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Circulating TGF-β1–Regulated miRNAs and the Risk of Rapid Progression to ESRD in Type 1 Diabetes

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We investigated whether circulating TGF-β1–regulated miRNAs detectable in plasma are associated with the risk of rapid progression to end-stage renal disease (ESRD) in a cohort of proteinuric patients with type 1 diabetes (T1D) and normal eGFR. Plasma specimens obtained at entry to the study were examined in two prospective subgroups that were followed for 7–20 years (rapid progressors and nonprogressors), as well as a reference panel of normoalbuminuric T1D patients. Of the five miRNAs examined in this study, let-7c-5p and miR-29a-3p were significantly associated with protection against rapid progression and let-7b-5p and miR-21-5p were significantly associated with the increased risk of ESRD. In logistic analysis, controlling for HbA_{1c} and other covariates, let-7c-5p and miR-29a-3p were associated with more than a 50% reduction in the risk of rapid progression ($P \leq 0.001$), while let-7b-5p and miR-21-5p were associated with a >2.5-fold increase in the risk of ESRD ($P \leq 0.005$). This study is the first prospective study to demonstrate that circulating TGF-β1–regulated miRNAs are deregulated early in T1D patients who are at risk for rapid progression to ESRD.

Diabetic nephropathy (DN) is characterized by a series of structural abnormalities affecting the function of the kidney (1). In addition to renal cell hypertrophy and glomerular basement membrane thickening, the accumulation of extracellular matrix proteins and mesangial cell expansion are key features of this disease that promote renal fibrosis (1). These structural changes lead to two separable clinical manifestations: increased urinary albumin excretion and the progressive decline of renal function (2). For 10–15% of all patients diagnosed with type 1 diabetes (T1D), it is this latter feature of this process that ultimately culminates in the onset of end-stage renal disease (ESRD) (2).

Transforming growth factor (TGF)-β1, a multifunctional cytokine, is an essential mediator of the pathogenesis of DN (3,4). In the setting of diabetes, high glucose levels stimulate the renal production of TGB-β1 and set in motion a signaling cascade that promotes fibrogenesis (5). During this process, TGF-β1 exerts its effects by upregulating several profibrotic factors, including various collagen genes, through activation of the Smad and mitogen-activated protein kinase signaling pathways (6–9). Recently, in vitro and in vivo models of DN have shown that TGF-β1 also positively or negatively regulates the expression of several microRNAs (miRNAs) that, in turn, amplify TGF-β1 signaling to further promote renal fibrosis (10–18). These studies clearly demonstrate that TGF-β1–regulated miRNAs are key downstream regulators of the TGF-β/Smad signaling pathway and, therefore, are important modulators of diabetic kidney disease.

miRNAs have recently been found to be detectable in a variety of human body fluids, including blood, saliva, and urine (19,20). miRNAs that are present in the circulation, including those in plasma and serum, are protected from endogenous RNase activity, allowing them to remain remarkably stable. Because of this, circulating miRNAs hold great promise to serve as potentially useful biomarkers to monitor pathophysiological changes and the prognosis disease (19,21,22). Moreover, because the deregulation of miRNAs also contributes to the development of various human diseases, these molecules are

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becoming attractive targets for miRNA-based therapeutic interventions (23).

Over the past few years, researchers have begun investigating the role of both circulating and urinary miRNAs in DN (24–28). In this study, for the first time, we examined the concentrations of circulating miRNAs involved in the TGB-β1 pathway in T1D patients who had normal renal function but were shown prospectively to be at extreme risk of rapid progression to ESRD.

RESEARCH DESIGN AND METHODS

Study Subjects

All study subjects included in this study were recruited while attending the Joslin Clinic in Boston, MA, using protocols and consent procedures approved by the Joslin Diabetes Center Institutional Review Board. All patients had baseline examinations that included standardized measurements of blood pressure and the collection of peripheral blood.

The proteinuric patients included in this study are members of the Joslin Proteinuria Cohort (29,30). Briefly, this cohort was ascertained between 1991 and 2004 from among ~3,500 adult T1D patients receiving long-term care at the Joslin Clinic during this period and was followed through 2011. All patients enrolled in this cohort were Caucasian and had persistent proteinuria, defined by a urinary albumin-to-creatinine ratio (ACR) ≥300 μg/mg in two of the last three measurements taken at least 1 month apart. The description of clinical characteristics for this cohort has previously been published (29,30). We used serum creatinine concentration and the Chronic Kidney Disease Epidemiology Collaboration formula to estimate renal function (estimated glomerular filtration rate [eGFR]) at study entry and during follow-up (31). For each patient, serial measures of serum creatinine were used to estimate the rate of eGFR decline (eGFR slopes) during this follow-up period using a general linear model as described by Skupien et al. (30). For the current study, we identified 38 proteinuric patients with normal renal function (eGFR ≥60 mL/min per 1.73 m^2) at enrollment and with the fastest rate of eGFR decline (i.e., rapid progressors). As a result of this rapid eGFR loss, the majority of these patients developed ESRD or reached chronic kidney disease (CKD) stage 4 during first 10 years of follow-up. From this same cohort, we selected 38 patients who maintained normal and stable renal function over the course of the follow-up period despite persistent proteinuria (i.e., nonprogressors).

