Overexpression of Serum Micro-RNA 152-3p in Type 2 Diabetes Mellitus with a Significant Elevation in Progressive Nephropathy

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Modulation of the immune inflammatory system has been implicated in the pathogenesis of type 2 diabetes mellitus (T2DM) and diabetic nephropathy (DN); nevertheless, many of the underlying mechanisms are still unknown. A possible role of micro-RNA 152-3p in T2DM and DN has been suggested due to its immunomodulatory effect on the innate immunity. This case control study aimed, first, to determine the possible role of micro-RNA 152-3p in the pathogenesis of T2DM and DN by evaluating its serum expression in T2DM and DN patients. Second, to assess the performance of serum micro-RNAs 16 and 24 as endogenous control in TaqMan assays of micro-RNA analysis by real time PCR in such disease. Quantification of the expression of micro-RNA 152-3p by qRT-PCR was performed using serum of 70 subjects enrolled in this study and grouped into 20 apparently healthy non-diabetic participants (control group), 15 patients with T2DM without nephropathy (DM group) and 35 diabetic patients with nephropathy (DN group). In diabetic patients with nephropathy (DN) (*P*<0.001), or without nephropathy (DM) (P 0.004), the expression of micro-RNA 152-3p demonstrated a significant elevation in comparison to the controls. Also, the level of micro-RNA 152-3p showed a positive correlation with HbA1c and the duration of diabetes mellitus. The severity of nephropathy as evaluated by markers of renal disease progression; estimated (e)GFR and albumin/creatinine ratio (ACR) revealed a significant correlation with the level of micro-RNA 152-3p. In the same context, serum level of micro-RNA 152-3p was elevated in diabetics with advanced stage of nephropathy (macroalbuminuria) versus the rest of diabetics (without albuminuria and with microalbuminuria). Two one sided T procedure provided a strong statistical support for equivalence of both micro-RNA s 16 and 24. In conclusion, such findings may indicate a pathologic role of micro-RNA 152-3p in Type 2 diabetes mellitus and in the progression of diabetic nephropathy.

Diabetic nephropathy (DN) is a significant microvascular complication of Diabetes mellitus (DM) and the leading cause of end stage renal disease (ESRD) [1]. Almost 20 to 40% of T2DM patients progress to nephropathy and almost 40% of those will develop ESRD [2]. The incidence of diabetic nephropathy is a complex, still unclear, process influenced by duration of diabetes, glycemic control, presence of hypertension, and genetic predisposition [3,4,5]. Many studies have demonstrated that renal inflammation and the immune system, particularly immunoglobulin and complement [6], cytokines (tumor necrosis factor-α, interleukins 1, 6, 18), immunomodulatory molecules (chemokines, adhesion molecules) [7], in addition to aberrant signalling pathways, may participate in the pathogenesis of diabetic nephropathy [8,9].

Micro-RNA s are small noncoding RNA, crucial in many pathophysiological processes including the control of renal reaction to hyperglycemia, and the development of chronic renal disease, such as DN [10,11]. Micro-RNA 152-3p has been found to have a regulatory role in the innate immune system, cell viability, angiogenesis, cell-cycle progression of Human GC cells,

and an inhibitory function in tumor growth and metastasis by targeting PIK3CA [12]. Moreover, few studies have speculated that miRNA expression might be implicated in the complex pathogenesis, diagnosis [13,14] and development of therapeutic tools for DN.

The current study aimed to determine the possible role of micro-RNA 152-3p in pathogenesis and progression of T2DM and DN by estimating its serum expression in patients with T2DM and DN in comparison to controls. In addition, to assess the performance of serum micro RNAs 16 and 24 as endogenous control in TaqMan assays of micro-RNA analysis by real time PCR in the afore-mentioned diseases.

Subjects and Methods

Study population

The current study recruited 50 patients diagnosed with type 2 diabetes mellitus (T2DM), based on the American Diabetes Association criteria 2018 [15], from the outpatient clinic of diabetes mellitus and internal medicine, Beni-Suef University Hospital. Diabetic patients were further grouped into 35 patients complicated with diabetic nephropathy (DN group) and 15 patients who had no DN manifestations (DM group). Diabetic nephropathy was defined according to Robles, et al, 2015 [16] as "diabetes with the presence of albuminuria, reduced glomerular filtration rate (GFR), or both". Consequently, in our study, the DN group included 30 patients evidenced by both the presence of albuminuria and reduced GFR < 60ml/min/1.73m^2 in addition to 5 patients presented with non-proteinuric nephropathy (GFR< 60ml/min/1.73m^2 only). Furthermore, diabetic patients, were categorized into 20 diabetics with no albuminuria, 15 DN patients with microalbuminuria [albumin creatinine ratio (ACR) 30-300 ug/mg creatinine] and 15 DN patients with macroalbuminuria (ACR above 300 ug/mg creatinine). In addition, 20 apparently healthy non-diabetic controls (control group) were enrolled from the blood donors, blood bank, Beni-Suef University Hospital.

