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Research article

A study on circulating micro RNAs in Egyptian diabetic patients with/without risk of cardiovascular complications

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Abstract

Egypt is ranked number nine among the top 10 countries of people with diabetes. The long term macro and microvascular diabetic complications are responsible for the diabetes- associated mortality. Thus, improving the quality of diabetic patients' life requires early prediction and prevention of chronic diabetic complications. Recently, microRNAs (miRNAs), which are short RNA molecules acting as regulators of protein expression in eukaryotic cells, had a great attention as novel potential biomarkers in several diseases. The objective of the current study was to evaluate serum levels of miR-126, mi-R133, miR-96 and miR-93 in Egyptian T2DM patients with/without cardiovascular complications. The present study compromised 18 healthy controls and 53 Egyptian diabetic patients divided into: 13 diabetic patients without diabetic cardiovascular complications and 40 patients with risk for diabetic cardiovascular complications. The expression levels of mature miRNAs were detected in all patients and controls sera using quantitative RT- PCR. Serum levels of miR-133, miR-126 and miR-93 were significantly reduced while serum miR-96 was significantly increased in T2DM patients without risk for cardiovascular complications compared to the healthy controls, which may play a role in developing diabetes and differentiating patients with a high likelihood of developing diabetes from healthy controls. Moreover, there was a significant decrease in miR-133 and miR-126 levels between the two diabetic groups, which suggests that both miRNAs could be useful for prediction of cardiovascular disease in T2DM Egyptian patients.

Introduction

Diabetes is a huge and growing problem, and costs to society are high and escalating all over the world. In Egypt, number of diagnosed diabetic cases was approximately 48,276 cases, while the number of diabetes related deaths were approximately 86,478 [1]. Egypt ranked number 9 among the top 10 countries of people with diabetes mellitus (DM) between the age (20-79) years old with 7.5 million patients [1]. Type 2 Diabetes (T2DM) is a chronic metabolic disease, characterized by a combination of resistance to insulin action and of an inadequate compensatory insulin secretory response, resulting in chronic hyperglycemia [2]. Diabetes is recognized as a potent and prevalent risk factor for heart disease. A specific diabetic cardiomyopathy, distinct from coronary arteriosclerosis was first proposed by Rubler *et al.,* [3]. Diabetic cardiomyopathy is an early complication of diabetes and is manifested by diastolic dysfunction followed by abnormalities in systolic function [4]. However, its underlying pathogenesis is partially understood. Hyperglycemia, being a hallmark of both type 1 and type 2 diabetes, increases the production of reactive oxygen species (ROS), alters the cellular redox status and causes rapid changes in membrane function, followed by contractile dysfunction within weeks in the diabetic heart [5].

In order to improve the quality of life of T2DM patients, prediction and prevention of chronic diabetic complications represent two major objectives. Thus, to date, specific biomarkers are needed for prediction, diagnosis and monitoring of diabetic complications. Recently, microRNAs (miRNAs) received great attention as new potential biomarkers in several diseases as cancer and diabetes. Compared to other biomarkers, miRNAs are less complex, very stable in biological fluids and conserved among different species. Moreover, the expression of some miRNAs is restricted to specific tissues leading to a much lower complexity and simpler targeting [6].

miRNAs are small 19–23 nucleotide RNA molecules that act as regulators of protein expression in eukaryotic cells by

inducing the translational arrest and degradation of messenger RNAs [7], through binding to the 3'-untranslated (3′ UTR) region of miRNAs and destabilizing them or inhibiting their translation[8].

Growing evidence indicates that miRNAs might be involved in the pathogenesis of diabetes [9, 10]. miR-96 and miR-126 directly target the insulin receptor substrate 1(IRS1) 3′ UTR, which is involved in insulin resistance under conditions of mitochondrial dysfunction in hepatocytes [11]. Likewise, miR-96 negatively regulates insulin exocytosis [12].