Additionally, 40 T1D patients with normoalbuminuria who maintained normal and stable renal function during 4–10 years of follow-up were randomly selected from the 2nd Joslin Kidney Study as a healthy reference subgroup (i.e., normoalbuminuric control subjects) (32). Briefly, the 2nd Joslin Kidney Study is a longitudinal investigation on the natural history of early diabetic nephropathy in nonproteinuric patients (determined by at least 2 ACR measurements during their 2 clinical visits preceding enrollment) with T1D attending the Joslin Clinic between 2003 and 2006. Within this cohort, 364 patients entered the study with normoalbuminuria and a median duration of T1D of >20 years. Among these patients, 249 had an HbA_{1c} persistently >7.4% yet maintained their normoalbuminuria and normal and stable renal function during 4–10 years of follow-up. We randomly selected 40 normoalbuminuric control subjects from this group to serve as a healthy reference subgroup for the current study.

RNA Isolation From Plasma Specimens

Peripheral blood was collected in EDTA tubes from all patients included in this study at the time of their enrollment. Briefly, all blood samples were centrifuged at 3,000*g* for 10 min. Plasma supernatant was then aliquoted into RNase-free tubes and stored at −80°C until analysis.

Plasma specimens from the 40 normoalbuminuric control subjects selected for this study were pooled and used for experiments to determine the baseline levels of TGF-β–regulated miRNAs in patients with T1D. Individual plasma specimens from these same normoalbuminuric patients, 38 rapid progressors, and 38 nonprogressors were used to isolate total RNA for determination of the levels of TGF-β–regulated miRNAs. Because of limited baseline sample availability, individual plasma samples collected during the follow-up period were used for the 38 nonprogressors included in this study. Importantly, the rate of eGFR decline maintained by these patients throughout their follow-up and at the time of the collection of the plasma samples used in this study was <3.3 mL/min per 1.73 m^2 per year.

Total RNA was isolated from 180 μL pooled plasma from 40 normoalbuminuric control subjects and 100 μL from individual plasma from 40 normoalbuminuric control subjects, 38 rapid progressors, and 38 nonprogressors using the Qiagen's miRNeasy Serum/Plasma kit (Qiagen, Valencia, CA).

For the pooled plasma sample from normoalbuminuric control subjects, 900 μL QIAzol reagent was added to 180 μL plasma followed by the addition of 3.5 μL of 1.6×10^8 copies/μL of a synthetic *Caenorhabditis elegans* miRNA (cel-miR-39-3p) exogenous normalization control. In the absence of established endogenous control miRNAs for normalization in human plasma, spiked-in RNAs, such as cel-miR-39-3p, have been shown to serve as stable reference normalization control subjects (19,33,34). The sample was then mixed thoroughly followed by the addition of 180 μL chloroform. After vortexing for 15 s, the sample was centrifuged at 12,000*g* for 15 min at 4°C. The aqueous phase containing the RNA was then transferred to a new collection tube, combined with 1.5 volumes of 100% ethanol, applied to the silica membrane of a miRNeasy MiniElute Spin column (Qiagen), and centrifuged at 10,000*g* for 15 s at room temperature. The retained RNA was then washed using buffers provided with the miRNeasy Serum/Plasma kit. First, 700 μL Buffer RWT was applied to the spin column, followed by centrifugation

at 10,000*g* for 15 s at room temperature. Next, 500 μL Buffer RPE was added, followed by centrifugation at 10,000*g* for 15 s at room temperature. The spin column was then washed with 500 μL of 80% ethanol, incubated at room temperature for 2 min, and centrifuged for 2 min at 10,000*g* at room temperature. High-speed centrifugation (20,000*g*) was performed for 5 min at room temperature to dry the silica membrane. RNA was eluted by applying 14 μL RNase-free water to the membrane followed by a 2-min incubation at room temperature and high-speed centrifugation for 1 min. The isolated RNA was stored at −80°C until further processing.

RNA isolation from all individual plasma samples was performed as described above with the following exceptions: 500 μL QIAzol reagent was added to 100 μL plasma followed by the addition of 3.5 μL of 1.6×10^8 copies/μL cel-miR-39-3p; all samples were then mixed thoroughly, followed by the addition of 100 μL chloroform. All isolated RNA samples were stored at −80°C until further processing.

miRNome Profiling in Pooled T1D Normoalbuminuric Control Sample

Reverse transcription of RNA isolated from pooled plasma from T1D normoalbuminuric control subjects was performed using the miScript II RT kit with miScript HiSpec Buffer (Qiagen). Isolated RNA (6 μL) from the pooled normoalbuminuric control subjects sample was used to prepare a 10-μL reverse transcription reaction as specified by the manufacturer. This was then incubated at 37°C for 60 min followed by 95°C for 5 min using a PTC-200 thermal cycler (MJ Research, Watertown, MA). The prepared cDNA was diluted five times using RNase-free water and stored at −20°C prior to further processing.

