

Evaluation of microRNA-192 in patients with diabetic nephropathy

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Background

Diabetic nephropathy (DN) is the leading cause of kidney failure. The role of microRNAs (miRs) which are endogenous RNA oligonucleotides that regulate gene expression in DN is not yet clearly established. This study was designed to evaluate the blood level of miR-192 and its relation to disease severity in patients with type 2 diabetes mellitus with and without DN evidenced by the presence of albuminuria.

Materials and methods

The study included 60 patients (37 women and 23 men) with type 2 diabetes mellitus and 20 healthy control participants. All were subjected to thorough history taking, clinical evaluation and measurement of glycosylated hemoglobin, creatinine and urinary albumin/creatinine ratio. miR-192 was quantified in blood using reverse transcription TaqMan microRNA assay.

Results

The study showed that the miR-192 levels were significantly higher in patients with lower estimated glomerular filtration rate and higher albumin/creatinine ratio.

Conclusion

These findings may help to find a new marker for early detection of DN and this could be used in the future as a novel therapeutic target for the treatment of DN.

Keywords:

albuminuria, diabetic nephropathy, microRNA-192, type 2 diabetes mellitus

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Introduction

It has been estimated that 1 in 12 of the world's population suffers from diabetes mellitus, with around 40% of them developing diabetes-related kidney disease [1]. The mechanism of development of diabetes complications is not yet clearly understood and a better way to understand this mechanism is needed to help in the development of new therapeutic strategies for these conditions [2]. Diabetic kidney disease is the most common cause of end-stage renal disease [3]. The current biomarker for the diagnosis of diabetic nephropathy (DN) is the presence of microalbuminuria. However, microalbuminuria does not always lead to progressive renal failure [4]. Moreover, some other conditions may lead to the elevation of urinary excretion of albumin such as urinary tract infection, exercise, fever, congestive heart failure, and marked hypertension [5]. Kidney injury in diabetes starts before the appearance of microalbuminuria, so new biomarkers are required for early detection of diabetic kidney injury. These new biomarkers may help to find alternative therapeutic approaches [6]. Due to the association between altered expression of microRNAs (miRs) and the development of

different diabetes complications, some miRs have been implicated in the development of such chronic conditions [2]. DN is the first complication of diabetes in which miRs were implicated; several miRs have been identified in cell and animal models of DN [7]. Transforming growth factor- β 1 (TGF- β 1) is a key cytokine playing an important role in the development of DN through the regulation of fibrosis and scarring of extracellular matrix [8]. MicroRNA-192 may promote DN progression by regulating TGF- β 1 signaling pathways and may be used as a potential biomarker in the diagnosis and as an early predictor for the progression of DN [9].

Materials and methods

A randomized case-control study that included 60 patients (37 women and 23 men) with type 2 diabetes mellitus (T2DM) and 20 age-matched and sex-matched healthy controls at Internal Medicine

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Department, Kasr Al-Ainy Hospital with a mean age of the patients and control groups 53.1 ± 3.9 and 52.0 ± 4.1 years, respectively ($P=0.279$).

All patients included were diagnosed with T2DM for greater than or equal to 5 years excluding patients with type1 diabetes mellitus, urinary tract infection, renal stones, or history of nephrotoxic drug usage in the last 3 months.

The patients were divided according to their urinary albumin/creatinine ratio (ACR; mg/g) into three groups, patients with ACR less than 30 mg/g were classified as normoalbuminuria, those with values more than 30 and less than 300 mg/g were defined as microalbuminuria, while the group with an ACR of more than 300 mg/g was classified as macroalbuminuria.

All procedures performed in this study were in accordance with the ethical standards of Cairo University Research Committee (the ethics committee has approved the submission of the manuscript without the IRB number) and with the 1964 Helsinki Declaration and its later amendments and informed consent were obtained from all individual participants included in the study.

All included participants gave their consent after being informed about the aim and procedures of the study. All participants were subjected to thorough history taking, clinical evaluation, and laboratory investigations including fasting plasma glucose, glycosylated hemoglobin (HbA1c), serum creatinine, and estimated glomerular filtration rate (eGFR) according to modification of diet in renal disease equation. Early morning urine sample taken for testing for ACR was repeated twice 3 months apart if the first sample was positive for microalbuminuria, patients have been instructed to refrain from heavy exercise 24 h before the test. miR-192 was quantified in blood using reverse transcription TaqMan miR assay.

