Journal of Diabetes & Metabolic Disorders (2021) 20:1631–1638
https://doi.org/10.1007/s40200-021-00914-z

RESEARCH ARTICLE

Salivary microRNA-126 and 135a: a potentially non-invasive diagnostic biomarkers of type- 2 diabetes

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Received: 9 July 2021 / Accepted: 4 October 2021 / Published online: 28 October 2021

Abstract

Purpose Emerging of miRNAs have illustrated the new mechanistic layer to regulate type 2 diabetes process and suggests a possible role of these RNAs in this defect. Thus, we designed this study to improve our understanding of salivary miRNA-126 and 135a expression utility as an easy of collection and non-invasive way in diabetic patients instead of blood sample.

Methods This case-control study was done on T_2D (n = 40) and healthy individuals (n = 40). The level of biochemical parameters were determined by enzymatic methods as well as glycosylated hemoglobin (HbA1c) was measured by immunoturbidimetry. We used the pooled whole stimulated saliva sample from cases and controls to assess the differentiation expression of miRNA 126 and 135-a with quantitative RT-PCR method. Unpaired Student's t test, Pearson's correlation coefficient and Receiver Operating Characteristic (ROC) analysis were used.

Results A correlation was observed between the level of HbA1c, glucose and lipid profiles (TG, TC, and LDL) in serum and whole stimulated saliva samples in T_2D patients compared to control ($p < 0.001$). miR-135a expression was considerably higher by 4.7-fold in T_2D compared to the control group (1.8-fold) ($p < 0.001$) while the miR126 expression was significantly decreased by 3.9-fold in T_2D compared to the controls (6.3-fold) ($p < 0.001$).

Conclusions The results of this case and control study showed that miR-135a and miR126 expression in saliva fluid as a reliable biomarkers and non-invasive approach in combination by change of lipid profiles, glucose and HbA1c may be used to monitor diabetic and non-diabetic patients, while further research is needed to investigate the relationship of these salivary miRNAs (miR135a, miR126) levels change on shifting the levels of clinical laboratory outcomes.

Keywords T_2D · miR135a · miR126 · Whole stimulated saliva

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Introduction

MicroRNAs (miRNAs) are small (~22 nucleotides) non-coding RNA species with specific actions, either mRNA degradation or translational repression of their target genes [1]. Of course they were discovered in Caenorhabditis elegant [2], but now their critical roles in diverse physiological cellular pathways like differentiation, proliferation and cell signaling has been approved. MiRNAs are also being increasingly implicated in several pathological states and suggesting that these disease modulators can be extended as novel disease diagnostic or prognostic biomarkers at an early stage [3–7].

Type 2 diabetes (T2D) is a heterogeneous clinical and genetic metabolic disease with complex multifactorial causes that leads to insulin resistance due to reduced response of peripheral tissues to insulin. The resulting hyperglycemia cause to increasing insulin synthesis and secretion, so this condition leads to pancreatic β-cell toxicity and cell death [8] . Emerging of miRNAs have illustrated the new mechanistic layer to regulate T_2D process as critical metabolic regulators and suggests a possible role of these RNA species in this defect. Various studies indicated alter the miRNome signatures during diabetes, and these, together with other evident confirmed the contribution of very specific miRNAs in diverse metabolic processes related to T_2D [9–12] . miRNA profiles in skeletal muscle of control and diabetic subjects were examined, 29 and 33 miRNAs were adjusted up and down, respectively [13].