Because small volumes of human plasma contain low amounts of RNA, preamplification of target miRNAs prior to quantification is required to accurately assess their expression. Highly multiplex, PCR-based preamplification reactions were performed using Qiagen's miScript Preamp PCR kit. As this kit amplifies up to 400 miRNA-specific cDNA targets in a single reaction, preamplification of the miRNAs included on Qiagen's miScript miRNA PCR Array Human miRNome (384-well [includes 1,066 miRNAs distributed over three 384-well plates], V16.0) was performed in three separate preamplification reactions using miScript PreAMP miRNome Primer Mixes (MBHS-16AZ, MBHS-16BZ, and MBHS-16CZ, respectively). For each reaction, 5 μL diluted cDNA from the pooled normoalbuminuric control subject sample was used in a 25-μL preamplification reaction using an miScript PreAMP PCR kit. Preamplification was performed using a PTC-200 thermal cycler and the following cycling conditions: 95°C for 15 min, 2 cycles of 94°C for 30 s, 55°C for 1 min, and 70°C for 1 min and 10 cycles of 94°C for 30 s and 60°C for 1 min. As per the manufacturer's recommendation, the preamplified cDNA from these three reactions were pooled together and then diluted fivefold using RNase-free water prior to being stored at −20°C.

After reverse transcription and preamplification, the levels of 1,066 miRNAs included on the miScript miRNA PCR Array Human miRNome were assayed in our pooled normoalbuminuric control sample by SYBR green–based quantitative RT-PCR using 0.25 μL diluted cDNA in a 10-μL reaction on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The following three-step cycling program was used: 95°C for 15 min and 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. Amplification results were analyzed with the SDS 2.4 software (Applied Biosystems). miRNome profiling of the pooled normoalbuminuric T1D control sample was performed in duplicate.

TGF-β–Regulated miRNA Analysis in Individual Plasma Samples

Profiling of five highly detectable TGF-β–regulated miRNAs (let-7b-5p, let-7c-5p, miR-21-5p, miR-29a-3p, and miR-29c-3p), along with c.el-miR-39-3p and two proprietary Qiagen control assays used to assess the efficiency of reverse transcription (miRTC and PPC), was performed using a custom miScript miRNA PCR array from Qiagen in individual plasma specimens from 38 rapid progressors, 38 nonprogressors, and 40 normoalbuminuric control subjects. Prior to quantification, reverse transcription of RNA from all individual samples was performed using fixed volumes of isolated RNA (1.5 μL for each individual sample) and the miScript II RT kit with miScript HiSpec Buffer (Qiagen) as described above.

Preamplification of let-7b-5p, let-7c-5p, miR-21-5p, miR-29a-3p, and miR-29c-3p, along with cel-miR-39-3p and two proprietary Qiagen controls used to assess the efficiency of reverse transcription (miRTC and PPC), was performed using diluted cDNA from 38 rapid progressors, 38 nonprogressors, and 40 normoalbuminuric control subjects; the miScript PreAMP PCR kit; and a custom miScript PreAMP Primer Mix. For each sample, 5 μL diluted cDNA was used in a 25 μL preamplification reaction as described above. The preamplified cDNAs were then diluted 20-fold using RNase-free water prior and stored at −20°C.

Profiling of let-7b-5p, let-7c-5p, miR-21-5p, miR-29a-3p, miR-29c-3p, and cel-miR-39-3p was performed using preamplified cDNA from these samples and a custom miScript miRNA PCR Array. SYBR green–based quantitative RT-PCR was performed in duplicate using an ABI 7900HT Fast Real-Time PCR System as described above. Amplification results were analyzed with the SDS 2.4 software.

Statistical Analysis

All statistical analyses were conducted in SAS for Windows, version 9.2 (SAS Institute, Cary, NC). Differences in clinical characteristics between study groups were tested using Student *t* test and a χ^2 test for continuous and categorical variables, respectively.

Cel-miR-39 was used for normalization to obtain relative levels of each TGF-β–regulated miRNA according

to the equation $2^{-\Delta Cq}$, where Δthreshold cycle (Cq) = average $Cq_{TGF\text{-}\beta1\text{-}regulated\ miRNA}$ − average $Cq_{cel\text{-}miR\text{-}39}$. Group-wise comparisons of differences in TGF-β1–regulated miRNA levels were first assessed by nonparametric Kruskal-Wallis and Mann-Whitney *U* tests, as appropriate. The effects of TGF-β1–regulated miRNA on the risk of rapid loss of renal function/the risk of proteinuria were then assessed using univariable and multivariable logistic regression analysis. Effect measures were expressed as the odds ratios (ORs) per 1-SD increase of normalized miRNA relative level. Multivariable analyses were adjusted for sex, age, HbA_{1c}, and duration of T1D. *P* values ≤0.01 (0.05/5 miRNAs) were considered statistically significant.