Collection of samples and storage

Five milliliters of venous blood were taken from each patient and was divided into two parts, one for the biochemical tests and the other for RNA extraction. The first part for the biochemical tests was used immediately while the second part for miR detection. Serum was separated and stored in RNase/DNase-free tubes at -80°C until RNA isolation.

MicroRNA extraction

MicroRNA (miR) was isolated using miR extraction kit supplied by mirVana™ miR Isolation kit

(Ambion, Carlsbad, California, USA) following the instructions of the manufacturer. In brief, the collected serum was mixed with 300 μl of binding buffer solution, and then ethanol was added to precipitate total RNA and eluted in nuclease-free water. Total RNA was then incubated with miR wash solutions without preamplification and then purified small RNA molecules eluted in 50–100 μl sterile RNase free water supplied with the kit. The samples were stored at -70°C until use.

Detection and quantification of microRNAs using quantitative real-time polymerase chain reaction

Complementary DNA (cDNA) was reverse transcribed from total RNA samples using specific miR stem-loop primers from the TaqMan miR assays and reagents from the TaqMan miR reverse transcription kit following the manufacturer's instructions. Amplification of cDNA carried out with the recommended reaction conditions were set according to the applied biosystems application note. It was held at 50°C for 2 min with an initial step of enzyme activation at 94°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60 v for 60 s. The qPCR analysis was performed using the TaqMan Universal Master Mix II, no UNG kit. Each reaction included cDNA template, TaqMan Universal Master Mix II, TaqMan Gene Expression Assay, and nuclease-free water.

Endogenous control

The endogenous control gene, noncoding miR-16, was used according to the applied biosystems application note. The difference of the threshold cycle (CT) between the target miR (miR-192) and miR-16 (ΔC_t) equivalent to the ratio of \log_2 -transformed absolute copy numbers was used to show the relative expression levels of miR-192.

Statistical methods

Data were entered on the computer using the 'Microsoft Office Excel Software' program (2010) for windows, then transferred to the Statistical Package for the Social Sciences software program, version 21 (SPSS; SPSS Inc., Chicago, Illinois, USA) to be statistically analyzed.

Data were summarized using range, mean, standard deviation, and median for quantitative variables or frequency and percentage for qualitative ones. Comparison between groups was performed using the Kruskal–Wallis test followed by pairwise comparisons through the Mann–Whitney test for quantitative variables while comparison for qualitative variables was performed through χ^2 -test. Spearman's

correlation coefficients were calculated to signify the association between different quantitative variables. Receiver-operating characteristics curve analysis was performed to explore the ability of miR-192 to differentiate between different groups. *P* values less than 0.05 were considered statistically significant.

Results

Sixty T2DM patients were enrolled in the study. Among them, 18 T2DM patients had normoalbuminuria, 23 were with microalbuminuria, and 19 participants were diagnosed with macroalbuminuria based on ACR classification.

There were no significant differences in gender and age distribution among all study groups (Table 1).

The mean eGFR was significantly lower in patients with macroalbuminuria and microalbuminuria than controls ($P<0.001$, <0.016 , respectively) with no significant difference between the control group and patients with normoalbuminuria ($P=0.67$). Serum creatinine was significantly higher in diabetic patients with macroalbuminuria than controls ($P<0.001$) with no significant difference between the control group and each of those with micro or normoalbuminuria ($P=0.154$, 0.082 , respectively) (Table 2).

There was no significant difference in mean HbA1c between diabetic patients with normoalbuminuria and those with microalbuminuria. The mean HbA1c was significantly higher in patients with macroalbuminuria than those with normoalbuminuria and microalbuminuria ($P<0.001$, 0.003 , respectively) (Table 2).

The level of miR-192 in serum samples from T2DM patients with different ACR levels was examined. The results showed that the level of miR-192 is more significantly elevated in patients with macroalbuminuria and microalbuminuria than those with normoalbuminuria. Significant positive correlation was noticed between miR-192 and A/C ratio in the whole study participants, patients with microalbuminuria and

those with macroalbuminuria ($P<0.001$) but no significant correlation was found in patients with normoalbuminuria ($P=0.097$) (Fig. 1).

A significant positive correlation between miR-192 and HbA1c in the whole diabetic group ($P<0.001$) is shown in Fig. 2. In addition, there was a significant negative correlation between miR-192 and eGFR. The miR-192 level was significantly upregulated in participants with lower eGFR ($P<0.001$) (Fig. 3).

No correlation existed between miR-192 with neither sex nor age ($P=0.679$ and <0.46 , respectively).