The analysis of cellular and chemical components of blood is commonly used in laboratories as diagnostic procedures, while saliva offers some valuable advantages. Saliva is non-invasively and potentially valuable for children and older adult's patients for the analysis because collection of the fluid is associated with fewer compliance problems compared to blood, and analysis of saliva may provide a cost-effective way to screen the large populations [14, 15]. Changes in microRNA expression in saliva have been investigated in various studies, such as Valentina Di Pietro et,al. study showed five MicroRNAs were significantly up-regulated in salivary samples of mild traumatic brain injury patients as a good classifiers of concussion [16], also there was reported salivary miRNAs were found in patients with pancreatic cancer that are not eligible for surgery to use of salivary miRNA as biomarker [17], Another study discovered and confirmed a panel of salivary miRNA biomarkers with valid clinical function for the diagnosis of gastric cancer [18]. It was reported in the concussive head trauma over 50% of the miRNAs expressed in CSF are also found in saliva, and nearly 10% undergo the same changes during this condition [19]. Zhang et al. presented that change in the expression level of miRNAs may be a potential underlying mechanism for development pleomorphic adenomas benign tumors [20], furthermore, Wiegand et al. detected that salivary microRNAs (miR-20b, −21 and 26b, miR-16, and − 134) change in response to the Trier Social Stress Test (TSST) [21] .

The role of miR126 and miR135a on emerging and promotion of diabetes complication with the specific pathway has been reported through serum, plasma and tissue samples [22–28], but no adequate studies have yet evaluated miRNA expression patterns in T2D saliva samples of T_2D patients. Saliva is an important physiological fluid that exhibits strong and stable expression of miRNAs [23, 28]. Therefore, we designed this study to improve our comprehend of salivary miRNA126 and 135-a expression level in diabetic patients compared to control as an easy of collection and non-invasive way.

Materials and methods

Research subjects

Subjects included 80 individuals, referred to the outpatient department in the Pasteur Hospital, Qazvin, Iran. 40 patients with T_2D were included in the test group and 40 healthy individuals were included in the control group. This study was approved by the Ethics Committee of Aja University of Medical Sciences and Qazvin University of Medical Sciences (code: IR.AJAUMS.REC.1397.056) and all methods were performed under relevant guidelines. Patients in both test and control groups signed informed consent forms before participating in the study. We used the World Health Organization (WHO) criteria for diagnosis T_2D as follows: fasting blood glucose (FG) levels 7.0 mmol/l, or a two-hour oral glucose tolerance test (OGTT)11.1 mmol/l in the presence of symptoms and g glycosylated hemoglobin (HBA1c) levels >6.5% [29]. We excluded people who were involved with a history of severe hepatic dysfunction, systemic disorder and evidence for malignant disease. We used following formulation to determine the sample size [30]: $n = Z^2_{1-\alpha/2} \times \partial / d^2$Confidence level was set at 95%, with corresponding z value of 1.96.

Expected standard deviation of parameter (s)

Desired precision (d)

Confidence level (which is usually set at 95%)

Whole stimulated saliva and serum collection

Venous blood samples were collected in the morning after 12 h fasting for participants. Before saliva collection, patients were asked to rinse their mouths with distilled water and relax for 5 min. To stimulate glandular salivary flow, patients received a 2% citric acid solution to posterior lateral surfaces of the tongue, applied bilaterally using a cotton swab for 5 s every 30 s. Stimulation with citric acid continued for 30-s intervals during the entire collection procedure [31]. After this process, the saliva samples were centrifuged separately at 3000 rpm for 20 min, and clear supernatants were stored at −80°C until use.

Whole stimulated saliva and serum biochemical parameters assessment

Whole saliva was assessed calorimetrically by a spectrophotometer and using affiliated kits (Ziest Chem Diagnostics, Tehran, Iran) for analysis of whole saliva clinical parameters. Fasting plasma glucose (FPG) was assayed via glucose oxidase method. The levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) were determined by enzymatic methods. Glycosylated hemoglobin (HBA1c) was measured by immunoturbidimetry. All above laboratory measurements were measured according to the manufacturer's instructions (ParsAzmoon, Karaj, Iran) by a Hitachi 512 Automatic Analyzer.

