.[11] So we conducted this study to investigate microRNA-21 as a potential marker of early nephropathy in T1DM.

Methods

Study design and population

A case-control study was carried out among patients with T1DM attending to diabetes and internal medicine clinics of Zagazig University hospitals from June 2017 to October 2018. The study was approved by the Institutional Ethics Committee and conformed to the Helsinki Declaration. The aim of the study was explained to patients, and informed consent was obtained. Patients were studied in three groups; Group I included healthy participants, Group II included patients with T1DM with <5 years duration, and Group III included patients with T1DM with >5 years duration with overt proteinuria (>300mg/day). Patients were excluded from the study if they suffered from other chronic systemic inflammatory diseases, autoimmune diseases, malignancy or concurrent use of corticosteroids. Type 2 diabetic patients were also excluded.

Demographic information was collected. The total number of participants was 340. Among them, 100 were Group I with comparable age and sex to other participants. Of the remaining 240 participants, Group II had 120 patients, and Group III 120 patients. Fundus examination was performed to confirm diabetic retinopathy in participants with proteinuria to confirm the diagnosis of DN.

All participants in this study were subjected to detail clinical examination. Blood pressure was measured with a mercury sphygmomanometer on the right arm with the patient in a sitting position after a rest of 5 minutes. Hypertension was defined as a systolic blood pressure >130 mmHg and/or a diastolic blood pressure >85 mmHg and/or the current use of antihypertensive medication. Fundus examination was done for diagnosis of diabetic retinopathy.

Venous blood samples were drawn in the morning after an overnight fast. All participants were subjected to routine investigations, including; serum creatinine (S. Cr), C-reactive protein (CRP) and HbA1C. All participants were instructed how to obtain a fresh, clean first morning urine specimen to exclude orthostatic proteinuria. Urine samples examined for urinary ACR. Early nephropathy defined urine ACR ≥30 mg/g, and overt nephropathy was defined as urinary ACR >300 mg/g. Albuminuria was measured by immune-turbidimetry. All positive cases were reexamined after three months to confirm diagnosis of chronicity. GFR was estimated by CKD-EPI equation as follows: CKD-EPI formula = 141 × min (Scr/κ, 1) × max (Scr/κ, 1) -1.209 × 0.993Age × 1.018 [if female] × 1. 159 [if black].[18]

Measurement of microRNA-21

Peripheral blood was collected in EDTA tubes and plasma supernatant was then aliquoted into RNase-free tubes. Total RNA was isolated using the Qiagen's miRNeasy Serum/ Plasma kit (Qiagen, Valencia, CA). The aqueous phase containing the RNA was then transferred to a new collection tube, combined with 1.5 volumes of 100% ethanol, applied to the silica membrane of a miRNeasy MiniElute Spin column (Qiagen), and centrifuged at 10,000g for 15 s at room temperature. The retained RNA was then washed using buffers provided with the miRNeasy Serum/Plasma kit.

miRNA profiling

cDNA was synthesized from 1ug of purified RNA using the mirVana qRT-PCR miRNA detection kit according to the manufacturer's instructions. qRT-PCR was performed using Stratagene, MX3000P quantitative PCR System *(Agilent technologies)* and analyzed using MxPro QPCR Software *(Agilent technologies).* PCR cycling conditions were as follows: 94°C for 10 minutes followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The primers used for detection of premature miR-21 are as follows: rat: forward primer, 5`-TGTCGGGTAGCTTATCAGAC-3`; reverse primer, 5`-TTCAGACAGCCCATCGACTG-3`.

For detection of mature miRNAs, mirVana qRT-PCR primer sets for hsa-mir-21 (Ambion) were used according to the manufacturer's protocol. mirVana qRTPCR primer sets for U6 (Ambion) were used for normalization. Data analyses were done by the comparative Ct method.[19]

Statistical analysis

Results were expressed as mean ± standard deviation (SD), analysis of variance by ANOVA and *post hoc* analysis

