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Potential value of circulating microRNA-126 and microRNA-210 as biomarkers for type 2 diabetes with coronary artery disease

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ABSTRACT

Background: Macrovascular complications are the main cause of morbidity and mortality among the diabetic patients. MicroRNAs (miRNAs), a family of small non-coding RNAs, play vital roles in the regulation of blood glucose level and the concurrent cardiovascular complications of type 2 diabetes. We hypothesized that plasma miR-126 and miR-210 are linked to coronary artery disease (CAD) in these diabetes patients.

Methods: Fasting blood samples were collected from 20 healthy volunteers and 100 patients with diabetes (54 patients without CAD and 46 patients with CAD). Plasma miR-126 and miR-210 expressions were assessed by quantitative real time PCR. Specificity and sensitivity of miR-126 and miR-210 to discriminate CAD with diabetes was determined by receiver operating characteristic curve analysis. Correlations between miR-126 and miR-210 and studied characteristics in diabetes patients with and without CAD were compared.

Results: Plasma relative expressions of miR-126 and miR-210 were 0.38 ± 0.03 and 5.3 ± 0.56 in diabetes alone vs. 0.08 ± 0.03 and 21.44 ± 0.97 in diabetes with CAD, respectively (both $p < 0.0001$). Levels of miR-126 and miR-210 significantly correlated with certain glycemic and lipid indices. The miRNAs significantly discriminated between diabetes with and without CAD at cut-off values of 0.055 (sensitivity 91.3%, specificity 100%) for miR-126 and of 17.59 (sensitivity 93.5%, specificity 100%) for miR-210.

Conclusion: Plasma miR-126 and miR-210 levels may be biomarkers for diabetes with or without CAD.

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MicroRNAs; MiR-126; MiR-210; type 2 diabetes; coronary artery disease

Introduction

Type 2 diabetes constitutes approximately 90–95% of all patients with diabetes worldwide and represents a growing epidemic. The International Diabetes Federation (IDF) listed Egypt among the world top 10 countries in the number of patients with diabetes [1]. Type 2 diabetes is a metabolic disorder characterized by insulin resistance and pancreatic β-cell dysfunction due to unsettled hyperglycaemia [2]. The American Heart Association considers diabetes as one of the six major controllable risk factors for cardiovascular disease [1]. Coronary artery disease (CAD) is a major cause of mortality in diabetics as >60% of patients die from cardiovascular diseases, whilst diabetes brings a two to threefold greater risk for developing [3].

MiRNAs are highly conserved small non-coding endogenous RNA molecules that have been proven to be involved in the regulation of key biological processes such as proliferation, differentiation, apoptosis and metabolism [4]. In addition, tumour miRNAs are involved in tumourigenesis and the development of various cancers [5]. They represent a promising novel class of biomarkers for various diseases such as CAD in diabetes [6]. MiR-126, abundantly expressed in endothelial cells, maintains endothelial homeostasis and vascular integrity via regulating several components of the vascular endothelial growth factor pathway [7]. MiR-210 has been strongly linked with the hypoxia pathway, and is up-regulated in response to hypoxia-inducible factors [8]. It is also over-expressed in cells affected by cardiac disease and tumours [9].

The current study aimed to assess levels of plasma miR-126 and miR-210 in type 2 diabetes with and without CAD and investigate their potential value as biomarkers. Our hypothesis is abnormal levels of plasma miR-126 and miR-210 in type2 diabetic patients with CAD.

Subjects and methods

The study enrolled 100 patients with type 2 diabetes (of whom 54 had CAD) from the outpatient clinic at the National Research Centre, Cairo, Egypt. Twenty

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apparently healthy volunteers recruited from paramedical personnel served as controls. Patients with type 1 diabetes, cancer, major organ failure, autoimmune diseases or sepsis were excluded. Diabetic patients were previously diagnosed and treated with oral hypoglycaemic and/or insulin, diagnosed by with American Diabetes Association criteria 2016 [10]. CAD was diagnosed by cardiac catheterization, with at least one major vessel with ≥ 50% stenosis.