All subjects were subjected to full clinical examination including standard anthropometric (height, weight, body mass index [BMI]), and history taking with special emphasis on diabetic symptoms, complications, treatment and family history. In the present study, patients were excluded if having any of the following criteria: metabolic and systemic disease other than T2DM, infection within the preceding month, medication that might affect glucose homeostasis, such as glucocorticoids, or urinary albumin excretion, such as angiotensin receptor blockers and/or angiotensin-converting enzyme inhibitors.

The protocol of the study was reviewed and approved by Faculty of Medicine, Beni-Suef University, Research and Ethical committee (approval # FWA00015574, July 2018).

Methodology

- Collection of samples and storage

Five milliliters of venous blood were withdrawn from each participant. Serum was separated and divided into two aliquots; one was used immediately for the biochemical tests and the other was stored in RNase/DNase-free tubes at −80°C until RNA extraction. Spot urine sample [midstream morning urine sample for urinary albumin creatinine ratio (ACR)]

- Laboratory investigations and biochemical tests

Spot urine samples: Midstream morning urine samples were collected and used for measurement of urinary albumin/creatinine ratio (ACR). In addition, Serum samples from each participant were analyzed for the following laboratory investigations; HbA1c, blood glucose level, lipid profile, creatinine, and calculation of estimated (e) GFR using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [17]

- Quantification of miRNA by real time quantitative PCR

A] Extraction and standardization of RNA: Total RNA including micro-RNA was extracted from serum of the study population by the Qiagen miRNeasy isolation kit (Catalog no. 217184, Qiagen, Germany), according to the manufacturer's instructions.

B] Quantification of miRNA 152-3p by quantitative reverse transcription qRT PCR assay: Transcription of cDNA from extracted RNA was done using TaqMan Micro-RNA Reverse Transcription kit (Catalog no. 4366596, Applied Biosystems, USA) according to the manufacturer's recommendations. In

brief, each 15- µl RT reaction consisted of 5 µl RNA sample, 7 µl master mix and 3 µl primer. Quantitative PCR was performed using specific probes and primers from TaqMan® MiRNA Assays (Applied Biosystems, USA) to measure the expression levels of hsa micro-RNA 152-3p (Assay ID 000475), hsa micro-RNA 24 (Assay ID 000402) and hsa micro-RNA 16 (Assay ID000391] were used. The performance of serum micro RNAs 16 and 24; as endogenous control in normalizing micro-RNA expression; was evaluated. Quantitative PCR was done under the following cycling conditions; enzyme activation for 10 minutes at 95 °C, denature at 95 °C for 15 seconds and Anneal/extend at 60 °C for 60 seconds for 40 cycles; using the Applied Biosystems® StepOnePlus™ Real-Time PCR System, USA. Relative quantification with hsa micro-RNA 16 as an endogenous control was done using the $2^{-\Delta\Delta Ct}$ equation [18].

Statistical Analysis

Statistical analysis of data was performed using Statistical Package for the Social Sciences (SPSS), IBM SPSS Statistics, version 19 (IBM Corp., Armonk, N.Y., USA). One-way analysis of variance (ANOVA) (Bronefferi post-hoc test) was used to test the difference in means of parametric data between 2 groups or more. Parametric data were presented in terms of mean and standard deviation (SD). Nonparametric variables were analysed using Mann Whitney U test. The differences between the means of groups of Categorical variables were analyzed using Chi square test and presented as frequency and percentages. Correlations between quantitative variables in two groups were calculated using the Pearson rank correlation. To test the equivalence of micro-RNA 16 and micro-RNA 24, we compared the variance of the 2 parameters and used the two one-sided test (TOST) procedure for dependent means since both measurements were taken for each subject [19]. The equivalence bounds of TOST test were predetermined at 14% of the pooled standard deviation, which is consistent with a small effect size. Determination of equivalence bounds was performed as previously described by Lakens, 2017 and Norman *et al*, 2003 [20,21].

