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The Association Between Circulating Levels of miRNA-181a and Pancreatic Beta Cells Dysfunction via SMAD7 in Type 1 Diabetic Children and Adolescents

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Background: miRNA-181a has been implicated in autoimmunity and apoptosis. Therefore, this study was conducted to explore its possible role in pancreatic beta-cells dysfunction. *Methods:* miRNA-181a expression was evaluated by real-time PCR in serum of 40 type 1 diabetic children and adolescents and 40 age- and gender-matched healthy controls. *Results:* miRNA-181a expression was significantly higher in diabetic children and adolescents and it was negatively correlated to fasting C-peptide and SMAD7 levels. *Conclusion:* miRNA-181a appears to play a potential role in pancreatic beta-cells dysfunction via SMAD7. J.Clin. Lab. Anal. **30**:727–731, 2016. © 2016 Wiley Periodicals, Inc.

Key words: C-peptide; miRNA; miRNA-181a; SMAD7; T1DM

INTRODUCTION

Diabetes mellitus is considered as the most common endocrine–metabolic disorder of childhood and adolescence. It is characterized by hyperglycemia that occurs when beta cells are unable to function properly or have been destroyed (1).

Type-1 diabetes mellitus (T1DM) is a chronic autoimmune disease that results from destruction of the beta cells of islets of Langerhans (2). In most western countries, T1DM accounts for over 90% of childhood and adolescent diabetes (3, 4). In Egypt, the estimated prevalence of T1DM in children and adolescents is 0.38/1,000 and the overall incidence is 3.5/100,000 (5).

Although autoimmunity is the predominant effector mechanism of T1DM, yet it may not be its primary cause (2) and it has been found that pancreatic beta cells death by apoptosis contributes significantly in T1DM (6).

In recent years, microRNAs (miRNAs), a family of short (average of 22 nucleotides long), naturally occurring, small antisense noncoding RNAs, have emerged as important posttranscriptional regulators of gene expression (3). They have since been discovered to be widely distributed, endogenous controllers of gene and protein expression by binding to the 3'-untranslated region of specific mRNAs and interfering with protein synthesis by inducing mRNA degradation or repressing translation (7, 8). Following the discovery of the first miRNA in *Caenorhabditis elegans* (9), the important roles of miRNAs in a variety of biological processes, including development, differentiation, apoptosis, lipid metabolism, and cancer have been discovered (10–13).

miRNA-181a is a member of miRNA-181 family. It has been found that miRNA-181a induced apoptosis through downregulation of the expression of anti-apoptotic proteins (14). Moreover, a recent study found that the downregulation of miRNA-181a significantly inhibited the H_2O_2-induced cellular apoptosis, reactive oxygen species (ROS) production, mitochondrial structure disruption, and activation of key signaling proteins in the mitochondrial apoptotic pathway (15).

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine regulating a variety of cellular processes such as apoptosis and immune response (16). It has been found that overexpression of SMAD7

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facilitated cell proliferation by antagonizing TGF-β-mediated antiproliferative gene responses and that most members of the TGF-β pathway are known to be targeted by one or more miRNAs (17).

Therefore, we conducted this study to evaluate the expression of miRNA-181a and to investigate the relation between miRNA-181a and the different clinical and laboratory parameters in type 1 diabetic children and adolescents. Additionally, the usefulness of microRNA-181a as a biomarker of residual pancreatic beta cells function was analyzed. The circulating levels of SMAD7 were also measured and correlated to the expression of miRNA-181a, aiming at clarifying the role of SMAD7 as a functional target of miRNA-181a in mediating pancreatic beta cells dysfunction.

MATERIALS AND METHODS

This study was conducted on 40 children and adolescents with uncomplicated T1DM recruited from Diabetes Clinic, Children's Hospital, Ain Shams University. They were 19 males (47.5%) and 21 females (52.5%). Their age ranged from 6.5 to 16 years with a mean age of 12.38 ± 2.75 years. Duration of diabetes ranged from 5 to 13 years (8.9 ± 2.3 years).

The control group consisted of 40 healthy children and adolescents matched in age and gender to the study group. They were 16 males (40.0%) and 24 females (60.0%). Their age ranged from 6 to 16 years with a mean age of 10.88 ± 3.23 years. All participants were subjected to history taking and thorough clinical examination.

This study has complied with the principles laid down in the Declaration of Helsinki, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, and recently amended at the 59th World Medical Assembly, Seoul, Korea, October 2008. The entire protocol was approved by institutional ethical committee. All parents or care givers provided signed informed consent for participation in the study as required.