Patients and healthy volunteers were subjected to detailed medical and family history and demographic and clinical data with emphasis on symptoms suggestive of CAD. Anthropometric measurements: weight, height and calculation of body mass index [weight (kg)/height (m^2)] were done. Twelve lead electrocardiography (ECG) was performed on all subjects, whilst cardiac catheterization was performed on those with symptoms and/or ECG findings suggestive of CAD. The study was approved by the ethical committee of the National Research Centre, Cairo, Egypt and was conducted according to the rules of Helsinki declaration for human studies. A written informed consent was obtained from all participants.

Eight hours fasting venous blood samples (6 mL) were collected from all subjects as follows: 2 mL blood was taken into NaF tubes for glucose determination, 2 × 2 mL into K-EDTA for HbA1c and miRNAs. For lipid profile, 4 mL was taken into non-gel serum tubes, were left for 45 min to clot, and then was centrifuged at 3000 × *g* for 15 min. Serum and plasma samples were stored at −40 °C till the time of use. Glycosylated Hb (HbA1c) was assayed by quantitative colorimetry (Stanbio Laboratory, Boerne, TX, USA), fasting blood glucose, total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c) and triglycerides (TG) by standard techniques (Olympus automatic analyser AU 2700, Olympus Diagnostics GmbH, Irish Branch, Lismeehan, Ireland). Low-density lipoprotein-cholesterol (LDL-c) was calculated by Friedewald formula (LDL = TC-TG/5-HDL) [11].

For miRNA-126 and miRNA-210 extraction and RT-quantitative PCR, RNA stabilization in the blood samples was ensured by mixing 0.5 mL aliquots of EDTA blood with 1.3 mL RNA later (Ambion, Austin, TX, USA) and stored at −80 °C until miRNA extraction. MiRNA-rich fraction was extracted from plasma specimens using the miRNeasyMini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNAs were quantified with NanoDrop ND-1000. For RT-PCR, extracted miRNA was reverse transcribed in reaction mixture containing miR-specific stem-loop RT primers using an Applied Biosystems™ TaqMan MicroRNA RT kit (Thermo Fisher Scientific), for one cycle only. Quantitative real time polymerase chain reaction (qRT-PCR) was performed using TaqMan microRNA assay (Applied Biosystems Life Technologies), for 40 cycles, in duplicate reactions containing the prepared cDNA and TaqMan specific primers for the target has-miR-126 (forward, UCGUACCGUGAGUAAUAAUGCG; reverse, GCGCAUGGUUUUCAUUAUUAC), for the target has-miR-210 (forward, CUGUGCGUGUGACAGCGGCUGA; reverse, GUCACACGCCACCCGUCCCCGA 3') and for the endogenous control RNU 6B (CGCAAGGATGACACGCAAA TTCGTGAAGCGTTCCATATTTTT) (Applied Biosystems Life Technologies) in Universal Master Mix without AmpErase UNG (Applied Biosystems, Foster City, CA). The cycling conditions were initial denaturation at 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 57 °C for 20 s, and 7 °C for 10 s. Only miRNAs with threshold cycle (Ct) < 35 were considered for subsequent analysis. Relative quantifications were calculated using $2^{-\Delta\Delta Ct}$ method [12].

GraphPad Prism®5 software was used for analysis of data. Clinical data were presented as mean and standard deviation (SD). Differences of the frequencies in the studied groups were analysed using chi-square test. One-way analysis of variance (ANOVA), followed by Tukey test was used for parametric data while, Kruskal–Wallis test followed by Dunns test was used for non-parametric data to explore the difference among studied groups. Correlation analysis was by Spearman's method. Receiver Operating Characteristic (ROC) curve was used to determine the cut-off values. The ordinal logistic regression (OLR) test was done using Minitab® 17 software package. *P*-value was considered statistically significant when $p < 0.05$ vs. corresponding control.

