Plasma Exosome MicroRNA Profiling Unravels a New Potential Modulator of Adiponectin Pathway in Diabetes: Effect of Glycemic Control

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Context: Type 2 diabetes is a chronic disease characterized by inadequate β-cell response to the progressive insulin resistance. MicroRNAs (miRNAs) are short, endogenous, noncoding RNAs representing a class of powerful gene expression modulators. Previous population studies observed a modulation of circulating miRNAs in diabetic patients; however, few data are presently available on miRNA modulation in diabetic patients naïve to pharmacological treatment as well as the effect of glycemic control on this.

Objective: We aimed at studying circulating miRNA expression in diabetic patients naïve to treatment and at investigating the influence on this of glycemic control.

Design: This was a case-control study.

Participants: Eighteen treatment-naïve diabetic patients with poor metabolic control and 12 control patients participated in the study.

Main Outcome Measures: Wide miRNA expression profiling was performed, and the expression of miRNAs found to be dysregulated was then validated by quantitative RT-PCR. Finally, algorithm-identified putative miRNA targets were evaluated by quantitative RT-PCR and ELISA.

Results: In diabetic patients, microarray analysis showed that four miRNAs are increased, whereas 21 miRNAs are decreased. Quantitative RT-PCR validation confirmed the significant up-regulation of miR-326 ($P = .004$) and down-regulation of let-7a ($P < .001$) and let-7f ($P = .003$). Notably, an inverse negative correlation was found between circulating miR-326 and its putative target adiponectin ($p = -0.479$, $P = .009$). After 12 months of antidiabetic treatment, quantitative RT-PCR data analysis showed that miR-326 levels were unaffected, whereas the levels of let-7a and let-7f were significantly increased.

Conclusions: Treatment-naïve, poorly controlled diabetic patients show a significant dysregulation of miRNAs involved in the regulation of the adiponectin pathway, a phenomenon that may be reversed, at least in part, by improved glycemic control. **(*J Clin Endocrinol Metab* 99: E1681–E1685, 2014)**

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Abbreviations: ADIPOR, adiponectin receptor; BMI, body mass index; HbA1c, glycated hemoglobin; miRNA, microRNA; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative real-time PCR.

MicroRNAs (miRNAs) are short sequences (~22 nucleotides) of endogenous RNA, which primarily act as posttranscriptional regulators of gene expression. Human genome is estimate to encode for approximately 1100 miRNAs that could modulate the expression of approximately 60% of genes (1). Moreover, miRNA deregulation is associated with several human diseases, including atherosclerosis and cardiovascular diseases (2). Remarkably, miRNAs have been found to be extremely stable in the circulation, underscoring their potential role not only as disease biomarkers but also as an important paracrine and endocrine communication system (3).

Previous studies have investigated the role of miRNAs in diabetes. Indeed, mice with selective *Dicer* deletion in pancreatic cells, hence unable to produce mature miRNAs, showed growth failure and usually died 3 days after birth (4). Moreover, many experimental works have elucidated the role of miRNAs in several molecular pathways crucial for glucose homeostasis and diabetic complications (5). Finally, some previous reports have investigated the expression of miRNAs in established diabetic patients (6–9); however, changes in the modulation of circulating miRNAs expression in newly diagnosed and poorly controlled diabetic patients are far from being thoroughly and fully investigated, and little is known about the influence of glycemic control on the miRNA modulation.

Materials and Methods

Study population

Eighteen treatment-naïve patients with type 2 diabetes [12 males, aged 57.2 ± 9.6 y, mean body mass index (BMI) 31.6 ± 5.1 kg/m^2, mean glycated hemoglobin (HbA1c) 9.6% ± 1.5%, mean plasma glucose 81 ± 16.4 mmol/L] and 12 nondiabetic subjects (six males, aged 48.5 ± 12.4 y, mean BMI 32.9 ± 5.4 kg/m^2, mean HbA1c 5.7% ± 0.5%, mean plasma glucose 39 ± 5.5 mmol/L) balanced for the main cardiovascular risk factors were enrolled into the study. We excluded patients with a history or clinical evidence of active inflammatory diseases, cancer, diabetic complications, liver/kidney dysfunction, and active treatment with statins or angiotensin receptor blockers [due to their influence on glucose metabolism (10, 11)]. Baseline patients' characteristics are reported in Supplemental Table 1. The study was approved by the local ethics committee and participants provided their written informed consent.