RESULTS

Study Groups and Their Clinical Characteristics

Clinical characteristics for the rapid progressors, nonprogressors, and normoalbuminuric control subjects included in this study are summarized in Table 1.

In comparison with the rapid progressor group, nonprogressors included more male subjects, had a longer duration of T1D, were older at baseline, and had higher systolic blood pressure. By design, both patient groups had urinary ACRs in the proteinuric range, although this was lower in nonprogressors. Both subgroups also had normal baseline eGFR. During follow-up, eGFR declined rapidly in the rapid progressors such that within 3–11 years, nearly all had either developed ESRD or reached CKD stage 4. All nonprogressors had no or minimal renal function loss during 7–20 years of follow-up; the majority were in CKD stage 1–2 at their last follow-up.

Normoalbuminuric control subjects included fewer males and were younger than the nonprogressor group and had the lowest blood pressure, HbA_{1c}, and ACR levels relative to both rapid progressors and nonprogressors. Baseline eGFR was also higher among patients in this group compared with both rapid progressors and nonprogressors, and their rate of eGFR loss was minimal and similar to that observed in nonprogressors.

Levels of Candidate TGF-β1–Regulated miRNAs in Plasma from T1D Patients

Previous studies have shown that several miRNAs, including let-7a/b/c, miR-2a, miR-21, the miR-29 family, miR-192, the miR-200 family, miR-215, miR-216a, miR-217, miR-377, miR-382, and miR-491, are altered in response to TGF-β1 in vitro or in various animal models (10–18,35–44). To establish the detectability of these and other miRNAs in plasma from T1D patients, we profiled 1,066 miRNAs included on Qiagen's miScript miRNA PCR Array Human miRNome in a pooled plasma sample derived from 40 healthy T1D normoalbuminuric patients. Among 22 TGF-β1–regulated miRNAs identified in the

Table 1—Clinical characteristics of rapid progressors, nonprogressors, and normoalbuminuric control subjects with T1D

	RP (*N* = 38)	NP (*N* = 38)	Normoalbuminuric control subjects (*N* = 40)	*P* (RP vs. NP)
Men, %	44.7	73.7	35.0	0.01
Age of diabetes diagnosis (years)	13.0 ± 9.0	11.3 ± 6.3	13.6 ± 7.1	0.33
Duration of diabetes (years)	22.1 ± 9.0	30.8 ± 9.3	18.3 ± 7.5	<0.0001
Age (years)	35.1 ± 7.8	42.1 ± 7.8	31.8 ± 10.4	0.0002
HbA_{1c} (%)	10.0 ± 1.6	9.6 ± 1.3	8.6 ± 1.0	0.17
Systolic BP (mmHg)	132.4 ± 18.1	141.8 ± 16.0	116.5 ± 11.5	0.02
Diastolic BP (mmHg)	81.3 ± 9.9	82.9 ± 14.9	70.6 ± 7.6	0.57
ACR (μg/mg), median (25th, 75th percentiles)	1,041.5 (385.4, 2,015.4)	496.8 (313.4, 868.3)	12.4 (10.5, 15.2)	0.005
Baseline eGFR (mL/min per 1.73m^2), median (25th, 75th percentiles)	100.3 (79.0, 115.3)	95.0 (80.7, 112.1)	116.5 (104.5, 126.4)	0.49
Duration of follow-up (years)	5.1 ± 2.8*	11.1 ± 5.0†	7.4 ± 1.6	<0.0001
Last follow-up eGFR, median (25th, 75th percentiles)	‡	87.2 (69.5, 103.5)	112.8 (100.0, 120.6)	
eGFR slope (mL/min per 1.73 m^2 per year)	−20.7 ± 14.8	−1.02 ± 0.9	−0.77 ± 1.1	<0.0001

Data are means ± SD unless otherwise indicated. All clinical characteristics are from baseline examinations with the exception of eGFR slope. BP, blood pressure; NP, nonprogressors; RP, rapid progressors. *Rapid progressors were observed throughout the duration of the follow-up period or until they reached ESRD. †As plasma specimens from nonprogressors collected at entry to the 2nd Joslin Kidney Study were limited, plasma samples collected during the follow-up period were used in this study. The duration of follow-up provided for nonprogressors corresponds to the time from the collection of the sample used in this study to the end of follow-up. ‡Thirty-three out of 38 (86.8%) patients reached ESRD during the follow-up period, 1 patient progressed to CKD stage 4, and the remaining 4 patients lost 50% of their baseline eGFR prior to being lost to follow-up.

literature, 12 of these miRNAs were found to be highly detectable (defined as a Cq value ≤30) in plasma from these patients (Supplementary Table 1). The TGF-β1–regulated miRNAs that were not detected in our pooled sample are also listed in Supplementary Table 1.