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA from subjects were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol and quantified with a Nano-drop spectrophotometer (ND-1000, Nano-drop Technologies, DE, USA). Ratios of OD 260/280 were between 1.9 and 2.0. The integrity of RNA samples was determined in a Bioanalyzer 2100 (Agilent, CA, USA). After isolation and quantification of total RNA, 5 ng of prepared RNA was used for a reverse transcription reaction using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific). Gene-specific primers to miR135a and miR126 were used in separate reactions. To compare the miRNAs expression level between the control and diabetic groups, we used SYBR Green (Applied Biosystems, CA, USA) and miRNA specific primers miR135a and miR126 and for the endogenous control RNU6B. The cycling conditions were as follows: initial denaturation at 95 C for 2 min, 40 cycles of 95·C for 10s, 57.C for 20 s, and 7. C for 10 s. We considered only miRNAs with Ct <35 for the next analysis and relative expression of each miRNAs was calculated through the $2-\Delta\Delta Ct$ method. ΔCt was calculated by subtracting average Ct values of the reference RNU6B from average Ct values of the target miR-126 and miR135a. ΔΔCt was then calculated by subtracting average ΔCt of controls from average ΔCt of cases (T2D). All experiments were done in triplicate and normalized to RNU6B.The sequence of primers are present in Table 1.

Statistical analysis

Unpaired Student's t test, Pearson's correlation coefficient and Receiver Operating Characteristic (ROC) analysis were used using SPSS software version 23 (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered as statistically significant. To analyse miR-135a and miR126 gene expression, the bootstrapping approach was applied (Pair Wise Fixed Reallocation Randomization Test) using REST 2009 software.

Results

Characteristics of subjects

The characteristic results showed 33% of the population and 67% were men and women, respectively. Means of ages (± SD) of control and diagnosed T2D group were 46 ± 1.4 and 47 ± 1.6, respectively. Findings also revealed that body mass index BMI (kg/M^2) in diagnosed T2D was 27.6 ± 1.3 and control group was 26.4 ± 1.9. The results illustrated that the levels of HbA1c, glucose and lipid profiles (TG, TC, and LDL) in serum and whole stimulated saliva samples were significantly higher in T_2D patients compared to control, whereas the mean of HDL level was considerably lower in T_2D patients to healthy individuals (Table 2 A, B).

Expression level of miR-135a and miR-126 in study groups

The results of the REST software indicated that the expression level of miR-135a in whole stimulated saliva of T_2D was about 2.5 times higher than control ($p<0.001$), while this value in T2D was about 1.5 times lower for miR126

Table 1 Sequence of primers used

Primers	Forward	Reverse
miR-135-a	5'ACACTCCAGCTGGGTATG GCTTTTTATTCCT3	5'GGTGTCGTGGAGTCGGCAA3
miR-126	5'UCGUACCGUGAGUAAUAAUGCG 3'	5'GCGCAUGGUUUUCAUUAUUAC3'
RNU6B	5'CGCAAGGATGACACGCAAA3'	5'TTCGTGAAGCGTTCCATATTTTT3'

Table 2 Clinical findings in control individuals and patients with T2DM in stimulated saliva (A) and serum (B) samples. Data are expressed as Mean ± SEM and analyzed by unpaired student's t test. P value of <0.05 is significant. Cycle Threshold (CT), Triglyceride (TG), Total Cholesterol (TC), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL)

A	Stimulated Saliva		
	Control	T2D	P value
Glucose (mg/dl)	1.3 ± 0.02	7.9 ± 0.1	0.0001
TG (mg/dl)	4.3 ± 1.8	25.2 ± 1.1	0.0001
TC (mg/dl)	9.6 ± 0.3	22.6 ± 0.7	0.0001
HDL (mg/dl)	6.6 ± 0.9	3.2 ± 0.8	0.0001
LDL (mg/dl)	6.8 ± 0.3	15.1 ± 0.5	0.0001
B	Serum		
	Control	T2D	P value
HBA1c (%)	4.0 ± 0.2	7.6 ± 0.3	0.0001
Glucose (mg/dl)	75.7 ± 7.5	162.8 ± 8.4	0.0001
TG (mg/dl)	102 ± 6.8	273 ± 13.2	0.0001
TC (mg/dl)	145 ± 6.1	285 ± 10.3	0.0001
HDL (mg/dl)	57 ± 3.2	33 ± 2.1	0.0001
LDL (mg/dl)	109 ± 5.6	164 ± 4.2	0.0001

