Acta Diabetologica (2019) 56:55–65
https://doi.org/10.1007/s00592-018-1216-x

ORIGINAL ARTICLE

Circulating miRNAs in diabetic kidney disease: case–control study and in silico analyses

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Received: 7 June 2018 / Accepted: 13 August 2018 / Published online: 30 August 2018

Abstract

Aims The aim of this study was to investigate a miRNA expression profile in type 1 diabetes mellitus (T1DM) patients with DKD (cases) or without this complication (controls).

Methods Expression of 48 miRNAs was screened in plasma of 58 T1DM patients (23 controls, 18 with moderate DKD, and 17 with severe DKD) using TaqMan Low Density Array cards (Thermo Fisher Scientific). Then, five of the dysregulated miRNAs were selected for validation in an independent sample of 10 T1DM controls and 19 patients with DKD (10 with moderate DKD and 9 with severe DKD), using RT-qPCR. Bioinformatic analyses were performed to explore the putative target genes and biological pathways regulated by the validated miRNAs.

Results Among the 48 miRNAs investigated in the screening analysis, 9 miRNAs were differentially expressed between DKD cases and T1DM controls. Among them, the five most dysregulated miRNAs were chosen for validation in an independent sample. In the validation sample, miR-21-3p and miR-378-3p were confirmed to be upregulated in patients with severe DKD, while miR-16-5p and miR-29a-3p were downregulated in this group compared to T1DM controls and patients with moderate DKD. MiR-503-3p expression was not validated. Bioinformatic analyses indicate that the four validated miRNAs regulate genes from PI3K/Akt, fluid shear stress and atherosclerosis, AGE-RAGE, TGF-β1, and relaxin signaling pathways.

Conclusions Our study found four miRNAs differentially expressed in patients with severe DKD, providing significant information about the biological pathways in which they are involved.

Keywords MicroRNAs · Diabetic kidney disease · Bioinformatics · Target prediction

Managed by Antonio Secchi.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00592-018-1216-x) contains supplementary material, which is available to authorized users.

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Introduction

Diabetic kidney disease (DKD) is a common microvascular complication that occurs in approximately 40% of patients with diabetes mellitus (DM), and it is caused by kidney cell dysfunction, leading to end-stage renal disease (ESRD) [1, 2]. This complication is clinically characterized by albuminuria and a progressive decline in the glomerular filtration rate (GFR) [1, 3]. Pathological alterations of DKD include glomerular hypertrophy, mesangial expansion, and tubulointerstitial fibrosis caused by accumulation of extracellular matrix (ECM) proteins, thickening of basement membrane, podocyte apoptosis, and foot process effacement [4, 5].

DKD is the cumulative result of inadequate glycemic control over years of DM; hence, patients with chronic hyperglycemia have a greater risk for developing this complication than those with a tight glycemic control [6, 7]. However, it has not yet been completely understood why some patients

with intensive treatment and dedicated compliance still develop DKD, while others do not develop it despite poor glycemic control [6]. Moreover, the predictive power of the laboratorial tests used for DKD diagnosis is apparently not sufficient for reliable identification of which patients are at high risk of developing DKD or progress to ESRD [2, 8]. Therefore, new biomarkers need to be identified to offer a more effective diagnosis, prognosis, and treatment of DKD patients.

MicroRNAs (miRNAs) are a class of short non-coding RNAs that regulate expression of more than 60% of protein-coding genes [9]. Evidence has shown that miRNAs can be transported between cells as well as circulate in body fluids constituting a new cell-to-cell communication mode [10, 11]. Moreover, miRNA expression profiles in body fluids usually reflect a tissue-specific injury [12], being associated with the development of several diseases [10, 13–15].

The association between miRNAs and kidney dysfunction was initially suggested by experimental studies showing that podocyte-specific knockout of dicer, an enzyme required for the production of mature miRNAs, caused proteinuria, podocyte foot effacement and apoptosis, glomerulosclerosis, and tubulointerstitial fibrosis [16]. Hyperglycemia is known to induce expression of several miRNAs in renal cells, which promotes the accumulation of ECM proteins related to fibrosis and glomerular dysfunction [16, 17]. Accordingly, over the past few years a number of circulating or urinary miRNAs have been reported as being dysregulated in specific stages of DKD [17–19]; however, results are still inconclusive.

Therefore, the aim of this study was to investigate a circulating miRNA expression profile in plasma of T1DM patients with different stages of DKD. Additionally, we carried out bioinformatic analyses to investigate the putative targets and biological pathways under regulation of the miRNAs potentially associated to DKD.

Methods

Study population and phenotype measurements

This case–control study was designed in accordance with STROBE guidelines [20], and included two independent samples of T1DM patients with DKD (cases) and without this complication (T1DM controls), called "screening" and "validation" samples. The screening sample comprised 23 T1DM controls and 35 DKD cases (18 with moderate DKD and 17 with severe DKD). The validation sample included 10 T1DM controls and 19 DKD cases (10 with moderate DKD and 9 with severe DKD). Additionally, a group of ten healthy subjects were included in both screening and validation samples.

All T1DM patients included in the study were from outpatient clinics at Hospital de Clínicas de Porto Alegre or Instituto da Criança com Diabetes at Grupo Hospitalar Conceição (Rio Grande do Sul, Brazil) and were recruited between August 2014 and September 2016. DKD was defined following the Kidney Disease Improving Global Outcomes (KDIGO) guidelines [21]. T1DM patients were divided into three groups: (1) patients without DKD and ≥10 years of T1DM [urinary excretion of albumin (UEA) < 30 mg/g and estimated GFR (eGFR) ≥ 60 ml/min/1.73 m^2; T1DM control]; (2) patients with moderate DKD (UEA 30–300 mg/g and/or eGFR 30–59 ml/min/1.73 m^2); and (3) patients with severe DKD (UEA > 300 mg/g and/or eGFR 1–29 ml/min/1.73 m^2). Therefore, patients having moderate or severe DKD constituted the case group. The inclusion criteria were age 18–40 years and T1DM was diagnosed based on the American Diabetes Association criteria [22]. Only white subjects were included to minimize stratification bias. Additionally, non-diabetic groups consisting of age- and gender-matched healthy blood donors recruited between August 2014 and September 2016 were included as controls to DM.

