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Plasma microRNA-192 expression as a potential biomarker of diabetic kidney disease in patients with type 2 diabetes mellitus

ABSTRACT

Background. Albuminuria is an early clinical indicator of diabetic kidney disease (DKD). However, it has several limitations. The aim of this study was to evaluate the plasma microRNA-192 (miRNA-192) expression and its diagnostic performance in patients with type 2 diabetes mellitus (T2DM) and DKD.

Methods. In this case-control study, 75 subjects were included into 3 groups: group (1): 20 patients with T2DM and UACR (urinary albumin creatinine ratio) < 30 mg/gm, group (2): 30 patients with T2DM and ACR ≥ 30 mg/gm, and group (3): 25 healthy controls. Patients were recruited from the outpatient clinic of the Diabetes unit at our institution. Real-Time Quantitative Reverse Transcription PCR was used to assess plasma miRNA-192 expression.

Results. Plasma miRNA-192 was significantly higher in T2DM patients with DKD compared to those with normal UAE. Additionally, in patients with T2DM, plasma miRNA-192 was positively correlated with UACR. The ROC curve analysis for miRNA-192 plasma expression in patients with T2DM, revealed that miRNA-192 had a good diagnostic performance (AUC = 0.778) to define T2DM patients with DKD.

Conclusion. Plasma expression of miRNA-192 was able to discriminate increased UAE among patients with T2DM; suggesting a promising role for miRNA-192 as a potential biomarker for DKD. (Clin Diabetol 2020; 9; 6: 454–460)

Key words: type 2 diabetes, diabetic kidney disease, albuminuria, microRNA, microRNA-192

Introduction

Diabetes mellitus (DM) is an expanding universal health problem; according to the International Diabetes Federation (IDF), the prevalence of DM worldwide is 8.3% expected to reach 9.8% by 2045 [1]. This continuously growing prevalence, is mainly attributed to the increase in type 2 diabetes mellitus (T2DM), the most common type of DM representing 90% of cases [2]. In Egypt, the prevalence of T2DM is around 15.6% among adults; thus Egypt is ranked the ninth country worldwide regarding the number of patients with T2DM [1].

Diabetic kidney disease (DKD) is not only the most frequent microvascular complication of DM, but also, it is the leading cause of end-stage renal disease (ESRD), accounting for 50% of cases [2].

Despite being an early clinical indicator of DKD, albuminuria, detected by urinary albumin creatinine ratio (UACR), has some limitations [3]. Diabetic patients may present with impaired renal function without significant increases in albuminuria [4]. Moreover, albuminuria is not a perfect prognostic indicator for DKD progression, as the degree of albuminuria does not closely correlate with the decrease in glomerular

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filtration rate (GFR) [5]. In addition, some structural alterations associated with DKD may precede albuminuria [6, 7]. Furthermore, 30% only of patients with moderately increased albuminuria (30–300 mg/gm) progress to overt nephropathy [8]. Accordingly, there is a call for identifying a biomarker which efficiently allows early diagnosis for more effective therapeutic interventions, and acts as a reasonable prognostic indicator for disease progression.

MicroRNAs (miRNAs) are highly conserved noncoding RNAs, consisting of 18–24 nucleotides and exerting their role in controlling human gene expression through post-transcriptional gene regulation or silencing [9]. Circulating miRNAs are characterized by high stability [10], reproducibility [11] and available detection by sensitive and specific quantitative real-time polymerase chain reaction (qRT-PCR) [12], therefore they are appealing biomarkers for a variety of diseases.

MicroRNA-192 is one of the most commonly expressed miRNAs in the renal cortex [13]. Several studies have reported an important role for miRNA-192 in the fibrogenesis process in DKD induced by transforming growth factor-β1 (TGF-β1). However, the results of these studies are conflicting. Owing to these contradicting reports about the role of miRNA-192 in identifying DKD, together with the need for an efficient diagnostic marker; we were directed to carry out the present study.

Subjects

In this case-control study 75 subjects were included and divided into 3 groups: group (1): 20 patients with T2DM without DKD, group (2): 30 patients with T2DM and DKD, and group (3): 25 healthy subjects of matched age and sex as a control group. T2DM and DKD were defined according to the diagnostic criteria of American Diabetes Association (ADA) [14, 15].

