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Inhibiting MicroRNA-503 and MicroRNA-181d with Losartan Ameliorates Diabetic Nephropathy in KKAY Mice

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Background: Diabetic nephropathy (DN) is the most lethal diabetic microvascular complication; it is a major cause of renal failure, and an increasingly globally prominent healthcare problem.

Material/Methods: To identify susceptible microRNAs for the pathogenesis of DN and the targets of losartan treatment, microRNA arrays were employed to survey the glomerular microRNA expression profiles of KKAY mice treated with or without losartan. KKAY mice were assigned to either a losartan-treated group or a non-treatment group, with C57BL/6 mice used as a normal control. Twelve weeks after treatment, glomeruli from the mice were isolated. MicroRNA expression profiles were analyzed using microRNA arrays. Real-time PCR was used to confirm the results.

Results: Losartan treatment improved albuminuria and the pathological lesions of KKAY mice. The expression of 10 microRNAs was higher, and the expression of 12 microRNAs was lower in the glomeruli of the KKAY untreated mice than that of the CL57BL/6 mice. The expression of 4 microRNAs was down-regulated in the glomeruli of the KKAY losartan-treated mice compared to that of the untreated mice. The expression of miRNA-503 and miRNA-181d was apparently higher in the glomeruli of the KKAY untreated mice, and was inhibited by losartan treatment.

Conclusions: The over-expression of miR-503 and miR-181d in glomeruli of KKAY mice may be responsible for the pathogenesis of DN and are potential therapeutic targets for DN.

MeSH Keywords: **Diabetic Nephropathies • Kidney Glomerulus • Losartan • MicroRNAs**

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Background

Diabetic nephropathy (DN) is the most lethal diabetic microvascular complication; it has become a major cause of renal failure and an increasingly globally prominent healthcare problem. The pathogenesis of DN is multi-factorial and consists of genetic and environmental factors that markedly stimulate intra- and extra-renal pathways. Genetic predisposition has been indicated as the decisive factor for the susceptibility and rate of progression to end-stage renal disease (ESRD) in diabetic patients. However, because this is a complex multiple-gene disease, the genetic mechanisms behind DN are not fully understood [1–3].

MicroRNAs (miRNAs) are a class of short non-coding RNAs that have been shown to have a regulatory role in gene expression [4]. MiRNAs exert their regulatory effects at the post-transcriptional level by binding to the 3'-untranslated region of their target mRNAs [5]. Recent evidence suggests that miRNAs may regulate the expression of key genes relevant to DN, but the role of miRNAs *in vivo* is still poorly understood [6–8].

Established diabetic nephropathy is characterized by mesangial expansion, which may be nodular, so-called Kimmelstiel-Wilson nodules, hyaline in both afferent and efferent arterioles, and markedly thickened GBM by electron microscopy. Podocyte loss may be a crucial contributor to this progressive sclerosis [9]. Diabetic injury also affects the tubulointerstitium. Tubular basement membranes thicken in parallel with GBM. Early interstitial inflammation with predominantly mononuclear cells is followed by later increased interstitial fibrosis and tubular atrophy [9,10]. KKAY mice with spontaneous type 2 diabetes are a widely used animal model in diabetic nephropathy research. These mice have clinical manifestations of hyperglycemia, impaired glucose tolerance, hyperinsulinemia, moderate obesity, hyperlipidemia, and proteinuria. Kidney damage in these mice is very similar to that which occurs during human diabetic nephropathy [11]. Because the glomerulus is a major target of injury in DN, the glomerular genomic and proteomic profile is very important. We recently carried out a proteomics study to explore the protein expression profile of glomeruli from KKAY mice treated with or without losartan (in press). We identified 57 proteins that were differentially expressed between the KKAY and C57BL/6 glomeruli at 20 weeks of age. The differential expression of the 75 kDa glucose-regulated protein (GRP75), the succinyl-CoA ligase subunit beta, and the ATP synthase subunit d in the KKAY glomeruli were inhibited by losartan treatment. In the present study we compared miRNA expression in the normal glomeruli of C57BL/6 mice with that of diabetic KKAY mice treated with or without losartan using the GeneChip® miRNAs Array. The aim was to identify candidate miRNAs that contribute to the pathogenesis of DN and to search for new treatment targets.

Material and Methods

Animals and drug treatment

Male KKAY mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, and male C57BL/6 mice for the control group were purchased from the Laboratory Animal Center, China Medical University. The mice were individually housed in plastic cages with free access to food and tap water throughout the experimental period. All mice were maintained in a room with controlled temperature ($23\pm 3^{\circ}\text{C}$) and humidity ($50\%\pm 20\%$) (China Medical University, Laboratory Animal Center SPF rodent housing facility) with a regular 12-h light/dark cycle according to the Chinese National Standard (GB 14925-2001). All animal studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee at China Medical University.

