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The expression profile of circANKRD36 and ANKRD36 as diagnostic biomarkers of chronic kidney disease in patients with type 2 diabetes mellitus

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Abstract

Background: The molecular mechanisms for chronic kidney disease (CKD) remain largely unknown and appear to be multifactorial. In the current study, we aimed to study the circulatory levels of circular ankyrin repeat domain 36 (circANKRD36) and *ANKRD36* in Egyptian patients with type 2 diabetes mellitus (T2DM) and CKD and to explore their associations with the progression of CKD. This cross-sectional controlled study enrolled 60 patients with T2DM and 40 controls. Real-time polymerase chain reaction (RT-PCR) and real-time quantitative PCR (RT-qPCR) analyses were used to detect the expression levels of circANKRD36 and *ANKRD36*.

Results: Our results detected that the relative expression levels of circANKRD36 and *ANKRD36* were significantly higher in patients with T2DM compared to controls. CircANKRD36 and *ANKRD36* were significantly overexpressed in patients with macroalbuminuria (0.2316 ± 0.096 , 0.0086 ± 0.0035 , respectively) compared microalbuminuria (0.1347 ± 0.032 , 0.0037 ± 0.0008 , respectively) as well as normoalbuminuria (0.1261 ± 0.018 , 0.0027 ± 0.0004 , respectively), $p < 0.001^*$.

Conclusion: The relative expression levels of circANKRD36 and *ANKRD36* were significantly increased in patients with T2DM more specifically in patients with diabetic nephropathy (DN) and macroalbuminuria.

Keywords: CKD, UACR, CircANKRD36, Gene expression, eGFR, RT-PCR

Background

Despite advances in the understanding of diabetes mellitus (DM) risk factors and progression, there has been a steady rise in the global prevalence of DM. Previous numerous studies have demonstrated by the end of 2030, the total number of patients with diabetes will increase and is expected to rise by 55% [1]. Chronic kidney disease (CKD) is a major health problem. It is considered one of the most predominant chronic complications of type 2 diabetes mellitus (T2DM), and several studies reported that ~30–40% of patients with DM had CKD [2].

Diabetic nephropathy (DN) is defined as persistently elevated urine albumin creatinine ratio >30 mg/g and persistently reduced estimated glomerular filtration rate (eGFR) <60 ml/min per 1.73 m² [3].

Accumulating studies have reported that the progression of CKD seems to be multifactorial, which may be due to interactions between genetic and environmental factors [4]. There are various epigenetic regulators including noncoding RNAs (ncRNAs) such as circular RNAs (circRNAs) [5]. Interesting studies presume that circRNAs regulate hyperglycemia-induced vascular endothelial cell dysfunction [6]. Likewise, another research suggested that hsa_circ_0054633 has a defensive mechanism against hyperglycemia hazards on

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endothelial functions through the miR-218/heme oxygenase-1 axes [7].

An overwhelming amount of evidence has demonstrated that epigenetics are interrelated with various diabetic microvascular complications development and progression, for example, retinopathy, nephropathy, and neuropathy. Consistent with this notion, a recent study explored the pathophysiological roles of circRNAs in patients with T2DM as well as in patients with CKD [8].

There is compelling evidence suggesting that early diagnosis of CKD decreases morbidity and mortality rates. Interestingly, microalbuminuria is the gold standard for the early diagnosis of DN. However, many studies have suggested that it is inadequate and has some drawbacks. At this time, recent technologies and novel biomarkers have been accepted for early diagnosis and progression evaluation of CKD in patients with T2DM [9]. The current study aims to evaluate the expression profile levels of circANKRD36 and *ANKRD36* in Egyptian patients with T2DM and CKD and to explore their associations with the progression of CKD.

Methods

Study population

The study enrolled 60 patients with T2DM and 40 matched controls of the same ethnic origin. T2DM patients were diagnosed according to the American Diabetes Association criteria, reported in 2020 [10]. We categorized T2DM patients according to the urine albumin creatinine ratio (UACR) into 3 subgroups; normal ($n=38$) was defined as a UACR persistently <30 mg/g, microalbuminuria ($n=15$) as a UACR between 30 and 300 mg/g, and macroalbuminuria ($n=7$) as a UACR >300 mg/g. All enrolled subjects underwent full history taking and clinical examination with special concern to other comorbidities including hypertension, cerebrovascular stroke, ischemic heart disease, current medications, duration of diabetes, and other diabetic complications. We calculated the body mass index (BMI). The eGFR was calculated by the CKD-EPI equation [11]. The exclusion criteria were for any patients with chronic liver diseases, autoimmune diseases, inflammatory diseases, and infections. We also excluded pregnant females to avoid false results of albuminuria.

Blood samples and biochemical analysis

Fasting plasma glucose (FPG), total cholesterol (TC), and triglycerides (TG) were measured by routine enzymatic methods (Spinreact). HDLc was determined after precipitation of apo B-containing lipoproteins. LDLc was calculated using the Friedewald formula [12]. The UACR measured in a spot urine sample is highly correlated with the 24-h urine albumin excretion [13].