Table 1: Demographic data and characteristics of the study groups

Group / Variable	Control *n*=100	Diabetic<5 years *n*=120	Diabetic >5 years *n*=120	*P*
Age (years)	39.46±3.66	37.9±7.52	42.5±6.46	NS
Gender				
Male%	48	58	56	
Female%	52	62	64	NS
Onset of diabetes (years)	-	3.4±0.99	8.8±6.37	<0.001
Systolic BP (mm Hg)	117.8±11.5	118.4±9.1	131.7±10.5[ab]	<0.001
Diastolic BP (mm Hg)	74.5±6.9	74.1±6.1	85.1±7.9[ab]	<0.001
CRP (mg/L)	2.9±2.5	5.1±1.1[a]	6.3±1.3[ab]	<0.001
HbA1C (%)	4.7±0.3	7.9±0.7[a]	8.8±0.8[ab]	<0.001
eGFR (ml/min/m^2)	111.5±6.5	112.8±8.2	95.6±7.1[ab]	<0.001
ACR (mg/gm)	11.5±2.3	15.4±4.6	178.6±58.6[ab]	<0.001
microRNA-21	0.0±00002	0.0016±0.0010[a]	0.0110±0.0002[ab]	<0.001

[a]A significant difference as compared to control group, NS: Non significant, $P<0.05$ is significant. [ab]A significant difference as compared to control group and diabetic <5 years, CRP: C-reactive protein, HbA1C: Glycated hemoglobin, eGFR: Estimated glomerular filtration rate, ACR: Albumin creatinine ratio

with LSD tests were applied for comparing differences among groups. Qualitative data were expressed in the form of numbers and percentages and comparison between data was performed by using the Chi-square test. The correlation between variables was calculated using the Pearson's and the Spearman correlation tests. Predictive values were assessed by the area under the curve/the receiver operator characteristic curve (AUC/ROC). The AUC/ROC was used to determine the discriminatory ability of microalbuminuria and miRNA 21 in detecting DN. The criterion for statistical significance was set at $P < 0.05$. All calculations were carried out using a standard statistical package (SPSS version 19, Inc., Chicago, USA).

Results

The existent studied number was 340 participants, their mean age was 39.7 ± 6.8, with males 160 (47%). Characteristics of the patients and the mean values of all parameters are outlined in Table 1. The age and gender did not differ between the 3 groups. Both systolic and diastolic blood pressures were significantly higher in group III than groups I, II, while HbA1C and CRP were significantly higher in group III than groups I, II and in group II than group I. ACR were significantly higher in group III than groups I, II. Lastly microRNA-21 was significantly higher, 8 folds in group II than group I and in group III than group II.

Correlates of microRNA-21 and other parameters

Age, duration of diabetes, blood pressure (BP), HbA1c and ACR were positively correlated with microRNA-21, while age of patients at diagnosis of diabetes and eGFR negatively correlated [Table 2]. Plasma miRNA-21 at a level of 0.01 had a greater sensitivity (94.1%) and specificity (100%) for identifying DN than ACR at level 45 mg/gm. sensitivity 88.2% and specificity 89% [Table 3].

Table 2: Correlates of microRNA-21 and other parameters

microRNA-21	*r*	*P*
Age	0.678	<0.001
Age of diagnosis	-0.565	<0.001
Duration of diabetes	0.897	<0.001
Systolic BP	0.565	<0.001
Diastolic BP	0.373	0.03
HBA1C	0.680	<0.001
CRP	0.178	NS
eGFR	−0.733	<0.001
ACR	0.935	<0.001

CRP: C-reactive protein, HbA1C: Glycated hemoglobin, eGFR: Estimated glomerular filtration rate, ACR: Albumin creatinine ratio. $P<0.05$ is significant. NS: Non significant

Discussion

Diabetic nephropathy is characterized by renal cell hypertrophy, GBM thickening, accumulation of extracellular matrix proteins and mesangial cell expansion, resulting in renal fibrosis. Early detection of DN has a pivotal role in the prevention of ESRD.[20] There is evidence that the microRNA21 was found deregulated in plasma and urine from patients with T1DM compared to matched controls.[21] miRNA-21 is known to induce fibrosis in many organs, including kidney[13] and promotes transforming growth factor-β-mediated effects on endothelial dysfunction,[14] therefore, miRNA-21 levels in plasma and urine of T1DM patients might serve as a potential non-invasive biomarker to identify patients with a high risk for ongoing endothelial dysfunction or future kidney injuries. The current study was conducted to investigate the level of miRNA-21 as a potential marker of early nephropathy in T1DM.