KKAY mice were divided into a non-treatment group ($n=10$) and a losartan-treated group ($n=10$). C57BL/6 mice were used as a control group ($n=10$). Losartan was administered at a dosage of 10 mg/kg/day in drinking water from 8 to 20 weeks. The dose of losartan was selected on the basis of previous studies that showed a significant renoprotective effect [12]. Fluid intake was measured every day. The losartan dosages were adjusted through the drinking water and by body weight.

Biochemical and metabolic parameters

Body weight (BW), fasting glucose, serum creatinine and urea nitrogen concentrations, and urinary albumin excretion were serially monitored every 4 weeks. Glucose levels in blood obtained from the retro-orbital sinus were measured using a Roche Glucotrend® 2 monitor (Roche, Germany). Blood pressure was measured by a non-invasive tail cuff and pulse transducer system (Softron BP-98A, Tokyo, Japan) after the mice were externally pre-warmed to 37°C for 10 min. Serum creatinine and urea nitrogen concentrations were enzymatically determined by a VITROS 950 automatic biochemistry analyzer (Johnson & Johnson, New Brunswick, NJ). Urinary albumin concentrations were examined by an immunospecific ELISA (Albuwell M kit; Exocell Inc., Philadelphia, PA), and all samples were individually adjusted for creatinine excretion (Creatinine Companion; Exocell Inc., Philadelphia, PA). For morphometric studies, the kidneys were fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. The 3- μm sections of paraffin-embedded tissues were stained with periodic acid-Schiff.

Isolating glomeruli and extracting total glomerular RNA of KKAY mice

Glomeruli were isolated from the KKAY and C57BL/6 mice at 20 weeks of age. Briefly, kidneys were perfused with ice-cold

PBS via the abdominal aorta to free the blood vessels of any remaining blood. Dynabeads in a concentration of 4×10^6 /ml PBS were perfused into the kidney at a constant rate of 7.4 ml/min/g kidney [13]. Kidneys were removed, chopped, and digested in collagenase A (1 mg/ml) at 37°C for 30 min with gentle agitation. The digested tissue was pressed through a 100 μ m cell strainer, followed by intermittent ice-cold PBS flushing. The filtered suspension was centrifuged at $200 \times g$ at 4°C for 5 min. The supernatant was discarded, and the pellet was dissolved in 2 ml PBS and transferred to a 2-ml tube. Glomeruli containing Dynabeads were separated from the renal tubules by a magnetic particle concentrator. The glomerulus RNA was extracted with Trizol. The purity ($A_{260/280} \geq 1.80$) and the quantity of extracted glomerulus RNA meet the requirements of Affymetrix miRNAs microarray experiments.

miRNA microarray

miRNA expression profiling was assessed by Affymetrix GeneChip® miRNAs arrays according to the manufacturer's instructions (Affymetrix). In brief, FlashTag RNA Labeling was produced by poly(A) tailing and ligation. RNA hybridization, washing, staining, and scanning were performed. The miRNA QC Tool was used for data summarization, normalization, and quality control.

Data analysis

To compare any 2 experiments, we used the GeneChip® software to conduct normalization and scaling of the data for each array, and pair-wise comparisons were made between the KKAY and C57BL/6 mice and between the KKAY non-treatment and losartan-treated KKAY mice. The screening criteria for the up-regulated miRNAs required that Detection in the B channel be TRUE and that the Ratio be more than 2. The screening criteria for the down-regulated miRNAs were that Detection in the A channel be TRUE and that the Ratio be more than 2. Hierarchical clustering of 3 groups was performed using all expression values from the microRNA after quality filtering. We used Cluster 3.0 software, with expression values median-centered per gene and clustered using the Pearson correlation distance and average linkage. The results were visualized using TreeView [14,15]. To determine the gene targets for the miRNAs, we used 5 leading miRNA target prediction algorithms (TargetScan 5.2, miRanda, miRbase, Diana microT 3.0, and EIMMo2).

Real-time reverse transcription (RT)-PCR

Key miRNAs were selected for validation using real-time PCR. TaqMan miRNA assays (Applied Biosystems, CA) were used for quantitative determination of miRNA expression according to the manufacturer's instructions. Briefly, 100 ng of total RNA were reverse transcribed using miRNA-specific stem-loop RT

primers, MultiScribe reverse transcriptase, RT buffer, dNTPs, and RNase inhibitor (Applied Biosystems) under the following conditions: 16°C for 10 min, 37°C for 30 min, and 65°C for 5 min. Real-time PCR was performed on the resulting complementary DNA (cDNA) using miRNA-specific TaqMan primers and TaqMan Universal PCR Master Mix in a 7500 real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. RNU6B (Applied Biosystems) was used as the internal control. Relative fold changes of gene expression were calculated by the $\Delta\Delta C_t$ method [14], and the values are expressed as $2^{-\Delta\Delta C_t}$. Data are presented as the expression level relative to the control group with the standard error of the mean of triplicate measures for each group.