Association of Plasma TGF-β1–Regulated miRNAs With the Risk of Rapid Progression to ESRD

To explore the relationship between plasma TGF-β1–regulated miRNAs and the risk of rapid progression to ESRD, we chose to focus on the five most highly detectable TGF-β1–regulated miRNAs identified in our normoalbuminuric control sample (Table 2) and examined their levels in baseline plasma specimens from 38 T1D patients at risk for rapid progression to ESRD (rapid progressors) and 38 T1D patients determined to have stable renal function (nonprogressors) that have been followed longitudinally at the Joslin Clinic.

Of the five TGF-β–related miRNA that were assayed, both let-7c-5p and miR-29a-3p were highly detachable in plasma collected from patients included in this study and had Cq values similar to the exogenous control (ranging from 13.4 to 19.9). The three additional miRNAs (let-7b-5p, miR-21-5p, and miR-29c-3p), while detachable, were present in much lower abundance than the endogenous control miRNA, let-7c-5p, and miR-29a-3p (Cq values ranging from 25.6 to 32.6).

The relative levels of let-7b-5p, let-7c-5p, miR-21-5p, miR-29a-3p, and miR-29c-3p were significantly different in baseline specimens from patients with proteinuria who subsequently lost renal function (i.e., became rapid progressors), those who maintained normal and stable renal function over the follow-up period (i.e., became nonprogressors), and normoalbuminuric control subjects (Kruskal-Wallis $P \leq 0.0003$) (Table 3 and Fig. 1).

Of these, let-7b-5p and miR-21-5p were found to be significantly upregulated in rapid progressors relative to nonprogressors ($P = 0.01$ and $P = 0.006$, respectively). Both miRNAs were also found to be highly correlated (Spearman $\rho \leq 0.74$) (Table 4). Conversely, while also highly correlated with each other (Spearman $\rho \leq 0.83$) (Table 4), let-7c-5p and miR-29a-3p were significantly downregulated in rapid progressors compared with nonprogressors ($P = 0.0002$ and $P = 0.0007$, respectively). The relative level of miR-29c-3p did not differ between rapid progressors and nonprogressors ($P = 0.68$). This miRNA was, however, significantly increased in these two patient groups relative to normoalbuminuric control subjects ($P = 0.0009$ and $P = 0.0003$, respectively).

These data suggest that four of the miRNAs examined in this study (let-7b-5p, let-7c-5p, miR-21-5p, and miR-29a-3p) are associated with the risk of rapid renal function decline experienced by rapid progressors, while miR-29c-3p, on the other hand, is associated with an increased risk of proteinuria. These relationships, and the effects of these miRNAs on these two phenotypes, were further assessed using logistic regression analysis.

For analyses of the miRNAs associated with rapid progression to ESRD, miRNA levels between the two nondecliner patient groups (i.e., nonprogressors and normoalbuminuric control subjects) and rapid progressors were compared (Table 5). In univariable analyses, the ORs for the risk of rapid progression to ESRD for a 1-SD increase in the relative level of plasma let-7b-5p was 2.51 (95% CI 1.42, 4.43; $P = 0.002$). Similarly, a 1-SD increase in the relative plasma level of miR-21-5p was associated with a 6.3-fold increase in the risk of rapid progression to ESRD (OR 6.33 [95% CI 1.75, 22.92; $P = 0.005$]). For both let-7c-5p and miR-29a-3p, a 1-SD increase in their relative plasma levels was associated with more than a 50% reduction in the risk of rapid progression to ESRD (OR 0.23 [95% CI 0.10, 0.52; $P = 0.0004$] and OR 0.38 [95% CI 0.20, 0.74; $P = 0.004$], respectively). The strength of each of these associations was not diminished after adjustments for sex, age, HBA_{1c}, and duration of T1D.

In a logistic model to assess the effect of miR-29c-3p on the risk of proteinuria, we compared this miRNA's relative level in normoalbuminuric control subjects with that in the two proteinuric patient groups (i.e., rapid progressors and nonprogressors). For this miRNA, although not statistically significant, a 1-SD increase in its plasma level was suggestive of a 1.7-fold increase in the risk of proteinuria (OR 1.73 [95% CI 0.96, 3.10; $P = 0.07$]).