Results

Clinical and laboratory data of study population

The study participants included 50 type 2 diabetic patients and 20 non-diabetic controls. Clinical and laboratory data of study population are summarized in Tables 1a and 1b.

Expression of serum micro RNA152-3P in the studied groups and its correlation with different parameters

Expression of serum micro-RNA 152-3P was significantly elevated in diabetic patients without nephropathy (DM group) and diabetic patients with nephropathy (DN group) when compared to the control group); P=0.004, P<0.001, respectively. However, there was no significant statistical difference between level of micro-RNA 152-3P in the serum of DM group versus DN group as shown in Figure1 and Table 2.

Assessment of the equivalence micro-RNA 16 and 24 as endogenous controls using quantitative reverse transcription PCR of micro-RNA analysis in serum of all patients.

The equivalence of micro-RNA 16 and micro-RNA 24 as internal endogenous controls was tested by the TOST (two one-sided test) procedure for dependent means as both measurements were taken for each subject. The equivalence bounds were predetermined at 14% of the pooled standard deviation. The pooled standard deviation was 2.92, yielding equivalence bounds of ± 0.4089. The observed equal variance was 8.64 and 8.41 for both micro RNAs 16 and 24, respectively. In addition, the results of the TOST procedure provided a strong statistical support for equivalence of both micro-RNA s 16 and 24. t = 9.0899, P < 0.0001.

Table 1a. Clinical features and laboratory parameters in the studied groups

(Units)	Control (n=20)	DM (n=20)	DN (n=30)	*P* value
Age (years)				
Mean ± SD	52.18 ± 8.025	50.33 ± 8.926	55.20 ± 7.112	NS
(95% CI for mean)	48.05- 56.30	45.39- 55.28	52.76- 57.64	
Systolic BP (mmHg)				
Mean ± SD	122.65 ± 14.37	138.67± 9.155	143.6 ± 13.148	<0.001
(95% CI for mean)	115.26 130.04	133.60 143.74	138.94 148.27	
Diastolic BP (mmHg)				
Mean ± SD	75.88 ± 5.073	85.33 ± 6.399	89.55 ± 8.326	<0.001
(95% CI for mean)	73.27-78.49	81.79- 88.88	86.59- 92.50	
HbA1c (%)				
Mean ± SD	5.071±0.5	8.01±1.15	8.24±1.1	
(95% CI for mean)	(4.81-5.329)	(7.37-8.65)	(7.83-8.66)	<0.001
urinary albumin/creatinine ratio (ACR) (ug/mg creatinine)				<0.001
Mean ± SD	15.1±4.16	19.833±4.18	311.86±312.7	
(95% CI for mean)	(12.95-17.24)	(17.51-22.15)	(204.42-419.3)	
Estimated glomerular filtration rate (e) GFR (ml/min/1.73m2)				<0.001
mean ± SD	103.78±26.29	78.56±13.11	53.59±12.67	
(95% CI for mean)	(90.264-17.30)	(71.0-85.82)	(49.23-57.94)	
Duration of DM (years)				
Mean ± SD		5.33 ± 2.38	7.51 ± 2.78	<0.01
(95% CI for mean)	____	(4.02-6.65)	(6.5-8.47)	

P>0.05 is not significant (NS)

Table 1b. Stages of chronic Kidney Disease (CKD)

	Control (n=20)	*DM (n=20)*	*DN (n=30)*
No clinical evidence of CKD	100%	100%	
Stages of CKD			
1			0%
2			34.3%
3			62.9%
4			0%
5			2.9%

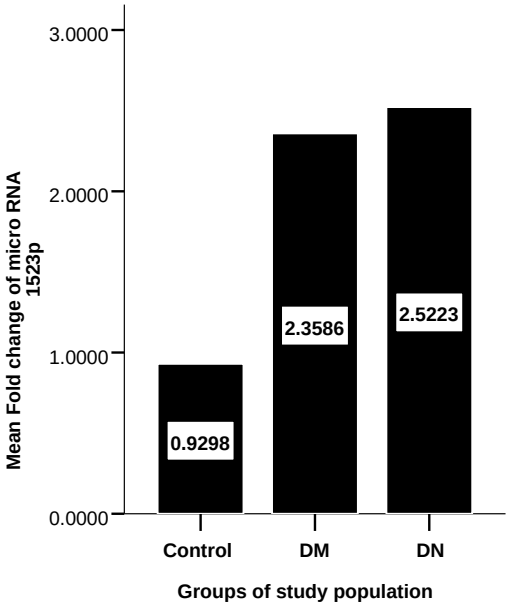

Figure 1. Expression of serum micro-RNA 152-3p along the groups of the studied population.