Moreover, several miRNAs have been identified in tissues in which diabetes complications occur [13]. However, whether these miRNAs are involved in the damage that appears in diabetes is yet to be established. Several studies demonstrated how differentially expressed miRNAs and their target genes in hearts of experimentally induced diabetic animals are playing important roles in diabetic subjects and deregulation of cardiac-enriched miRNA levels have importance in the development of cardiac dysfunction including diabetic cardiomyopathy [14, 15].

Among the miRNAs most consistently associated with diabetes was miR-126. This miRNA has previously been shown to be highly enriched in endothelial cells and endothelial apoptotic bodies and to govern the maintenance of vascular integrity, angiogenesis, and wound repair [16]. Literature data from different laboratories indicates that diabetic complications are often associated with changes in the levels of certain miRNAs in various tissues, including the heart under different pathophysiological conditions. Among these, the first miRNA that was shown to change its expression level in diabetic heart was miR-133 [17]. It was shown that miR-133 plays a critical role in regulating myogenesis and the change of miR-133 levels in heart is associated with cardiac hypertrophy [18]. Horie *et al.*, found that miR-133 over expression lowered GLUT4 levels and reduced insulin-induced glucose uptake in cardiomyocytes [19].Cardiac tissue from the diabetic mice displayed significant down regulation in miR-133a expression [13]. Despite the critical role of vascular endothelial growth factor (VEGF) in microvascular complications of diabetes, the regulatory role of miRNAs on VEGF remains unknown. However, a study showed that miR-93 regulates VEGF expression in experimental models of diabetes both in vitro and in vivo [20]. miR-96 downregulates insulin secretion by decreasing the expression of nucleolar complex protein 2 (Noc2), which is required for insulin exocytosis [12].

The present study aimed to evaluate serum levels of miR‑126, miR-133, miR-96 and miR-93 in T2DM Egyptian patients and their relative contribution to cardiovascular complications.

Experimental

Study design

The present study compromised 53 Egyptian patients (25 males and 28 females) with T2DM, recruited from the clinical pathology department at National institute for Diabetes and Endocrinology (NIDE), Kasr EL Einy, Cairo, Egypt. In addition to 18 healthy control volunteers (8 males and 10 females) did not suffer from any disease. Before inclusion all the study subjects underwent careful physical examination, detailed history, and laboratory investigations to exclude any condition that may interfere with the studied parameters. Definition and selection of T2DM were done according to American Diabetes Association criteria [21]. Patients were divided into 2 groups: **Group I:** 13 T2DM patients without any clinical, biochemical and/or instrumental evidence of diabetic chronic complications, **Group II:** 40 patients with risk for diabetic cardiovascular complications **(**presence of hypertension and/or dyslipidemia). The healthy controls in this study were volunteers with a similar age range as the patients. The patients and controls included in the present study provided their written informed consent and the study was approved by the research ethics committee of the General Organization for Teaching Hospitals and Institutes and the National Research Centers (approval No. IDE00140 on 30/1/2012)

For all subjects in the study, the following data were collected: age, gender, body mass index (BMI), duration of diabetes, presence of hypertension and/or dyslipidemia, glucose, blood pressure and lipid-lowering therapy (with indication of the class of drug). Table 1 shows main clinical parameters.

Serum Collection and RNA Extraction

After 12 hours of fasting, blood samples were collected and centrifuged. The serum was collected, divided into 3 aliquots for each sample and immediately stored at −80˚C pending for RNA extraction. Glycemic indices [fasting blood glucose (FBG) and glycated hemoglobin (HbA1c)], Serum creatinine, and lipid profile [total cholesterol (TC), triacylglycerol (TAG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C)] were measured for all participants. Glucose measurements were carried out using the hexokinase method [22]using a Bayer ADVIA® 1650 analyzer, while HbA1c, was analyzed by ion-exchange HPLC technique [23] using Bio-Rad D-10 Hemoglobin testing system. TC, HDL-C, LDL-C, and TAG were determined using direct enzymatic methods (Greiner Diagnostic GmbH, Germany). The atherogenic ratios (TC/ HDL-C and LDL-C / HDL-C) were calculated.