DISCUSSION

This is the first study to demonstrate that miRNAs involved in the TGB-β1 pathway are deregulated very early in T1D patients who are at risk for rapid progression to ESRD. It expands upon a growing body of literature that highlights the role of these miRNAs in TGB-β1–mediated fibrogenesis in diabetic kidney disease. While several recent studies have examined urinary miRNA

Table 2—Summary of TGF-β1–regulated miRNAs detected in plasma from normoalbuminuric control subjects who were selected for examination in rapid progressors and nonprogressors

miRNA	Cq	Reported TGF-β1 effect on miRNA expression	Reference(s)
let-7b-5p	24.23	Downregulated	Wang et al., 2014 (16)
let-7c-5p	25.67	Downregulated	Brennan et al., 2013 (35)
miR-21-5p	19.88	Upregulated	Zhong et al., 2011 (45)
miR-29a-3p	22.53	Downregulated	Du et al., 2010 (10); Qin et al., 2011 (40); Wang et al., 2012 (43)
miR-29c-3p	21.79	Downregulated	Qin et al., 2011 (40); Wang et al., 2012 (43)

Table 3—Analysis of circulating TGF-β1–regulated miRNA levels in rapid progressors, nonprogressors, and normoalbuminuric control subjects

miRNA	Relative miRNA levels*			Kruskal-Wallis ANOVA *P*	Mann-Whitney *U* test *P* (RP vs. NP)
	RP	NP	Normoalbuminuric control subjects		
let-7b-5p	$8.0 \times 10^{-4} \pm 9.8 \times 10^{-4}$	$3.4 \times 10–4 \pm 4.2 \times 10^{-4}$	$1.9 \times 10^{-4} \pm 2.6 \times 10^{-4}$	0.0003	0.01
let-7c-5p	1.7 ± 1.6	3.1 ± 2.1	3.7 ± 2.7	<0.0001	0.0002
miR-21-5p	$9.9 \times 10^{-4} \pm 1.4 \times 10^{-3}$	$2.2 \times 10–4 \pm 3.8 \times 10^{-4}$	$5.6 \times 10^{-5} \pm 6.0 \times 10^{-5}$	<0.0001	0.006†
miR-29a-3p	2.17 ± 3.05	3.5 ± 2.4	4.2 ± 2.7	<0.0001	0.0007
miR-29c-3p	$1.1 \times 10^{-3} \pm 1.3$ x10^{-3}	$1.3 \times 10^{-3} \pm 1.8 \times 10^{-3}$	$6.2 \times 10–4 \pm 1.2 \times 10^{-3}$	0.0003	0.68‡

Data are means ± SD. NP, nonprogressors; RP, rapid progressors. *The relative level of each miRNA was calculated according to the equation $2^{-\Delta Cq}$, where ΔCq = average $Cq_{TGF\text{-}\beta1\text{–}regulated\ miRNA}$ – average $Cq_{c.el\text{-}miR\text{-}39}$. †miR-21-5p was significantly upregulated in both rapid progressors ($P < 0.0001$) and nonprogressors ($P = 0.02$) compared with normoalbuminuric control subjects. ‡miR-29c-3p was significantly increased in both rapid progressors ($P = 0.0009$) and nonprogressors ($P = 0.0003$) compared with normoalbuminuric control subjects.

profiles in DN patients, ours is the most extensive study to date of circulating miRNAs in plasma specimens (24–28). Moreover, a major strength of our study design over previous studies is its use of a well-characterized cohort of T1D patients that have been followed longitudinally over the course of 7–15 years of follow-up.

Our study examined the levels of five circulating TGF-β1–regulated miRNAs in baseline plasma specimens taken from T1D patients who were found to be either at risk for or protected against rapid progression to ESRD. The strongest association that we observed was with the protective effects of let-7c-5p. Consistent with this finding, Brennan et al. (35) recently reported that let-7c overexpression mimics the fibrosuppressant effects of lipoxin A4, a lipid mediator involved in the resolution of acute inflammatory responses, in human proximal tubular epithelial cells. Downregulation of let-7c results in TGF-β1–mediated induction of several effectors of fibrosis, including collagen type I, α1 (COL1A1); collagen type I, α2 (COL1A2); and thrombospondin (THBS1).