Table 2. Relative quantification (RQ) of serum Micro RNA152-3p in different studied groups.

RQ of Micro-RNA 152-3p (fold change)	Control	DM	DN	*P* value
				P < 0.001
mean± SD	0.93±0.42	2.36±0.85	2.52±1.52	*P*1 =0.004
(95% CI for mean)	(0.71-1.15)	(1.89-2.82)	(1.99-3.04)	*P*2 <0.001
				*P*3 =1

P within the 3 groups. *P*1 control vs DM, *P*2 control vs DN, *P*3 DM vs DN

No significant difference of the expression of serum micro-RNA 152-3p was detected pairwise between the following groups: diabetics without albuminuria, DN with microalbuminuria and DN with macroalbuminuria (Figure 2). The only significant elevation in serum micro-RNA 152-3p level (*P*=0.05) was detected in diabetics with macroalbuminuria (advanced or progressive stage of nephropathy) in comparison to the rest of diabetics (without albuminuria and with microalbuminuria) (Figure 3).

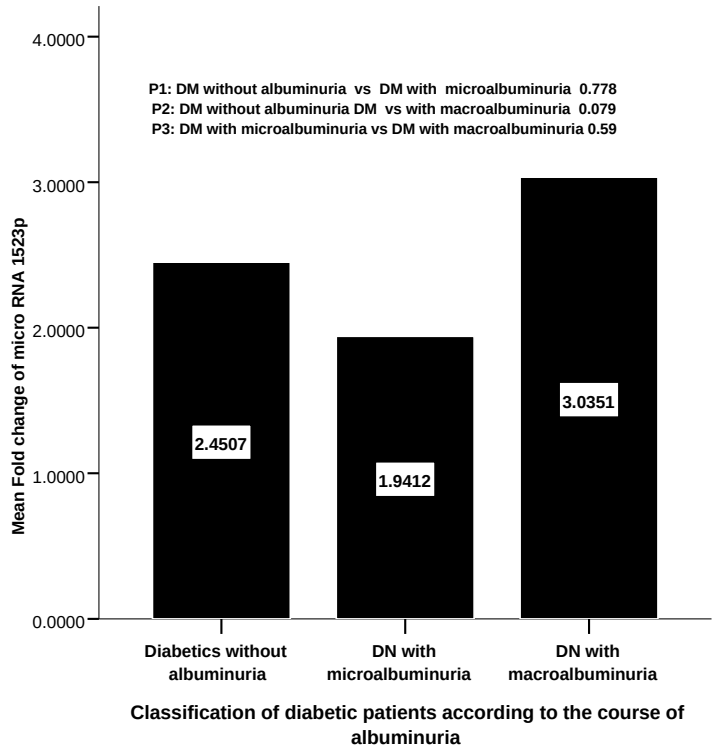

Figure 2. Expression of serum micro-RNA 152-3p according to the stage of albuminuria

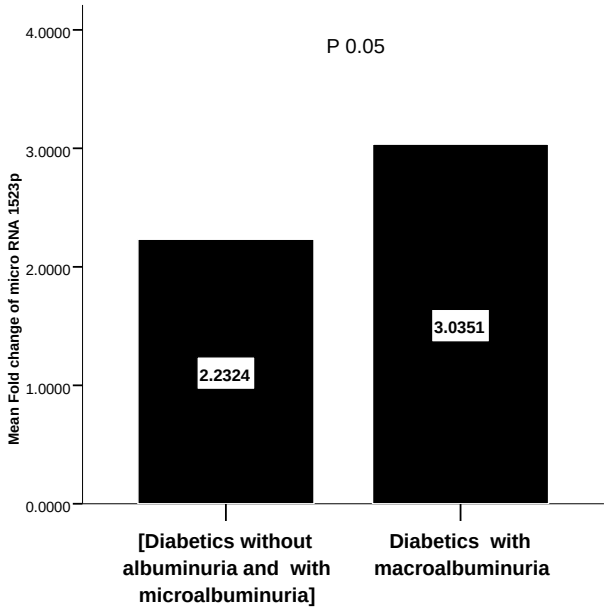

Figure 3. Elevation of serum micro-RNA 152-3p with progressive albuminuria

All study participants were grouped according to the estimated GFR, expression of micro-RNA 152-3p was significantly higher in the group of reduced GFR ≤60ml/min/1.73m^2 in comparison to the group with GFR >60ml/min/1.73m^2 (Figure 4).