All patients underwent physical and laboratory evaluations, as previously described [23]. Serum creatinine was measured by the Jaffé reaction and UAE by immunoturbidimetry (Sera-Pak immuno microalbuminuria, Bayer, Tarrytown, NY, USA). Estimated GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [24]. The study protocol was approved by Ethic Committees in Research from Hospital de Clínicas de Porto Alegre and Grupo Hospitalar Conceição/Instituto da Criança com Diabetes, and all patients gave their informed consent in writing.

RNA extraction

Peripheral blood samples of all subjects were collected in the morning with at least 8 h of fasting. After collection, the non-hemolyzed samples were centrifuged at 3500 rpm for 15 min at 4 °C, and plasma aliquots were stored at − 80 °C until expression analyses. Total RNA was extracted from 450 µl of plasma using the MiRVana PARIS miRNA Isolation Kit (Ambion, Thermo Fisher Scientific, DE, USA). Purity and concentration of RNA samples were evaluated using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

Quantification of miRNA expression using custom TaqMan array miRNA cards

Relative expression of 48 miRNAs was analyzed in plasma of all subjects of the screening sample using Custom TaqMan Array MicroRNA cards (Thermo Fisher Scientific), which contain probes for 45 target miRNAs and for three

reference genes [*RNU44, RNU48*, and *small nuclear RNA U6* (*U6snRNA*)], as described in the Supplementary Table 1. Selection of these miRNAs was performed by searching in the miRWalk 2.0 database [25] those miRNAs possibly associated with DKD and also based on the available literature [26, 27].

Quantification of miRNA expression was done in three separate reactions. First, total RNA (10 ng) was reverse-transcribed into specific miRNAs using the TaqMan miRNA Reverse-Transcription (RT) kit and Multiplex RT Pool Set (Thermo Fisher Scientific). Second, cDNAs of the 48 miRNAs of interest were pre-amplified with the TaqMan PreAmp Master Mix 2x kit and Custom PreAmp Primer Pool (Thermo Fisher Scientific). Third, RT-qPCR reactions were done in a Vii™ 7 Fast Real-Time PCR System Thermal Cycler (Thermo Fisher Scientific), using the TaqMan Low Density Array (TLDA) block. Relative expressions were calculated using the $2^{-\Delta\Delta Cq}$ method [28]. For all analysis, the *U6snRNA* was used as the reference gene. Data are shown as *n*-fold changes in relation to a calibrator sample. More details about miRNA quantifications can be found in Supplementary Material 1.

Validation of Custom TaqMan Array miRNA results using individual RT-qPCR

Five miRNAs were chosen for confirmation of their expressions in the validation sample using individual RT-qPCR, accordingly to the MIQE guidelines [29]. The criteria for choosing these miRNAs were: (1) those miRNAs with the highest differences in *n*-fold changes between cases and controls of the screening sample; and/or (2) number of miRNA targets involved in pathways related to DKD pathogenesis, which was assessed by bioinformatic analysis. Quantifications of the five miRNAs were performed using the $2^{-\Delta\Delta Cq}$ method and the *U6snRNA* gene as the reference gene [29], and are shown as *n*-fold changes in relation to the calibrator sample. Detailed description of this analysis can be found in the Supplementary Material 1.

In silico identification and analysis of putative miRNA target genes

Those dysregulated miRNAs associated with DKD in both screening and validation samples were then submitted to bioinformatic analyses to investigate their putative target genes and to find possible biological pathways under their regulation (Supplementary Fig. 1). For these analyses, we used both validated data and computational prediction tools. Experimentally validated miRNA-target interactions were collected from miRTarBase v6.1 [30], starBase v2.0 [31], and Tarbase v7.0 [32] databases. Computationally predicted interactions were collected from TargetScan v7.1 [33], Diana MicroT-CDS [34], and Microcosm v5.0 [35] databases. For more details about these analyses refer to Supplementary Material 1. Lists of unique validated and predicted miRNA-target gene interactions were combined for use in the subsequent functional enrichment analysis.

We investigated the functional enrichment of the retrieved target genes using pathways annotation from KEGG Pathway Database [36] and statistical methods implemented in the package clusterProfiler in R/Bioconductor environment [37]. Significance for KEGG pathways enrichment was estimated using a hypergeometric test and adjusted to account for multiple hypothesis testing using the false discovery rate (FDR) procedure implemented in the *q*-value R package [38]. Pathways with a q-value < 0.05 were considered strongly enriched for the inputted list of target genes; nonetheless, as this analysis is sensitive to the number of targets retrieved for a given miRNA, pathways with P values < 0.1 and biological plausibility were also taken into consideration in the interpretation of miRNA functional roles. Functional similarities between miRNAs of interest were analyzed using the Jaccard similarity coefficient (JC), as described in the Supplementary Material 1.

Statistical analysis

Variables with normal distribution are shown as mean ± standard deviation (SD). Variables with skewed distribution were log-transformed before analyses and are presented as median (25th–75th percentiles). Categorical data are shown as percentage. Clinical and laboratory characteristics as well as miRNA expressions were compared among groups using one-way ANOVA or χ^2 tests, as appropriate. Correlations between quantitative variables were analyzed using Pearson's correlation tests. All analyses were performed using the SPSS statistical package (v.20.0) for Windows (SPSS Inc, Chicago, IL). P values < 0.05 were considered statistically significant.

