The Expression Profile of Circular ANKRD36 as a Genetic Biomarker for Type 2 Diabetes Mellitus and Related Cardiometabolic Risk Factors

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Abstract
Background: Circular RNAs (circRNAs) are a class of non-coding RNAs (ncRNAs) with covalently closed single-stranded structures. The epigenetic regulatory role of ncRNAs in diabetes-associated cardiometabolic diseases is still evolving. This study aimed to investigate whether the relative expression level of circ. ankyrin repeat domain 36 (circANKRD36) are altered in Egyptian patients with T2DM and whether they correlated with other cardiometabolic risk factors.

Methods: A case-controlled study was conducted on forty patients with T2DM, and 20 healthy subjects were included in the study. Diagnosis of T2DM was based on ADA Criteria. The expression profile of circANKRD36 was measured using quantitative real-time (qRT) PCR.

Results: The relative expression levels of circANKRD36 were significantly increased in patients with T2DM compared to control. p<0.001*. The relative expression levels of circANKRD36 were significantly positively correlated with cardiometabolic risk factors and linear regression test revealed that hba1c as well as BMI, were independently correlated with epigenetic markers. Our results revealed that the sensitivities and the specificities of circANKRD36 expression levels to diagnosis of T2DM were 87% and 99.15.

Conclusion: The relative expression levels of circANKRD36 were significantly increased in patients with T2DM. Also, the relative expression levels of circANKRD36 were significantly positively correlated to cardiometabolic risk factors.

Keywords: T2DM; circANKRD36; gene expression, cardiometabolic r
Introduction

Globally, non-communicable diseases (NCDs) are expected to contribute to over three quarters of all deaths in 2030, one of the most important NCDs is diabetes mellitus (DM) [1]. Worldwide, the number of individuals with DM is expected to rise by 48%, from 425 million in 2017 to 629 million by 2045 [2].

Diabetes is a metabolic disorder characterized primarily by chronic hyperglycemia, which results from the inability of pancreas to produce and/or secrete enough insulin and/or resistance to insulin in the peripheral tissues [3]. Recent studies explored that the pathogenesis of T2DM is complex, and the chronic hyperglycemia leads to changes in gene expression. Epigenetic modification factors play a very important role in diabetes mellitus, and these modifications can be passed from one organism to its progeny. Epigenetic factor encompasses cytosine methylation, histone modification and noncoding RNAs (ncRNAs) [4].

CircRNA, a type of closed circular RNA molecule belongs to the ever-growing class of naturally occurring ncRNAs and mostly are derived from precursor-mRNA by back-splicing which is a non-canonical event. CircRNAs are mainly formed from exons or introns, by two kinds of splicing, exons reverse splicing of exons and introns. They are well-preserved, broadly distributed in eukaryotes and highly stable [5]. CircRNAs are actively involved in the development of many diseases like diabetes and their mechanism of action is an important area, and their differential expression plays an important role in the disease development. In initiation and progression of diabetes a number of circRNAs, i.e., circRNA_0054633, circHIPK3, circANKRD36 and circRNA11783-2 have been observed to be involved. [6] circANKRD36 has been reported to be related to inflammatory response in type 2 diabetes. In the present study, we examined whether circANKRD36 relative expression levels are altered in Egyptian patients with T2DM and whether they correlate with other cardiometabolic risk factors.

2. Subjects and methods

2.1. Subjects

Forty patients with T2DM and 20 healthy subjects were included in the study. Diagnosis of T2DM was based on ADA Criteria [7]. (Fasting plasma glucose > 7 mmol/L, 2 hours’ glucose challenge plasma glucose > 11.1 mmol/L or HbA1c > 6.5%) a, 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. 6321). All enrolled subjects underwent full history taking and clinical examination with special concern to other comorbidities including hypertension, obesity, ischemic heart disease, current medications, duration of diabetes, and other diabetic complications. We calculated the body mass index (BMI). The exclusion criteria were for any patients with chronic liver diseases, autoimmune diseases, inflammatory diseases, and infections.
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 [8].