Total RNA was extracted by using a total RNA Extraction Kit (Cat. No. BSC52S1); supplied by Bioer Technology Co., China. The RNA reverse transcription kit is HiSenScript kit (Cat. No. 25014); supplied by iNtRON Biotechnology, Inc., South Korea, and it was used for cDNA synthesis from the total RNA. The RNA quality was quantified by A260 using UV/spectrophotometer. RT-PCR technique was applied by using StepOne Plus™ System (Applied Biosystems Inc., USA). RT-PCR was done in 20 μ l as a final volume containing cDNA (5 μ l), 10 μ l of 1X Quantitect sybr green with low ROX PCR master mix (Cat.No.RT500S; Enzynomics, Republic of Korea), and pmol/ml each primer (0.5 μ l for each) of circANKRD36 or *ANKRD36* and was completed by 4 μ l DdH₂O according to the manufacturer's instructions.

The primers of circANKRD36 or *ANKRD36* were designed by Primer 3 (<http://www-genome.ut.ee/>), and according to previous research [14], their sequences are listed in Table 1.

Amplification protocol consisted of initial denaturation with polymerase activation at 95°C for 15 min, then 40 cycles of denaturation 94°C for 15 s; annealing at 55 °C for 30 s and extension at 70°C for 30 s. Expression levels of each gene were normalized to β -actin. The relative gene expression of the target *ANKRD36* was analyzed and calculated using RT-PCR and the Livaks $2^{-\Delta\Delta C_t}$ equation relation to β -actin as internal control genes. The C_t (threshold cycle) is a relative measure of the concentration of the target in the PCR reaction. $\Delta\Delta C_t = [C_t(\text{target, normal}) - C_t(\text{I.C, normal})] - [C_t(\text{target, HCC}) - C_t(\text{I.C, HCC})]$.

Statistical analysis

Analysis of data was performed using SPSS v.21. The data were expressed using descriptive statistics (mean \pm standard deviation) and were analyzed using the *t* test. Comparisons between the two groups were performed using chi-square (χ^2). We tested the association between the expression profile of circANKRD36 and *ANKRD36* with UACR and other studied variables in patients with CKD and detected the independent variables by linear regression. Receiver operating characteristics (ROC) tested the diagnostic powers of circANKRD36 and its target gene *ANKRD36* levels among studied subjects.

Results

Study was conducted on 60 Egyptian patients with T2DM—42 were females (70%), 18 were males (30%), and their mean age was 43.63 ± 12.17 years—in addition to 40 healthy subjects as control—25 were females (62.5%), 15 were males (37.5%), and their mean age was 41.55 ± 10.45 years. The diabetic and control groups were matched for age, sex, and smoking. According to the current study results, patients with T2DM had

Table 1 Primers of circANKRD36 or ANKRD36

Gene	Forward primer(5'-3')	Reverse primer (5'-3')
circANKRD36	GGAGGCCACAAGTGATGAGA	CCTGGTGGTTTCTCAGAAGAC
ANKRD36	CGAGGAAGATTCTGTTTGGGT	CCTTCTCATCACTTGTGGCC
β -actin	TTCCTTCTGGGCATGGA	GAGGAGCAATGATCTTGA

higher values of metabolic risk factors and impairment of kidney function compared to the control group as shown in Table 2, $p < 0.001^*$.

General characteristics of diabetic patients

The results revealed that 38 patients had normal UACR, 15 patients had microalbuminuria, and 7 patients had macroalbuminuria. Thus, about 36.7% of patients with T2DM had DN. There was a statistically significant long duration of diabetes as well as higher values of BMI, waist/hip ratio, diastolic blood pressure, TG, and UACR in patients with macroalbuminuria compared to other subgroups. Regarding diabetic treatment and diabetic vascular complications, there was a statistically significant difference between the studied groups, $p < 0.001^*$, Table 3.

Relative expression of circANKRD36 and mRNA expression level of ANKRD36 in the studied groups

The results of the current study show significantly higher values of circANKRD36 relative expression level in the T2DM group compared to the control group (0.219 ± 0.061 , vs 0.111 ± 0.013 ; $p < 0.001$). Regarding the mRNA expression level of ANKRD36, it was higher in

the T2DM than that in the control group (0.006454 ± 0.0018 , vs. 0.0033 ± 0.004 , $p < 0.001$), Table 2.

Relative expression of circANKRD36 and mRNA expression level of ANKRD36 in patients with T2DM

Our results show that patients with macroalbuminuria (0.2316 ± 0.096) had statistically significantly higher values of the relative expression level of circANKRD36 compared to patients with microalbuminuria (0.1347 ± 0.032) and patients with normoalbuminuria (0.1261 ± 0.018), Fig. 1a, $p < 0.01$. Regarding the mRNA expression level of ANKRD36, patients with macroalbuminuria (0.0086 ± 0.0035) had statistically significantly higher values of the relative expression level of circANKRD36 compared to patients with microalbuminuria (0.0037 ± 0.0008) and patients with normoalbuminuria (0.0027 ± 0.0004), Fig. 1b, $p < 0.01$.