Isolation of total RNA from human serum

Total RNA, was extracted from human serum using miRNeasy kit (Qiagen) according to the manufacturer's instructions with some modifications. Briefly, 200 µl of plasma was mixed with 5 volumes Qiazole lysis reagent, placed at room temperature for 5 min. Then 100 µl chloroform was added and the mixture was centrifuged at

12000 rpm for 15 min. followed by separation on RNeasy Mini spin column as specified by manufacturer's instructions. RNA was eluted in 30 ul of nuclease-free water and then subjected to downstream reactions. The final RNA products were quantified by absorbance measurements at 260 nm (A260) and 280 nm (A280). The A260/A280 values were higher than 1.6 for all of the samples.

Quantitative polymerase chain reaction (qPCR)

The expression of mature miRNA was detected by using the Qiagen technology. In brief, 4 µl RNA was reverse transcribed to cDNA using the miScript II RT Kit (Qiagen, *Chatsworth, CA, USA*) following the manufacturer's instructions. The miScript SYBR® Green PCR Kit (Qiagen *Chatsworth, CA, USA*) was used to perform the qPCR with the manufacturer provided miScript Universal Primer and miScript Primer Assay in Rotor-Gene Q (QIAGEN Hilden, Germany).

Urine samples

Fresh early morning urine spot sample was collected from each patient for measurement of microalbuminuria (turbidimeteric assays) [24] and urine creatinine [25] using ADVIA® 1650 clinical chemistry system, Siemens, Germany to calculate Microalbuminuria (expressed as A/C ratio mg/g creatinine).

Statistical analysis

The relative quantity (RQ) of each miRNA was calculated using the comparative threshold cycle (ΔCt) method with the equation $2^{-\Delta CT}$, where ΔCT = Ct miRNA - Ct SNORD 68 [26]. The results are expressed as median of the fold change values. Data were analyzed using Prism 5 software, version 5.00 (GraphPad Software, San Diego, CA, USA). ANOVA test was used to draw comparisons between groups.

Results and Discussion

Results

Patient and control subjects characteristics

The number of patients and healthy controls used for this study and their ages, gender, BMI, glycemic indices and lipid profile are shown in Table 1. Age, Gender and BMI distribution were not significantly different from the control healthy group. As expected, the diabetic without and with risk to CVD patients had a significantly higher levels of fasting blood glucose (155.5±12.2 & 224.60 ±13.60 at P<0.05 & P<0.001, respectively) and HbA1c (8.44 ±0.74& 913±0.41 at P<0.05 & P<0.01, respectively) compared to the healthy control. T2DM with risk to CVD group showed significant elevation in the level of both TC and TAG compared to the healthy control and as a result, risk ratio 1 and risk ratio 2 were also significantly increased (8.46±0.82 & 6.46±0.66, respectively at P<0.05) compared to the healthy control group. Moreover, ACR, was significantly increased in diabetic group with risk for CVD compared to both healthy control and diabetic group at P<0.001.

Table 1. Demographic data and laboratory characteristics of the studied groups

Parameter	Healthy control (N= 18)	Diabetic patients (N= 13)	Diabetic patients with risk for CVD (N=40)
Age (years)	52.2±1.64	48.5±1.83	48.12 ±1.46
BMI(Kg/m²)	25 ± 0.55	30.24 ±1.7	30.30 ±0.77
FBG (mg/dL)	75.8±2.1	155.5±12.2*	224.6±13.6***##
HbA1c (%)	5.28 ±0.14	8.44 ±0.74*	9.13±0.41**
ACR(mg/g Cr.)	10.61±0.99	21.64±0.94	49.75±3.19***###
TC (mg/dL)	151.2±8.8	179±8.8	212.3±9.8** #
TAG (mg/dL)	94.1±20	178.8±9.1**	193.6±10.6***
Ratio 1	2.84 ±0.45	5.55 ±0.98	8.46 ±0.82*
Ratio 2	2.8 ± 0.34	3.33 ± 0.73	6.46 ±0.66*#

*,** and ***: compared to control group at P<0.05 , P<0.01 and P<0.001, respectively.
#,## and ###: compared to diabetic group at P<0.05 , P<0.01 and P<0.00, respectively. Values are expressed as mean ± SEM.
Using parametric ANOVA test followed by Tukey-Kramer multiple comparisons test.