Results

Clinical and laboratory characteristics of the subjects included in the study

Characteristics of T1DM controls, DKD cases, and healthy subjects are summarized in Table 1. Considering that distributions of these characteristics were similar between subjects of the screening and validation samples (data not shown), results are shown in Table 1 for both samples analyzed together. DKD cases, T1DM controls, and healthy subjects did not differ significantly regarding age, gender, and mean body mass index (BMI). HbA1c levels were higher in patients with DKD compared to the other groups. Patients

Table 1 Clinical and laboratory characteristics of all subjects included in the study considering the screening and validation samples together

Characteristic	T1DM controls ($n=33$)	Moderate DKD ($n=28$)	Severe DKD ($n=26$)	Healthy subjects ($n=20$)	P value*
Age (years)	25.2 ± 5.5	22.3 ± 5.0	26.8 ± 6.1	24.6 ± 5.9	0.247
Gender (% male)	55.6	58.8	51.8	54.9	0.814
BMI (kg/m^2)	23.4 ± 2.9	23.6 ± 3.6	22.8 ± 3.7	25.7 ± 3.8	0.174
HbA1c (%)	8.4 ± 1.1[a]	10.1 ± 2.1[b]	9.3 ± 1.8[b]	5.3 ± 0.3 [c]	0.0001
Hypertension (% yes)	11.1[a]	11.8[a]	71.4[b]	0.0	0.0001
Age at T1DM diagnosis (years)	9.0 (5.0–12.0)	6.0 (3.0–10.0)	6.0 (6.0–9.0)	—	0.608
Duration of T1DM (years)	15.0 (13.0–19.0)[a]	13.5 (10.0–21.0)[a]	19.0 (12.0–26.0)[b]	–	0.003
Insulin dose (unit/kg/day)	0.8 ± 0.2	0.9 ± 0.3	0.7 ± 0.3	–	0.442
Triglycerides (mg/dl)	74.0 (50.0–107.0)	110.5 (63.5–148.2)	114.0 (63.0–215.0)	–	0.068
Total-CT (mg/dl)	177.4 ± 33.4	193.6 ± 79.0	186.4 ± 42.9	–	0.727
HDL-CT (mg/dl)	49.1 ± 11.3	58.5 ± 14.9	62.1 ± 27.5	–	0.173
Creatinine (µg/dl)	0.8 (0.6–0.9)[a]	0.9 (0.8–1.2)[a]	5.1 (1.7–8.1)[b]	–	0.0001
eGFR (mL/min per 1.73 m^2)	120.0 (109.0–127.0)[a]	120.0 (89.0–128.0)[a]	13.5 (6.0–61.5)[b]	–	0.0001
UAE (mg/g)	5.5 (3.3–9.1)[a]	77.0 (46.0–201.1)[b]	565.6 (303.4–3057.9)[c]	–	0.0001
Urea (mg/g)	36.0 (32.0–37.0)	42.0 (38.0–46.0)	105 (67.5–138.5)	–	0.124
Diabetic retinopathy (%)	7.7[a]	21.4[a]	78.6[b]	–	0.0001

Variables are shown as mean ± SD, median (25th–75th percentiles) or %, as appropriate

CT cholesterol, *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *HbA1c* glycated hemoglobin, *UAE* urinary albumin excretion

*P values were computed using χ^2 or ANOVA, followed by post hoc multiple comparison tests (residual analysis or Tukey's tests, respectively), as appropriate. Analyses with significant differences are indicated as follows: means, medians or % indicated by different letters differed from the other groups at $P<0.05$

with severe DKD showed higher prevalence of hypertension and diabetic retinopathy compared to the other diabetic groups.

Expression profile of 45 miRNAs in subjects from the screening sample

Expression of 45 circulating miRNAs was evaluated in plasma of T1DM controls and DKD cases from the screening sample. Of these 45 miRNAs, 40 (88.9%) had detectable expression in plasma in more than 10% of the samples, allowing reliable statistical analyses (Fig. 1; Table 2). Nine of these 40 miRNAs were differentially expressed between T1DM controls and DKD cases (Table 2). Of note, miR-16-5p, miR-29a-3p, and miR-204-5p were significantly downregulated in patients with moderate and severe DKD compared to T1DM controls, while miR-503-5p was upregulated in moderate and severe DKD. Interestingly, miR-21-3p and miR-378a-5p were upregulated only in patients with severe DKD compared to the other groups, whereas miR-141-3p, miR-192-5p, and miR-215-5p were downregulated in patients with severe DKD (Table 2).

Expression of the nine dysregulated miRNAs in DKD patients was also compared between T1DM controls and healthy subjects to investigate if these miRNAs could be associated with T1DM per se (Table 3). As a result, we found that miR-16-5p and miR-29a-3p were upregulated in healthy subjects compared to T1DM controls ($P=0.0001$ and $P=0.039$, respectively); thus, expression of these miRNAs progressively decreased from healthy controls to T1DM controls and DKD patients. Expressions of the other seven dysregulated miRNAs were not different between T1DM controls and healthy subjects (Table 3).

Validation of the five selected miRNAs by RT-qPCR

Out of the nine miRNAs differentially expressed among the analyzed groups of the screening sample, we choose five miRNAs to validate by RT-qPCR in the validation sample (miR-16-5p, miR-21-3p, miR-29a-3p, miR-378a-5p, and miR-503-5p). As depicted in Fig. 2a, c, miR-16-5p and miR-29a-3p were downregulated in patients with severe DKD compared to moderate DKD and T1DM control groups, although the comparison of miR-29a-3p expression between severe DKD and moderate DKD groups did not reach formal statistical significance ($P=0.090$). MiR-21-3p and miR-378a-5p were increased in severe DKD patients compared to patients with moderate DKD and T1DM controls (Fig. 2b, d). In contrast with the array data, miR-503-5p expression was not significantly different among the analyzed groups (Fig. 2E); therefore, this miRNA was not validated.