Correlations between relative expression of circANKRD36 and the mRNA expression level of ANKRD36 with clinical and laboratory characteristics in patients with CKD

The current study revealed that the relative expression of circANKRD36 and the mRNA expression level of ANKRD36 were significantly positively correlated with

Table 2 Anthropometric and biochemical characteristics of the studied groups

Variables	Control group (n =40)	T2DM patients (n =60)	P value
Body mass index (kg/m ²)	22.18 \pm 1.289	36.03 \pm 4.26	$< 0.001^*$
Waist/hip ratio	0.864 \pm 0.121	1.17 \pm 0.31	$< 0.001^*$
Systolic blood pressure	117.5 \pm 8.40	149.7 \pm 20.54	$< 0.001^*$
Diastolic blood pressure	75.6 \pm 4.589	92.92 \pm 14.33	$< 0.001^*$
Total cholesterol (mg/dl)	184.3 \pm 19.90	222.88 \pm 28.1	$< 0.001^*$
Triglycerides (mg/dl)	175.26 \pm 31.019	276.16 \pm 58.6	$< 0.001^*$
LDL cholesterol (mg/dl)	100.08 \pm 23.067	124.91 \pm 32.4	$< 0.001^*$
HDL cholesterol (mg/dl)	56.48 \pm 4.87	35.25 \pm 5.63	$< 0.001^*$
FPG (mg/dl)	89.72 \pm 6.304	220.97 \pm 30.04	$< 0.001^*$
HbA1c (%)	5.63 \pm 0.624	9.59 \pm 2.206	$< 0.001^*$
eGFR (mL/min)	93.37 \pm 7.56	76.19 \pm 13.2	$< 0.001^*$
Serum creatinine (mg/dl)	0.916 \pm 0.27	1.46 \pm 0.48	$< 0.001^*$
UACR (mg/g)	20.72 \pm 2.304	122.97 \pm 103.04	$< 0.001^*$
Relative expression of circANKRD36	0.111 \pm 0.013	0.219 \pm 0.061	$< 0.001^*$
mRNA expression level of ANKRD36	0.0033 \pm 0.004	0.006454 \pm 0.0018	$< 0.001^*$

T2DM Type 2 diabetes mellitus, FPG Fasting plasma glucose, HbA1c Hemoglobin, A1c Estimated glomerular filtration rate, UACR Urine albumin: creatinine ratio, circANKRD36 Circular ankyrin repeat domain 36

* $P < 0.05$

Table 3 Laboratory and anthropometric parameters of diabetic patients

Variables	Normal (n=38)	Microalbuminuria (n=15)	Macroalbuminuria (n=7)	P1	P2	P3
Duration of diabetes (years)	8.864 ± 3.1	10.87 ± 3.31	15.17 ± 6.	ˆ0.001*	ˆ0.001*	ˆ0.001*
Body mass index (kg/m ²)	35.18 ± 1.289	37.4 ± 4.3	38.2 ± 4.26	ˆ0.001*	ˆ0.001*	0.891
Waist/hip ratio	0.964 ± 0.121	1.27 ± 0.31	1.27 ± 0.31	ˆ0.001*	ˆ0.001*	ˆ0.001*
Systolic blood pressure	140.5 ± 8.43	145.7 ± 12.2	140.7 ± 14.3	0.878	0.943	0.970
Diastolic blood pressure	85.6 ± 4.58	99.9 ± 14.3	105.9 ± 14.3	ˆ0.001*	ˆ0.001*	0.839
Total cholesterol (mg/dl)	216.3 ± 19.9	232.8 ± 25.1	234.8 ± 48.1	ˆ0.001*	0.128	0.914
Triglycerides (mg/dl)	175.3 ± 31.1	266.4 ± 37.6	285.3 ± 44.6	ˆ0.001*	ˆ0.001*	0.800
LDL cholesterol (mg/dl)	111.1 ± 26.2	114.5 ± 36.4	130.9 ± 37.4	0.093	0.135	0.824
HDL cholesterol (mg/dl)	36.9 ± 4.87	32.25 ± 1.5	31.25 ± 3.4	ˆ0.001*	0.055	0.694
FPG (mg/dl)	227.1 ± 16.3	231.9 ± 31.4	265.9 ± 41.3	0.077	0.886	0.289
HbA1c (%)	8.63 ± 1.4	9.59 ± 2.3	10.59 ± 1.5	ˆ0.001*	0.222	0.447
eGFR (mL/min)	93.37 ± 7.5	76.19 ± 13.2	76.19 ± 13.2	ˆ0.001*	0.135	0.772
Serum creatinine (mg/dl)	1.216 ± 0.27	1.46 ± 0.9	2.36 ± 1.2	0.250	ˆ0.001*	ˆ0.001*
UACR (mg/g)	25.7 ± 3.3	183.97 ± 58.1	358.7 ± 94.3	ˆ0.001*	ˆ0.001*	ˆ0.001*
Medication						
Diet	8 (21.1%)	7 (46.7%)	6 (85.7%)	ˆ0.001*	ˆ0.001*	ˆ0.001*
Oral	8 (21.1%)	7 (46.7%)	5 (71.4%)	ˆ0.001*	ˆ0.001*	ˆ0.001*
Insulin	19 (50%)	9 (60%)	5 (71.4%)	ˆ0.001*	ˆ0.001*	ˆ0.001*
Vascular complications						
Retinopathy	14 (36.8%)	8 (53.3%)	7 (100%)	ˆ0.001*	ˆ0.001*	ˆ0.001*
Neuropathy	20 (52.6%)	10 (66.7%)	5 (71.4%)	ˆ0.001*	ˆ0.001*	ˆ0.001*
Stroke	6 (15.8%)	8 (53.3%)	6 (85.7%)	ˆ0.001*	ˆ0.001*	ˆ0.001*
CHD	14 (36.8%)	8 (53.3%)	7 (100%)	ˆ0.001*	ˆ0.001*	ˆ0.001*