Expression pattern of microRNAs (126, 133, 93 and 96) in serum

Table 2 gives the median (interquartile range [IQR]) of the fold change values of the four tested microRNAs (126, 133, 93 and 96) in the serum of the T2DM patients with and without risk for CVD as well as healthy control subjects. Serum levels of both miR-126 and miR-133 were significantly reduced in T2DM patients without risk for CVD at p< 0.05 and in T2DM patients with risk for CVD at p< 0.001, compared to healthy control subjects. As well both miRNAs serum levels were reduced significantly in T2DM patients with risk for CVD at p<0.05, compared to T2DM patients without risk for CVD, as shown in Figure 1 & Figure 2.

Regarding miR-93, Figure 3 shows that there was a significant decrease in serum levels of miR-93 of T2DM patients with and without risk for CVD at p< 0.001, compared to healthy control group. While there was no significant difference between serum miR-93 of both T2DM groups. On the other hand, serum miR-96 levels were significantly increased in T2DM patients with and without risk for CVD compared to the healthy control group at p<0.05 and p<0.001, respectively, with no significant difference between the two diabetic groups, Figure 4.

Discussion

Since 2015, approximately 35.4 million people, or 9.1% of adults aged 20-79, are living with diabetes in the Middle East and North Africa region. Diabetes was responsible for 342,000 deaths.

Table 2. Levels of microRNAs (miR-126, miR-133, miR-93 and miR-96) in the serum of the studied groups

Parameter	Healthy control (N= 18)	Diabetic patients (N= 13)	Diabetic patients with risk for CVD(N= 40)
miR-126	29.10(10.43-43.8)	6.70 (1.01-10.6) *	0.34 (0.10-1.13) ***#
miR-133	28.60(11.0-43.48)	2.90 (2.74-4.30) *	1.26 (0.52-1.92)***#
miR-96	19.30(16.58-23.18)	27.98(26.85- 30.49) ***	24.48(19.13-29.45)*
miR-93	25.16(11.07-42.75)	4.36(2.60- 6.53) ***	5.30(2.64-7.60) ***

*,*** :significant from control healthy group at p<0.05and p<0.001, respectively.
#: Significant from Diabetic group at p<0.05.
Values are expressed as median (IQR).
Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons test.
IQR: interquartile range

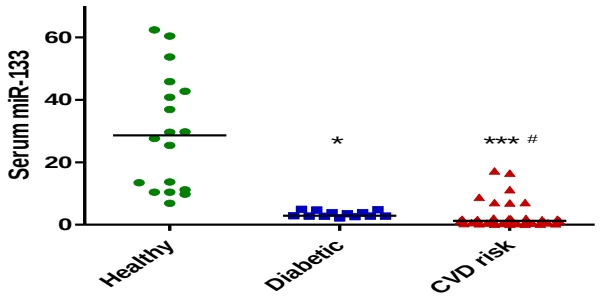

Figure 1. Serum miR-133 levels in diabetic patients with and without risk for CV complications
*,*** :significant from control healthy group at p<0.05 and p<0.001, respectively.
#: Significant from Diabetic group at p<0.05.
Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons test.
Horizontal lines represent the median of the distribution.