For the subsequent analyses, both screening and validation samples were analyzed together to increase the statistical power [T1DM controls ($n=33$), moderate DKD ($n=28$),

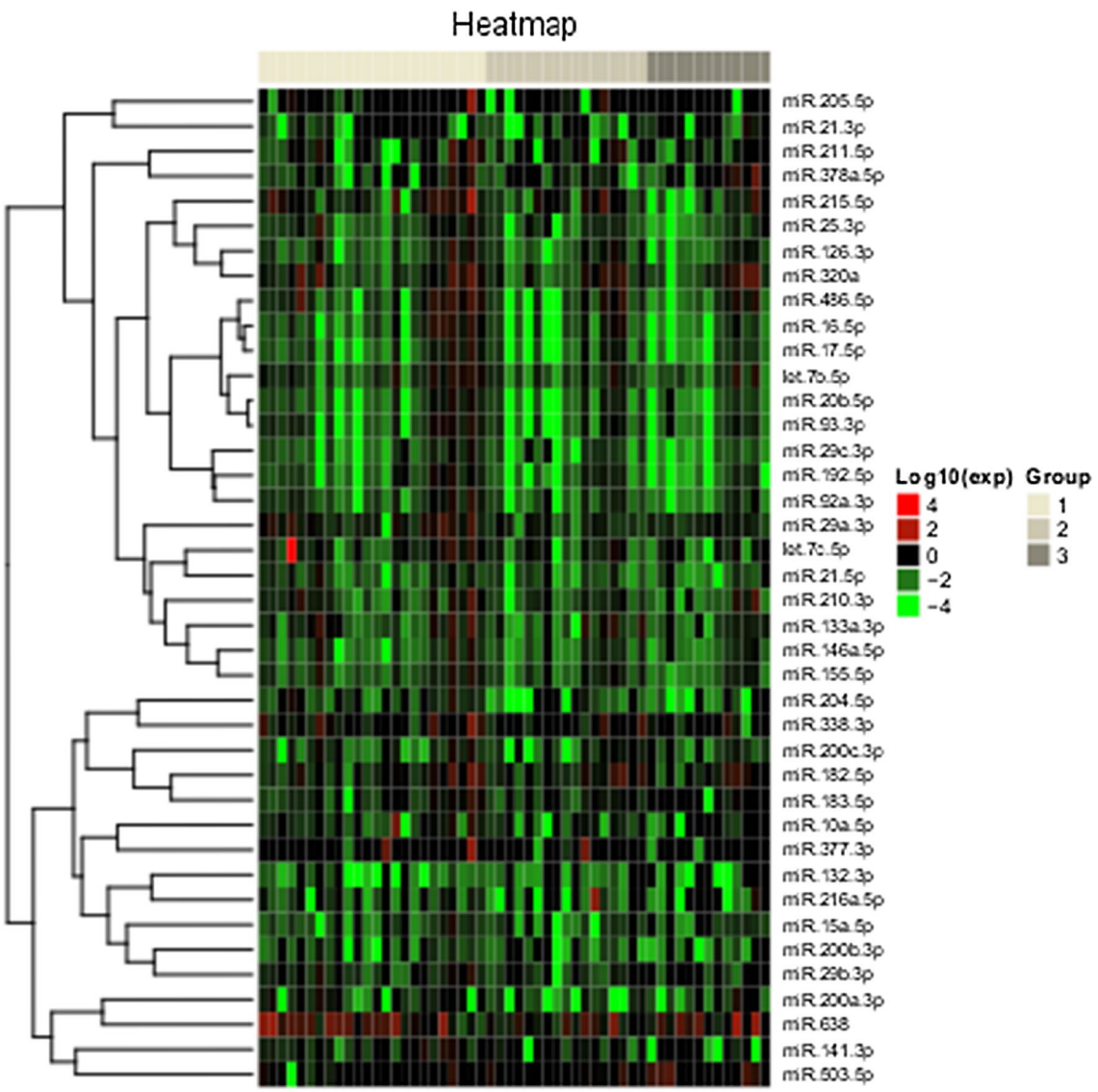

Fig. 1 Distinct miRNA expression patterns in T1DM controls and in patients with moderate or severe DKD. Heatmap of the 40 miRNAs expressed in subjects of the screening sample, derived from the array analysis. Each column represents an individual sample and each row represents an individual miRNA. Expression levels of miRNAs are shown in red (for upregulated miRNAs) and green (for downregulated miRNAs), with brighter shades indicating higher fold differences ($\log_{10}$ *n*-fold change values) in relation to the calibrator sample. Absence of difference in expression levels is represented in black. Hierarchical clustering was performed using Pearson uncentered distance metric with complete linkage. Group 1, T1DM controls; group 2, moderate DKD; and group 3, severe DKD.

and severe DKD ($n=26$)]. We evaluated correlations among the four dysregulated miRNAs validated in this study (miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p) and DKD-related measurements (eGFR, creatinine, and UAE levels), as well as HbA1c levels. MiR-21-3p and miR-378a-5p expressions were negatively correlated with eGFR ($r=-0.633$, $P=0.004$; and $r=-0.455$, $P=0.044$, respectively), while miR-378a-5p expression was positively correlated with UAE levels ($r=0.338$, $P=0.049$) in DKD cases. MiR-21-3p was also positively correlated with serum creatinine levels ($r=0.616$, $P=0.004$). MiR-16-5p and miR-29a-3p did not correlate with DKD-related measurements ($P>0.05$), and none of the 4 validated miRNAs correlated with HbA1c levels ($P>0.05$).

Target prediction and functional enrichment analysis for the four dysregulated miRNAs investigated in the validation sample

Target prediction of the four validated miRNAs (hsa-miR-16-5p, hsa-miR-29a-3p, hsa-miR-21-3p, and hsa-miR-378a-5p) was performed using six distinct databases of

Table 2 MiRNA expression in the screening sample obtained using Custom TaqMan Array MicroRNA cards