Blood sample collection and RNA extraction

Whole blood was drawn into EDTA tubes and separated in plasma and peripheral blood mononuclear cells (PBMCs). ExoQuick exosome precipitation solution (System Biosciences) was used to precipitate plasma exosomes, and then the RNeasy kit (QIAGEN) was used to extract miRNAs. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare), and total RNA enriched for small RNAs was isolated using Trizol (Invitrogen) according to the manufacturer's protocol.

Evaluation of microRNA expression

For miRNome analysis, two homogeneous samples were created: one by pooling 2 μL of RNA from exosomes of diabetic patients and the other by pooling RNA from exosomes of control subjects. After reverse transcription (mirCURY LNA universal cDNA synthesis kit; Exiqon), the reaction was performed on a serum/plasma focus miRNA PCR panel 384 well (V1.R) (Exiqon) using the ABI 7900HT (Applied Biosystems). The most stable miRNAs (miR-425 and miR-423–5p) were identified by NormFinder and GeNorm, validated on each sample, and used as normalizators for circulating miRNAs. After miRNome analysis, the selected miRNAs were measured on samples from each single subject by quantitative real-time PCR (qRT-PCR) (Exiqon). For miRNA evaluation from PBMCs, 1 μg of RNA was used for reverse transcription, and then qRT-PCR was carried out to quantify the expression of miR-326, let-7a, and let-7f. Relative quantification values were calculated using the equation $2^{-\Delta\Delta Ct}$.

Evaluation of targets

Putative targets were individuated in silico by target prediction algorithms (Targetscan, PicTar, miRanda) (Supplemental Table 2). One microgram of total RNA from PBMCs was reverse transcribed by using a high-capacity cDNA reverse transcription kit (Applied Biosystems), and then qRT-PCR was performed to measure the expression level of *AdipoR1, AdipoR2*, and *APPL1* (Applied Biosystems). We used 18S as reference gene, and relative quantification values were calculated using the equation $2^{-\Delta\Delta Ct}$. Plasma adiponectin levels were determined by a commercially available double-antibody sandwich ELISA assay (RayBio), using a Spectramax 190 (Molecular Devices).

Statistical analyses

Statistics were calculated using SPSS 16 (SPSS Inc). Log_{10}-transformed values were used for all computations to approximate a Gaussian distribution. A Student *t* test was used to compare differences between groups, with Welch correction when appropriate. Correlations were estimated by Spearman correlation test. Simultaneous-entry multiple linear regression analysis was conducted with plasma adiponectin levels as the dependent variable. Binary logistic regression analysis was performed as previously described (12). Differences were considered significant at a two-tailed value of $P < .05$.

Results

Identification of differentially expressed circulating microRNAs

After microarray evaluation of pooled samples, we found a dysregulation (>5 fold-change) of 25 miRNAs in diabetic patients (Figure 1A). Notably, among these miRNAs, we found that miR-326, miR-186, miR-21, miR-126, miR-16, let-7a, let-7f, and let-7g have predicted targets involved in the pathophysiology of diabetes. Thus, the expression of these miRNAs was further confirmed by

