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Microrna 192 Gene Expression in Type II Diabetic Nephropathy

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ABSTRACT

Background: Diabetic nephropathy (DN) is the common cause of kidney failure in patients with diabetes mellitus. MicroRNAs (miRNAs) are short non-coding RNAs of about 22 nucleotides which recently have been shown to play vital roles in mammalian gene expression. **Aim of the study:** was to investigate the role of miRNA-192 in the pathogenesis of diabetic nephropathy and disease progression. Patients and Method: Sixty five patients with uncontrolled diabetes mellitus, they were subdivided into; thirty nine patients with normoalbuminuria (<20mg/L); their ages ranged between 48-67 years and the onset of disease between 1-5 years; twenty six patients with microalbuminuria (20-200 mg/L), their ages ranged between 47-66 years and the onset of disease between 5-15 years, in addition to twelve apparently healthy individuals as control; their ages ranged between 51-67 years. Serum Transforming growth factor *beta* (TGF-β), Interleukin 18 (IL-18) were determined using ELISA technique, the expression level of miRNA-192 in whole blood using (RT-PCR) was determined, other biochemical parameters as fasting plasma glucose (FPG), glycated haemoglobin (HbA1c), lipid profile and creatinine were estimated using commercial available kits. Patients were given written contest. **Results:** The level of miRNA-192 expressions was significantly lower in microalbuminuria group when compared to normoalbuminuria group. Serum level of IL-18 and TGF-β were significantly higher in both patient groups when compared to control group and their levels were significantly higher in microalbuminuria group than normoalbuminuria group. **Conclusion:** Together with TGF-β1 and IL-18, miRNA- 192 may not only be used as molecular biomarker in diabetic microvascular complications but also as early marker of alterations in specific biological processes in the kidney.

Keywords: Diabetic nephropathy, miRNA-192, IL-18, TGF-β.

INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) has increased significantly, especially in developed countries [(1)]. Many studies have speculated that diabetes mellitus causing microvascular and macrovascular pathological conditions could result in various complications leading to a sever morbidity in T2DM subjects [(2)]. Approximately, 40% of T2DM patients develop diabetic nephropathy [(3)]. Diabetic nephropathy (DN) is a progressive kidney disease caused capillaries damage of the kidneys' glomeruli [(4)]. Its prevalence is rising in developed countries, and it was considered one of the primary causes for end-stage renal disease in diabetic patients [(5)]. Microalbuminuria is a widely-used as an early marker for nephropathy in diabetic patients [(6)]. TGF-β families are essential for regulation of cellular growth, differentiation and apoptosis, as well as immune suppression [(7)]. TGF-β1 has been known as a key mediator in extracellular matrix formation [(8)]. In fibrosis and in tissue remodelling during disease progression indifferent organs, Up-regulation of TGF-β1 is informed to be necessary [(9)], in which glomerular fibrosis in the kidney is included [(10)]. Inflammatory cytokines implicated in the pathogenesis of diabetes play a significant role in several renal disorders development and progression [(11)], including diabetic nephropathy [(12)]. Effects of inflammatory cytokines on renal disease are involved the expression of different molecules, intraglomerular abnormalities, alteration of extracellular matrix, apoptosis and necrosis, endothelial permeability, oxidative stress [(13)], which cause the development of microvascular diabetic complications as neuropathy, retinopathy, and nephropathy [(14)]. IL-18 is a member of the IL-1 family and was primarily described as an interferon gamma inducing factor [(15)]. It has been associated with obesity [(16)], insulin resistance [(17)], and Dyslipidaemia [(18)].Circulating levels of IL-18 have consistently been reported to be elevated in patients with T2DM in different studies[(19)], and have also been suggested to participate in microangiopathy such as nephropathy in T2DM [(20)]. MicroRNAs (miRNAs) are endogenous ubiquitous non-coding single-stranded (ss) RNA transcripts, frequently of 19–25 nucleotides in length that alter the differentiation, growth, apoptosis and proliferation of

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cells by interfering with protein synthesis by either inducing mRNA degradation or repressing translation [21]. Specifically, miRNAs are expressed in many diseases and different cancers such as diabetes, hepatic cancer, prostatic cancer, breast cancer, gastric cancer, squamous cell carcinoma, lymphoma, colon cancer, and lung cancer. Serum miRNAs phenotypes have the potential to become new kinds of diagnostic markers. MiRNA-192 is highly expressed in kidney especially in renal cortex. Many studies have confirmed that miRNA-192 played important roles in the fibrosis of kidney and liver, but the effect of miRNA-192 in DN are still controversial [22]. So our study was aimed to investigate the role of miRNA-192 in the pathogenesis of diabetic nephropathy and disease progression.

