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CLINICAL STUDY

MiR-126 and miR-146a as markers of type 2 diabetes mellitus: a pilot study

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ABSTRACT

INTRODUCTION: Despite known risk factors for developing type 2 diabetes mellitus (T2D), the research community still tries to discover new markers that would widen our diagnostic and therapeutic approach to diabetes. Therefore, research on microRNA (miR) in diabetes thrives. This study aimed to assess the utility of miR-126, miR-146a, and miR-375 as novel diagnostic markers for T2D.

METHODS: We examined relative quantities of miR-126, miR-146a, and miR-375 in the serum of patients with established type 2 diabetes mellitus (n = 68) and compared these with a control group (n = 29). We also undertook a ROC analysis of significantly changed miR to examine their use as a diagnostic test.

RESULTS: MiR-126 ($p < 0.0001$) and miR-146a ($p = 0.0005$) showed a statistically significant reduction in patients with type 2 diabetes mellitus. MiR-126 also proved to be an exceptional diagnostic test in our study cohort, with high sensitivity (91 %) and specificity (97 %). We did not find any difference in our study groups' relative quantities of miR-375.

CONCLUSION: The study proved a statistically significant reduction of miR-126 and miR-146a in patients with T2D *(Tab. 4, Fig. 6, Ref. 51)*. Text in PDF *www.elis.sk*

KEY WORDS: microRNA, epigenetics, genomics, type 2 diabetes mellitus, miR-126, miR-146a and miR-375.

Introduction

The increasing incidence and prevalence of type 2 diabetes mellitus (T2D) raise legitimate concerns, as it is one of the most important diseases worldwide in terms of morbidity and mortality. The rising incidence is present mainly in Asian countries, namely India and China (1). Although the diagnostic algorithm for diabetes is relatively specific and straightforward, several patients are still not diagnosed in time, leading to complications and higher mortality. Efforts to identify risk factors that would predict the development of diabetes have resulted in recognizing pre-diabetic conditions and treating these patients in some countries. Other risk factors are also recognized, some of which can be targeted before irreversible metabolic changes occur, and thus this could be used to prevent the development of diabetes (2). Despite these known factors, there is considerable interindividual variability in the incidence of T2D. Research into other risk markers and factors, such as adipokines, inflammatory markers, microRNAs (miR), and many others, brings new possibilities for diagnostic, therapeutic, and preventive approaches to such patients. This study aims to assess selected miR in patients with T2D, namely miR-126, miR-146a, and miR-375, and to examine their role as a possible novel diagnostic marker of T2D.

Methods

Participants

We included outpatients and inpatients who regularly visited the diabetes center or were admitted to the Department of Internal Medicine I from 2017 to 2021. The diagnosis of T2D was based on standard criteria (repeated measurements of fasting glycemia $\geq$ 126 mg/dL (7 mmol/L) or 2 hours glycemia $\geq$ 200 mg/dL (11.1 mmol/L) on oral glucose tolerance test). Patients with any ongoing acute disease or hyperglycemic medication were excluded. Control probands were recruited from healthy volunteers in whom an oral glucose tolerance test excluded diabetes. All study participants were thoroughly educated about the procedures and diagnostic tests and gave informed consent in written form. The Ethics Com-

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mittee of Jessenius Faculty of Medicine, Comenius University, approved the study protocol.

Sample collection and processing

All peripheral blood samples were drawn under standardized conditions in the morning after overnight fasting. Subsequently, we centrifuged these samples for 3 minutes at 4000 rpm. The supernatant in a volume of 1 ml was pipetted into pre-prepared 2 ml test tubes with a stabilizing agent, followed by manual mixing. All these procedures were executed within 2 hours of collection. Afterwards, we stored all the samples at a temperature of –80 °C.

RNA isolation

MiR isolation was performed using miRNeasy Serum/Plasma Advanced Kit reagent according to the manufacturer's recommendations. Proteinkinase K was added to the test tube, and then the samples were incubated for 30 minutes at 55–60 °C. Buffer solution and alcohol were then added to the sample tube. The solution was bound to a solid silica reagent which was further washed with an alcohol buffer and finally eluted with an optimal elution solution.