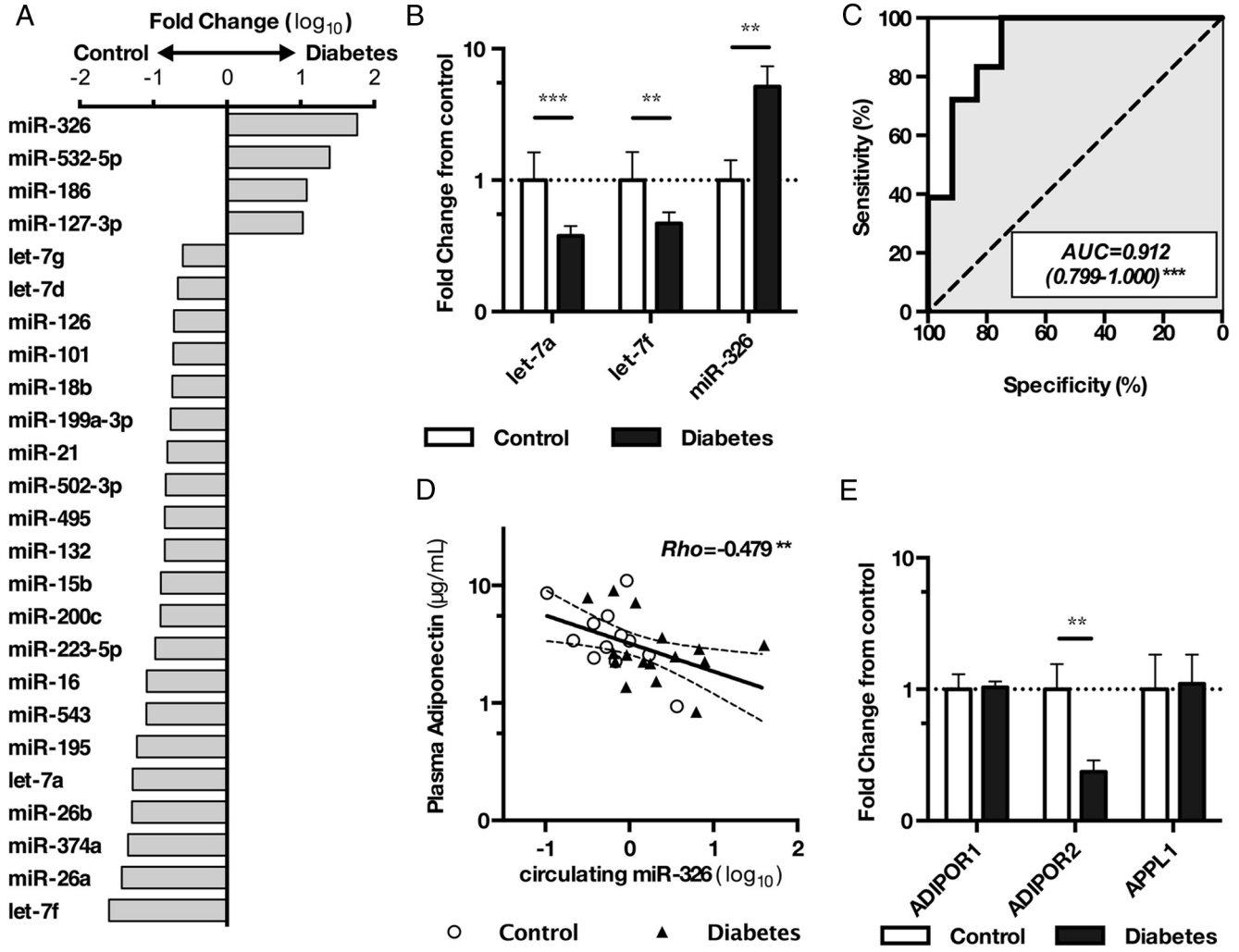

Figure 1. Circulating microRNA profile in diabetic patients. A, miRNome analysis for circulating microRNAs in pooled samples. Schematic representation of circulating microRNAs plasma exosomes as modulated by diabetes. The x-axis indicates the difference in expression level on a $\log_{10}$ scale when compared with control subjects. The y-axis represents modulated microRNAs. B, Validation of selected microRNA expression by qRT-PCR reveals significant differences in let-7a, let-7f, and miR-326 levels. Data are shown as fold change relative to control group (set as 1.0). C, Receiver-operating characteristic curve derived from the logistic regression model developed on circulating miR-326, let-7a, and let-7f levels. AUC, area under the curve. D, Correlation between circulating levels of miR-326 (on x-axis) and plasma adiponectin concentration (y-axis). E, Evaluation of the potential targets by qRT-PCR on PBMCs. Data are shown as fold change relative to control group (set as 1.0). *, $P < .05$; **, $P < .01$; ***, $P < .001$.

qRT-PCR on individual plasma samples. Among the investigated miRNAs, miR-326 ($P = .004$), let-7a ($P < .001$), and let-7f ($P = .003$) showed a statistically significant difference in the diabetic patients group (Figure 1B). A logistic regression analysis was performed in order to confirm that concomitant modulation in circulating miR-326, let-7a, and let-7f was significantly associated with diabetes in our population. The overall model resulted statistically significant, and the derived receiver-operating characteristic curve demonstrated a good accuracy of this model for identifying the presence of diabetes in our study population (area under the curve = 0.912, 0.799–1.000; $P < .001$) (Figure 1C). Remarkably, this miRNA pattern remained independently associated with diabetes, even after correction for age, BMI, and sex.