Patients were recruited from the outpatient clinic of the Diabetes and metabolism unit at Alexandria Main University hospital, in the period between December 2018 and August 2019. Patients with acute illness at time of the study, hepatic disease, cardiovascular disease, hematological disorders, malignancy, systemic chronic inflammation, history of hemodialysis or renal transplantation and patients using nephrotoxic drugs or corticosteroids were excluded.

An informed consent was obtained from each patient after explaining the nature and the aim of the study. The current study was done according to the Ethical Principles for Medical Research Involving Human Subjects defined in the Helsinki Declaration in 1975 (revised in 2008). The approval of the ethics committee of Faculty of Medicine, Alexandria University was obtained in 2018.

Methods

Laboratory investigations

Fasting plasma glucose (FPG), fasting insulin, glycated haemoglobin (HbA_{1c}) and UACR were determined by commercial enzymatic methods. Insulin resistance was calculated on the basis of the homeostasis model assessment of insulin resistance (HOMA-IR), using the following formula: [HOMA-IR = (fasting insulin in uIU/L $\times$ fasting glucose in mg/dL)/405] [16].

Molecular analysis: Relative quantification of miRNA-192 expression using Real-Time qRT-PCR [17] was done through 3 steps:

I. Total RNA extraction: Purification of cell-free total RNA from plasma, which includes small RNAs as miRNAs, was done using the miRNeasy Serum/Plasma Kit (Qiagen, Germany). Exogenous oligonucleotide (cel-miR-39) was added in order to monitor miRNA analysis (RNA extraction and reverse-transcription real time PCR).
II. Real-time qRT-PCR, in 2 steps:
    1. Reverse transcription (RT): complementary DNA was synthesized from purified RNA samples using the miScript II RT Kit (Qiagen, Germany) according to the manufacturer's protocol.
    2. Real-time PCR quantification of mature miRNA-192: using target-specific miScript Primer Assays (forward primers) (Qiagen, Germany) and the miScript SYBR Green PCR Kit (Qiagen, Germany), which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix.
III. Calculation relative quantification of miRNA-192 was determined using comparative CT method ($2^{-\Delta\Delta CT}$) normalized to RNU6B as an endogenous control.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Chi-square test for categorical variables, to compare between different groups. Mann-Whitney test for abnormally distributed quantitative variables, to compare between two studied groups. F-test (ANOVA) for normally distributed quantitative variables, to compare between more than two groups, and post hoc test (Tukey) for pairwise comparisons. Kruskal Wallis test for abnormally distributed quantitative variables, to compare between more than two studied groups, and post hoc (Dunn's multiple com-

Table 1. Comparison between the studied groups according to demographic data

	uACR [mg/gm]		Control (n = 25)	Test of sig.	P
	< 30 (n = 20)	≥ 30 (n = 30)			
Sex					
Male	11 (55%)	16 (53.3%)	12 (48%)	$\chi^2 = 0.254$	0.881
Female	9 (45%)	14 (46.7%)	13 (52%)		
Age (years)	48.75 ± 2.94	47.43 ± 3.24	46.48 ± 3.93	F = 2.458	0.093
Diabetes duration (years)	5.50 (1–12)	7.0 (1–16)	–	U = 237.0	0.211

χ2 — Chi square test; F — F for ANOVA; test U — Mann Whitney test; P — P value for comparing between the studied groups
Qualitative data were described using number and percentage
Normally Quantitative data was expressed using Mean ± SD
Abnormally Quantitative data was expressed using Median (Min–Max)