Results

There was no significant difference in age and sex between the three groups (Table 1), but there were differences in glycemic control and lipid profile between patients with or without CAD. MiR-126 was significantly lower by 13.1 and 2.8-fold in diabetes patients with and without CAD, respectively, compared to controls. Moreover, patients with CAD had lower miR126 levels than patients without CAD. miR-210 was significantly higher by 21.4 and 5.3-fold in diabetes patients with and without CAD, respectively, compared to controls and in patients with CAD compared to those without CAD.

In those with diabetes alone, there was a significant inverse correlation between miR-126 and fasting glucose and HbA1c (Table 2). As regards miR-210, there were significant correlations with lipid profile. In patients with CAD, miR-126 and miR-210 correlated with fasting glucose, HbA1c and lipid profile. The two miRNAs correlated inversely with each other in diabetes ($r = 0.32$, $p = 0.018$) and in diabetes plus CAD ($r = -0.64$, $p < 0.001$).

Ordinal logistic regression analysis revealed a significant association between groups and miR-126 and HDL-c ($p < 0.05$): as miR-126 and HDL-c decreased, subjects were more likely to be diabetic with CAD. This analysis also revealed a significant association between groups and miR-210, BMI, fasting glucose, HbA1c, TC, TG and LDL-c ($p < 0.05$): with increases in these indices, subjects more likely to be diabetic with CAD.

Table 1. Demographic and clinical characteristics of the studied groups.

	Controls ($n = 20$)	Diabetes ($n = 54$)	Diabetes with CAD ($n = 46$)	*P*-value
Age (years)	58.1 ± 1.1	56.5 ± 7.7	57.0 ± 6.2	0.48
Sex (male/female)	11/9	29/25	23/23	0.9
BMI (kg/m^2)	23.2 ± 0.2	30.7 ± 5.3[a]	29.7 ± 3.5[a]	<0.001
Duration (years)	–	10.8 ± 7.8	11.2 ± 5.2	0.34
Fasting glucose (mmol/L)	4.5 ± 0.3	7.7 ± 1.4[a]	9.9 ± 4.5[a, b]	<0.001
HbA1C (%)	4.8 ± 0.4	8.3 ± 1.1[a]	9.4 ± 1.0[a,b]	<0.001
TC (mmol/L)	4.2 ± 0.2	5.2 ± 0.5[a]	6.2 ± 1.3[a,b]	<0.001
TG (mmol/L)	1.2 ± 0.1	1.4 ± 0.2[a]	1.8 ± 0.6[a,b]	<0.001
HDL-c (mmol/L)	1.3 ± 0.1	1.0 ± 0.6[a]	0.9 ± 0.3[a]	<0.001
LDL-c (mmol/L)	2.4 ± 0.2	3.6 ± 0.8[a]	4.6 ± 1.4[a,b]	<0.001
MiR-126	1.01 (0.77–1.34)	0.36 (0.18–0.45)[a]	0.04 (0.02–0.05)[a,b]	<0.001
MiR-210	1.01 (0.97–1.49)	5.22 (1.37–7.50)[a]	20.7 (18.62–22.65)[a,b]	<0.001

Notes: CAD: coronary artery disease; BMI: body mass index; HbAlc: glycosylated haemoglobin; TC: total cholesterol; TG: triglycerides; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; MiR: MicroRNA; Data presented as mean ± SD or median with IQR. P values by ANOVA followed by Tukey test or Kruskal–Wallis test followed by Dunn's test.
[a]Different from the control group.
[b]Different from diabetes alone.

Table 2. Correlation of miR-126 and miR-210 with demographic and biochemical parameters.