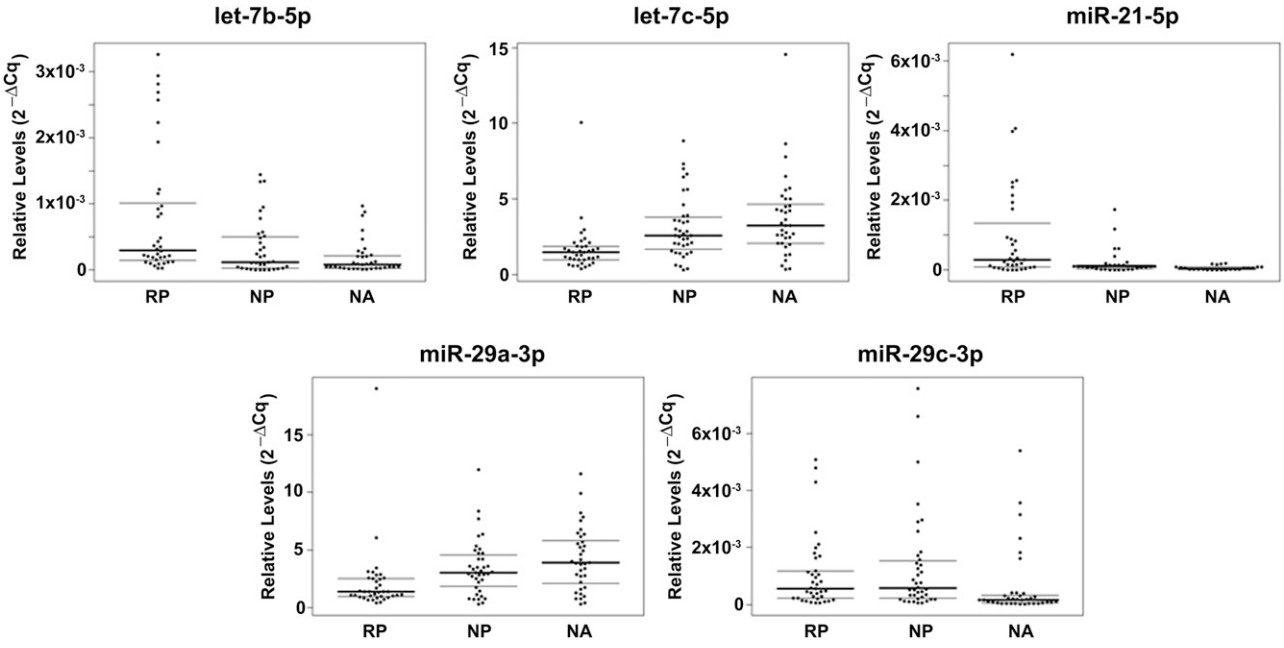

Figure 1—Relative levels of TGF-β1–regulated miRNAs in plasma from rapid progressors, nonprogressors, and normoalbuminuric control subjects. The relative levels of let-7b-5p, let-7c-5p, miR-21-5p, miR-29a-3p, and miR-29c-3p were significantly different among patients with proteinuria who either lost (i.e., rapid progressors) or maintained (i.e., nonprogressors) renal function over the follow-up period and normoalbuminuric control subjects (Kruskal-Wallis $P \leq 0.0003$) (Table 3). miR-192-5p levels were similar among all patients examined in this study (Kruskal-Wallis $P = 0.15$) (Table 3). Mann-Whitney *U* test *P* values from comparisons between rapid progressors and nonprogressors are provided. Horizontal bars indicate the median (bold) and first and third quartile in each group. Normalized relative levels of each miRNA are presented in the form $2^{-\Delta Cq}$, and cel-miR-39-3p was used as a stable reference normalization control. NA, normoalbuminuric control subjects; NP, nonprogressors; RP, rapid progressors.

Table 4—Spearman rank order correlation coefficients between TGF-β1–regulated miRNA levels

miRNA	let-7b-5p	let-7c-5p	miR-21-5p	miR-29a-3p	miR-29c-3p
let-7b-5p	1	0.08	0.74	0.04	0.61
let-7c-5p		1	−0.10	0.83	0.18
miR-21-5p			1	0.03	0.65
miR-29a-3p				1	0.26
miR-29c-3p					1

miR-29a-3p was also found to be protective against the loss of renal function in patients from our study. Previous studies have shown that this miRNA is downregulated in response to TGB-β1 in a variety of human and nonhuman cell lines, including human and rat proximal tubule epithelial cells, mouse mesangial cells, and human podocytes (10,40,43). miR-29a and other members of the miR-29 family negatively regulate the expression of several fibrotic genes, including a number of collagen genes (e.g., COL1A1 and COL1A2 and collagen type IV, α1, -2, and -3), via a Smad3-dependent mechanism (10,40,41,43). In addition to these in vitro models, and in agreement with our observation in patients at risk for renal function decline, miR-29a has also been shown to be markedly decreased in kidney tissue in rodent models of diabetic renal fibrosis (10,43). Similar findings have also been noted in nondiabetic mouse models, suggesting that aberrant miR-29 levels are common to both diabetic and nondiabetic kidney disease (10,43).

Our findings that plasma let-7b-5p and miR-21-5p are associated with an increased risk of rapid progression to ESRD in patients with T1D are supported by data from a number of prior in vitro and in vivo studies of DN (15,16,18,37,45,46). Both let-7b-5p and miR-21-5p have been shown to target genes directly implicated in renal function decline. For example, in cultured human podocytes, Schaeffer et al. (46) demonstrated that hyperglycemia-induced let-7b expression reduces levels of laminin-β2 (LAMB2), an extracellular matrix glycoprotein critical to normal podocyte function. Similarly, upregulation of miR-21 has been reported to contribute to fibrotic scarring by directly targeting matrix metallopeptidase-9 (MMP-9) (15,16,18,37,45). Interestingly, anti–miR-21 therapy decreases tissue inhibitor of metalloproteinase 1 (TIMP1), collagen IV, and fibronectin protein levels and reduces glomerular basement membrane thickening, suggesting that miR-21 is a potential therapeutic target against the progression of DN (15,18).