miRNA	T1DM controls ($n=23$)	Moderate DKD ($n=18$)	Severe DKD ($n=17$)	P*
hsa-let-7b-5p	0.248 (0.065–1.356)	0.166 (0.002–0.387)	0.072 (0.010–0.186)	0.360
hsa-let-7c-5p	0.349 (0.037–0.941)	0.215 (0.045–0.521)	0.104 (0.003–0.653)	0.150
hsa-miR-10a-5p	0.153 (0.038–0.591)	0.079 (0.003–0.553)	0.009 (0.001–0.136)	0.145
hsa-miR-15a-5p	0.069 (0.006–0.157)	0.070 (0.001–0.138)	0.086 (0.009–0.200)	0.424
hsa-miR-16-5p	0.219 (0.039–1.000)[a]	0.054 (0.0008–0.391)[b]	0.010 (0.003–0.119)[b]	0.014
hsa-miR-17-5p	0.071 (0.009–0.374)	0.050 (0.001–0.463)	0.012 (0.001–0.123)	0.453
hsa-miR-20b-5p	0.089 (0.014–0.315)	0.041 (0.0005–0.302)	0.019 (0.003–0.089)	0.376
hsa-miR-21-3p	0.043 (0.0003–0.145)[a]	0.011 (0.0001–0.076)[a]	1.026 (0.339–1.587)[b]	0.003
hsa-miR-21-5p	0.017 (0.004–0.070)	0.058 (0.017–0.156)	0.091 (0.026–0.298)	0.110
hsa-miR-25-3p	0.116 (0.045–0.485)	0.116 (0.027–0.258)	0.023 (0.003–0.125)	0.067
hsa-miR-29a-3p	1.112 (0.397–1.757)[a]	0.371 (0.119–0.480)[b]	0.291 (0.126–0.614)[b]	0.001
hsa-miR-29b-3p	0.199 (0.103–0.332)	0.057 (0.031–0.265)	0.031 (0.014–0.120)	0.161
hsa-miR-29c-3p	0.076 (0.008–0.162)	0.060 (0.003–0.194)	0.007 (0.0005–0.045)	0.123
hsa-miR-92a-3p	0.148 (0.022–0.401)	0.084 (0.016–0.255)	0.016 (0.005–0.142)	0.156
has-miR-93-5p	0.156 (0.013–0.407)	0.065 (0.0008–0.515)	0.018 (0.007–0.207)	0.550
hsa-miR-126-3p	0.110 (0.018–1.562)	0.094 (0.011–0.689)	0.040 (0.009–0.417)	0.471
hsa-miR-132-3p	0.005 (0.0001–0.03)	0.006 (0.003–0.013)	0.0005 (0.0001–0.004)	0.065
hsa-miR-133a-3p	0.189 (0.026–0.918)	0.082 (0.008–1.234)	0.129 (0.014–0.426)	0.522
hsa-miR-141-3p	0.203 (0.075–0.955)[a]	0.040 (0.006–0.626)[a]	0.0001 (0.0001–0.112)[b]	0.001
hsa-miR-146a-5p	0.099 (0.010–0.197)	0.095 (0.008–0.281)	0.023 (0.008–0.239)	0.919
hsa-miR-155-5p	0.134 (0.027–0.355)	0.129 (0.027–0.443)	0.015 (0.007–0.356)	0.156
hsa-miR-182-5p	0.446 (0.101–1.210)	0.461 (0.155–2.861)	0.406 (0.020–2.939)	0.899
hsa-miR-183-5p	0.124 (0.075–0.980)	0.141 (0.045–0.502)	0.272 (0.001 - NO)	0.586
hsa-miR-192-5p	0.060 (0.033–0.418)[a]	0.051 (0.002–0.329)[a, b]	0.010 (0.0001–0.112)[b]	0.010
hsa-miR-200a-3p	0.173 (0.005–0.805)	0.047 (0.0001–0.193)	0.056 (0.004–0.084)	0.475
hsa-miR-200b-3p	0.034 (0.006–0.108)	0.011 (0.001–0.029)	0.005 (0.001–0.049)	0.426
hsa-miR-200c-3p	0.126 (0.009–0.361)	0.099 (0.004–0.586)	0.070 (0.019–0.208)	0.878
hsa-miR-204-5p	0.090 (0.017–0.224)[a]	0.009 (0.0001–0.032)[b]	0.004 (0.0001–0.028)[b]	0.001
hsa-miR-205-5p	0.327 (0.019–11.925)	0.0001 (0.0001–2.112)	NA	0.168
hsa-miR-210-3p	0.212 (0.015–0.602)	0.149 (0.021–0.302)	0.033 (0.007–0.468)	0.508
hsa-miR-211-5p	0.048 (0.008–1.317)	0.059 (0.002–1.455)	0.047 (0.010–0.223)	0.907
hsa-miR-215-5p	0.499 (0.143–1.964)[a]	0.340 (0.097–1.144)[a]	0.022 (0.0001–0.385)[b]	0.021
hsa-miR-216a-5p	0.062 (0.006–0.450)	0.003 (0.0001–0.075)	0.004 (0.0001–0.015)	0.179
hsa-miR-320a	0.469 (0.101–1.049)	0.452 (0.042–1.595)	0.171 (0.044–2.358)	0.715
hsa-miR-338-3p	0.835 (0.210–5.019)	0.565 (0.132–4.689)	0.501 (0.058–1.483)	0.361
hsa-miR-377-3p	14.480 (0.007 - NO)	0.150 (0.001 - NO)	NA	0.575
hsa-miR-378a-5p	0.052 (0.008–0.157)[a]	0.087 (0.014–0.316)[a]	2.812 (0.795–4.475)[b]	0.008
hsa-miR-486-5p	0.211 (0.044–1.245)	0.119 (0.007–0.764)	0.025 (0.007–0.543)	0.336
hsa-miR-503-5p	0.112 (0.0001–0.354)[a]	1.574 (0.977–2.549)[b]	5.676 (4.952–6.504)[b]	0.006
hsa-miR-638	5.442 (0.582–15.909)	1.005 (0.324–4.044)	4.646 (0.470–26.433)	0.269

Data are shown as median (25th–75th percentiles) of n-fold values. Values with significant differences are indicated by different letters ($P<0.05$), while similar values are indicated by the same letters

NO non-observed (for these groups, it was not observed any subject with expression higher than the 50th percentile), *NA* non-available (no expression value was observed for this group)

**P* values were obtained using one-way ANOVA followed by Tukey post hoc tests using the log-transformed variable

miRNA-gene interactions. Using the strategy described in the "Methods" Section, 1643 genes were identified as putative targets of these four miRNAs analyzed together, although only 116 genes were found to be modulated by two or more miRNAs (Supplementary Fig. 2).