Blood Sample Collection and Processing

Venous blood samples (5 ml) were aseptically withdrawn from all the participants after an overnight fasting for 12–14 h and divided into two portions as follows: 2.0 ml of blood was placed in an ethylenediaminetetraacetic acid (EDTA) containing tube for complete blood picture using coulter B66, Miami, Florida, and the determination of glycated hemoglobin (HbA1c) using Helena GLYCO-Tek affinity column method (Helena Laboratories, Beaumont, Texas). The remaining 3.0 ml of blood was used for separation of serum. The separated serum samples were kept frozen at −80°C until used in the determination of total cholesterol, HDL-cholesterol, and triglycerides according to the manufacturers' instructions of standard enzymatic kits (Randox Laboratories, Crumlin, UK), C-peptide according to the manufacturer's instructions of Immuno-Biological Laboratories (IBL) enzyme-linked immunosorbent assay (ELISA) kit (Immuno-Biological Laboratories, Inc., Minneapolis, MN), SMAD7 according to the manufacturer's instructions of Biocompare ELISA kit (Atlanta, GA), and miRNA-181a expression using quantitative reverse transcription-PCR. LDL-cholesterol levels calculated using Friedewald equation. Urinary albumin excretion (UAE) was determined in early morning fasting urine samples as albumin-to-creatinine ratio by an immuno-turbidimetric method performed on COBAS Integra 800 (Roche Diagnostics, Mannheim, Germany).

Quantitative Reverse Transcription PCR (qRT–PCR) of miRNA-181a

Total RNA was isolated using miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA). The miRNAs were reversely transcribed into cDNAs according to the manufacturer's instructions of miScript II RT Kit (Qiagen, Valencia, CA). Each reaction mixture of qRT–PCR contained 10 μl of RT product (normalized to 500 ng), 12.5 μl of SYBR GREEN PCR Master Mix, 0.5 μM of each primer, and deionized water to a total volume of 25 μl. Reactions were run with the following thermal cycling parameters: 95°C for 5 min followed by 35 cycles of 95°C, 57°C, and 75°C for 10, 30, and 30 sec, respectively. Each sample was normalized based on its endogenous U6 RNA content. Relative expression of miRNA-181a was presented as fold expression in relation to the control sample; the actual values were calculated using the $2^{-\Delta\Delta Ct}$ equation, where $\Delta\Delta Ct$ = [Ct miRNA-181a – Ct U6 RNA] (diabetic sample) [Ct miRNA-181a – Ct U6 RNA] (control sample).

STATISTICAL ANALYSIS

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 19, SPSS, Inc., Chicago, IL). Results were expressed as mean ± standard deviation (SD). Differences between continuous variables were analyzed using Student's *t*-test. Correlation between different variables was performed by Pearson. Statistical significance was set at a value of $P < 0.05$. Receiver operating characteristic (ROC) curve was used to discriminate positive from negative results. It determined the threshold value for optimal sensitivity and specificity, which was constructed by calculating the true-positive fraction (sensitivity percent) and false-positive fraction (100-specificity) of markers at several cutoff points. Sample size was calculated using the CaTS-Power Calculator

TABLE 1. Vital Signs and Anthropometric Measures of the Studied Groups

Parameters	Diabetic group Mean ± SD (range)	Control group Mean ± SD (range)	*P*
Pulse	75.65 ± 6.83 (60–90)	76.25 ± 8.89 (65–95)	0.736
Systolic BP	106.50 ± 6.62 (100–120)	109.25 ± 7.56 (100–120)	0.087
Diastolic BP	70.13 ± 6.45 (60–85)	71.63 ± 5.24 (60–80)	0.257
Weight (Kg)	33.40 ± 6.44 (22–49)	33.58 ± 6.72 (22–49)	0.906
Height (m)	1.44 ± 0.15 (1.18–1.68)	1.40 ± 0.13 (1.14–1.60)	0.189
BMI (Kg/m^2)	18.14 ± 3.13 (16.4–24.08)	17.13 ± 1.91 (15.4–22.37)	0.083
Waist circumference (cm)	60.93 ± 4.63 (51–70)	61.13 ± 4.59 (54–70)	0.850

$P > 0.05$ is nonsignificant.

(www.sph.umich.edu/csg/abecasis/CaTS). The power of study was 80% and relative risk for power calculation was set at 2.

RESULTS

The vital signs and anthropometric measures of all participants are shown in (Table 1). *N*-fold miRNA-181a expression ($P < 0.001$) and HbA1C ($P < 0.001$) were significantly higher in type 1 diabetic children and adolescents compared to healthy controls. On the other hand, SMAD7 ($P < 0.001$) and fasting C-peptide ($P < 0.001$) were significantly lower in type 1 diabetic children and adolescents compared to healthy controls (Table 2).

The correlation coefficients between *N*-fold miRNA-181a expression and the investigated laboratory parameters revealed significant positive association between *N*-fold miRNA-181a expression and HbA1c ($r = 0.627$, $P = 0.012$), triglycerides ($r = 0.520$, $P = 0.047$) and total cholesterol ($r = 0.668$, $P = 0.007$), and significant negative association with fasting C-peptide ($r = -0.391$, $P = 0.013$) and SMAD7 ($r = -0.384$, $P = 0.014$) in type 1 diabetic children and adolescents (Table 3).