T2DM Type 2 diabetes mellitus, FPG Fasting plasma glucose, HbA1c Hemoglobin, A1c Estimated glomerular filtration rate, UACR Urinary albumin-creatinine ratio, circANKRD36 Circular ankyrin repeat domain 36

* $P < 0.05$

duration of diabetes, diastolic blood pressure, TG, LDL, FPG, HbA1c, creatinine, and UACR and were significantly negatively correlated with eGFR, $p < 0.001$, Table 4.

Linear regression analysis in patients with CKD

A linear regression analysis test was done to assess the main independent parameters associated with the relative expression of circANKRD36 and the mRNA expression level of ANKRD36. Our results showed that eGFR as well as UACR were independently correlated with both epigenetic biomarkers, $p < 0.001$, Table 5.

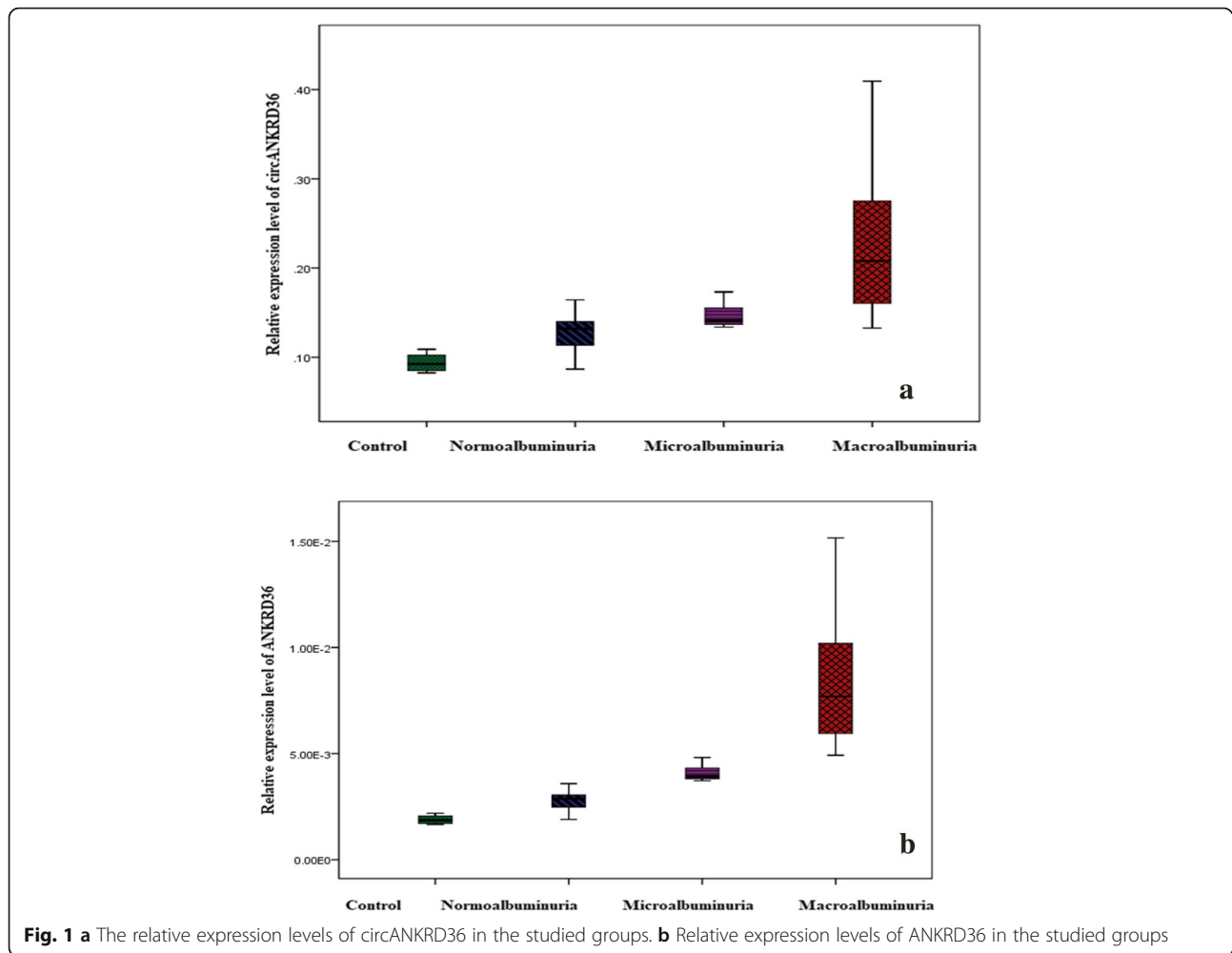
Accuracy of relative expression of circANKRD36 and mRNA expression level of ANKRD36 for discriminating patients with T2DM from the control group by ROC analysis