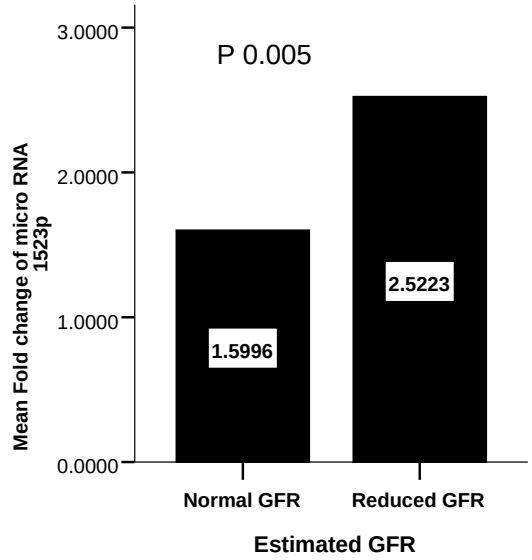

Figure 4. Expression of serum micro-RNA 152-3p according to the estimated GFR.

Expression of micro-RNA152-3p showed a significant positive correlation with the duration of DM, HbA1c and ACR, and a significant negative correlation with eGFR as shown in Table 3. Also, triglycerides analysis was correlated with each of eGFR (r - 0.639, *P*<0.001), ACR (r=.560 *P*<0.001), and RQ of serum micro-RNA 152-3p (r 0.430, *P*<0.001)

Table 3 Correlations of serum micro RNA152-3p expression with different laboratory and clinical parameters.

Laboratory parameters	Relative quantification (RQ) of serum micro-RNA 152-3p	
	r	*P* value
Duration of DM	0.406	<0.001
HbA1c	0.441	<0.001
Triglycerides	0.430	<0.001
ACR	0.398	0.001
eGFR	-0.408	0.001

r= correlation coefficient. Correlation is significant at the 0.01 level (2-tailed). *P*<0.05 is significant.

Discussion

Micro-RNA152-3p had been investigated in regulation of glucose metabolism and insulin resistance [22] and thus could have a possible role in the pathogenesis of T2DM in addition to its possible role in identifying new mechanisms in the pathophysiology of DN [23]. In the present study, the micro-RNA 152-3p level was significantly overexpressed in diabetic patients with nephropathy (DN group) and without nephropathy (DM group) when each of these groups was compared to the non-diabetic controls $P<0.001$, $P = 0.004$, respectively. However, no such significant discrimination in the level of the micro-RNA could be detected between the diabetics with nephropathy and without nephropathy. Consequently, it could be argued that micro-RNA 152-3p was not a suitable marker for DN since its expression was not significantly different between the two groups (DN and DM) and the increase in its level in the two diabetic groups versus the control was only attributed to T2DM, and not to diabetic nephropathy, pathogenesis. On the other hand, it is well noted that the histological abnormalities occur before micro-albuminuria and DN are recognizable. Therefore, by the time the microalbuminuria is detectable, renal tissue damage and inflammation have already existed [24,25]. As such, the elevation in the level of micro-RNA 152-3p earlier in the DM group could have been mediated by the initiation and development of renal damage even before the DN diagnosis is achievable by microalbuminuria. The clarification of such assumption needs a large prospective study to investigate whether the high level of micro-RNA 152-3p patients is predictive to a later diabetic nephropathy incidence.