Reverse transcription and real-time quantitative PCR (qRT-PCR)

Reverse transcription was performed using a C 1000TM Thermal Cycler with reagent and plastic TaqMan MicroRNA Assays and Taqman MicroRNA RT kit, containing microRNA RT primers of selected miR. The isolated miR was diluted with DEPC-treated water to achieve the appropriate concentration of 2 ng/μL before reverse transcription. The Master Mix solution was vortexed and, by centrifugation, got to the bottom of another set of test tubes. Subsequently, 4.67 μL of Master Mix solution and 3.33 μL of the diluted sample with an RNA concentration of 2 ng/μL were pipetted into the appropriate number of 0.2 ml volume test tubes according to the number of samples examined. The RT primers for miR-126, miR-146a, miR-375, and endogenous controls were also separately mixed, and then 2μL of these reagents were added to the prepared test tubes with the other components. The tubes thus filled were sealed, vortexed, centrifuged briefly, and finally left on ice for 5 minutes. The thermocycler was set according to the manufacturer's recommendations, and the volume of the total PCR reaction was set to 10 μL. Subsequent reverse transcription products were immediately processed by qRT-PCR or stored in a freezer at –20 °C. Ct values of selected miR were determined using 20X TaqMan MicroRNA Assays Mix reagent with fluorescently labeled TM probe on an IQ5 Multicolor Real-Time PCR Detection System. In the case of frozen samples, these were first thawed on ice. Subsequently, the Master Mix was prepared in a 1.5 ml tube, mixed gently, and centrifuged briefly. The 96-well PCR plate was labeled, and 18.67 μL of Master Mix solution and 1.33 μL of reverse transcription sample were pipetted into each well. In two wells, water was pipetted instead of the sample as a negative control. The plate was carefully covered with a foil to prevent evaporation of the samples during amplification, and the amplification program was set according to the manufacturer's recommendations. The reaction volume was adjusted to 20 μL. The amplification output was a Ct value for each of the miR examined, at which the fluorescence value exceeded the thresholds and was further analyzed. Ct values from 19–29 indicate a high presence of the target miR. Medium amounts represent Ct values from 30–35, and above 35 represent a minimum amount of miR in the examined sample.

Endogenous control

After a literature review, several endogenous controls were selected, examined for 20 samples, and their expression and uniformity were determined. RNU6B and miR-1233 were examined in this manner. MiR-1233 was finally determined as the endogenous control, as it was more stable in the samples and did not correlate with any other parameters examined.

Relative quantification

The relative quantification of miR was performed by comparing Ct values, comparing the relative change in the amount of the selected miR in the examined sample with the endogenous control and with the control sample. First, the ΔCt was calculated, subtracting the selected miR Ct value from the endogenous control's Ct values. The ΔΔCt was then calculated, which is a subtraction of the average ΔCt of the endogenous control from the individual ΔCt of the selected miR. Finally, a mathematical calculation was performed, where ΔΔCt values are inserted into the formula for calculating the relative amount of specific miR – relative quantity (RQ) = $2^{-\Delta\Delta Ct}$.

Statistical analysis

Statistical analysis was performed by the "JMP starter" program. Quantitative data were tested for normality (the Shapiro–Wilk test was used for this testing); then, a t-test (normal distribution) or a Mann-Whitney U test (asymmetric distribution) was used for the assessment of quantitative differences in selected miR

Tab. 1. Characteristics of the study groups.

	Control	T2D
Number (n)	29	68
Male (n/%)	16 (55%)	31 (46%)
Female (n/%)	13 (45%)	37 (54%)
Age (yr)	63±12.5	69±6.7
Age range (yr)	43–89	47–87
BMI (kg/m^2)	23±1.6	31±5.7

BMI – body mass index; T2D – type 2 diabetes mellitus; n – number; yr – years

Tab. 2. Characteristics of the patients with type 2 diabetes mellitus.