We investigated the potential diagnostic value of the relative expression of circANKRD36 (Fig. 2a) and the mRNA expression level of ANKRD36 (Fig. 2b) by the ROC test. When we discriminate patients with T2DM from the control group, the cutoff values were 0.142 and 0.0048, respectively, and the AUC was 0.932

(95% CI = 0.875–0.989) and 0.945 (95% CI = 0.887–0.992); additionally, the sensitivities and the specificities were 90% and 93% for circANKRD36 and 88.3% and 87.5% for the mRNA expression level of ANKRD36, respectively.

Accuracy of relative expression of circANKRD36 and the mRNA expression level of ANKRD36 for discriminating patients with microalbuminuria from patients with normoalbuminuria by ROC analysis

We further investigated the potential diagnostic value of the relative expression of circANKRD36 (Fig. 3a) and the mRNA expression level of ANKRD36 (Fig. 3b) by the ROC test. When we discriminate patients with microalbuminuria from patients with normal UACR group, the cutoff values were 0.1338 and 0.0054, respectively. The AUC was 0.762 (95% CI = 0.615–0.909) and 0.895 (95% CI = 0.7609–1.000). Additionally, the sensitivities and the specificities were 81.8% and 69.4% for circANKRD36 and 85.7% and 87.7% for the mRNA expression level of ANKRD36, respectively, $p < 0.001$.



Accuracy of relative expression of circANKRD36 and mRNA expression level of ANKRD36 for discriminating patients with macroalbuminuria from patients with microalbuminuria by ROC analysis

The relative expression of circANKRD36 (Fig. 4a) and the mRNA expression level of ANKRD36 (Fig. 4b) were done by the ROC test. When we discriminate patients with microalbuminuria from patients with normal UACR group, the cutoff values were 0.1782 and 0.0064, respectively. The AUC was 0.819 (95% CI =0.561–1.000) and 0.781(95% CI =0.611–0.909). In addition, the sensitivities and the specificities were 80.1% and 80.7% for circANKRD36 and 85.7% and 87.7% for the mRNA expression level of ANKRD36, respectively, $p < 0.001$.

Discussion

Diabetes is a common chronic metabolic disease. Mounting evidence has elaborated that the major causes of morbidity and mortality in the diabetic population are retinopathy, nephropathy, cardiomyopathy, neuropathy, and atherosclerosis. These observations suggested that

the macro- and microvascular affections are key features of such complications [15]. Growing evidence has illustrated that low-grade inflammatory changes associated with increased production of inflammatory mediators may be a key mechanism in the pathogenesis of chronic diabetic complications [16].

In the present study, we observed that out of sixty Egyptian patients with T2DM, twenty-two patients had DN with microalbuminuria ($n=15$) and seven patients had macroalbuminuria. Thus, about 36.7 % of patients with T2 diabetes mellitus had DN.

Similar to our result, a study conducted by Zelmanovitz et al. observed that about 30% of patients with T2DM develop clinically overt nephropathy [17]. DN is a multifactorial disorder that results from the interaction between environmental and genetic factors. Hyperglycemia, hypertension, and proteinuria are the main insults that cause structural abnormalities in a DN [18].

According to Elnajjar et al. study, about 78% of all the patients studied had DN. This study was conducted on both patients with T1DM and T2DM. Thus, the high

Table 4 Correlations between relative expression of circANKRD36 and the mRNA expression level of ANKRD36 with clinical and laboratory characteristics in patients with CKD

Variables	circANKRD36		ANKRD36	
	r	p	r	p
Duration of diabetes (years)	0.460	*0.001*	0.511	<0.001*
Body mass index (kg/m ²)	0.246	0.058	0.044	0.741
Waist/hip ratio	0.139	0.290	0.102	0.440
Systolic blood pressure	0.207	0.112	0.056	0.666
Diastolic blood pressure	0.282	*0.001*	0.380	*0.001*
Total cholesterol (mg/dl)	0.117	0.372	0.180	0.058
Triglycerides (mg/dl)	0.383	*0.001*	0.346	*0.001*
LDL cholesterol (mg/dl)	0.371	*0.001*	0.453	<0.001*
HDL cholesterol (mg/dl)	-0.039	0.770	-0.081	0.537
FPG (mg/dl)	0.459	*0.001*	0.522	*0.001*
HbA1c (%)	0.345	*0.001*	0.508	*0.001*
eGFR (mL/min)	-0.484	*0.001*	-0.654	*0.001*
Serum creatinine (mg/dl)	0.567	*0.001*	0.493	<0.001*
UACR (mg/g)	0.589	*0.001*	0.840	<0.001*

T2DM Type 2 diabetes mellitus, FPG Fasting plasma glucose, HbA1c Hemoglobin, A1c Estimated glomerular filtration rate, UACR Urinary albumin-creatinine ratio, circANKRD36 Circular ankyrin repeat domain 36 *P < 0.05

prevalence of DN among their studied groups could be due to the inclusion of patients with T1DM [19]. However, in our study, we enrolled results in patients with T2DM only. In most type 2 diabetics, the prevalence of DN ranges from 30 to 50% [20].