According to the study design, the 3 studied groups were compatible regarding demographic data. As generally

Table 3: Validity of ACR and microRNA21 in prediction of DN

Variables	Cutoff	AUC	*P*	PPV	NPV	Sensitivity	Specificity	Accuracy
ACR	45	1.0	0.001	78.9%	96.9%	88.2%	89%	88.2%
miRNA-21	0.01	1.0	0.001	100%	97.1%	94.1%	100%	98%

ACR: Albumin creatinine ratio, PPV: Positive predictive value, NPV: Negative predictive value. P <0.05 is significant

expected, microalbuminuria usually start to appear after 5 years of diabetes duration and then progressively increased afterward, which appear compatible with many previous reports.[3,22] Arterial hypertension is a main risk factor in the development and progression of diabetic nephropathy.[23] In additional individuals with T1DM, blood pressure levels are usually normal at diagnosis, and the onset of hypertension is closely correlated with the onset of DN.[24] In the current study, we found, all normoalbuminic patients were normotensive and the onset of microalbuminuria started with rising blood pressure ≥130/80 mmHg, which appears compatible with previous studies.

Similarly, hyperglycemia is a contributing factor in diabetic nephropathy as HbA1C significantly higher in microalbuminuria group than normoalbuminuria group. Hyperglycemia induces renal damage directly or through hemodynamic mechanisms. It induces activation of protein kinase C, increases production of advanced glycosylation end products, stimulates intrinsic glomerular cells to produce TGF-β1, which contributes to glomerular sclerosis and tubulointerstitial damage.[25-27] There is evidence that microRNAs is modulated by hypoxia and high glucose in endothelial cells cultured *in vitro*.[28] It is not surprising that upregulation of this microRNA-21 leads to mitochondrial dysfunction and oxidative stress and these data may provide novel clues about the inhibition of microRNA-21 as a new therapeutic approach to protect against cellular oxidative injury in glucose variability and diabetes[29] In addition, the positive correlation of microRNA-21 level with CR*P* values may suggest already ongoing inflammatory events in the kidney of T1DM patients; these results are generally compatible with other studies.[11]

In the current study, we demonstrated that in contrast to healthy subjects, microRNA-21 level was significantly up-regulated in the plasma of the T1DM patients and start to rise early within the first 5 years from the onset of diabetes even before the appearance of microalbuminuria then rises with progression of DN and decline of GFR. In addition, we observed that microRNA-21 at level ≥0.01 can predict microalbuminuria. Such results allow us to postulate that T1DM not only induced overexpression of microRNA-21, but also microRNA-21 may be an early marker of DN with a good index of severity. These results are compatible with other studies, which showed that circulating microRNA-21 level was significantly up-regulated in the plasma and urine of the T1DM patients.[11]

There is a concept that microalbuminuria is a marker of endothelial dysfunction.[30] MicroRNA-21 may be involved in pathogenesis of endothelial dysfunction and endothelial-to-mesenchymal transition,[14] this may explain the current results of rising microRNA-21 before onset of microalbuminuria in T1DM patients <5 years as microRNA-21 involved in pathogenesis of microalbuminuria, so unregulated earlier before the appearance of microalbuminuria. Moreover, we found plasma microRNA-21 level link with a procession of nephropathy and progression of CKD in T1DM, this finding can be explained by reports of other studies, which found that microRNA-21 was involved in pathogenesis of DN by down regulating target smad7 that led to deposition of collagen-IV and collagen-I resulting in glomerular basement membrane (GBM) thickened and mesangial matrix hyperplasia also miR-21 is known to induce fibrosis in kidney[13] through modulation of TGF-β1 signaling.[14] Targeting microRNA-21 may be beneficial for early detection and prognostic biomarker of nephropathy and progression of CKD in T1DM patients.

Conclusion

Plasma microRNA-21 can serve as an early marker for diagnosis of DN even before appearance of microalbuminuria and has greater sensitivity and specificity for identifying DN than ACR. Also, plasma microRNA-21 level links with the progression of nephropathy in T1DM.

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Conflicts of interest

There are no conflicts of interest.

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