SUBJECTS AND METHODS

This study has been conducted on 65 patients attended at National Institute of Diabetes and Endocrinology, Cairo, Egypt, in the period from January to March, 2014. They were divided into two groups according to urinary albumin concentration (UAC); first group 39 patients with normoalbuminuria (<20mg/L); they were 17 males and 22 females, their ages ranged between 48-67 years and the onset of disease between 1-5 years. Second group 26 patients with microalbuminuria (20-200 mg/L); they were 11 males and 15 females, their ages ranged between 47-69 years and the onset of disease between 5-15 years. With 12 apparently healthy individual, 7 males and 5 females; their ages ranged between 51-67 years and represent control group. Patients were on oral hypoglycaemic drugs. Acute illness at time of the study, systemic chronic inflammation, and history of superficial or deep venous thrombosis, liver disorders, malignancy, thrombocytosis, and known tendencies to coagulation abnormalities, hypertension, and cardiovascular disease were excluded. None of the patients were under anticoagulant therapy or any drug that affect urinary albumin excretion rate. Approval had been taken from the research ethics committee of General Organization of Teaching Hospitals and Institutes. An informed consent was obtained from all patients and normal control subjects.

10 ml of peripheral venous blood were drawn from each subject after an overnight fasting (8-12 hours) for determination of fasting plasma glucose (FPG), glycated haemoglobin (HbA1C), lipids profile (total cholesterol, low density lipoprotein (LDL-C), high density lipoprotein (HDL-C), triglycerides), urea, creatinine, interleukin 18 (IL-18) and transforming growth factor β (TGF-β) concentrations. Available commercial kits were used for determination of fasting plasma glucose (FPG) by glucose oxidase reaction; glycated haemoglobin (HbA1c) by affinity chromatography method, lipid profiles concentrations by calorimetric assay, as well as glomerular filtration rate (GFR) was calculated using MDRD equation. Urine samples were collected for the determination of urine albumin concentration by turbidimetric multiagent microalbumin assay(Abbott Diagnostics, US). Both Serum IL-18 and TGF-β were measured using an enzyme linked Immunosorbent assay method (mybiosource.com, San Diego, USA respectively).

MiRNA-192 extraction and expression

Real time PCR: Expression of miRNA-192 was determined using real time polymerase chain reaction. Primer sets for each gene are listed in (Table1).The kit used is High Pure RNA Isolation kit **(Roche Diagnostics GmbH, Roche Applied Science Mannheim, Germany)**. According to the manufacture procedures where specific pre-lysis buffer was added to 500 μL of whole blood. The samples were stored in -80℃ until use.11μL of miRNAs was used for reverse transcription using the Transcriptor First Strand cDNA synthesis kit by **(Roche Diagnostics GmbH, Roche Applied Science Mannheim, Germany)** according the manufacture procedures in which the template-primer mixture was denaturated by heating for 10 min at 65^0C, then the reaming components of RT mix were added for final volume 20μL, the reaction was incubated for 10 min at 25^0C followed by 60 min at 50^0 C; inactivation of the Transcriptor reverse transcriptase was done by heating to 85^0C for 5 min. ***Amplification*** of miRNA-192 was done using Light Cycler Fast Start DNA master ^{plus} SYBR Green I **(Roche Diagnostics GmbH, Roche Applied Science Mannheim, Germany)** following the instruction provided by using 5μL of cDNA, 2μL PCR primer mix, 4μL master mix and PCR grade water with the following program; denaturation at 95^0C for 10s , annealing at 66^0C for 10s and extension at 72^0C for 25s and number of cycles were 45 cycles. To confirm that the desired PCR product has been amplified a melting curve analysis after PCR may be performed. If PCR generated one

amplicon, melting curve analysis will show only one peak. ***Melting curve analysis***95°C for 0 sec, 65°C for 60 sec then 95°C for 0 sec; followed by a single cooling cycle 40°Cfor 30 sec. Real-time data were analysed with LC software, version4.0 (Roche Molecular Diagnostics, USA).