Our study revealed clear evidence that in patients with macroalbuminuria, there was a statistically significant long duration of diabetes as well as higher values of

BMI, waist/hip ratio, diastolic blood pressure, TG, and UACR compared to patients with normoalbuminuria and microalbuminuria.

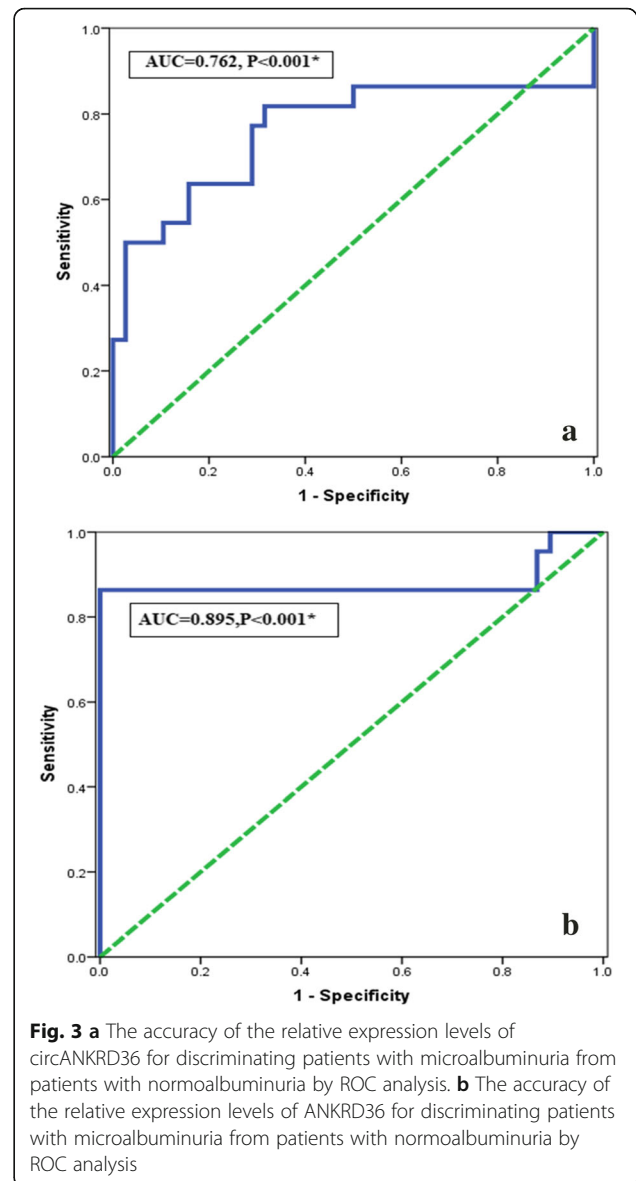
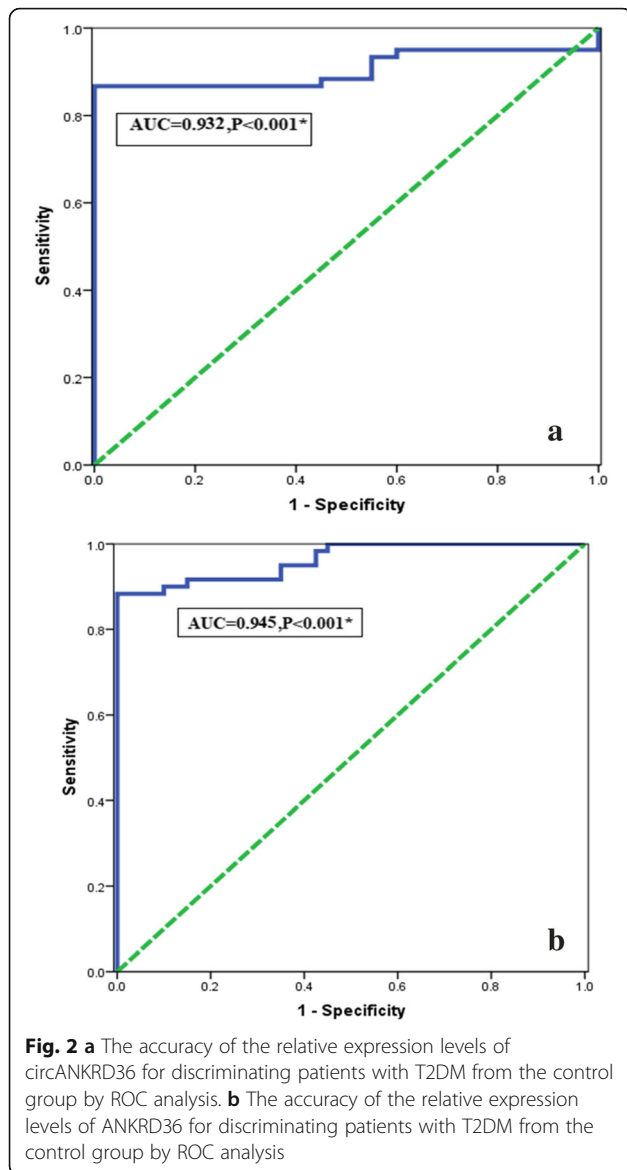
Similar to our results, a study conducted by Elnajjar et al. revealed that there were statistically significant long duration of diabetes, higher systemic blood pressures, an evident decrease in eGFR, poor glycemic control, and obesity in patients with DN compared to patients without DN [21]. In agreement with our results, Kathryn et al. observed that there were significant correlations between the increasing duration of diabetes and the development of microproteinuria [22].