Individually, 983 target genes were found for hsa-miR-16-5p, 56 for hsa-miR-21-3p, 689 for hsa-miR-29a-3p,

Table 3 MiRNA expression in T1DM controls and healthy subjects of the screening sample

miRNA	T1DM controls ($n=23$)	Healthy subjects ($n=10$)	P*
hsa-miR-16-5p	0.219 (0.039–1.000)	1.098 (0.644–1.583)	0.0001
hsa-miR-21-3p	0.043 (0.0003–0.145)	0.079 (0.009–0.741)	0.949
hsa-miR-29a-3p	1.112 (0.397–1.757)	1.450 (1.167–1.935)	0.039
hsa-miR-141-3p	0.203 (0.075–0.955)	2.295 (0.097–NO)	0.256
hsa-miR-192-5p	0.060 (0.033–0.418)	0.211 (0.074–0.588)	0.096
hsa-miR-204-5p	0.090 (0.017–0.224)	0.037 (0.002–0.185)	0.794
hsa-miR-215-5p	0.499 (0.143–1.964)	0.415 (0.225–7.873)	0.161
hsa-miR-378a-5p	0.052 (0.008–0.157)	2.091 (0.274–6.580)	0.245
hsa-miR-503-5p	0.112 (0.0001–0.354)	NA	-

Data are shown as median (25th–75th percentiles) of *n*-fold values

NO non-observed (for these groups, it was not observed any subject with expression higher than the 50th percentile), *NA* non-available (no expression value was observed for this group)

*P values were obtained by Student's t tests, using the log-transformed variable

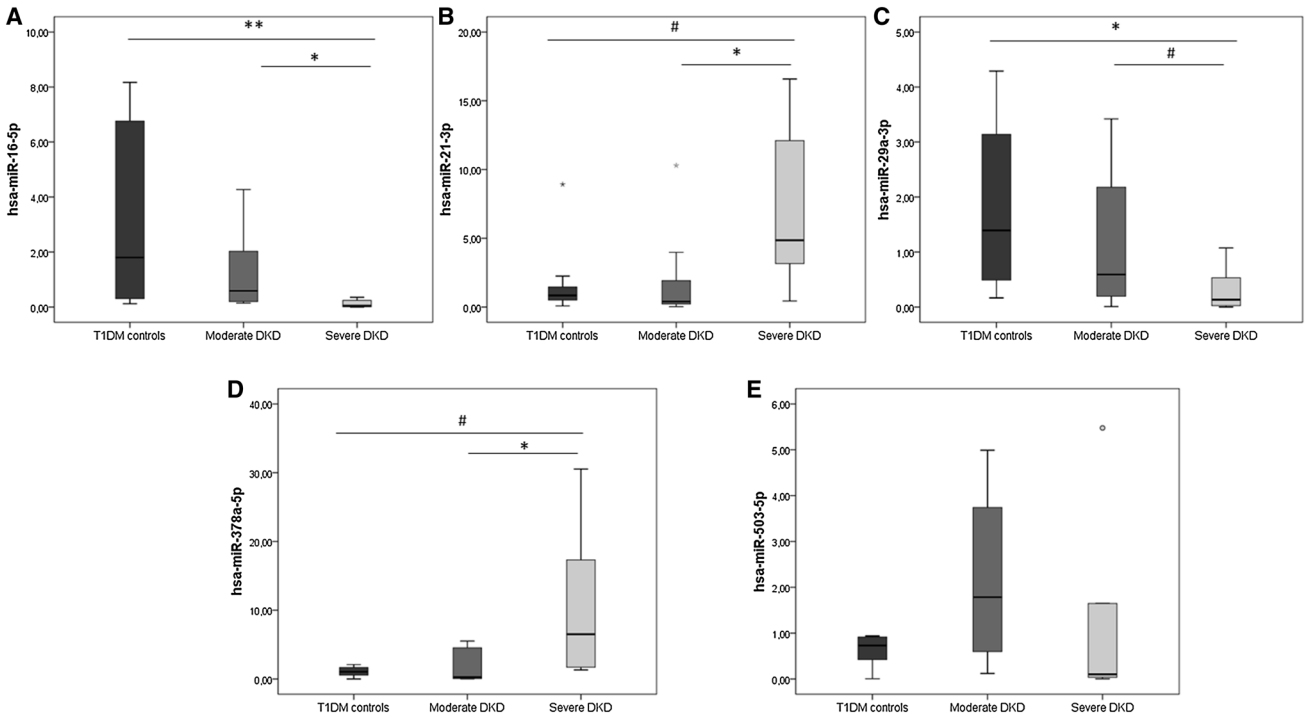

Fig. 2 Dysregulated miRNAs in plasma from T1DM controls and patients with moderate or severe DKD of the validation sample. Relative expressions of **a** miR-16-5p, **b** miR-21-3p, **c** miR-29a-3p, **d** miR-378a-5p, and **e** miR-503-5p were evaluated using RT-qPCR. Results are expressed as *n*-fold changes in relation to the calibrator sample ($\Delta\Delta C_q$ method), using the *U6 snRNA* as the reference gene, and are shown as median (25th–75th percentiles). P values were obtained using one-way ANOVA tests with Tukey's post hoc tests. *$P<0.050$, **$P<0.010$ and $^{\#}P<0.100$

and 34 for hsa-miR-378a-5p (Supplementary Table 2 and Supplementary Fig. 2), with some miRNAs targeting the same genes. Among these targets, 1196 were from experimentally validated interactions while 566 were predicted interactions (Supplementary Table 3).

We next compared the list of putative target genes shared among the four validated miRNAs by computing the JC in a pairwise fashion (Fig. 3a). The highest JC was found for the comparison between hsa-miR-16-5p and hsa-miR-29a-3p (0.065), whereas the smallest non-zero JC value was detected between hsa-miR-16-5p and hsa-miR-378a-5p (0.002), indicating that these four miRNAs target few genes in common.