	Diabetes ($n = 54$)				Diabetes with CAD ($n = 46$)			
	miR-126		miR-210		miR-126		miR-210	
	r	*P*	*r*	*P*	*r*	*p*	*r*	*p*
Age	0.04	0.744	0.19	0.171	−0.07	0.631	0.27	0.074
BMI	0.01	0.978	−0.20	0.139	0.03	0.829	−0.01	0.940
Disease duration	0.11	0.439	−0.01	0.931	0.15	0.219	−0.08	0.595
Fasting glucose	−0.67	<0.001	0.15	0.274	−0.92	<0.001	0.56	0.006
HbA1c	−0.68	<0.001	0.15	0.267	−0.81	<0.001	0.48	0.001
TC	−0.27	0.052	0.43	<0.001	−0.48	0.001	0.46	0.001
TG	−0.03	0.849	0.53	<0.001	−0.20	0.189	0.52	<0.001
HDL-c	0.09	0.500	−0.79	<0.001	0.41	0.005	−0.50	<0.001
LDL-c	−0.17	0.226	0.68	<0.001	−0.46	0.001	0.41	0.004

See Table 1 for abbreviations. *r*: Spearman correlation coefficient.

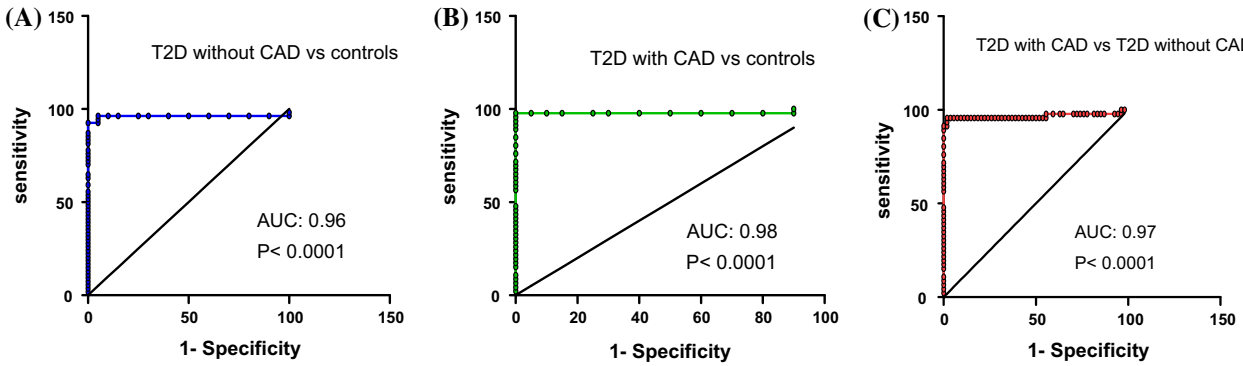

Figure 1. ROC curves of plasma miR-126.
Notes: A, Type 2 diabetes (T2D) without CAD vs. controls; B, T2D with CAD vs. controls; C, T2D with CAD vs. T2D without CAD.

Sensitivity and specificity of miR-126 and miR-210 for diabetes with/without CAD assessed by ROC curves showed abilities to discriminate between patients with and without CAD from healthy controls at cut-off value of 0.57 (sensitivity 97.8%, specificity 95%), 0.65 (sensitivity 96.3%, specificity 95%), respectively, for miR-126 and of 9.2 (sensitivity 97.8%, specificity 100%), 1.12 (sensitivity 87%, specificity 100%), respectively, for miR-210. ROC curves of both miRNAs significantly discriminated diabetes with CAD patients from those without CAD at cut-off value of 0.055 (sensitivity 91.3%, specificity 100%) for miR-126 and of 17.59 (sensitivity 93.5%, specificity 100%) for miR-210 (Figures 1 and 2, respectively).