Regarding glycemic control, it was notable that in our observation patients with microalbuminuria had higher HbA1c values compared to patients with normoalbuminuria levels. Similar results were described in the ADVANCE Collaborative Group [22]. Nevertheless, the Veteran’s Affairs Diabetes Trial (VADT) observed that intensive therapy did not lead to a reduction in retinopathy or major nephropathy outcomes compared with standard therapy, but this study was for a short duration [23]. Mounting evidence has elaborated that BMI values were higher in patients with DN [18, 24, 25].

Despite the limited knowledge on the molecular mechanisms underlying the pathophysiology of microvascular complications of T2DM in particular CKD, emerging scientific evidence has indicated that the dysregulated epigenetic is associated with risk and progression of diabetic microvascular complications and could be used as predictors and diagnostic genetic markers of T2DM and CKD.

Table 5 linear regression analyses to test the influence of the main independent variables against relative expression of circANKRD36 and the mRNA expression level of ANKRD36 levels (dependent variable) in patients with CKD

Model	Unstandardized coefficients		Standardized coefficients	t	P	95% C.I.	
	B	SE				Beta	Lower bound
CircANKRD36							
(Constant)	0.009	0.058		0.151	0.881	-0.108	0.0126
eGFR	0.042	.013	0.406	3.288	0.002	0.016	0.068
S. creatinine	0.000	0.000	0.054	0.514	0.610	-0.001	0.001
UACR	0.000	0.000	0.440	3.336	0.002	0.000	0.000
TG	0.001	0.001	0.211	1.034	0.306	-0.001	0.002
ANKRD36							
(Constant)	-0.001	0.002		-0.636	0.528	0.006	0.003
eGFR	0.002	0.000	0.385	3.817	0.000	0.001	0.003
S. creatinine	-1.100E-6	0.000	-0.006	-0.075	0.941	0.000	0.000
UACR	1.012E-5	0.000	0.555	5.144	0.000	0.000	0.000
HbA1c	2.228E-5	0.000	0.151	0.907	0.369	0.000	0.000
TG	0.002	0.002	0.245	1.025	0.310	-0.002	0.007

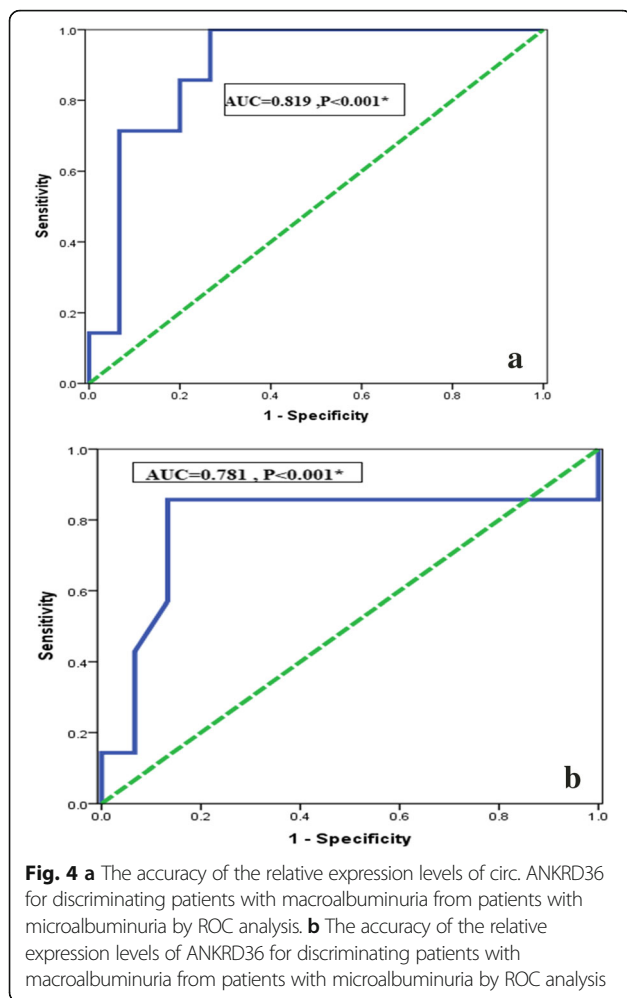


Emerging evidence demonstrated that about 30% of patients with T2DM develop clinically overt nephropathy. Consequently, early detection of DN is very useful for preventing progression to renal failure. Due to the high stability of ncRNAs in body fluids, they could be used as a noninvasive diagnostic biomarker for DN. Thus, the current study aimed to investigate the expression profile levels of circANKRD36 and *ANKRD36* in Egyptian patients with T2DM and DN and to explore their associations with the risk and the progression of DN.