Moreover, significant correlations were found between HbA1c and miR-326 ($\rho = 0.580$, $P = .004$) and let-7a ($\rho = -0.439$, $P = .028$) and between plasma glucose levels (evaluated at blood sampling time) and miR-326 ($\rho = 0.408$, $P = .025$), let-7a ($\rho = -0.489$, $P = .006$), and let-7f ($\rho = -0.373$, $P = .043$).

Evaluation of potential target expression of dysfunctional microRNAs

In silico analysis showed miR-326 to target adiponectin, adiponectin receptor (ADIPOR)-1, ADIPOR-2, and APPL-1, a molecule involved in the intracellular pathway of insulin signaling. Thus, plasma adiponectin levels were evaluated by ELISA, and a significant inverse correlation was found with circulating miR-326 ($\rho = -0.479$, $P =$

.009) (Figure 1D). Then a multiple regression analysis was performed with plasma adiponectin as the dependent variable. This analysis yielded a model in which circulating miR-326 was an independent predictor of plasma adiponectin levels ($\beta = -0.503$, $P = .015$), irrespective of age, BMI, and sex. Finally, we also analyzed the gene expression of *ADIPOR-1, ADIPOR-2*, and *APPL-1* in PBMCs by qRT-PCR. Interestingly, we found that the expression of *ADIPOR-2* was significantly reduced in patients with diabetes ($P = .003$), whereas no significant differences were found in *ADIPOR-1* and *APPL-1* (Figure 1E).

Evaluation of intracellular microRNAs

Next, to evaluate whether modulation of circulating miRNAs reflects changes in intracellular miRNAs expression, we analyzed the expression of let-7a, let-7f, and miR-326 in the PBMCs of control and diabetic patients. For these comparison studies, we also included *RNU6*, a small nucleolar RNA frequently used as an intracellular reference gene in miRNA studies, as an additional housekeeping for the normalization of qRT-PCR data. Interestingly, the expression of the studied miRNAs in PBMCs did not correlate with the miRNA pattern in plasma. Furthermore, intracellular levels of these miRNAs did not shown significant differences between the groups. These findings suggest that miRNA dysregulation in diabetes occurs in circulating miRNAs, whereas the intracellular miRNA system may be influenced by different processes.

Effect of glycemic control on circulating microRNA

According to the current standard of care (13), patients underwent glucose-lowering treatment and then were clinically reevaluated after 12 months. In detail, all patients were treated with lifestyle changes plus metformin with or without other oral antidiabetic agents (dipeptidyl peptidase IV inhibitors and glynides). Ten patients accepted to perform this study follow-up. One patient did not respect the therapeutic program, and his glycemic control got even worse (HbA1c rose from 8.0% to 8.5%); for this reason, he was excluded by the final analysis. The remaining patients showed a clear improvement in their glucose control as reflected by the significant reduction in HbA1c levels (Figure 2A). As a matter of fact, the mean HbA1c dropped from 10.0% ± 1.3% (89 ± 14.2 mmol/mol) to 6.4% ± 0.8% (47 ± 8.9 mmol/mol). Notably, patients with a significant improvement in their glucose control showed also a concomitant significant increase in the circulating level of both let-7a ($P = .002$) and let-7f ($P = .001$). On the other hand, no significant changes were found in the circulating miR-326 (Figure 2, B–D).