Table 1: List of primers sequences applied in real time-PCR.

Genes	Forward primer	Reverse primer
GAPDH	CCCCGGTTTCTATAAATTGAGC	CACCTTCCCCATGGTGTCT
MiRNA-192	CUGACCUAUGAAUUGACAGCC	CUGCCAAUUCCAUAGGUCACAG

Relative quantification analysis:

The analysis compare two ratios: 1) the ration of target miRNA-192 to a reference housekeeping gene (GAPDH) in an unknown sample and 2) the ratio of the same two sequences in a standard sample called calibrator. The result is expressed as a normalized ratio. The analysis uses the sample's crossing point (cp), the efficiency of the reaction, the number of cycles completed.

The study was done after approval of ethical board of Al-Azhar university and an informed written consent was taken from each participant in the study.

***Statistical analysis*:** Data were assessed with Graph Pad prism software using Student t test, Pearson's test. All results were expressed as means± standard deviation of the mean and *p* value less than 0.05 was deemed to be statistically significant.

RESULTS

Sixty-five uncontrolled T2DM participants were enrolled in the study to determine the role of miRNA-192 in DN pathogenesis. Among them, 39 T2DM patients were normoalbuminuria and their ages ranged between 48-67 years (57.18 ± 5.12) and the onset of disease between 1-5 years and 26 of them were microalbuminuria; their ages ranged between 47-69 years (57.35 ± 6.12) and the onset of disease between 5-15 years along with 12 apparently healthy subjects as control their ages ranged between 51-67 years (55.58 ± 5.37). There were significance differences in all metabolic parameters among groups except for age in demographic and metabolic parameters (Table 2).

Table 2: **Demographic and metabolic characteristics of the studied groups, data represented as mean ±SD.**

	Control group (n=12)	Normoalbuminuria group I (n=39)	Microalbuminuria Group II(n=26)
Demographic parameters			
Sex (%) **Male** **Female**	 **58.3** **41.7**	 **43.6** **56.4**	 **42.3** **57.7**
Age(years)	**55.58 ± 5.37**	**57.18 ± 5.12**	**57.35 ± 6.12**
BMI(kg/m^2)	**28.64 ± 5.13**	**34.76 ± 3.18^a**	**34.99 ± 4.17^a**
Metabolic parameters			
FPG (mg/dl)	**94.17 ± 8.02**	**229.49 ± 32.51^a**	**240.65 ± 43.88^{ab}**
HbA1c%	**4.64 ± 0.85**	**6.97 ± 1.38^a**	**7.41 ± 1.04^a**
Creatinine (mg/dl)	**0.75 ± 0.16**	**0.78 ± 0.16**	**0.98 ± 0.47^{ab}**
Urea (mg/dl)	**34.58 ± 7.57**	**39.79 ± 8.51**	**42.77 ± 8.79^a**
GFR (ml/min per 1.73 m^2)	**98.25±15.66**	**93.08±13.19**	**83.15±26.91**
UAC (mg/L)	**10.40 ± 2.28**	**12.70 ± 4.58**	**70.45 ± 14.85^{ab}**
TG (mg/dl)	**131.50 ± 30.77**	**152.72 ± 29.50^a**	**164.65 ± 42.06^{ab}**
T-Cholesterol (mg/dl)	**152.75 ± 11.35**	**156.23 ± 15.59**	**185.53 ± 23.28^{ab}**
HDL (mg/dl)	**43.33 ± 5.47**	**40.92 ± 2.78**	**36.62 ± 5.22^{ab}**
LDL (mg/dl)	**84.57 ± 10.30**	**93.72 ± 9.76^a**	**109.18 ± 17.64^{ab}**
TGF-β (pg/ml)	**87.94 ± 8.35**	**245.98 ± 20.90^a**	**302.32 ± 16.30^{ab}**
IL-18 (pg/ml)	**80.44 ± 9.13**	**194.94 ± 18.78^a**	**209.67 ± 15.45^{ab}**

Data are expressed as means ± standard deviation or percentage. FPG, fasting plasma glucose HbA1C, glycated hemoglobin; GFR, glomerular filtration rate, UAC, urinary albumin concentration; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein [a]p: statistically significant differences (p< 0.001 and <0.05) vs. control group; [b]p<0.001 and <0.05 vs. normoalbumin group.