In agreement to our results, Bijkerk *et al,* 2015 [26] observed a significant increase in plasma levels of micro-RNA 152-3p in both type I diabetes mellitus (DM1) patients; with preserved renal function and with diabetic nephropathy when compared to study controls, however no significant difference was detected in the level of the micro-RNA between the diabetic nephropathy patients and DM1 patients with good renal function [26]. Similar results were detected in both studies, even though micro-RNA was measured in serum of T2DM patients in our study, in contrast to plasma of DM1 patients in Bijkerk et al, 2015 study [26]. On the contrary to our results, plasma expression of micro-RNA 152-3p was significantly different in T2DM patients without nephropathy when compared to patients with diabetic nephropathy in a study by Roux et al, 2018 [23]. This discrepancy in findings could be related to the increased percentage of patients with macroalbuminuria (100%) in the first stage of Roux *et al* study [23] in contrast to 50% in our study. High prevalence of patients with macroalbuminuria in the study by Roux et al might have resulted in the increased level of micro-RNA 152-3p in the DN group, as was reported in our study as well. To our knowledge, Roux et al, 2018 [23] was the only study to assess the level of micro-RNA 1523-p in type 2 DM and DN patients but the study lacked a nondiabetic control group.

Our results supported the idea of the association of micro-RNA 152-3p with diabetic nephropathy in three main aspects: first, the significant difference in the expression of micro-RNA 152-3p between DN and the nondiabetic control group ($P < 0.001$), in addition to the higher expression of micro-RNA 152-3p in patients with reduced e GFR P= 0.005. Secondly, the

observed correlation between serum micro-RNA 152-3p with ACR in a positive direction (p= 0.001) and with eGFR in a negative line (*P*= 0.001) in all the studied population. Third, an additional significant elevation micro-RNA 152-3p level was detected in DN patients with macroalbuminuria compared to the rest of the diabetics (*P*=0.05). Since macroalbuminuria is a presenting feature in advanced or progressive stage of DN [27], it could be noted that micro-RNA 152-3p was significantly increased with the progress of DN. Also, the notion that persistent macroalbuminuria is considered as a predictor of ESRD [28,29.30], might highlight a possible predictive role of micro-RNA 152-3p due to its association with macroalbuminuria. To summarize, elevated micro-RNA 152-3p might be associated with DN progression and might have a possible prognostic role. Large-scale follow up studies are needed to confirm these findings. Still, carefulness is needed in the interpretation of circulating miRNA results [31]. Elevated levels of circulating miRNA might be due to a reduced clearance in patients with diseased kidney, although Neal *et al*, 2011[32] contradicted this speculation [32].

Two previous studies found an association of circulating micro-RNA 152-3p with diabetes mellitus type2 and obesity [33], as well as with type 1 DM in children [34]. The analysis in our study investigated the linkage between T2DM and micro-RNA 152-3p and revealed a significant increase of the serum micro-RNA 152-3p expression in DM group when compared to nondiabetics (control group), and also a positive correlation was observed between serum micro-RNA 152-3p with both the level of HbA1c levels ($p < 0.001$) and the duration of diabetes ($P < 0.001$).

The role of 152-3p in regulation of glucose metabolism and insulin resistance was explored by Ofori et al, 2017 [22], showed that micro-RNA -152 impacts glucose-stimulated insulin secretion via regulation of ATP levels through negative modulation of pyruvate dehydrogenase and glucokinase proteins in the pancreatic beta cells [22]. Micro-RNAs have been identified as indirect modifiers of inflammation-derived insulin resistance, by regulating the action of innate immune cells in insulin target cells [35]. In the same context, Liu *et al*, 2016 [36] reported that miR-152 had the ability of preventing lipopolysaccharide- induced upregulation of major histocompatibility complex (MHC) II expression and dendritic cells (DCs) initiated antigen-specific $CD4^+$ T cell production by modulation of Calcium/calmodulin-dependent protein kinase IIa (CaMKIIa) [36, 37]. Thus, miR-152 had negative regulatory role in the immune system with an important emphasis on the innate immunity [36]. Additionally, Wang *et al*, 2016 [38] found that micro-RNA 152 facilitates hepatic glycogen synthesis via modulation of the protein kinase B/glycogen synthase kinase (AKT/GSK) pathway by targeting phosphatase and tensin homolog (PTEN)[38].

The current study detected an association between each of triglycerides, ACR and level of micro-RNA 152-3p in the whole study population. Many studies supported the linkage between triglycerides and ACR [3,39] and suggested that dyslipidemia plays a crucial role in the onset and development of kidney disease in diabetic patients [40].

In conclusion, overexpression of serum level of micro-RNA 152-3p in T2DM patients, in absence or presence of nephropathy manifestations, might provide an insight to its implication in any of the

following: an underlying pathogenesis of diabetes mellitus, the onset of kidney damage in T2DM before detection of microalbuminuria, in addition to, the possible involvement in progress of DN.

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