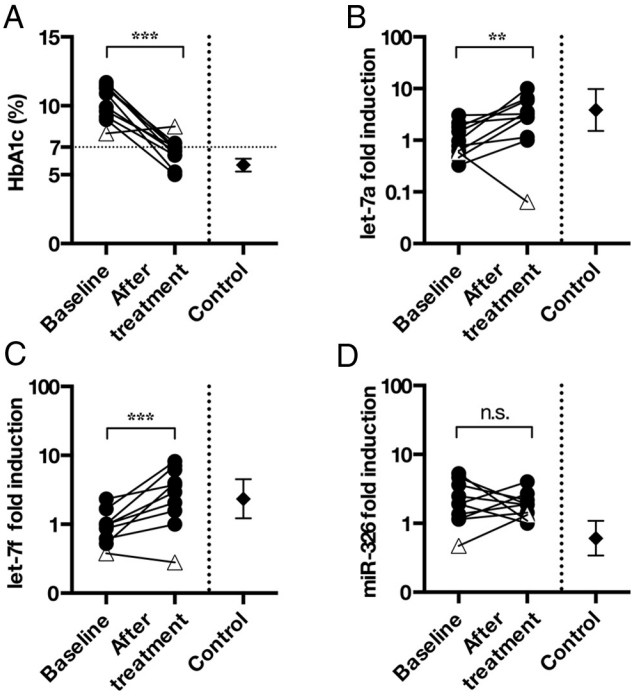

Figure 2. Effects of glycemic control on microRNA expression. Treatment resulted in a significant improvement of glycemic control, as confirmed by drop in HbA1c levels (A) and in significant increases of both let-7a (B) and let-7f (C). No differences were found in circulating miR-326 levels (D). The white triangles represent the data of the only noncompliant patient. As a reference, the mean and 95% confidence interval of HbA1c and circulating miRNA levels of control subjects is reported. No significant differences for the circulating levels of let-7a and let-7f were found between diabetic patients after treatment and control subjects.

Discussion

To the best of our knowledge, in this paper we provide the first evidence of the following: 1) poorly controlled type 2 diabetes is associated with a decrease of let-7a/let-7f and an increase of miR-326 circulating levels; 2) circulating miR-326 levels are inversely correlated with its putative target adiponectin; and 3) glycemic control as obtained by pharmacological therapy may restore let-7a and let-7f (but not miR-326) levels.

The let-7 miRNA family has already been linked to diabetes in previous studies (14). Indeed, several of the genes modulated by this miRNA family have been associated with type 2 diabetes in a genome-wide association study (14, 15). In the present study, we describe a decrease in the circulating levels of two members of the let-7 family (let-7a and let-7f) in diabetic patients and demonstrate that they can be restored by improved metabolic control. Prospective studies on a larger population, however, will be needed to better clarify the clinical relevance of miRNA changes in diabetic patients and the efficacy of different antidiabetes strategies.

Our data appear to be in contrast with in vitro and in animal studies showing that let-7 may reduce pancreatic insulin secretion and induce insulin resistance in peripheral

skeletal muscles (14). However, this is only an apparent discrepancy because we have demonstrated that circulating levels of let-7a and let-7f are independent on their intracellular pool, thus suggesting that circulating levels may not necessarily reflect the expression levels in liver and skeletal muscles. Additional studies on animal models will help to definitively clarify this discrepancy.

Our paper also provides the first evidence of the involvement of miR-326 in type 2 diabetes. Despite the fact that it plays a potential key role in adipocyte differentiation (16), so far miR-326 has been found up-regulated only in lymphocytes from type 1 diabetes patients with positive autoantibodies (17). In our study, not only did we find that miR-326 levels were increased in diabetic patients, but we also demonstrated an inverse correlation between circulating miR-326 and its predicted target adiponectin. Adiponectin is the main hormone secreted by white adipose tissue, and studies on murine model (18) and population studies (19) as well as a recent Mendelian randomization study (20) have all demonstrated an inverse correlation between adiponectin and insulin resistance. Moreover, in our study we also found that gene expression of ADIPOR-2, another predicted target of miR-326, was lower in the PBMCs of diabetic patients. Thus, higher levels of circulating miR-326 in diabetic patients might represent a new potential modulator of the adiponectin pathway. Further in vitro and in vivo mechanistic studies will be needed to confirm this view.

Finally, the observation that the concomitant modulation of these three miRNAs is strongly and independently associated with diabetes represents an interesting finding to be investigated in future studies on larger populations of patients affected by both type 1 and type 2 diabetes. We believe that our findings, although based on associations, descriptive in nature, and obtained in a limited number of individuals are potentially important because they point out the effect of diabetes and glycemic control on the expression levels of circulating miRNAs and suggest a new potential molecular target for adiponectin pathway regulation in humans.

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