Discussion

The development of biomarkers that can identify diabetes patients at risk of CAD can improve the care of such patients. MiRNAs have roles in the epigenetic regulation of key metabolic, inflammatory and antiangiogenic pathways in diabetes that may be associated with long term complications [13]. Our results revealed a significant decrease of plasma miR-126 levels in diabetic patients with and without CAD compared to controls as well as between diabetic patients with CAD compared to diabetic patients without CAD. These results are in accordance with many studies [6,13–15] although one

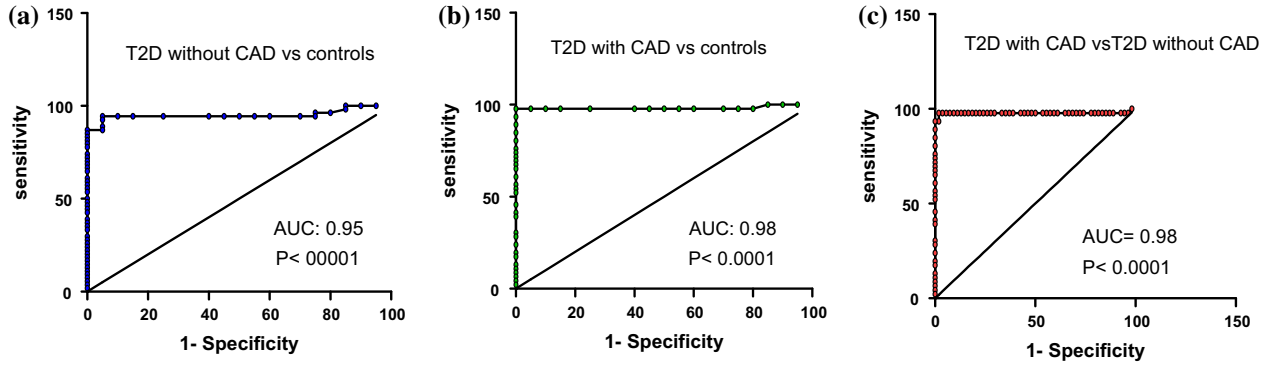

Figure 2. ROC curves of plasma miR-210.
Notes: A, Type two diabetes (T2D) without CAD vs. controls; B, T2D with CAD vs. controls; C, T2D with CAD vs. T2D without CAD.

showed no changes in miR-126 levels between diabetic patients and controls [16]. In patients with stable CAD, Jansent and colleagues reported a significant association between high miR-126 in circulating microvesicles and lower major adverse cardiovascular events [17]. Moreover, the current study revealed that the expression levels of miR-126 were negatively associated with fasting glucose and HbA1c in both patient groups. Notably, the miR-126 ROC curve significantly discriminated between both patient groups and controls as well as between diabetes patients with and without CAD, in accordance with others [6,14].

The downregulation of miR-126 in diabetes patients might be caused by hyperglycaemia that reduces the miR-126 content of endothelial apoptotic bodies, or prolonged hyperglycaemia may lead to further decrease of endothelial miR-126. These findings might relate to impaired peripheral angiogenic signalling in patients with diabetes due to the loss of endothelial protective role of miR-126 [13]. The epigenetic effect of miR-126 is encoded within an intron of Egfl7 and it is enriched in endothelial cell. Its expression has been shown to be partly driven by vascular-associated Ets transcription factors. These transcription factors play important roles in vasculogenesis, angiogenesis, inflammation, and remodelling [18]. For example, miR-126 promotes vascular endothelial growth factor (VEGF) signalling by suppressing two negative regulators of the VEGF pathway [19]. The lower plasma miR-126 in diabetes patients with CAD may be explained by its depletion from endothelial cells during hyperglycaemia resulting in its decrease in plasma [13] as well as circulating miR-126 was consumed during transcoronary passage [20].