than control group ($p < 0.001$) (Fig. 1). These outcomes illustrated that the expression of miR135a and miR126 in T_2D patients were respectively up and down-regulated. In addition, the receiver operating characteristic (ROC) curve analyses showed the considerably differences between the change of miR-126 (Cut off: 26.9, AUC: 0.007) and miR135 (Cut off: 25.6, AUC: 1) expression in whole stimulated saliva (Fig. 2). Significant differences between biochemical parameters such as a HbA1c, Glucose, TG, TC, LDL and HDL were also observed in stimulated saliva and serum samples as the most important clinical factors which may be used to discriminate and diagnostic patients involved with T_2D (Table 3) ($P < 0.0001$).

A direct correlation is shown between HbA1c and miR126 expression in whole stimulated saliva, whereas HbA1c levels in contrast to HDL and miR135a expression. On the other side, the expression level of miR135a in whole stimulated saliva of T_2D showed the inverse correlation with LDL, TG, TC, BMI, and glucose, while it had a significant positive correlation with HDL. In addition, results showed a direct correlation between miR126 expression levels in whole T2D-stimulated saliva with TG, TC, LDL, BMI and glucose, but had a considerable negative correlation with HDL (Table 4).

Discussion

The Pearson's correlation coefficient analysis showed that the level of HBA1c, glucose and lipid profiles (TG, TC, and LDL) were significantly higher in T_2D patients compared to control and there was seen a significant correlation between the level of lipid profiles and glucose in serum compared to whole stimulated saliva was seen, which was match with previous studies that concluded a positive correlation between the changing level of glucose in saliva compared to plasma and serum in patients with T_2D [32, 33]. Tatsuya Machida et al. study result suggested miR-1246 and miR-4644 in salivary exosomes can be used as biomarkers for diagnosis of pancreatobiliary tract cancer [34] .

Previous studies have shown that miR-126 levels in plasma are reduced by glucose-dependent methods. This association suggests that increased plasma glucose might result in the decrease delivery of miR-126 to monocytes, which in turn contributes to vascular endothelial growth factor (VEGF) resistance and endothelial dysfunction. MiR-126 regulates the expression of several proteins with prominent roles in multiple diseases, including the anti-inflammatory TOM1 (target of Myb1), the growth factor VEGF-A, and the cell cycle regulatory and signaling protein IRS-1 (insulin receptor substrate 1) [27, 35, 36] . Surprisingly the Pearson's correlation coefficient analysis indicated the down-regulation of miR126 had a positive direct correlation with

Table 3 Receiver operating characteristic curve analysis

	Serum					Stimulated Saliva				
Clinical Parameters	Cut off	AUC	% Sensitivity	% Specificity	P value	Cut off	AUC	% Sensitivity	% Specificity	P value
HbA1c (%)	50.6	0.934	90	90	0.0001					
Glucose (mg/dl)	115	0.982	100	100	0.0001	4.0	1	100	100	0.0001
TG (mg/dl)	172	0.997	95	95	0.0001	14.7	1	100	100	0.0001
TC (mg/dl)	227	0.993	95	95	0.0001	19.2	1	100	100	0.0001
HDL (mg/dl)	44	0.012	90	90	0.0001	4.9	0.021	95	95	0.0001
LDL (mg/dl)	145	0.968	95	95	0.0001	10.6	0.991	95	95	0.0001
miR135a						26.9	0.007	95	95	0.0001
miR126						25.6	1	100	100	0.0001