Discussion

Type 2 diabetes mellitus (T2DM) is one of the most important global public health problems. The mechanism responsible for the development of diabetes complications still needs to be better understood in order to develop new therapeutic strategies for these chronic conditions [2]. DN is the leading cause of renal failure resulting in renal replacement therapy worldwide [10]. The main clinical features of DN are persistent albuminuria and progressively declined GFR [11]. Although microalbuminuria is considered the gold standard for the diagnosis of early DN, its ability to detect precisely the disease progression is still unsatisfactory [12]. For that reason, exploring more sensitive markers for monitoring the progression of DN could enable earlier diagnosis and more efficient intervention.

MicroRNAs are short noncoding endogenously produced RNAs of about 20–22 nucleotides in length playing an important role in post-transcriptional gene expression [13]. A physiological role has been recognized for several miRs in tissues in which diabetes complications occur [2].

In the present study, the miR-192 blood level is upregulated in participants with more progressive DN compared with T2DM patients with normal albuminuria. It is apparent that the blood level of

Table 1 Clinical information of participants recruited in this study

	Control	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	<i>P</i> value
Male	10	7	10	6	0.741
Female	10	11	14	13	
Age (years)	52.0±4.1	53.3±2.8	52.8±4.6	54.3±3.9	0.279
Creatinine (mg/dl)	0.68±0.12	0.73±0.11	0.76±0.14	0.88±0.11	<0.001
eGFR (ml/min)	115.2±16.9	101.2±18.8	99.6±19.0	80.5±11.7	<0.001
HbA1c	5.1±0.4	7.5±0.7	7.7±0.6	8.4±0.8	<0.001

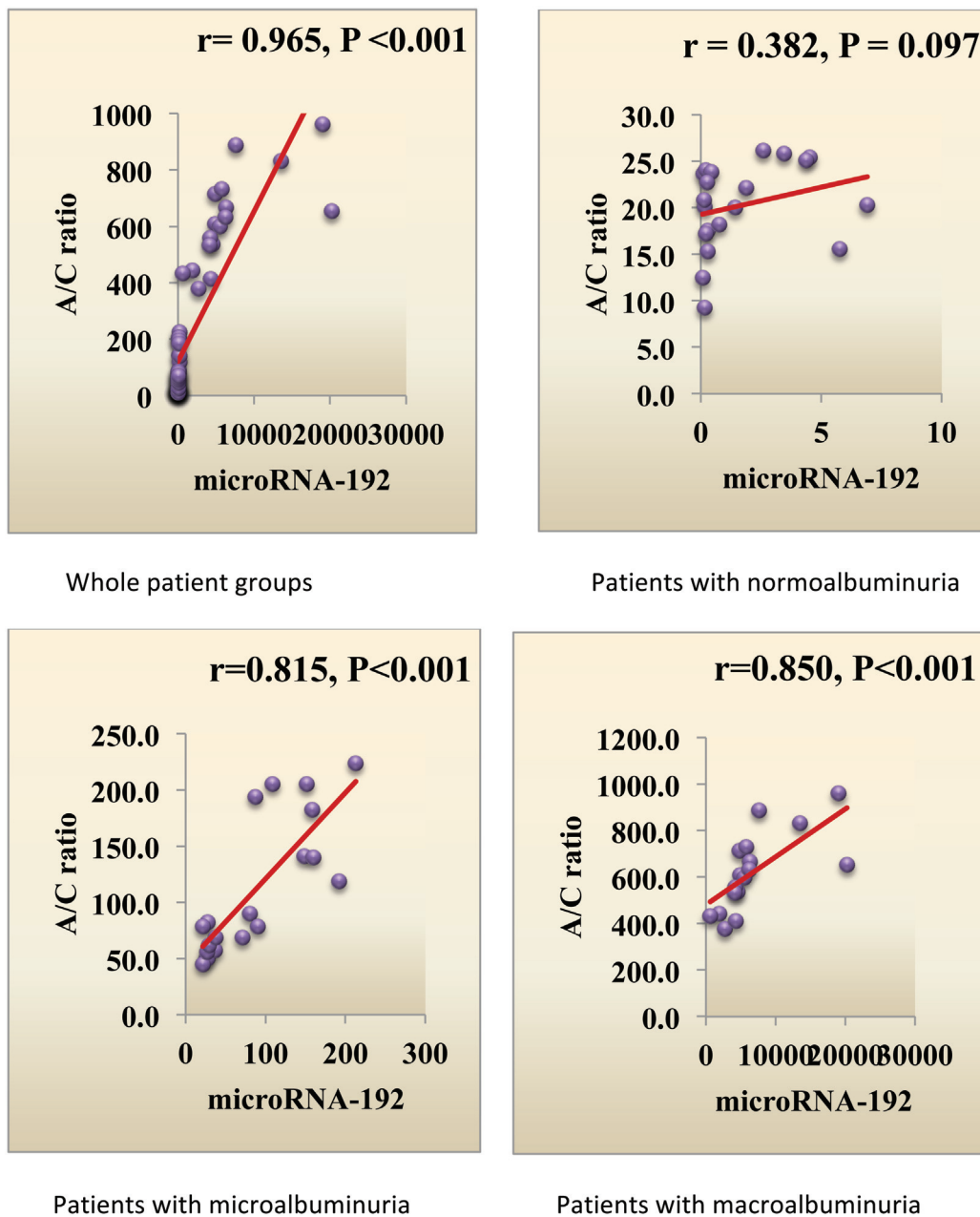
eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin.