Number (n)	68	Total bilirubin (μmol/L)	13.3±6.4
Male (%)	31 (56%)	Triglycerides (mmol/L)	1.94±1.0
Female (%)	37 (54%)	Total cholesterol (mmol/L)	5.0±1.0
Glycaemia (mmol/L)	7.5±1.51	HDL cholesterol (mmol/L)	1.3±0.4
Creatinine (μmol/L)	86.9±34.1	LDL cholesterol (mmol/L)	3.1±1.0
Uric acid (μmol/L)	352.6±90.0	GGT (μkat/l)	0.5±0.4
HbA_{1c} (% DCCT)	5.4±1.3	AST (μkat/l)	0.4±0.2
Diabetes length (yr.)	12±7.4	ALT (μkat/l)	0.4±0.1

ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGT – gamma-glutamyl transferase; HbA_{1C} – glycated hemoglobin estimated according to Diabetes Control and Complications Trial; HDL – high density lipoproteins; LDL – low density lipoproteins.

Tab. 3. Results of relative quantification of microRNAs.

	n	miR-126	miR-146a	miR-375
Control[a]	29	1.03 (0.92–1.14)	1.07 (0.89–1.26)	1.18 (0.75–1.61)
T2D[a]	68	0.17 (0.10–0.25)	0.61 (0.49–0.73)	1.21 (0.93–1.50)
Fold change[b]		6.06	1.75	0.96
p		< 0.0001	0.0005	0.1584

[a]The table shows medians of relative quantities with the 5th and 95th percentile shown in brackets. [b]The fold change represents the ratio of relative quantities of the control and T2D groups. n – number; miR/microRNA – micro ribonucleic acid; T2D – type 2 diabetes mellitus

between compared groups of patients. The Chi-square test was used to compare categorical variables. Subsequently, the method "Receiver Operating Characteristic" (ROC) was used, according to which the suitability of the diagnostic marker for differentiation of healthy and sick probands can be assessed. In this way, we analyzed the significance of all three examined miR. The result is an 'Area Under a Curve' (AUC) value, which expresses the probability that the proband is correctly assigned to the corresponding group based on the investigated characteristics. This is more likely the closer the AUC is to 1. This method can also determine the sensitivity and specificity of a test for a given characteristic. $p < 0.05$ was determined as a statistically significant difference.

Results

Patients and controls

During the study period, we were able to enroll 68 patients with T2D (31 men, 37 women, mean age 69 years, ranging from 47 to 87 years) and 29 controls (16 men, 13 women, mean age 63 years, ranging from 43 to 89 years) matching study inclusion and exclusion criteria. The baseline characteristics of the enrolled subjects are reported in Table 1 (patients versus controls) and Table 2 (T2D patients). Patients with T2D had a mean value of glycated hemoglobin ($HbA1_C$) 5.4 ± 1.3 % DCCT. T2D duration was 12 ± 7.4 years. Controls were slightly younger ($p = 0.09$) and had significantly lower body mass index in comparison with T2D patients (23.0 ± 1.6 vs 31.0 ± 5.7 kg/m^2; $p < 0.05$) (Tabs 1 and 2).

Relative quantification of microRNAs

There was a statistically significant difference in the relative quantity of miR-126 between probands in the control group and with T2D. We found a decrease in the quantity of miR-126 in probands with T2D ($p < 0.0001$). MiR-146a also showed a statistically significant reduction in patients with T2D ($p = 0.0005$). MiR-375 was present in the control group and the group with T2D in comparable quantities without a statistically significant difference. These results are shown in Table 3 and Figures 1–3.

ROC analysis

ROC analysis determined the sensitivities and specificities of the significantly altered miRs by themselves and the combination of miR-126 and miR-146a. The results are summarized in Table 4. The ROC curves for each miR are shown in Figures 4 and 5, and Figure 6 shows the ROC curve for the combination of miR-126