The results presented herein are innovative as this study performs a robust estimation of the expression levels of circANKRD36 and *ANKRD36* in patients with T2DM in particular patients with DN. Expression of circANKRD36 and *ANKRD36* were significantly higher in patients with T2DM

compared to healthy control. Intriguingly, patients with macroalbuminuria had statistically significantly higher values of the relative expression level of circANKRD36 and *ANKRD36* compared to patients with microalbuminuria and patients with normoalbuminuria levels.

In agreement with the present study, Fang et al. confirmed that the expression level of circANKRD36 is upregulated in patients with T2DM. Moreover, the level of *ANKRD36* which is the host gene of circANKRD36 was upregulated in the T2DM group compared to the general population [26]. Previously, it was shown that *ANKRD36* is the host gene of circANKRD36 and the level was increased in the T2DM group as the circ-RNAs can positively regulate their parent genes at the transcriptional level and post-transcriptional level [27–29].



The current study revealed that the relative expression of circANKRD36 and mRNA expression levels of *ANKRD36* were positively correlated with duration of diabetes, diastolic blood pressure, TG, LDL, FPG, HbA1c, creatinine, and UACR and were significantly negatively correlated with eGFR. Currently, several pieces of evidence exist, demonstrating that miRNAs are involved in the regulation of vascular inflammation [30]. Furthermore, it has been also proposed that circRNAs influence the cardiometabolic risk factors which have a deleterious effect on blood vessels and can lead to the development of endothelial dysfunction and hence diabetic vascular complications [31].

Until the time of writing this article, only one experimental study was conducted to illuminate the roles of circRNA in CKD progression. In this study, circRNA_15698 upregulates *TGFB1* expression and promotes ECM protein production. Thus, the inhibition of circRNA_15698 could delay the progression of CKD [32].

The current study aims to assess the correlations between circANKRD36 and *ANKRD36* relative expression in patients with DN. We observed that, there were

significant positive correlations between the levels of both genes and duration of diabetes, diastolic blood pressure, TG, LDL, FPG, HbA1c, creatinine, and UACR. However, there were significant negative correlations between both epigenetics and eGFR. A linear regression test was done to investigate the main independent parameters associated with the relative expression of circANKRD36 and the mRNA expression level of *ANKRD36*, and we observed that eGFR as well as UACR were independently correlated with both epigenetic biomarkers. Similar results were observed in a significant positive correlation between circANKRD36 and blood glucose as well as HbA1c [26].

ROC analysis was used to clarify the diagnostic power of circANKRD36 and the mRNA expression level of *ANKRD36* for discriminating patients with T2DM from the control group. Our results revealed that the sensitivities and the specificities were 90% and 93% for circANKRD36 besides 88.3% and 87.5% for the mRNA expression level of *ANKRD36*. As regards, the differentiation of patients with microalbuminuria from patients with normoalbuminuria, the sensitivities and the specificities were 81.8% and 69.4% for circANKRD36 as well as 85.7% and 87.7% for the mRNA expression level of *ANKRD36*, respectively. We attempted to discriminate patients with macroalbuminuria from patients with microalbuminuria, the sensitivities, and the specificities were 80.1% and 80.7% for circANKRD36 in addition 85.7% and 87.7% for the mRNA expression level of *ANKRD36*, respectively.

Conclusion

The current study revealed that about 36.7% of patients with T2DM had DN. The relative expression levels of circANKRD36 and *ANKRD36* were significantly higher in patients with T2DM and DN more specifically in patients with macroalbuminuria.

Abbreviations

BMI: Body mass index; CircANKRD36: Circ. ankyrin repeat domain36; CircRNAs: Circular RNAs; CKD: Chronic kidney disease; DM: Diabetes mellitus; DN: Diabetic nephropathy; eGFR: Estimated glomerular filtration rate; FPG: Fasting plasma glucose; NcRNAs: Noncoding RNAs; RT-qPCR: Real-time quantitative PCR; TC: Total cholesterol; TG: Triglycerides; T2DM: Type 2 diabetes mellitus; UACR: Urine albumin: creatinine ratio

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Authors' contributions

NMR and MHS collected patients' samples and clinical data. ASE and MAEA prepared the sample for laboratory investigations. NMR wrote the paper. Statistical analysis, interpretation of data, and preparation of the paper for international submission were done by NMR. Critical revision of the manuscript was performed by all of the authors. The authors have read and approved the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author (nrashad78@yahoo.com) upon reasonable request.

Declarations**Ethics approval and consent to participate**

The enrolled subjects accepted their participation in the study and gave their written consent. The study protocol was approved by the Ethics Committee of Faculty of Medicine, Zagazig University, and the reference number was IRB (Ethics number. 5144).

Consent for publication

The authors declare that they received consents for publication from all the participants.

Competing interests

The authors declare that they have no competing interests.

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