Table 2 Comparison of the different patient groups regarding serum creatinine, estimated glomerular filtration rate and glycosylated hemoglobin

Pairwise comparisons	Groups					
	Control to normoalbuminuria	Control to microalbuminuria	Control to macroalbuminuria	Normoalbuminuria to microalbuminuria	Normoalbuminuria to macroalbuminuria	Microalbuminuria to macroalbuminuria
Creatinine	0.154	0.082	<0.001	0.655	<0.001	0.005
eGFR	0.067	0.016	<0.001	0.524	0.001	<0.001
HbA1c	<0.001	<0.001	<0.001	0.357	0.001	0.003

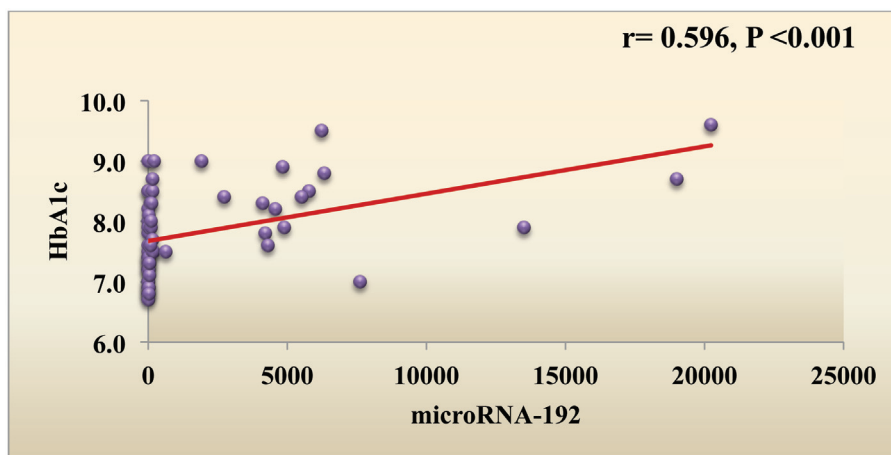
eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin.

Figure 1



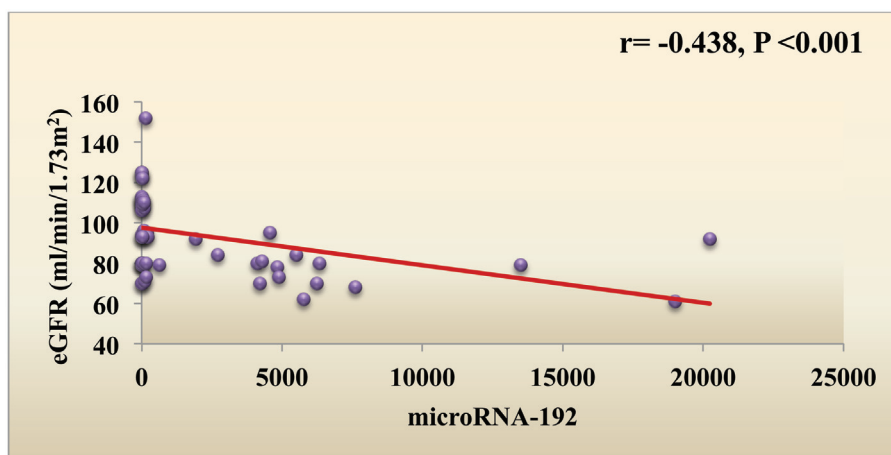
Expression of serum microRNA-192 in type 2 diabetic patients with differential A/Rs. MicroRNA-192 was examined in individual samples pooled for use in microRNA profiling by qRT-PCR, using microRNA-16 as an endogenous control. *r*, Spearman's correlation coefficient. $P < 0.05$, significant.