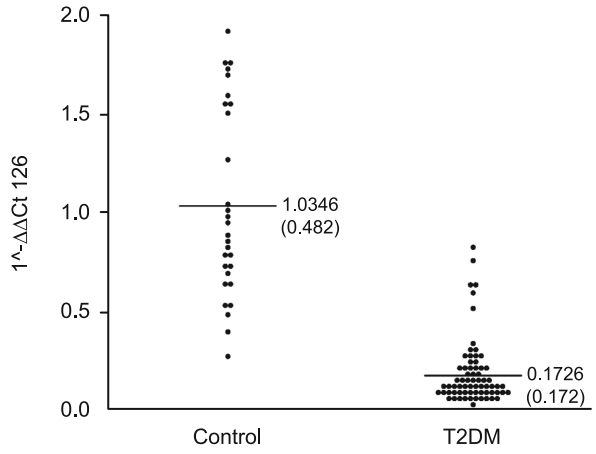

Fig. 1. Difference of the relative quantities of miR-126 in the control and T2DM groups.

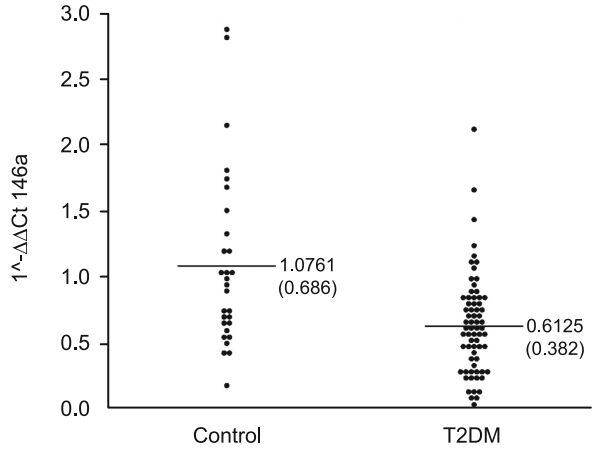

Fig. 2. Difference of the relative quantities of miR-146a in the control and T2DM groups

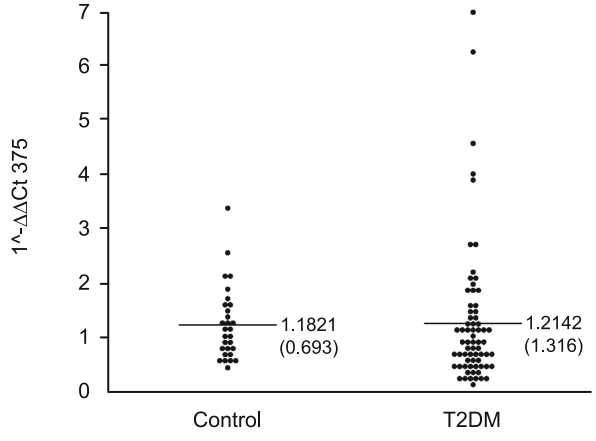

Fig. 3. Difference of the relative quantities of miR-375 in the control and T2DM groups.

Tab. 4. ROC analysis for significantly changed microRNAs.

n=97	miR-126	miR-146a	Combination of miR-126 and miR-146a
Sensitivity	91%	83%	100%
Specificity	97%	65%	90%
AUC	0.976	0.725	0.7975
p	< 0.0001	0.0009	< 0.0001

AUC – area under a curve; miR/microRNA – micro ribonucleic acid

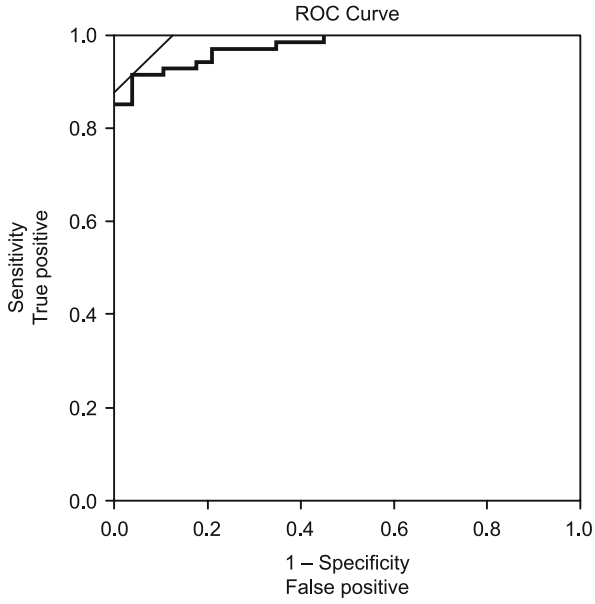

Fig. 4. ROC curve for miR-126.