****$P < 0.0001$

Table 4 Correlation between clinical parameters in serum and stimulated saliva samples among patients with T2DM and expression level of miR-135a and miR-126 compared to findings in the control group. Data were analyzed by Pearson's correlation coefficient analysis test

		Serum					Stimulated saliva							
Serum		FBS	TG	TC	HDL	LDL	Glucose	TG	TC	HDL	LDL	miR135a (CT)	miR126 (CT)	BMI
	HBA1c	0.73**	0.74**	0.78**	−0.76**	0.59**	0.77**	0.77**	0.79**	−0.74**	0.77**	−0.58**	0.69**	0.71**
	FBS		0.81**	0.81**	−0.78**	0.65**	0.84**	0.87**	0.87**	−0.79**	0.84**	−0.72**	0.82**	0.72**
	TG			0.89**	−0.89**	0.77**	0.93**	0.94**	0.95**	−0.92**	0.94**	−0.83**	0.89**	0.88**
	TC				−0.92**	0.81**	0.89**	0.89**	0.91**	−0.85**	0.89**	−0.71**	0.83**	0.85**
	HDL					−0.77**	−0.84**	−0.85**	−0.88**	0.81**	−0.86**	0.69**	−0.77**	−0.82**
Stimulated saliva	LDL						0.74**	0.75**	0.77**	−0.71**	0.80**	−0.52**	0.78**	0.71**
	Glucose							0.98**	0.96**	−0.90**	0.93**	−0.86**	0.95**	0.88**
	TG								0.97**	−0.91**	0.94**	−0.85**	0.94**	0.89**
	TC									−0.90**	0.95**	−0.83**	0.90**	0.89**
	HDL										−0.89**	0.76**	−0.87**	−0.86**
	LDL											−0.79**	0.91**	0.85**
	miR135a (CT)												−0.78**	−0.80**
	miR126 (CT)													0.86**

**$P < 0.01$

increasing the level of HbA1c, glucose and lipid profiles except for HDL and these results maybe can explain the role of miR-126 in progression T_2D diseases and related symptoms as it was approved that the expression level of miR-126 in HepG2 cell can be connected to the expression of glucose–lipid (fasting blood glucose, total cholesterol, triglyceride, low-density lipoprotein and high-density lipoprotein) genes [37] . Previous studies on diabetic diseases have revealed circulating miR-126 levels in the blood of patients with T_2D were reduced [27, 38, 39], as the results of present study showed this phenomenal. Other investigations have shown an increase in miR-126 levels in the blood samples of patients with diabetic nephropathy (DN) with type 1 diabetes (T1D) [40] and in the urine samples of patients with DN T2D [41] compared with controls while these results is in contrast to the results of present study, although this discrepancy maybe justified by the variation between not only type of diabetes but also the kind of samples. Down-regulation of miR-126 has been shown to be associated with high glucose concentrations in human umbilical vein endothelial cells and disrupts the functional characteristics of endothelial progenitor cells in diabetic patients by signaling vascular endothelial growth factor (VEGF) [42].

Studies have revealed that the down-regulation of forkhead box O1 (FOXO1) and increasing level of cyclooxygenase-2 (COX-2) genes in db/db VSMC (Vascular Smooth Muscle Cells) mice was related to increase of miR135-a expression inside the vascular smooth muscle during hypertension [43–45], also increasing the expression of miR135-a in serum and renal tissue from diabetic nephropathy patients can led to development of micro-albuminuria and renal fibrosis by targeting the transient receptor potential cation channel, subfamily C, member 1 (TRPC1) to prevent Ca^{2+} entry into cells may be a mechanism whereby miR-135a promotes renal fibrosis in diabetic kidney injury [24]. The expression level of miR135a in salivary sample of T_2D patients was higher than control and noteworthy that the up-regulation expression of miR135a is inverse to the lipid profiles and glucose, although it showed the positively direct relation to HBA1c and HDL, as it was observed, up-regulation of miR135a terminated to decreasing the level of free cholesterol (FC), total cholesterol (TC) and cholesterol ester (CE) in atherosclerosis (AS) diseases by targeting lipoprotein lipase mRNA [46]. Results of various studies noted that increasing the expression level of miR-135a in diabetes status was related to decreasing the expression of IRS2 at the level of mRNA and protein but also miR-135a level was elevated in the human diabetic skeletal muscle and these results suggested that IRS2 mRNA is the main target of miR-135a for binding to regulate skeletal muscle insulin signaling pathway [22, 25] .