Our study revealed a significant increase of plasma miR-210 in diabetic patients with and without CAD compared to controls. In type 1 diabetes, Nielsen and colleagues reported increased serum miR-210 [21]. Conversely, Nesca and colleagues reported reduced miR-210 in the islet cells of prediabetic and overtly diabetic mice and an increase in apoptosis of pancreatic cells after downregulation of miR-210 [22]. The link between miR-210 and diabetes may be mediated by HIFs, where dysregulation in HIFs plays a role in the pathogenesis of several diseases including diabetes and cardiovascular diseases [23]. Also, there is an association between diabetes and hypoxia where, intermittent periods of hypoxia associated with obstructive sleep apnea may play a pathogenic role in insulin resistance and diabetes [24]. HIF is responsible for the overexpression of miR-210 which is considered one of the most responsive and influential miRNAs regulated by HIF. MiR-210 affects cellular processes, including regulation of cell cycle, function of mitochondria, apoptosis and angiogenesis [25]. In the current study, patients with diabetes and CAD showed higher levels of miR-210 than in diabetes alone. Li and colleagues reported increased expression of hsa-miR-210 in the intima layer of patients with atherosclerosis [26] whilst Chen and colleagues reported increased miR-210 expression in the aorta of mice with high-fat diets [27]. Proangiogenic and anti-apoptotic roles of miR-210 may act through regulation of different genes; ephrin A3 (Efna3), Protein tyrosine phosphatase 1B (Ptp1b), death-associated protein kinase 1(Dapk1) and connective tissue growth factor (Ctgf) which were predicted to be the putative target genes of miR-210, where its over expression leads to suppression of these genes [28]. Efna3 is involved in inhibition of angiogenesis and its suppression is vital for stimulation of tubulogenesis and angiogenesis [29]. Ptp1b is involved in induction of apoptosis, where its inhibition by siRNA significantly decreased apoptosis in cardiomyocyte [30]. Dapk1, which encodes a pro-apoptotic serine/threonine kinase, is critical for regulating the cell cycle, apoptosis and metastasis, mainly functioning in the early stages of eukaryotic programmed cell death [31]. Ctgf is a secreted cysteine-rich protein with different roles in angiogenesis, chondrogenesis, osteogenesis, tissue repair, cancer and fibrosis, with enhanced expression in cardiac myocytes and fibroblasts after myocardial infarction [32]. Despite several studies reported anti-apoptotic role of miR-210 [33,34], others suggested a pro-apoptotic function of miR-210 by targeting the anti-apoptotic gene BCL2 and PDK1 [35,36].

Notably, Abdul-Maksoud et al. report low serum levels of miR-210 in rheumatoid arthritis that correlated inversely with TNF-α and IL-1β, out-performing several routine indices (ESR, CRP, rheumatoid factor), and reflecting disease activity, and so might also serve as a non-invasive biomarker.

The current study revealed associations between miR-126, miR-210 and glycaemia and lipid profiles which suggest a potential role for these miRNAs in metabolism. The ROC curve of miR-210 significantly discriminated between both patient groups and controls as well as between diabetes patients with and without CAD. The limitations of our study were that all participants were selected from one hospital and some level of selection bias could not be avoided, and the small number of controls. Further studies are recommended to understand the mechanism of action of miR-126 and miR-210 as well as to assess their possible roles as therapeutic targets for such disease.

This work represents an advance in biomedical science because it reports miR-126 and miR210 as potential biomarkers for diabetes with and without CAD.

Summary table

What is known about this subject:

- Diabetes brings a two to threefold greater risk for cardiovascular disease
- Plasma miRNAs are potential noninvasive biomarkers for the diagnosis and prognosis of many diseases.
- Circulating miR-126 is increased in several cardiovascular diseases.

What this study adds

- MiR-210 and miR-126 are significantly inversely correlated with each other in diabetes and in diabetes plus CAD
- MiR-210 levels are reduced in diabetes, and reduced further in diabetes plus CAD, whilst miR-126 levels are raised in diabetes, and raised further in diabetes plus CAD.
- ROC curves indicate both miRNAs can discriminate diabetes from healthy controls, and diabetes from diabetes plus CAD.

Disclosure statement

No potential conflict of interest was reported by the authors.

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