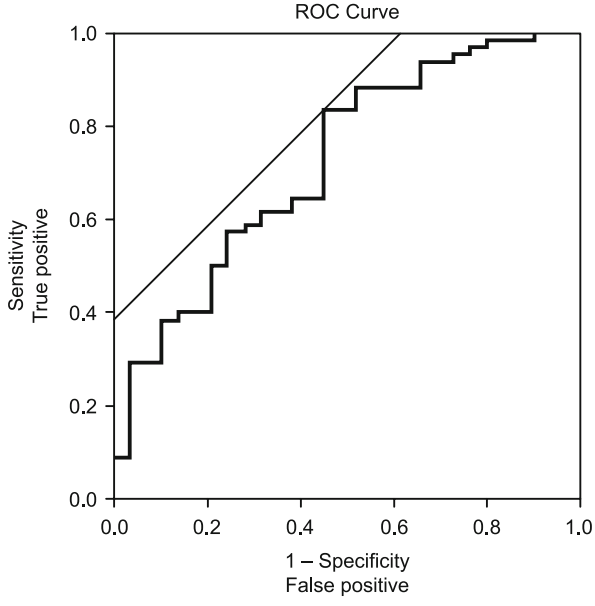

Fig. 5. ROC curve for miR-146a.

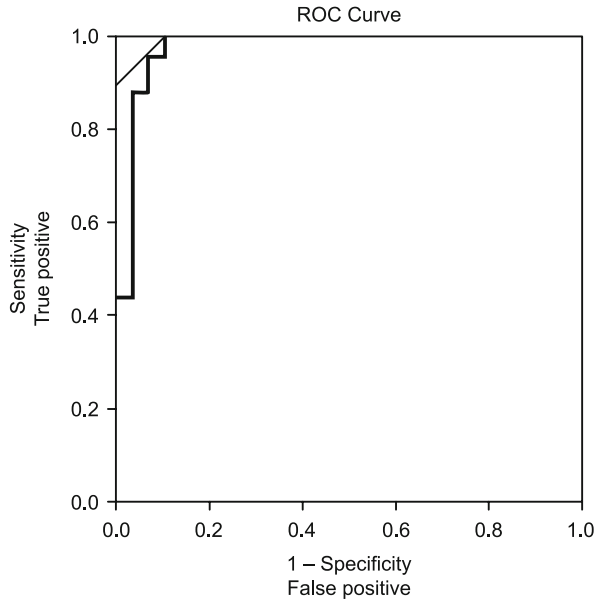

Fig. 6. ROC curve for the combined use of miR-126 and miR-146a.

and miR-146a. Based on the calculated values, it can be assessed that the miR-126 test has an excellent differentiation potential to determine T2D with a sensitivity of 91 % and a specificity of 97 % in our study groups. Adding the examination of miR-146a increases the sensitivity to 100 % but slightly reduces the test's specificity to 90 % (Tab. 4, Figs 4–6).

Discussion

Choosing a suitable endogenous or exogenous control to normalize the examined microRNAs' representation is crucial in determining the proportion of microRNAs in individual studies. This is where the most significant discrepancies arise between the various studies, which can clarify the often-contradictory results. In our study, we selected miR-1233 as the endogenous control based on its stable presence in all samples examined. Reference genes of small nuclear RNAs, such as RNU6B, are often used as a control. However, they did not have sufficient stability in our samples, which was consistent with findings from other studies where plasma or serum microRNA assays were performed (3). The microRNAs themselves can also be chosen as the endogenous control, as was done in our study. For example, miR-1228 was used for the relative quantification of other microRNAs in a previously published study (4). The addition of synthetic microRNAs from other organisms to samples followed by qRT-PCR can also be used as a control for data normalization (5). Despite a relatively large selection of possible endogenous or exogenous controls, in some studies, it was not possible to find a control molecule that is stable in all samples, as often, even these may correlate with one of the factors examined (6). Summarizing, we have confirmed the possibility of relative microRNA quantification using endogenous microRNA; however, the issue of optimal quantification of mi-

croRNAs is not concluded and might explain differences in results obtained in so far published studies.