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 total RNA. The RNA quality was quantified by A260 using UV/spectrophotometer. RT-PCR technique was applied by using StepOnePlusTM System (Applied Biosystems Inc., USA). RT-PCR was done in 20 μl as a final volume containing cDNA (5μl), 10 μl of 1X Quantitectsybr green with low ROX PCR master mix (Cat.No. RT500S; Enzynomics, Republic of Korea), pmol/ml each primer (0.5 μl for each) of circANKRD36 or ANKRD36 and was completed by 4μl DdH2O according to the manufacturer’s instructions. The primers of circANKRD36 was designed by Primer 3 (http://www-genome.ut.ee/) and according to previous research [9] their sequences are listed in this 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−ΔΔCt equation relation to β-actin as internal control genes. The Ct (threshold cycle) is a relative measure of the concentration of the target in the PCR reaction.

\[ ΔΔCT = [CT(target,normal)−CT(I.C,normal)]−[CT(target,HCC)− CT(I.C,HCC)] \]

Statistical analysis

Analysis of data was performed using SPSS v.26. The data were expressed using descriptive statistics (mean ± standard deviation) and were analyzed using the "t" test. The correlation between the relative expression level of circANKRD36 and other studied parameter were assessed by Pearson correlation test. We tested the association between the relative expression level of circANKRD36 and other studied variables in patients with T2DM and detected the independent variables by linear regression. Receiver operating characteristics (ROC) tested the diagnostic powers of circANKRD36 levels among studied subjects.

3. Results

The diabetic and control groups were matched for age and sex. Patients with T2DM (20 males and 20 females) had higher values of metabolic risk factors compared to the control group (9 males and 11 females). The metabolic risk factors for example systolic blood pressure, diastolic blood pressure, BMI, waist/hip ratio, total cholesterol and...
triglycerides, fasting plasma glucose, HbA1c, HDL cholesterol, and LDL cholesterol. p <0.001 as shown in table 2. Compared to the control group, p < 0.001.

**The relative expression of circANKRD36 level in the studied groups.**

Our results show that diabetic patients had statistically significant higher values of the relative expression level of circANKRD36 compared to the control group (0.519±0.262 vs 1.71±0.513, p <0.001*, as shown in table 2.

**Correlations between relative expression of circANKRD36 in patients with T2DM**

Our results demonstrated that the relative expression of circANKRD36 was significantly positively correlated with systolic blood pressure, diastolic blood pressure, BMI, total cholesterol and triglycerides, fasting plasma glucose, and HbA1c (P<0.001), Table 3.

**Linear regression analyses in patients with T2DM.**

Linear regression analysis test was done to assess the main independent parameters associated with relative expression of circANKRD36 levels. Our results showed that hba1c as well as BMI, were independently correlated with both epigenetic biomarkers, P<0.001, Table 4.

**The accuracy of relative expression of circANKRD36 for discriminating patients with T2DM from the control group by ROC analysis.**

We investigated the potential diagnostic value of the relative expression of circANKRD36 by the ROC test. When we discriminate patients with T2DM from the control group, the cutoff values were 1.103 and the AUC was 0.997 (95% CI =0.988-1.000) additionally, the sensitivities and the specificities were 87% and 99.1%, P<0.001, figure 1.

**Table 1:** The primer sequences of circANKRD36 and ANKRD36

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>circANKRD3</td>
<td>GGAGGCCACAAGTGATGAG</td>
<td>CCTGGTGTTTCTCAGAAGAC</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>TTCTTTCTGGCGATGGA</td>
<td>GAGGAGCAATGATCTTTGA</td>
</tr>
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</table>

**Table 2:** Anthropometric and biochemical characteristics of the studied groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group (n =20)</th>
<th>Patients with T2DM (n =40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.18±1.189</td>
<td>37.03±4.96</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.864±0.011</td>
<td>1.07±0.21</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>117.8±9.40</td>
<td>148.8±19.44</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Variables</td>
<td>circANKRD36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.583</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.615</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.613</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>0.764</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>LDL-c</td>
<td>0.525</td>
<td>0.01*</td>
<td></td>
</tr>
<tr>
<td>HDL-c</td>
<td>-0.022</td>
<td>0.885</td>
<td></td>
</tr>
<tr>
<td>FPG</td>
<td>0.900</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.882</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
</tbody>
</table>

* Significant P value (P < 0.05).

Figure (1): The accuracy of the relative expression levels of circANKRD36 for discriminating patients with T2DM from control group by ROC analysis.
Table 4: Linear regression analyses to test the influence of the main independent variables against relative expression of circANKRD36 level (dependent variable) in patients with T2DM

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>t</th>
<th>p</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE</td>
<td>Beta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>0.013</td>
<td>0.013</td>
<td>0.95</td>
<td>5</td>
<td>0.349</td>
</tr>
<tr>
<td>CircANKRD36</td>
<td>0.012</td>
<td>0.001</td>
<td>0.178</td>
<td>3.62</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMI</td>
<td>3.359</td>
<td>0.006</td>
<td>0.000</td>
<td>.010</td>
<td>0.99</td>
</tr>
<tr>
<td>TG</td>
<td>0.001</td>
<td>0.007</td>
<td>0.958</td>
<td>37.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FPG</td>
<td>6.572</td>
<td>0.003</td>
<td>0.012</td>
<td>0.21</td>
<td>0.832</td>
</tr>
</tbody>
</table>