Serum level of TGF-β, IL-18

Concentration of both TGF-β and IL-18were significantly differed among the studied groups (p<0.001). In both patients groups -normoalbuminuria and microalbuminuria- had significantly higher TGF-β and IL-18 concentrations when compared to the control group. Also, their concentrations were higher in the microalbuminuria group when compared the normoalbuminuria group (p<0.001) (Table 2).

Fold expression of miRNA-192

The fold expression of miRNA-192 in group I and II were significantly higher when compared to control (28.03 ± 6.39andand13.28 ± 3.66 vs. 1.00 ± 0.02, P<0.001). Furthermore, miRNA-192 expression in group II was significantly lower than group I (13.28 ± 3.66 vs. 28.03 ± 1.023 respectively, P<0.001) (Fig. 1).

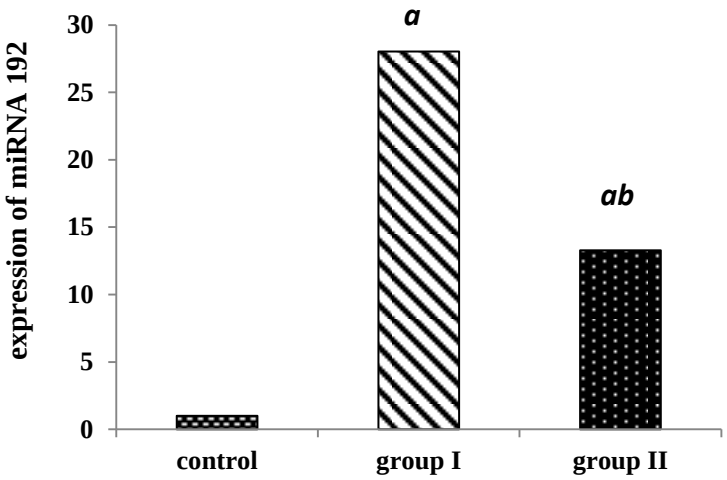

Fig.1:fold expression of miRNA-192 in three studied groups, [a]p<0.001 when compared with control group, [b]p<0.001 when compared with group I

Pearson's correlation analysis: there was significant positive association between IL-18 and*HbA1C (%)* in normoalbuminuria group (r= 0.596, p =0.001) (Table 2). As well as, TGF-β showed significant negative association with *TG (mg/dl)* (r=-0.532, p=0.001) (Table 3). No other significant correlation was observed.

Table 3: Pearson's correlation between serum IL-18 and TGF-β and others variables in normoalbumin and microalbumin groups.

parameters	Normoalbumin group				Microalbumin group			
	IL-18		TGF-β		IL-18		TGF- β	
	R	p	r	P	R	p	r	P
FPG (mg/dl)	0.055	0.742	-0.064	0.697	0.047	0.818	-0.135	0.509
HbA1C (%)	***0.596***	***0.001****	-0.006	0.973	0.276	0.172	-0.023	0.910
Creatinine (mg/dl)	0.010	0.951	0.170	0.300	0.089	0.667	-0.016	0.938
TG (mg/dl)	0.169	0.303	***-0.532***	***0.001****	-0.254	0.211	-0.119	0.562
Cholesterol (mg/dl)	0.084	0.610	-0.206	0.209	0.189	0.356	-0.162	0.429
LDL (mg/dl)	-0.048	0.771	0.122	0.459	-0.103	0.616	0.119	0.562
HDL (mg/dl)	-0.172	0.297	0.108	0.514	-0.231	0.257	0.094	0.649
GFR (ml/min per 1.73 m2)	0.035	0.8324	-0.253	0.116	-0.2409	0.236	-0.215	0.296
IL-18 (pg/ml)	----	-----	-0.097	0.557	----	-----	-0.114	0.579

FPG, fasting plasma glucose ;HbA1C, glycated haemoglobin; TG, triglyceride LDL, low density lipoprotein; HDL, high density lipoprotein; GFR, glomerular filtration rate, p significance at <0.05

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Roc analyses in both patients groups