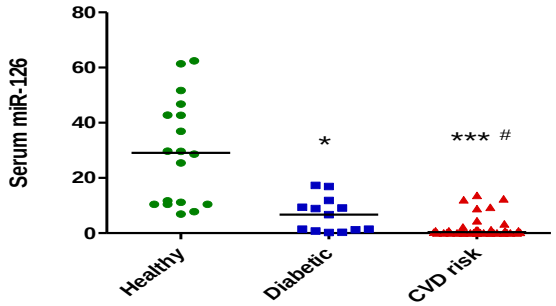

Figure 2. Serum miR-126 levels in diabetic patients with and without risk for CV complications
*,*** :significant from control healthy group at p<0.05 and p<0.001, respectively.
#: Significant from Diabetic group at p<0.05. Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons test.
Horizontal lines represent the median of the distribution.

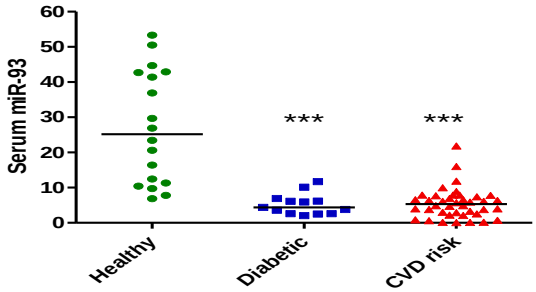

Figure 3. Serum miR-93 levels in diabetic patients with and without risk for CV complications
*** :significant from control healthy group at p<0.001. Using Kruskal-Wallis Test,(Nonparametric ANOVA) followed by Dunn's Multiple Comparisons test.
Horizontal lines represent the median of the distribution.

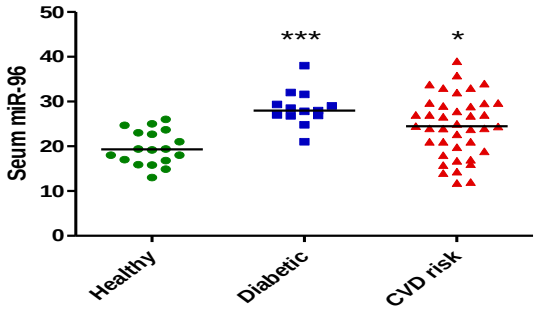

Figure 4. Serum miR-96 levels in diabetic patients with and without risk for CV complications
*,***:significant from control healthy group at p<0.05 and p<0.001, respectively.
Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple, Comparisons test.
Horizontal lines represent the median of the distribution.

These early deaths may be the result of a combination of factors: the rapidly changing environments and lifestyles in the region, late diagnoses and health systems that are not equipped to provide optimal management to the increasing numbers of people with diabetes [1]. Almost all forms of diabetes are invariably characterized by microvascular complications in the renal glomerulus, peripheral nerve and the retina and macrovascular complications such as hypertension and heart valve defects which later on manifest as cardiac hypertrophy [27]. Altered lipid profile was reported in DM[28] and it has been shown that the dyslipidemia predisposes diabetic patients to cardiovascular complications, especially the coronary heart diseases [29, 30]. Regarding lipid profile, in the present study, TC, TAG levels and atherogenic ratios (ratio 1& ratio 2) showed significant increase in diabetic patients with risk for CVD compared to healthy control group. Presence of Microalbuminuria doubles the risk for a cardiovascular