The overall diagnostic performance of *N*-fold miRNA-181a expression for pancreatic beta cells dysfunction was assessed by ROC curve analysis and the best cutoff value was 2.45 (Fig. 1). Table 4 shows sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of *N*-fold miRNA-181a expression in determination of pancreatic beta cells dysfunction.

DISCUSSION

A number of miRNAs have been identified in the extracellular environment. As they may regulate a significant portion of the transcriptome and proteome, considerable attention has focused on miRNAs as mediators or biomarkers of illness (18).

Oxidative stress is involved in beta cells destruction (19). It is recognized as a mediator in the development of macrovascular or cardiovascular complications in T1DM (20).

miRNA-181a has been implicated in apoptosis (14,15). Researchers found that H_2O_2 induced upregulation of miRNA-181a resulting in apoptosis of cardiac myocytes. They also demonstrated that the use of anti-miRNA-181a blocked H_2O_2-induced apoptosis by regulating mitochondria-related apoptotic pathways (15). In another study, researchers found that the transfection of primary chronic lymphocytic leukemia cells with mature mimics

TABLE 2. Laboratory Results of the Studied Groups

Parameters	Diabetic group Mean ± SD (range)	Control group Mean ± SD (range)	*P*
Hemoglobin (g/dl)	11.53 ± 1.80 (9.9–13.4)	11.33 ± 1.19 (9.2–13.5)	0.445
Total leucocytic count/cmm	8.80 ± 1.56 (4.3–12.1)	8.08 ± 2.07 (4.3–12.5)	0.084
Platelets count/cmm	275.03 ± 56.95 (176–400)	251.53 ± 74.09 (155–398)	0.116
Triglycerides (mg/dl)	77.95 ± 18.09 (65–134)	73.08 ± 13.3 (57–98)	0.174
Total cholesterol (mg/dl)	158.73 ± 29.55 (106–190)	149.6 ± 22.68 (109–161)	0.125
HDL (mg/dl)	41.28 ± 5.10 (31–53)	40.20 ± 5.93 (32–51)	0.387
LDL (mg/dl)	81.42 ± 17.51 (60–120)	87.72 ± 20.06 (64–150)	0.138
UAE (mg/g urinary creatinine)	20.82 ± 4.18 (15–29)	19.49 ± 3.46 (15–26)	0.123
HbA1c (%)	11.48 ± 1.79 (9.0–16.0)	5.37 ± 0.59 (4.4–6.2)	<0.001**
Fasting C-peptide (ng/ml)	0.33 ± 0.14 (0.09–0.45)	2.05 ± 0.87 (0.5–3.2)	<0.001**
SMAD7 (ng/ml)	0.45 ± 0.09 (0.11–2.1)	4.35 ± 1.65 (1.0–9.9)	<0.001**
N-fold miRNA-181a	7.67 ± 2.58 (1.22–11.7)	1.22–11.7 (0.98–5.3)	<0.001**

** $P < 0.01$ is highly significant.

TABLE 3. Correlation Between N-fold miRNA-181a and the Studied Parameters

Parameters	Diabetic group		Control group	
	r	*P*	*r*	*P*
Age (years)	−0.107	0.505	0.201	0.213
Weight (Kg)	−0.008	0.958	0.119	0.465
Height (m)	−0.07	0.662	0.216	0.181
BMI (Kg/m^2)	−0.076	0.636	0.059	0.717
Pulse	−0.099	0.542	0.129	0.428
Systolic blood pressure	−0.152	0.349	−0.404	0.135
Diastolic blood pressure	0.098	0.546	−0.154	0.343
Hemoglobin (g/dl)	−0.061	0.709	0.155	0.340
Total leucocytic count/cmm	0.259	0.107	0.045	0.784
Platelets count/cmm	0.306	0.055	−0.140	0.389
Triglycerides (mg/dl)	0.520*	0.047	0.139	0.394
Total cholesterol (mg/dl)	0.668**	0.007	−0.0167	0.302
HDL (mg/dl)	0.204	0.208	−0.115	0.480
LDL (mg/dl)	0.080	0.625	0.090	0.759
UAE (mg/g urinary creatinine)	0.040	0.805	−0.222	0.168
HbA1c (%)	0.627*	0.012	−0.206	0.202
Fasting C-peptide (ng/ml)	−0.391*	0.013	0.251	0.146
SMAD7 (ng/ml)	−0.384*	0.014	−0.13	0.456

*Correlation is significant at the 0.05 level.
**Correlation is highly significant at the 0.01 level.

of miRNA-181a increased apoptosis (14). In addition to its role in apoptosis, miRNA-181a has been implicated in autoimmunity via regulation of a number of phosphatases known to negatively control T-cell sensitivity (21).