In contrast to our findings for the majority of miRNA examined in this study, miR-29c-3p was not associated with either the risk of or protection against rapid progression to ESRD. This miRNA was, however, increased in patients with proteinuria, irrespective of renal function decline, relative to those with persistent normoalbuminuria. These empirical data challenge the conventional model that regards DN as a disease that advances sequentially through characteristic stages defined by increasing levels of albuminuria followed by the development of renal decline (47). Furthermore, this intriguing finding supports our hypothesis that albuminuria and renal decline are uncoupled phenotypes (32).

Interestingly, miR-29c expression has been shown to be increased in both in vitro and in vivo models of DN (48). As demonstrated by Long et al. (48), miR-29c levels are significantly increased in kidney glomeruli from *db/db* diabetic mice compared with control *db/m* littermates. Moreover, miR-29c expression is also significantly increased in both kidney podocytes and kidney microvascular endothelial cells in response to hyperglycemic conditions. Importantly, in contrast to control *db/db* mice, Long et al. further demonstrated that knockdown of miR-29c with a chemically modified antisense oligonucleotide significantly reduced albuminuria in *db/db* mice in vivo.

Some limitations of our study should be considered. First, our study is incapable of determining the source of the TGF-β1–regulated miRNAs that were differentially present in plasma from rapid progressors and

Table 5—Logistic regression analysis of circulating TGF-β1–regulated miRNA levels and the risk of rapid progression to ESRD

	Unadjusted		Adjusted‡	
miRNA	OR (95% CI)†	*P*	OR (95% CI)†	*P*
let-7b-5p	2.51 (1.42, 4.43)	0.002	2.38 (1.31, 4.06)	0.004
let-7c-5p	0.23 (0.10, 0.52)	0.0004	0.23 (0.10, 0.53)	0.0006
miR-21-5p	6.33 (1.75, 22.92)	0.005	5.87 (1.68, 20.46)	0.006
miR-29a-3p	0.38 (0.20, 0.74)	0.004	0.39 (0.20, 0.76)	0.00.6

The rapid progressor group is the reference group. For estimation of the effects of these miRNAs on rapid progression to ESRD, rapid progressors were compared with the combined nonprogressor group (i.e., nonprogressors and normoalbuminuric control subjects). †Effect measures are expressed as the ORs per SD increase of normalized relative miRNA level. ‡Multivariable analyses were adjusted for sex, age, HbA_{1c}, and duration of T1D.

nonprogressors. Circulating miRNAs are largely thought to be a by-product of cell death contributed by organs throughout the body (49). Although each of the miRNAs examined in our study are reported to have expression in the kidney (50), while intriguing, whether the kidney is the major contributor of the plasma miRNAs that we found to be associated with increased risk of rapid progression to ESRD remains unclear. Further studies are necessary to investigate this issue. Second, the current study only investigated a subset of miRNAs that have been reported to be regulated by TGF-β1 in various models of DN. It is likely that other miRNAs in the TGF-β1/Smad pathway, as well as those in other pathways, could have important roles in the risk of rapid progression to ESRD seen in patients with T1D. Many of these miRNA, while lowly detectable in plasma, may be more abundant in serum, urine, or other biofluids. Third, along this same line, the current study focused solely on TGF-β1–regulated miRNAs identified in peer-reviewed publications. While our findings show that the majority of miRNAs from this modest, yet well-defined, set are deregulated very early in T1D patients who are at risk for rapid progression to ESRD, whether other miRNAs beyond those investigated in this study are similarly deregulated, and perhaps more strongly associated with the risk of rapid progression to ESRD, is unclear. We anticipate that the findings presented in this study will likely serve as a springboard for further studies aimed at investigating this question. Lastly, we have previously acknowledged that the patients included in this study lack direct measurements of GFR (32). Although less accurate, the serum creatinine–based estimates of GFR used in this study adequately approximate the baseline renal function of the included patients, and serial measures of these estimates are able to distinguish rapid progressors from nonprogressors.

Our use of a well-characterized cohort of T1D patients that have been followed longitudinally for more than a decade allows us to begin to assess the predictive utility of various biomarkers, including miRNAs, in determining which patients might be most at risk for or protected against rapid progression to ESRD. Our findings suggests that TGF-β1–regulated miRNAs detectable in plasma could be preclinical indicators of early renal decline and, therefore, might have utility in identifying patients most at risk for renal function decline and progression to ESRD. Pending further studies, therapeutic augmentation of these, and perhaps other, miRNAs may prove useful in inhibiting fibrogenesis and modifying the risk of renal function decline in T1D.

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