The overall performance of TGF-β, IL-18, and miRNA-195 was assessed by ROC curve analysis. The best cut-off values for serum TGF-β in group I and group II were 157.8 and 185.55 respectively) ($p<0.001$) with100% sensitivity and 100 % specificity producing area under the curve (AUC) 1.00 (Fig. 2 and 3). For IL-18 the best cut-off points were 116.2 and 185.5 in group I and II respectively) ($p<0.001$) with 100%sensitivity and 100% specificity producing AUC= 1.00 (Fig. 4 and 5). On the other hands, the best cut-off point for miRNA-195 was 27.39 (P=0.005*) with 53.8% sensitivity and 100% specificity producing AUC= 0.776, while for group II the best cut-off value was16.86) (P=0.042*) with 83.3% sensitivity and 100% specificity producing AUC= 0.946 respectively (Fig 6 and 7).

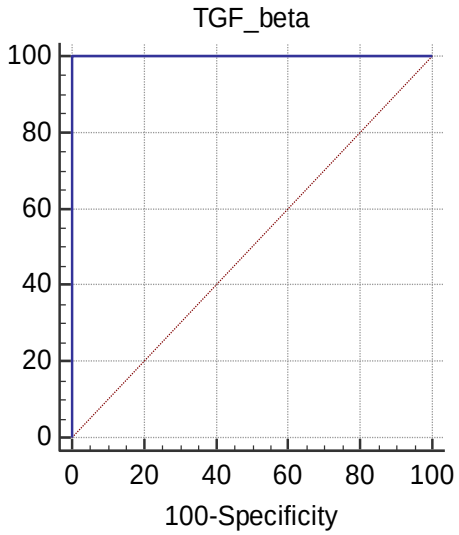

Fig.2: ROC curveof TGF-β in group I

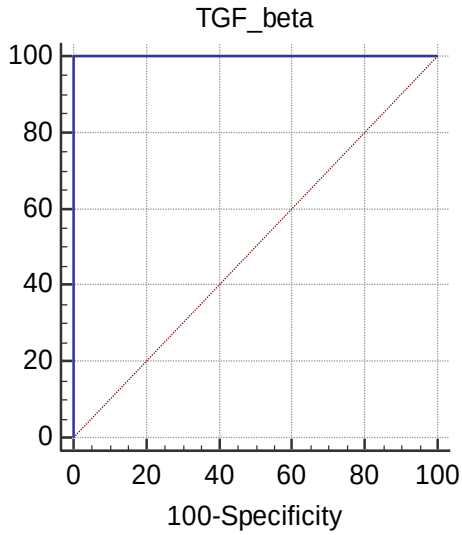

Fig.3: ROC curveof TGF-β in group II

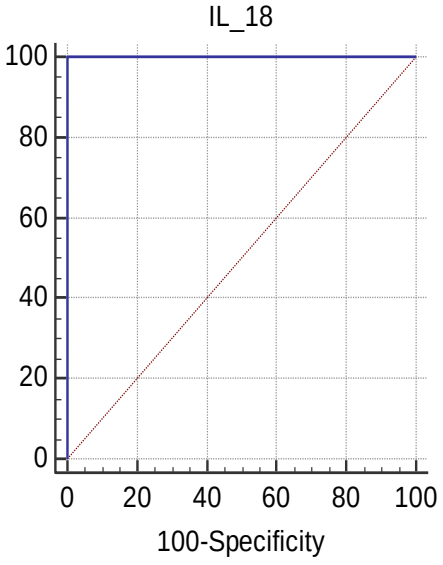

Fig.4: ROC curveof IL-18 in group I

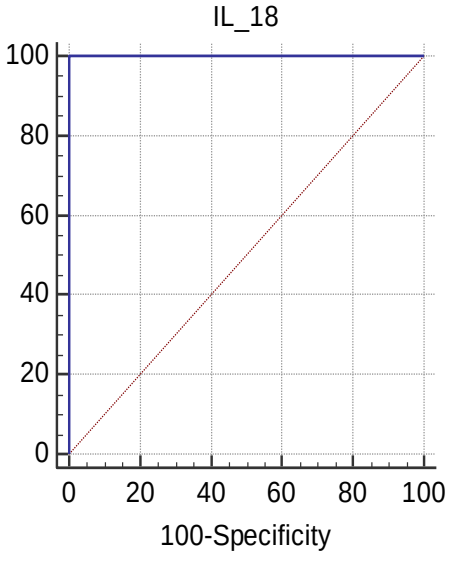

Fig.5: ROC curveof IL-18 in group II

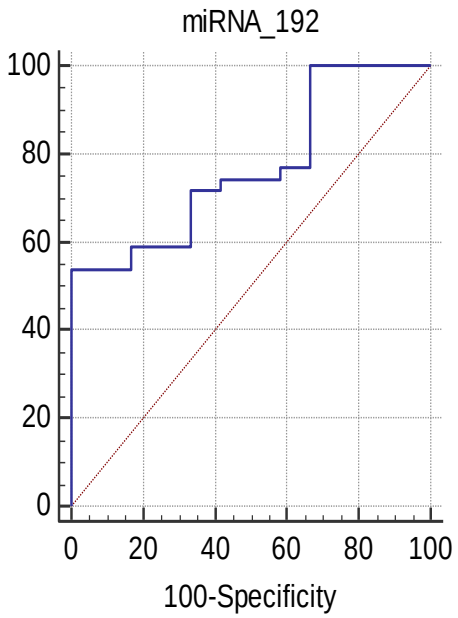

Fig.6: ROC curve of miRNA-192 in group I

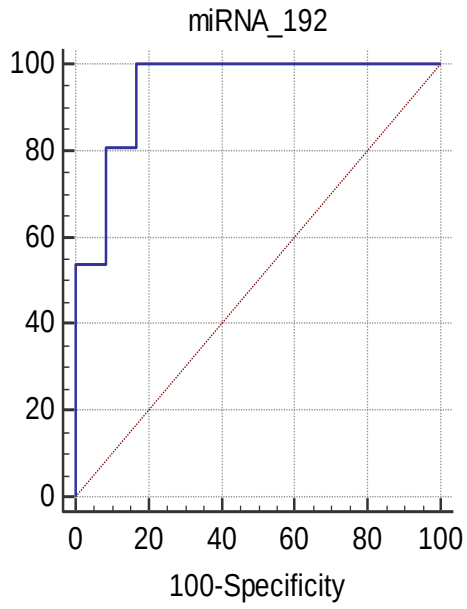

Fig.7: ROC curve of miRNA-192 in group II

DISCUSSION

Diabetic nephropathy (DN) pathogenesis is very complicated including glucose and lipid metabolic disorders, change of haemodynamic, oxidative stress, and cytokines, which can cause thickening of glomerular basement membrane, accumulation of extracellular matrix, glomerular sclerosis, damage of filtration membrane, renal tubule atrophy, and renal interstitial fibrosis. These pathological