Based on the previous results, our study evaluated the expression of miRNA-181a in peripheral blood of children and adolescents with T1DM. Our results revealed overexpression of miRNA-181a among diabetic children and adolescents compared to healthy controls. We found significant positive correlation between the expression of miRNA-181a and triglycerides and total cholesterol in the diabetic group, which indicates that miRNA-181a correlates with parameters of lipid metabolism in T1DM. Furthermore, our results revealed significant positive correlation between the expression of miRNA-181a and the levels of HbA1c of diabetic group.

Although no enough studies were found as regards the expression of miRNA-181a in T1DM, our findings were in agreement with (21, 22) who found an upregulation of miRNA-181a in serum of diabetic patients and with Klöting et al. (23) who found that the expression of miRNA-181a was positively correlated with HbA1c levels in obese subjects.

As connecting peptide (C-peptide) is used as a marker of endogenous insulin production and reflection of

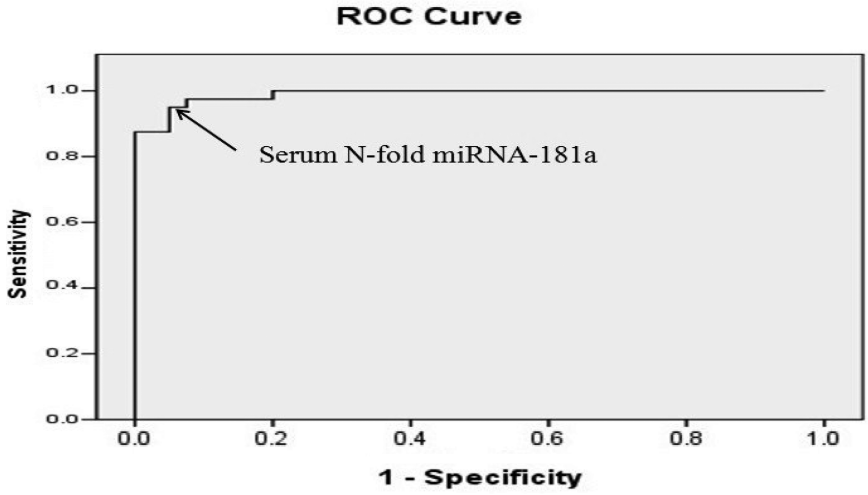

Fig. 1. The ROC curve analysis for *N*-fold miRNA-181a expression in the diabetic group versus healthy group to calculate the best cutoff value. Area under the curve is 0.989, standard error is 0.006, and 95% confidence limits are 0.977–1.001. Arrows denote cutoff point at 2.45.

TABLE 4. Sensitivity and Specificity of *N*-Fold miRNA-181a in Detection of Pancreatic Beta Cells Dysfunction

Variables False-negative (n, %) False-positive (n, %)	Sensitivity	Specificity	NPV	PPV	Accuracy
N-fold miRNA-181a expression: False-negative (1, 2.5%) False-positive (4, 10%)	97.3%	90.7%	97.5%	90%	94%

Total number of cases ($n = 80$), diabetic group ($n = 40$), and healthy group ($n = 40$).

pancreatic beta cells function (24). We assessed residual beta cells function through the measurement of fasting C-peptide in our diabetics. We found that the mean level of C-peptide was significantly decreased among diabetics compared to healthy controls. Furthermore, the expression of miRNA-181a was negatively correlated with C-peptide levels.

Our results revealed that overexpression of miRNA-181a in diabetic children and adolescents was associated with significant reduction in the mean level of SMAD7. This comes in concordance with the study done by Parikh et al. (25), who demonstrated that miRNA-181a activated TGF-β via suppression of SMAD7. Consequently, our data confirmed SMAD7 as a functional target of miRNA-181a and we therefore postulated that miRNA-181a may induce its damaging effect to pancreatic beta cells via the negative regulation to SMAD7.

The ROC curve was used to determine the best cutoff value for *N*-fold miRNA-181a expression to discriminate between healthy and diabetic children and it was 2.45. Applying this cutoff value, the sensitivity and specificity of miRNA-181a were 97.3% and 90.7%, respectively. The relatively high false-positive results (10%) of *N*-fold miRNA-181a expression may be an indicator of its predictive role for beta cells dysfunction.

In conclusion, the control of pancreatic beta cells apoptosis is important for the prevention and treatment of T1DM. The concordance between increase expression of miRNA-181a and decrease residual beta cells function supports the potential role of this miRNA during disease progression of T1DM. Assessment of circulating levels of miRNA-181a may be a potential valuable future tool for treatment planning and monitoring of intervention therapies designed to preserve/regenerate beta cells function in T1DM.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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