event in patients with T2DM even after adjusting for the usual risk factors and predicts target organ damage, left ventricular dysfunction, stroke, and myocardial infarction [31]. miRNAs are found to be critical in the development and progression of DM, currently emerging reports also associate altered levels of a range of miRNAs with these diabetic complications [27]. Circulating miRNAs are very stable [32]. Consequently, their utility as ideal candidate biomarkers to identify disease initiation and progression, with findings of other diseases such as cancer and CVD [33]. It was found that there are distinct profiles of circulating miRNAs in patients with DM and T2DM complications compared to non-diabetic patients [34]. Studies have shown that specific miRNA profiles are correlated to DM pathology; while all candidate miRNAs are involved in regulating insulin production machinery, insulin sensitivity, glucose homeostasis, or lipid metabolism implicated in T2DM pathology [35, 36]. Various miRNAs play a crucial role to regulate homeostasis of tissues where diabetic complications occur [37]. The contribution of miRNAs in DM, suggests that these small RNA species might be distinct and critical in this complex disease and they or their inhibitors may be exploited as targets for therapeutic interventions for prediction and prevention of chronic diabetic complication. Thus, in the current study serum levels of four miRNAs (miR-126, miR-133, miR-93 and miR-96) were estimated in T2DM patients with and without risk for cardiovascular complications. Regarding serum levels of the estimated miRNAs, our results showed that the serum levels of both miR-133 and miR-126 were significantly reduced in T2DM patients with and without risk for CVD compared to the healthy controls. Moreover, both miRNAs showed significant decrease in diabetic patients with risk for CVD with respect to diabetic group. This came in accordance with Mishra *et al.,* who demonstrated that down regulation of miRNA-133 levels lead to cardiac hypertrophy and that blocking of miRNA-133 function in vivo caused marked and sustained cardiac hypertrophy [38]. Also, a study by Gallagher *et al.,* revealed down regulation of miRNA-133 in diabetes [39]. On the other hand, our results disagree with Xiao *et al.,* who showed over-expression of miR-133 in the hearts of diabetic rabbits [27]**.** Regarding miR-126, it is highly enriched in endothelial cells, and plays a pivotal role in maintaining endothelial homeostasis and vascular integrity [16]. Zhang *et al.*, reported that miR-126 expression was already decreased before the manifestation of T2DM in agreement with our finding in the Egyptian patients [40]. Another study by Zhang *et al.,* showed significant reduction of miR-126 in plasma samples of T2DM susceptible individuals and T2DM patients [41]. A previous study indicated an association between miR-126 and T2DM and that high glucose significantly reduced the miR-126 content in endothelial apoptotic bodies. Moreover, there was a gradual decrease in plasma levels of miR-126 across categories of normal glucose tolerance, impaired fasting glucose/impaired glucose tolerance and manifest DM [42]. The significant decrease in serum miR-133 and miR-126 in diabetic patients with risk for CVD from the diabetic patients with no evidence of complications in the present study may suggest that they could be related to the predisposition to CVD.

miR-93 was reported to be under-expressed in T1DM patients compared to control samples [43]. miR-93 negatively regulates the vascular endothelial growth factor (VEGF). High levels of VEGF have been associated with the pathogenesis of several inflammatory diseases, mainly microvascular diabetic complications. [20]. Considering our study, miR-93 was significantly decreased in T2DM patients with and without risk for CVD with no significant difference between the two diabetic groups. Which is consistent with Long *et al.*, who identified miR-93 to be a "signature miRNA" in hyperglycemic conditions [20]. On the other hand, miRNA-96 was significantly high in T2DM patients with and without risk for CVD compared to the healthy controls. A study showed that miR-96 is over expressed in DM and it has a key role in controlling the level of several critical machinery components governing insulin secretion [12]. Moreover, studies showed that miR-96 and miR-126 directly target the insulin receptor substrate 1(IRS1) 3` UTR, with reduction in its level involved in insulin resistance under conditions of mitochondrial dysfunction in hepatocytes [11, 44].

The complexities in the pathogenesis of DM make this more challenging. Moreover, emerging evidence suggests that miRNAs are differentially expressed, and indeed have a potential causative role, in diabetes and its related cardiovascular complications.

Conclusion

The significant change in circulating miR-133, miR-126, miR-93 and miR-96 in T2DM patients with no risk for diabetic complications suggests that, a blood signature of these miRNAs accurately differentiate patients with a high likelihood of developing diabetes from healthy controls. Moreover, the significant reduction in serum miR-126 and miR-133 levels between the two diabetic groups suggests that both miRNAs could be utilized as biomarkers for prediction of CVD in T2DM patients.

From this point of view, large-scale assessment of circulating miRNA levels using high throughput technologies will surely provide new specific markers relevant for clinicians.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

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