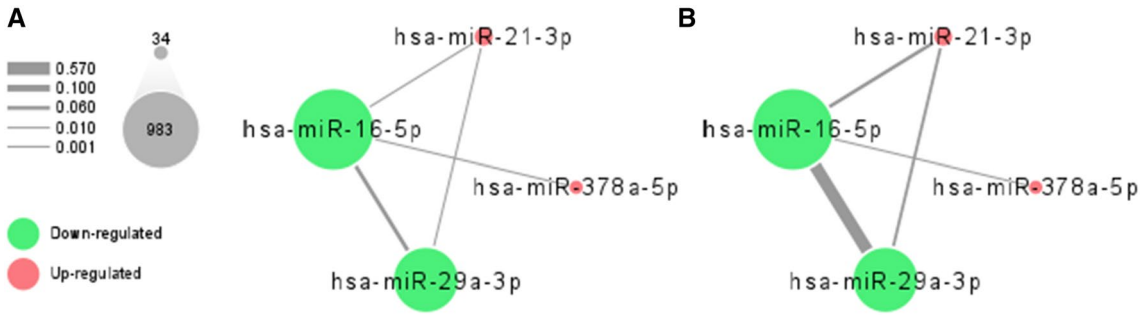

Fig. 3 Jaccard coefficients (JC) computed in terms of **a** number of target genes and **b** number of pathways for miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p. Solid lines connecting molecules represent the existence of shared targets or pathways (JC > 0). Node size represents number of targets or pathways for each miRNA; edge width is proportional to the overlap between miRNAs targets or pathways measured by the JC; and nodes are colored based on the differential expression of the miRNA in DKD cases: green represents those miRNAs downregulated in DKD patients; while pink represents upregulated miRNAs

To explore the biological pathways possibly affected by the four validated miRNAs, we carried out functional enrichment analysis of their target genes using pathways maps from the KEGG Database. A total of 130 significant pathways were enriched for these miRNAs (Supplementary Table 4). We also analyzed functional enrichment of the four miRNAs of interest grouped by their expression profile (up- or downregulation), and observed that many of the enriched pathways consist of genes well known to be related to DKD pathogenesis, such as PI3K/Akt, p53, AMPK, fluid shear

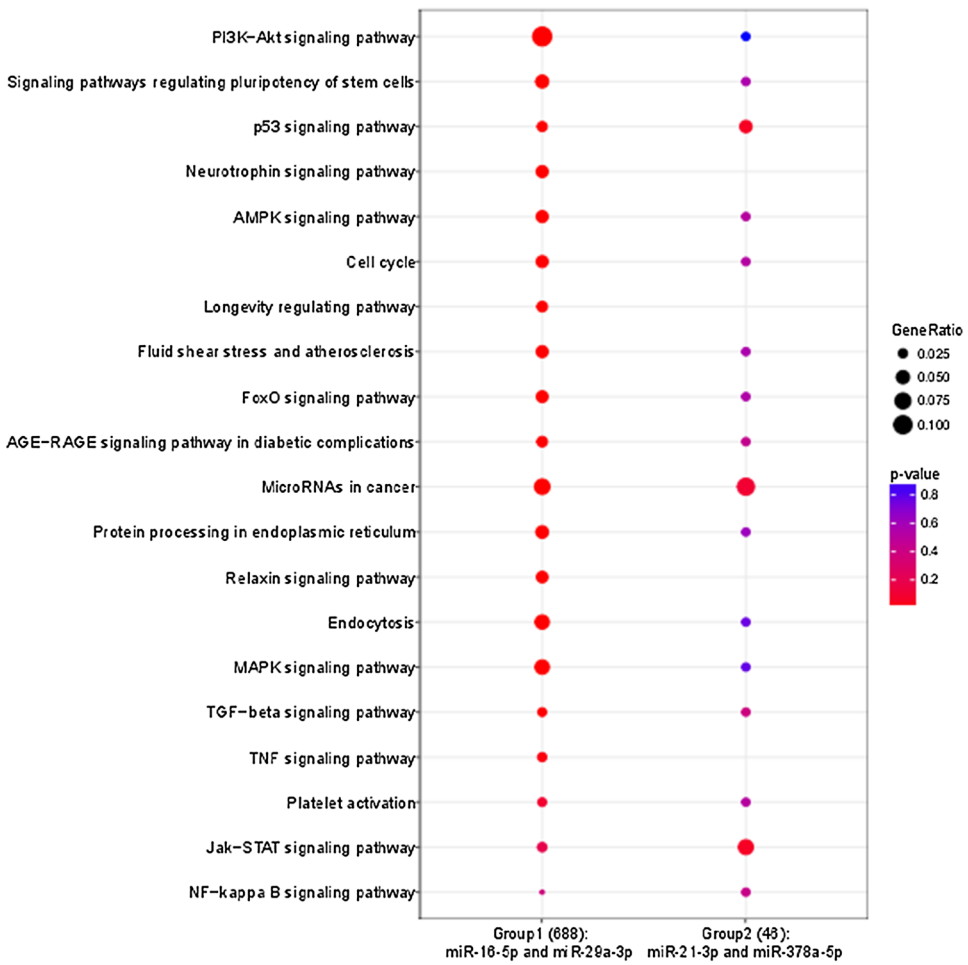

Fig. 4 KEGG pathways potentially regulated by miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p, according to their expression profile. Downregulated miRNAs in DKD patients: miR-16-5p and miR-29a-3p. Upregulated miRNA in DKD patients: miR-21-3p and miR-378a-5p. The size of the circles denotes the ratio of target genes involved in a given pathway (GeneRatio) and its color represents the pathway's *q*-value. *Q*-values: *P* values corrected for multiple tests using the Benjamini–Hochberg procedure, as described in the "Methods" Section

stress and atherosclerosis, endocytosis, protein processing in endoplasmic reticulum, AGE-RAGE, TGF-β1, and relaxin signaling pathways (Fig. 4).

We next compared the similarity among the four miRNAs of interest in terms of their enriched pathways using the pairwise JC (Fig. 3b). Despite the low overlap among target genes of these miRNAs, as shown in Fig. 3a, a substantial overlap in the list of regulated pathways was found for some pairs of miRNAs, especially among hsa-miR-16-5p and hsa-miR-29a-3p (Fig. 3b). This may indicate that these miRNAs act in shared pathways through distinct mechanisms and targets, thus having complementary roles in their modulation.