Table 2. Comparison between the study groups according to the studied parameters

	uACR [mg/gm]				
	< 30 (n = 20)	≥ 30 (n = 30)	Control (n = 25)	Test of sig.	P
FPG [mg/dL]	156 (72–263)	210.50 (105–325)	87 (75–99)	H = 46.43*	< 0.001*
		$P_1 = 0.039$*, $P_2 < 0.001$*,$P_3 < 0.001$*			
F insulin [uIU/mL]	14.7 (2.3–23.4)	17.8 (7.27–42)	7.90 (1.20–41.50)	H = 11.025*	0.004*
		$P_1 = 0.122$, $P_2 = 0.132$, $P_3 = 0.001$*			
HOMA-IR	4.14 (1.39–12.96)	9.91 (3.46–22.6)	1.70 (0.20–9.80)	H = 34.14*	< 0.001*
		$P_1 = 0.008$*, $P_2 = 0.007$*, $P_3 < 0.001$*			
HbA_{1c} (%)	9.98 ± 2.30	9.56 ± 2.41	5.29 ± 0.14	F = 44.004*	< 0.001*
		$P_1 = 0.452$, $P_2 < 0.001$*,$P_3 < 0.001$*			
MicroRNA 192	2.08 (1.0–2.99)	3.12 (1.36–4.80)	0.82 (0.23–1.05)	H = 53.74*	< 0.001*
		P1 = 0.025*, P2 < 0.001*, P3 < 0.001*			

F — F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey); H — H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Dunn's for multiple comparisons test); P — P value for comparing between the studied groups; P_1 — P value for comparing between < 30 and ≥ 30; P_2 — P value for comparing between < 30 and control; P_3 — P value for comparing between ≥ 30 and control; *Statistically significant at P ≤ 0.05
Normally Quantitative data was expressed using Mean ± SD
Abnormally Quantitative data was expressed using Median (Min–Max)

parisons test) for pairwise comparisons. Spearman coefficient to correlate between two distributed abnormally quantitative variables. Receiver operating characteristic curve (ROC) It is generated by plotting sensitivity (TP) on Y axis versus 1-specificity (FP) on X axis at different cut off values. The area under the ROC curve (AUC) denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test. The ROC curve allows also a comparison of performance between two tests. Significance of the obtained results was judged at the 5% level.

Results

The 3 study groups were age- and sex-matched and there was no statistically significant difference between the 2 groups of diabetic patients regarding diabetes duration (Table 1).

The FPG and HOMA-IR were significantly lower in T2DM patients with normal UAE compared to those with increased UAE (P = 0.039 and P = 0.008 respectively). On the contrary, there was no significant difference between both groups regarding HbA_{1c} (P = 0.452).

Regarding the plasma expression of miRNA-192 was significantly higher in diabetic patients with normal and increased UAE compared to the controls (P < 0.001). Moreover, plasma miRNA-192 expression was significantly higher in T2DM patients with DKD (UACR ≥ 30) compared to T2DM patients with normal UAE (P = 0.025). (Table 2, Fig. 1).

Additionally, in patients with T2DM, the plasma expression of miRNA-192 was positively correlated with FPG (r = 0.598, P < 0.001), HOMA-IR (r = 0.565, P < 0.001), diabetes duration (r = 0.450, P < 0.001) and UACR (r = 0.506, P < 0.001) (Table 3).