changes cause increasing in urinary albumin excretion rate, slowly progressive proteinuria, and renal dysfunction in clinical practice [(23)]. Recently, Studies found that podocytopathy is an important cause of diabetic nephropathy. The deterioration of podocytes causes weakness of the glomerular filtration charge barrier and albuminuria is induced. Albuminuria can increase the extracellular matrix and emphasize renal fibrosis. Moreover, the regulation of extracellular matrix generation by the damaged podocytes is disturbed and causes TGF-β1 increase in DN [(24)].

TGF-β is a cytokine that mediates the fibrosis and inflammation of kidney. TGF-β1 can promote the synthesis of extracellular matrix; prevent its degradation and accumulation by promoting the adhesion between cells and matrix [(25)]. TGF-β1 increase not only in the late stage, but also indicated in the early stage of diabetic nephropathy. Expression of TGF-β1 mRNA significantly increased in renal biopsy from diabetic patients [(26)]. Abnormality in glucose metabolism and increase the hemodynamic change in the early phase of diabetes mellitus is the major factor that causes the increase of TGF-β1 expression in diabetic nephropathy. In addition, the local rennin-angiotensin system activation cause over secretion of angiotensin II which is also a factor of increasing TGF-β1 under high glucose condition. Also, glycosylated products can stimulate the expression of TGF-β1, causing kidney damage [(27)].IL-18 is involved in the direct induction of renal injury in diabetic nephropathy as it induces dendritic cells accumulation in the glomerulus leading to endothelial dysfunction formation [(28)].