Figure 2



Plot of microRNA-192 expression against glycosylated hemoglobin (HbA1c) levels, Spearman's correlation coefficient analysis showed significant association between serological microRNA-192 level and HbA1c levels in type 2 diabetes mellitus patients.

Figure 3



Plot of microRNA-192 expression versus MDRD eGFR. Spearman's correlation coefficient = -0.438. $P < 0.001$.

miR-192 is related to the stage of DN as the level of miR-192 is significantly higher in patients with macroalbuminuria than in patients with microalbuminuria. A significant negative correlation was also found between miR-192 and eGFR. These results agree with Hamdia *et al.* [14], who found a significantly higher level of blood miR-192 in diabetics than in nondiabetics with again significantly higher levels in patients with long-standing disease and in patients with retinopathy and nephropathy than the newly diagnosed patients without microvascular complications. Cai *et al.* [15] found that miR-192 and miR-205 levels were significantly higher in the sera obtained from patients with primary focal segmental glomerulosclerosis and correlated with proteinuria and interstitial fibrosis. Significantly increased expression of miR-192 was observed in glomeruli isolated from streptozotocin-induced

diabetes and diabetic db/db mouse in parallel with increased TGF- β 1 and collagen 1a2 (Col1a2) levels [16]. miR-192 is also upregulated in the kidneys of oszther models of renal fibrosis (unilateral ureteral obstruction in mice and a rat model of remnant kidney disease) and in tubular epithelial cells treated with TGF- β 1 in a Smad3-dependent manner [17]. The possible mechanisms of how miR-192 causes renal fibrosis and the pathogenesis of diabetic kidney disease have been found to be through targeting the E-box repressor Smad-1 interacting protein (Zeb2), which binds E-box enhancer elements in the Col1a2 gene and then promotes collagen deposition in response to TGF- β 1 [18]. Enhanced TGF- β 1 expression in renal cells promotes fibrosis and hypertrophy during the progression of DN. TGF- β 1 levels were found to be upregulated by miR-192 or micRNA200b/c in mouse mesangial cells (MCs). Inhibitors of miR-192

decreased the expression of miR-200b/c, Col1a2, Col4a1, and TGF- β 1 in mouse mesangial cells, and in mouse kidney cortex [17]. In addition, miR-192 can activate Akt kinase via increased expression of other miRs as miR-216a and miR-217. Akt activation in mouse MCs results in ECM gene expression, apoptosis inhibition, and hypertrophy which are major changes in diabetic kidney disease [19].

In the current study, a significant positive correlation is present between miR-192 and the degree of glycemia (HbA1c). These results agree with the information that elevated glucose causes significant alterations in proximal tubular epithelial cells (PTC) gene expression and is implicated as an important causal stimulus in DN [20,21]. In previous studies upregulation of miR-192 in mesangial cells and tubular epithelial cells (TECs) had been demonstrated after treatment with high glucose, advanced glycation end products (AGE), and TGF- β 1. Several studies demonstrated that the elevation of renal miR-192 is highly correlated with the diabetic condition [12,22]. Moreover, Putta *et al.* [23] found that specific reduction of renal miR-192 decreases renal fibrosis and improves proteinuria.

In contrast to the above, other studies have suggested that the relationship between miR-192 and renal fibrosis is complicated. Krupa *et al.* [24] found that miR-192 in human renal biopsies was significantly lower in patients with advanced DN, correlating with tubulointerstitial fibrosis and low GFR. The contrasting findings highlight the complex nature of miRs research. Some of the differences may relate to models and/or experimental conditions; however, another explanation is that some effects of miRs are likely to be indirect in nature [2]. MicroRNA-based therapeutic strategies show great potential, with promising results and few side effects [25]. It was demonstrated that the injection of locked nucleic acid-modified anti-miR-192 into the mouse model of DN results in the attenuation of functional indices of renal fibrosis and hypertrophy, specifically collagens, TGF- β 1, and Akt activation, suggesting that anti-miR therapies can be developed in the future for human diabetic renal disease [19,24]. The mitotic inhibitor, paclitaxel (used in cancer chemotherapy) has downregulated miR-192 resulting in attenuated fibrotic damage in a rat model of remnant kidney disease [26].

Conclusion

The results of our study suggest a possible role of miR-192 in the pathogenesis and progression of diabetic

kidney disease in humans. Also, blood miR-192 may be a useful biomarker for predicting the development and the stage of diabetic kidney disease. Owing to its role in the pathogenesis and progression of diabetic kidney disease miR-192 may provide a novel therapeutic target for preventing the progression of DN.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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