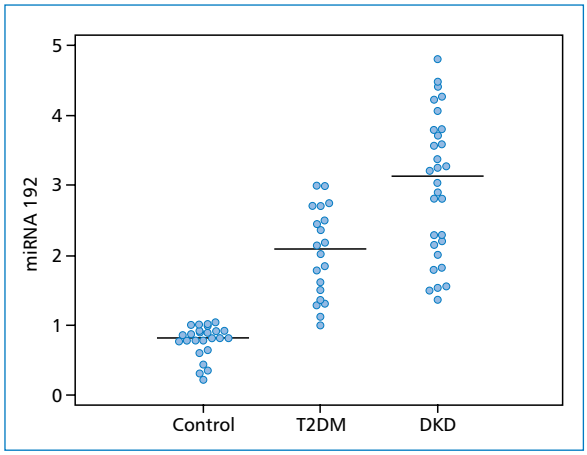

Figure 1. Comparison between the studied groups according to microRNA-192

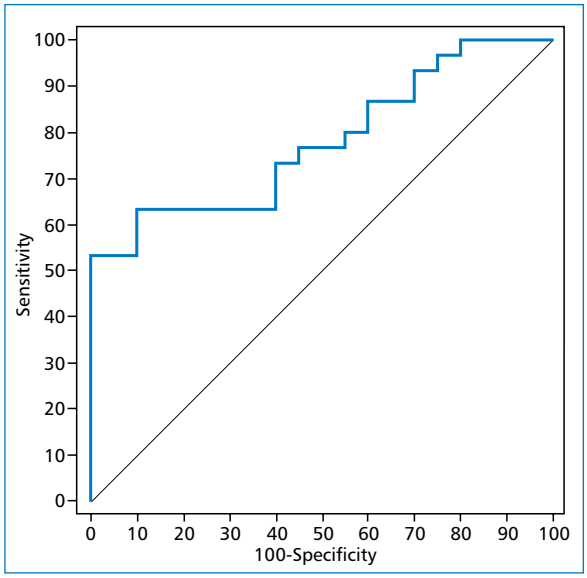

Figure 2. ROC curve for microRNA-192 to predict T2DM patients with uACR ≥ 30 mg/gm

Furthermore, the ROC curve analysis for miRNA-192 plasma expression in patients with T2DM, revealed that miRNA-192 had a good diagnostic performance (AUC 0.778, 95% C.I 0.652–0.904) to discriminate T2DM patients with DKD from those with normal UAE. Also, according to the ROC curve, at a cutoff value > 2.7549, plasma miRNA-192 expression had 63.33% sensitivity and specificity was 90% (Fig. 2).

Discussion

DKD is the most frequent diabetic microvascular complication and the most common cause of chronic kidney disease worldwide [18]. Despite being the most widely used test for early detection of DKD, albuminuria has multiple drawbacks [5]. This triggers exploring new biomarkers for the identifying early diagnosis and prognosis of DKD.

MicroRNA- 192 is among miRNAs which are highly expressed in the human kidneys, and it has an important role in normal kidney function [19]. An important role for miRNA-192 in the fibrogenesis process in DKD has been suggested in several studies. However, the results of these studies are inconsistent.

Kato et al. [20], in 2007, provided the first landmark report about the role of miRNA in DKD, as they found that miRNA-192 levels significantly increased in glomeruli of diabetic mice parallel to the increased TGF-β1 and collagen 1a2 levels.

In 2010, Kato and colleagues [21] found that in mouse mesangial cells, TGF-β1 was upregulated by miRNA-192. In addition, inhibition of miRNA-192 decreased the expression of miR-200b/c, collagen 1a2, collagen 4a1 and TGF-β1 in mouse mesangial cells, and in mouse kidney cortex.

In line with these results, Putta et al. [22] reported that in in cultured glomerular mesangial cells and in glomeruli from diabetic mice, TGF-β1 upregulated miRNA-192. Furthermore, they found that miRNA-192

Table 3. Correlation between miRNA 192 and different studied parameters

	miRNA 192					
	ACR < 30 mg/gm (n = 20)		ACR ≥ 30 mg/gm (n = 30)		Total cases with T2DM (n = 50)	
	r_s	P	r_s	P	r_s	P
FPG [mg/dL]	0.598*	0.005*	0.535*	0.002*	0.598*	< 0.001*
HbA_{1c} (%)	–0.154	0.518	–0.328	0.077	–0.244	0.087
DM duration (years)	0.542*	0.013*	0.339	0.067	0.450*	0.001*
uACR [mg/gm]	0.189	0.426	0.247	0.189	0.506*	< 0.001*
HOMA-IR	0.293	0.210	0.608*	< 0.001*	0.565*	< 0.001*

r_s — Spearman coefficient; *Statistically significant at P ≤ 0.05

increased collagen expression through targeting the E-box repressors Zeb1/2. Additionally, locked nucleic acid, an inhibitor of miRNA-192, significantly increased Zeb1/2 and decreased expression of collagen, TGF-β1 and fibronectin in the kidneys of diabetic mice. Moreover, inhibition of miRNA-192 decreased proteinuria in these mice.

Contrariwise, Krupa and colleagues [23] reported that decreased miRNA-192 expression was associated with tubulointerstitial fibrosis and low GFR in tissues of renal biopsies from patients with DKD. Moreover, reduced miRNA-192 expression in proximal tubular cells was observed after treatment with TGF-β1.

The observed contradictory between the results of the aforementioned studies may be attributed to the different models, cell lines and time points that were used. It also can be suggested that these discrepancies may indicate a cell-type-specific regulation; such that upregulated glomerular miRNA-192 enhances matrix deposition, whereas miRNA-192 downregulation in renal tubules, facilitates epithelial to mesenchymal transition [24].