The results of the ROC curve analysis indicate that changes in the expression levels of miR135a, miR126 and HBA1c and other laboratory results may use whole stimulated saliva to assess the status of T2D disease. Al-Kafaji et al. found a direct correlation between miR375 and miR9 with presence of pre-diabetes and T_2DM as well as a significant association of miR-375 but not miR-9, with development of T_2DM independently of age, sex, BMI, mean blood pressure, HBA1c, total cholesterol, triglyceride, and LDL [9].

Recent investigation has concluded that miRNA-146a and miRNA155 in saliva provide reliable, non-invasive, diagnostic and prognostic biomarkers that can be used to monitor periodontal health status among diabetic and non-diabetic patients [47]. Our previous studies results indicated miR135a was expressed up-regulated in serum and plasma samples from various types of diabetic patients (T_2D, IGT, IFG and GDM) compared to healthy individuals, Honardoost et al. also showed that miR-135a was a key regulator of skeletal muscle growth by regulating the IRS / PI3K pathway in diabetic status [25]. All of these data confirm the important role of miR-135a and 126 in the progression and development of diabetes and its associated side effects, and agree with our findings on the up and down regulation of miR135a and 126, respectively, and the clinical outcomes of T2D patients. While it is worth noting that the most important advantage of the present study over previous research on the expression level of these microRNAs in diabetes is that we had used stimulated saliva instead of blood samples derivatives as non-invasive and accessible approach could serve as a biomarkers in clinical evaluation to monitor health status among diabetic and non-diabetic patients since the microRNA changes in saliva samples from type-2 diabetic patients have not been adequately studied.

Conclusion

The results of the present study showed that altering the expression level of salivary miRNAs (miR135a, miR126) in saliva fluid as a reliable biomarkers and non-invasive approach in combination by change of lipid profiles, glucose and HbA1c may be used to monitor diabetic and non-diabetic patients, while further research is needed to investigate the relationship of these salivary miRNAs (miR135a, miR126) levels change on shifting the levels of clinical laboratory outcomes that may help to improve the sensitivity of salivary miRNA testing in T_2D. Improved sensitivity would be critical if these miRNAs approach will to be applied in clinical settings of monitoring diabetes status to understand its complication process at the genetically level.

Acknowledgements We would like to thank the clinical staff of pathobiology laboratory in Pasteur Hospital (Qazvin province, Iran) for their technical assistance.

Author contributions Conceptualization and methodology: Y.K.M., I.M.D., and MR.M.D. Material preparation: E.K., and MR.S; Investigation: Y.K.M., I.M.D. and S.H..; Data collection: E.K., Y.K.M., and S.H.; Writing—original draft preparation: Y.K.M., I.M.D., Writing—review and editing: Y.K.M., I.M.D., MR.M.D. And MR.S.

Funding This study was funded by Aja University of Medical Sciences and Qazvin University of Medical Sciences.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval The current study was carried out according to Helsinki Declaration and was approved by the ethics committee of AJA University of Medical Sciences (IR.AJAUMS.REC.1397.056).

Informed consent Informed consent was obtained from all individual participants included in the study.

Study limitations The most important limitation in writing our manuscript was that we could not find reports in regard to the assessment of the expression level of Micro RNAs in saliva samples as biomarkers in diabetes.

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