The presence of miRNAs in the serum or plasma seems promising to use as biomarkers for identification of disease initiation and progression. It was recently recognized a certain members of circulating miRNAs in patients with diabetes mellitus and complications of type 2 diabetes mellitus compared to non-diabetes mellitus patients [(29)]. Furthermore, miRNAs which involved in regulating insulin production, insulin sensitivity, glucose homeostasis, or lipid metabolism implicated in T2DM pathology [(30)].

TGF-β1 could control the process of renal fibrosis by up regulation or down regulation of several

miRNAs including miRNA-192 [31]. ***Krupa et al.*** [32] found that miRNA-192 expression was inhibited by TGF-β1 in human proximal tubular cells (PTCs) and its insufficiency connected with acceleration of renal fibrosis and reduction of GFR in DN. Furthermore, the expression of miRNA-192 was lower when the duration of the disease was longer.

Wang et al. [33] also reported that the expression of miRNA-192in rat PTCs, mesangial cells, and human podocytes was decreased by TGF-β1, moreover, the biopsy form diabetic patients showed that they exhibited lower level of miRNA-192.There are two transcription factors namely zinc finger E-box binding homeobox-1 (Zeb1) and Zeb2 that located downstream of TGF-β1 signalling pathway can suppress E-cadherin and control renal fibrosis. Overexpression of miRNA-192 could inhibit the TGF-β1 signalling pathway and the expression of Zeb1 and Zeb2 and then prevented the kidney from fibrosis. So it was reported thatTGF-β1 inhibit the expression of miRNA-192 that targeted Zeb1/2 to activate TGF-β1 signalling pathway and accelerate renal fibrosis in DN [32].

In our study, we found a significant increase in IL-18 in both patients groups when compared with control ($p<0.001$) and its level was significantly higher in microalbuminuria group than normoalbuminuria group ($p<0.001$).Also, we detected a significant positive association between IL-18 and HbA1C ($p<0.001$). Our results were consistent with ***Nakamura et al.*** [34] ***and Madhaet al.*** [35]**.**We also detected that serum level of TGF-β was significantly higher in both patients groups when compared with control ($p<0.001$), and in microalbuminuria group was higher in normoalbuminuria group ($p<0.001$).These results in agreement with ***Yehia et al.*** [36] and ***Xiaoyuet al.*** [22]**,** and we revealed that the level of miRNA-192 expressions was significantly lower in microalbuminuria group when compared with normoalbuminuria group and this in agreement with ***Xiaoyuet al.*** [22]and ***Krupa et al.*** [32]. Concluding that a decrease in miRNA-192 is associated with increased renal fibrosis in vivo and the expression of miRNA-192 was lower when the duration was longer.

However, there are different studies with opposite conclusions. These studies found a significant expression of miRNA-192 increased in mesangial cells due to high glucose level, and it has an vital role in the kidney disease pathogenesis as it amplify TGF-β1 signalling [37]. MiRNA- 192 was found to be expressed in the kidney, and its expression is up regulated in *streptozotocin*-induced diabetes mellitus and db/db mice [38] and miRNA-192 increase in parallel with increased TGF-β1. Also, ***Hung-Yuet al.*** [39] reported that miRNA-192 was significantly higher in overt proteinuria than in normoalbumin and microalbumin groups. Moreover, level of miRNA-192 exhibited good sensitivity to diagnose progression of diabetic nephropathy between patients with microalbuminuria and overt proteinuria. Inhibition of miRNA-192can reduces proteinuria and renal fibrosis with improving the renal function. The possible mechanisms include Smad and Akt signalling pathways [40].

CONCLUSION

The current study revealed that elevated serum TGF-β1 level and IL-18 in patients with diabetes is associated with a high risk of nephropathy and miRNA-192 together with TGF-β1 and IL-18 could possibly reflect the pathogenesis of diabetic nephropathy to some extent. The three parameters are significantly varied in early stage of diabetic nephropathy indicating that they may be useful for early diagnosis of the disease.

However, there were ***several limitations in this study*** first: the sample size is relatively small. Second, renal biopsy should be done to explain the correlation between miRNA level and renal histopathological condition, such as degree of renal fibrosis. Lastly, follow-up period is recommended to identify the precise interpretations for the importance of tested miRNA during diabetic nephropathy progression.

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