Conflicting results regarding the role miRNA-192 in DKD were not only reported in studies of cultured tissues and mice, but also studies involving patients with DKD revealed similar contradiction.

In the current work, the results showed that plasma miRNA-192 was significantly higher in T2DM patients with DKD compared to those with normal UAE. Moreover, in patients with T2DM, plasma miRNA-192 was positively correlated with UACR. The ROC curve analysis for miRNA-192 plasma expression in patients with T2DM, revealed that miRNA-192 had a good diagnostic performance to define T2DM patients with DKD.

In line with our results, Saadi et al. [25], demonstrated that serum miRNA-192 was significantly higher in diabetic patients with lower GFR and higher UACR.

Chien et al. [26] reported that there was no significant difference in serum miRNA-192 between T2DM subjects with and without DKD. However, serum miRNA-192 was significantly higher in patients with markedly increased UAE than in patients with moderately increased UAE.

Conversely, in a study of patients with T2DM with different levels of UAE, Jia et al. [27] reported that miRNA-192 levels were significantly higher in urine extracellular vesicles of patients with moderately increased UAE compared to normoalbuminuric and control subjects. Moreover, miRNA was positively correlated with albuminuria and TGF-β1 in patients with normal and moderately increased UAE. Additionally, the ROC curve analysis showed AUC of 0.802 for miRNA-192 in discriminating T2DM patients with normal UAE from those with moderately increased UAE. However, miRNA-192 levels in urine extracellular vesicles was decreased in patients with markedly increased UAE.

On the other hand, in disagreement with the results of the current study, Ma et al. [13] found that miRNA-192 in patients with markedly increased UAE was significantly lower than those with moderately increased UAE. Additionally, miRNA-192 was in patients with moderately increased UAE compared to those with normal UAE. Furthermore, the expression of miR-192 was negatively correlated with TGF-β1.

Comparably, in study by Al-Kafaji and colleagues, miRNA-192 expression was 2.4-fold lower in the microalbuminuric patients compared to the normoalbuminuric group. Moreover, it was significantly lower by 19-folds in patients with macroalbuminuria compared to the normoalbuminuric patients. Additionally, the AUC of the ROC curve for miRNA was 0.70 regarding detection of increased UAE [28].

Similarly, A. El-Monem et al. [29] found that miRNA-192 expression was significantly lower in T2DM patients with microalbuminuria than those with normoalbuminuria. Microalbuminuria in patients with T2DM was accompanied by significantly higher serum level of IL-18 and TGF-β. Moreover, the ROC curve of miRNA-192 in patients with microalbuminuria showed very good performance with AUC of 0.946.

Despite their conflicting results, the current work together with the aforementioned studies suggest a significant importance for miRNA-192 in identifying DKD. Nevertheless, further research should be carried out on larger number of patients with different ethnicities and different stages of DKD in order to define clearly the role of miRNA-192 in pathogenesis of DKD and its ability to diagnose and predict the clinical course of DKD.

The current work proved a high specificity for miRNA-192. Different methods have been endorsed by international guidelines to screen for DKD. Spot urine sample UACR is a simple easy method for screening, but many limitations are there. In addition to the intraday variability and the need for repeated measures for conformation, false-positive rates were found to increase with age approaching 30%, so it is considered a poor predictor of quantitative AER, and so, should not be used as a diagnostic test [30].

Again, Although GFR is commonly accepted as the best overall index of kidney function, it is generally reduced after widespread structural damage, so its sensitivity to early detect renal damage is questionable. it has been reported to underestimate the renal function in some populations, especially in patients with near-normal renal function [31].

Conclusion

In this case-control study, the plasma expression of miRNA-192 was significantly higher in T2DM patients with DKD compared to those with normal UAE. Additionally, in patients with T2DM, the plasma expression of miRNA-192 was positively correlated with albuminuria and displayed good diagnostic performance in discriminating patients with DKD in T2DM. Thus, the plasma expression of miRNA-192 was able to discriminate increased UAE among patients with T2DM; suggesting a promising role for miRNA-192 as a potential biomarker for DKD.

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Conflict of interest

The authors declare that there is no conflict of interest.

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