Discussion

Significant efforts have been made to identify biomarkers that could clinically detect early stages of DKD and the progressive decline in kidney function [17, 19]. Circulating miRNAs are not affected by glomerular filtration and dialysis since they circulate in body fluids complexed to proteins, such as Argonaute-2, or packaged into microvesicles, exosomes, or apoptotic bodies [39, 40]; potentially constituting ideal biomarkers of DKD. Therefore, we evaluated the expression profile of 45 circulating miRNAs in DKD cases and T1DM controls. In the screening sample, we found nine miRNAs dysregulated in plasma of patients with different stages of DKD (miR-16-5p, miR-21-3p, miR-29a-3p, miR-141-3p, miR-192-5p, miR-204-5p, miR-215-5p, miR-378a-5p, and miR-503-5p). Five of these miRNAs were chosen for validation in an independent sample. Among them, miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p maintained the expression trend observed in the screening sample, but miR-503-5p was not confirmed to be associated with DKD.

MiR-16-5p and miR-29a-3p were downregulated in patients with severe DKD compared to the other groups of patients. In line with our results, Baker et al. [41] reported that miR-16-5p was downregulated in renal proximal tubules of DKD patients compared to healthy subjects. Moreover, this miRNA was also downregulated in serum of patients with nephrotic syndrome [42]. In contrast, Neal et al. [43] showed that this miRNA was upregulated in plasma of non-diabetic patients with ESRD receiving dialysis compared to patients with chronic kidney disease or with normal renal function. Interestingly, miR-16-5p is regulated by vasopressin in kidney inner medullary collecting duct cells from rats [44]. MiR-16-5p targets *VEGFA* [45], an important vascular growth factor for maintaining the integrity of the glomerular filtration barrier as well as a survival factor for the podocyte [46].

MiR-29 family members (including miR-29a-3p) have potent anti-fibrotic activities, mainly by targeting different types of collagen via TGF-β1-Smad3-dependent mechanisms, which leads to decreased ECM accumulation and, consequently, decreased renal fibrosis [47–49]. MiR-29a-3p is downregulated by TGF-β1 in a variety of cell lines, including human and rat renal proximal tubule epithelial cells, mouse mesangial cells, and human podocytes [48–50], which might predispose to DKD. Accordingly, miR-29a knockdown in diabetic mice promoted histone deacetylase activity, decreasing nephrin expression, and thus, causing podocyte apoptosis, proteinuria, and renal dysfunction [50]. Hence, diabetic mice overexpressing miR-29a showed improved renal function and podocyte viability and decreased glomerular fibrosis compared with wild-type mice [50]. In agreement with the present study, Pezzolesi et al. [18] found that miR-29a-3p was downregulated in plasma of fast-progressors to ESRD compared to non-progressors.

The present study also indicates that miR-21-3p and miR-378a-5p are upregulated in plasma of DKD patients. MiR-21-3p was reported to be increased in post-transplant renal biopsies of patients with acute kidney injury compared to matched allografts without any pathology, 12 days after engraftment [51]. However, the molecular mechanisms behind the association of this miRNA with kidney injury are still unclear. Bang et al. [52] showed that miR-21-3p is produced by cardiac fibroblasts and acts as a potent paracrine-acting miRNA that induces cardiomyocyte hypertrophy. Therefore, there is a possibility that this miRNA might also lead to hypertrophy of renal cells. Accordingly, miR-21-3p is also induced by TGF-β1 in primary parenchymal lung fibroblasts, affecting their function [53]. Interestingly, in a colorectal cancer cell line, miR-21-3p targeted E-cadherin and Smad7, leading to epithelial-to-mesenchymal transition promotion [54]. It is known that E-cadherin decreases collagen production and, consequently, ECM accumulation and fibrosis in renal cells [4]; thereby, there is a possibility that miR-21-5p may be triggering renal fibrosis by targeting E-cadherin. By targeting Smad7, a TGF-β1 negative regulator [4], miR-21-3p may also amplify the TFG-β1 signaling cascade during renal fibrosis.

MiR-378a-5p was originally reported to be expressed in a number of cancer cell lines [55], and has been shown to directly affect *VEGFA* expression by competing with miR-125a for the same seed-region in *VEGFA* mRNA, causing upregulation of this vascular growth factor [56]. Xing et al. [57] demonstrated that mesenchymal stromal cells overexpressing rno-miR-378a-5p had increased expression of *VEGFA, PDGF-β* and *TGF-β1*, which have a recognized role in kidney dysfunction. Further studies are necessary to clarify by which mechanisms miR-378-5p predisposes to DKD.

In conclusion, the present study identified a set of nine miRNAs dysregulated in T1DM patients with different stages of DKD in the screening analysis. Four of them had

their results validated in a different sample, confirming that miR-21-3p and miR-378a-5p are upregulated in patients with severe DKD, while miR-16-5p and miR-29a-3p are downregulated in this group of patients. Additionally, these miRNAs regulate genes from several important pathways involved in mechanisms associated with DKD development, such as podocyte injury, proteinuria, mesangial proliferation, extracellular matrix accumulation, and endothelial cell injury. Experimental validation of the putative targets and perturbed pathways under their regulation, as well as further investigation of these miRNAs in different populations, are needed to confirm their role in DKD pathogenesis.

Author contributions TSA designed the study, acquired and analyzed the data, and drafted the manuscript. MRM performed the bioinformatics analysis and reviewed the manuscript. ACB, MP, BT, and LHC interpreted the data and reviewed the manuscript. DC supervised the study, analyzed the data, and drafted the manuscript. All authors approved the final version.

Funding This study was supported by Grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant number: 482525/2013-4), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (Edital FAPERGS/CNPq PRONEX 12/2014: 16-2551-0000476-5), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and Fundo de Incentivo à Pesquisa e Eventos at Hospital de Clínicas de Porto Alegre (Grant number: 14–0213). D. Crispim, L. H. Canani and T. S. Assmann are recipients of scholarships from CNPq.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Hospital de Clínicas de Porto Alegre research committee (number of approval 14–0213) and with the 1964 Helsinki Declaration and its amendments or comparable ethical standards.

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

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