4. Discussion

Previous numerous studies have demonstrated that T2DM has reached epidemic proportions worldwide, and its prevalence is rising [1]. In addition, the advanced stages of T2DM, patients often experience various complications. Therefore, early diagnosis and intervention are immediately needed. However, current diagnostic methods show various inefficiencies markers for the early diagnosis of T2DM. To improve this situation, researchers have explored the association between genetic variants and early-onset T2DM [10,11].

Despite the limited knowledge on the molecular mechanisms underlying the pathophysiology of T2DM growing evidence highlights the complex disease etiologies and the most important of which is chronic hyperglycemia, which may contribute to its pathogenesis by causing changes in gene expression, inflammation, and oxidative stress [15]. One of the most important functions of circRNAs is their role as “miRNA sponges,” which competitively bind miRNAs to generate post-transcriptional regulation, the early stages of T2DM, most patients are asymptomatic, and they rarely visit hospitals to seek diagnosis and therapy. To facilitate early detection of T2DM, we investigated the
expression profile of circANKRD36, and its target gene levels in Egyptian patients for early detection of T2DM and explored their associations with cardiometabolic risk factors.

Our study revealed clear evidence that in patients with T2DM, there were higher values of systolic and diastolic blood pressure as well as BMI, waist/hip ratio, TC, LDL, TG, FPG, and HbA1c, compared to control group. In agreement with our results, Dyck et al. study observed that higher level of HbA1c in patients with T2DM compared to general populations [12]. In contrast, a study by Tapp et al. found a non-significant association between waist circumference and diabetes [13].

Even Though the state of the present knowledge of circRNAs biology is at a very early stage, mounting evidence points to their role as master regulators of gene expression in many diseases including metabolic disorders. In line with this observation, a growing number of studies revealed the dysregulation of circRNAs in association with the pathophysiology of several diseases such as diabetes, hypertension, cardiovascular diseases (CVD), and other metabolic perturbations [14-16].

The results presented here are innovative as this study performs a robust estimation of the expression of circANKRD36 in patients with T2DM in comparison with healthy control for early detection of T2DM. We found that the expression levels of circANKRD36 were significantly increased in patients with T2DM compared to healthy control, with a positive correlation between the levels of epigones and cardiometabolic risk factors. For example, obesity and hyperglycemia.

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 [17]. Previously, it was shown that ANKRD36 is the host gene of circANKRD36, and the level was upregulated in the T2DM group as the circRNAs can positively regulate their parent genes at the transcriptional level and post-transcriptional level [18-20].

Cardiometabolic risk factor for example obesity has also been reportedly linked with epigenetic modulation of genes and protein cascades in insulin signaling [21,22]. Furthermore, altered expression of lncRNAs has been associated with poor glycemic control, IR, accelerated cellular senescence, and inflammation in diabetes patients [23].

Up to now, no studies have specifically exploited the role of circRNAs in obesity-IR-T2DM settings, but this scenario will likely change in the future as alterations in many circRNAs in association with IR have been noticed. For instance, circHIPK3 has been shown to contribute to hyperglycemia and IR via sponging miR-192-5p and upregulating FOXO1 [24].

For further evaluation of our results, we performed a linear regression test to investigate the main independent parameters associated with the relative expression of
circANKRD36 levels and we observed that hba1c as well as BMI, were independently correlated with both epigenetic biomarkers. Similar results were observed a significant positive correlation between circANKRD36 and blood glucose as well as Hba1c [17].

To better elucidate the diagnostic power of circANKRD36 for discriminating patients with T2DM from the control group by using ROC analysis. Our results revealed that the sensitivities and the specificities were 87% and 99.15.

**In conclusion,**
The relative expression levels of circANKRD36 were significantly elevated in patients with T2DM compared to control groups. There were significantly positive correlations between the relative expression levels of circANKRD36 and cardiometabolic risk factors.

**Declarations**

**Ethics approval and consent to participate.**
Written informed consent was taken from all of the participants after explaining details and benefits as well as risks to them. The ethical committee of Faculties of Medicine, Zagazig University approved the current study.

**Consent for publication:** The authors declare that they received consent for publication from all the participants.

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

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**References**