MiR-126 is the most frequently examined microRNA associated with T2D (7–9). The results mainly indicate a reduced amount of this microRNA in the serum, plasma, whole blood, and polymorphonuclear cells of diabetic patients, but there were also conflicting ones (10–14). Regarding the etiology and pathogenesis of T2D, miR-126 is mainly associated with regulating endothelial cells and perhaps plays a role in protecting against atherosclerosis (10, 15–18). MiR-126 has also been investigated as a possible marker of improved diabetes compensation (8). Similar results were observed by Catanzaro et al., who monitored the presence of miR-126 in plasma in patients with T2D before and after 15 months of sitagliptin treatment. The authors of this study reported that miR-126 was increased after treatment in patients with better diabetes compensation, but these data were inconsistent among other studies (19, 20). MiR-126 was also associated with all-cause long-term mortality of patients with T2D in one study (21) and diabetic complications in several more (22–25). In our study, we observed a statistically significant reduction of miR-126 in the serum of T2D patients compared to the control group, which is recorded in the Central European population for the first time and is consistent with previous findings in other investigated populations (26, 27). In the case of distinguishing healthy probands from probands with T2D, miR-126 proved to be an excellent discriminating marker with high sensitivity and specificity, which indicates a possibility of the future use of the test in clinical practice.

MiR-146a is the second most frequently examined microRNA in patients with T2D (28). It is mainly associated with the inflammation process through the regulation of NF-κB with the subsequent development of chronic subclinical inflammation in case of a decrease in its amount (29, 30). MiR-146a expression was significantly decreased in serum, plasma, whole blood, and polymorphonuclear cells, similar to miR-126 (12, 31–34). Still, some studies have shown inconsistent findings (35, 36). The role of miR-146a in the genesis of diabetic complications was also evaluated in several studies (37–41). In our study, we observed a statistically significant decrease in the serum of probands with T2D compared to the control group; however, to a lesser extent than miR-126. Nevertheless, this is the first time such a change in the quantity of this microRNA was confirmed in T2D patients in the Central European population. By analyzing the ROC curve, we found the ability of miR-146a to differentiate healthy subjects from patients with T2D with a sensitivity of 83 % and a specificity of 65 %, which were lower compared to miR-126. There was a minimal shift in test characteristics with a slight increase in sensitivity at the expense of specificity when examining both microRNAs.

MiR-375 was consistently positively correlated with T2D in serum, plasma, and whole blood (42–44). Zhu et al. demonstrated in their meta-analysis that miR-375 is the most increased of all other microRNAs in patients with T2D (45). This observation was confirmed in a meta-analysis by Villard et al. that found the most significant fold change in the amount of miR-375 between controls and patients with T2D. In addition, miR-375 levels correlated with a longer duration of the disease, which may indicate the pathophysiological importance of this microRNA in the development of diabetes (46). Several studies have also linked miR-375 to diabetic retinopathy (47–49). MiR-375 was also researched as a possible biomarker of T2D development (50–51). However, the relative quantity of miR-375 was not statistically significantly changed in our study. Therefore, our results did not confirm the presumed usefulness of miR-375 determination in the discrimination between T2D and normal glucose metabolism.

Limitations

Regarding the structure of our study, the control group was at low risk for the development of diabetes. Therefore, our results need validation on a larger cohort to better represent a distinct high-risk population. As mentioned, another limitation is using different control molecules for the relative quantification of miR in studies; hence, the results of different studies are difficult to compare. Finally, the overall sample size in our study is limited. All these limitations should be considered when interpreting the results obtained in our study.

Conclusion

Our study found a statistically significant difference in relative quantities of miR-126 and miR-146a (decreased) in patients with T2D. Additionally, MiR-126 had high sensitivity and specificity for the diagnosis of T2D. Although our results support future research on the role of selected microRNAs as a marker for the diagnosis of T2D, more research in this area will be needed for final conclusions.

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