Statistics

The quantitative data are expressed as the mean \pm SEM. The statistical analysis was performed using SPSS 22.0 software. Pair-wise comparisons were performed using the *t* test. $P < 0.05$ was considered statistically significant.

Results

Losartan ameliorated urinary albumin excretion and pathological injury of type 2 diabetic KKAY mice

KKAY mice developed higher body weight, blood glucose levels, hypertension, and urinary microalbumin/creatinine ratios at 20 weeks of age than C57BL/6 mice of the same age ($P < 0.05$). The predominant pathological findings in KKAY mice were segmental and diffuse mesangial expansion, glomerular basement membrane thickening, and even glomerulosclerosis. Treatment with 10 mg/kg/day losartan markedly improved albuminuria levels and pathological lesions in KKAY mice. Losartan treatment also significantly reduced the systolic blood pressure of KKAY mice (Table 1, Figure 1).

Hierarchical clustering of miRNA profiles

Of the 609 mouse miRNAs present in the GeneChip® miRNAs Arrays, 184 miRNAs were discovered to be expressed in the KKAY and C57BL/6 glomeruli. We then performed a hierarchical clustering analysis of the miRNA data to further investigate the potential similarities and differences between miRNA profiles (Figure 2).

Comparison of miRNA expression profiles between glomeruli of type 2 diabetic KKAY mice and C57BL/6 mice

Twenty-two miRNAs were expressed differentially between the glomeruli of the KKAY and C57BL/6 mice at 20 weeks of

Table 1. Biochemical and metabolic parameters at 20 weeks of age.

	C57BL/6 mice (n=10)	KKAY non-treatment group (n=10)	KKAY Losartan-treated group (n=10)
Body weight (g)	30.32±1.3	46.88±2.61*	45.08±1.96*
Random blood sugar (mmol/L)	11.07±1.26	29.62±3.99*	30.41±3.83*
Urinary albumin: creatinine ratio (mg/g)	49.37±18.77	728 ±177.19*	539.71±100.23* [#]
Mean systolic blood pressure (mm Hg)	89.4±2.5	116.5±3.2*	107.3±2.8* [#]
Serum urea nitrogen (mmol/l)	11.03±2.97	12.30±3.18	9.50±2.74
Serum creatinine (μmol/l)	32.67±4.51	30.33±5.82	35.20±17.68

Data are expressed as the Mean ±SEM. * $P < 0.05$ vs. C57BL/6 group; [#] $P < 0.05$ vs. KKAY non-treatment group.

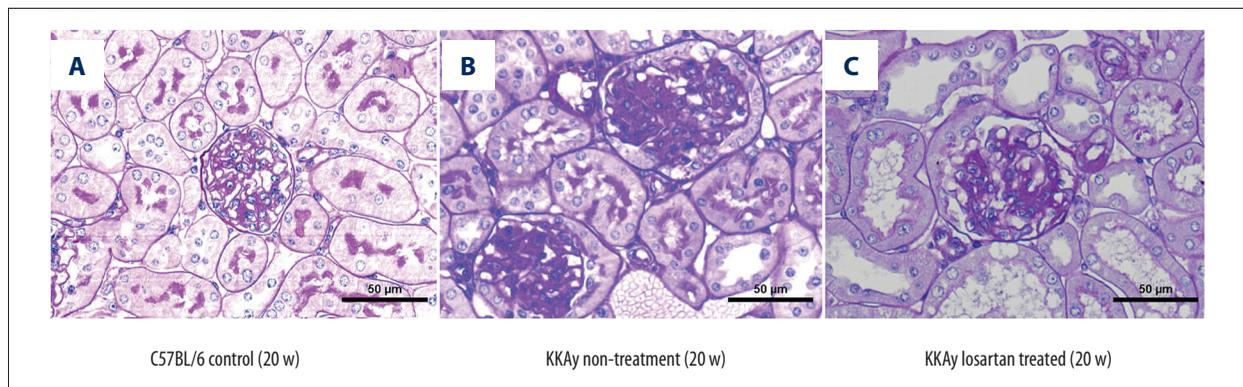


Figure 1. The predominant pathological findings in KKAY mice were segmental and diffuse mesangial expansion, glomerular basement membrane thickening, and even glomerulosclerosis. Losartan treatment improved pathological lesions of type 2 diabetic KKAY mice at 20 weeks of age (PAS' staining ×400).

age. Ten miRNAs, including miR-503, miR-669, miR-181d, and miR-29b, were up-regulated (Table 2), and 12 miRNAs, including miR-16, miR-194, and miR-200a-c, were down-regulated in the glomeruli of the KKAY mice compared to that of the C57BL/6 mice (Table 3).

Comparison of miRNA expression profiles between glomeruli of non-treated KKAY mice and losartan-treated KKAY mice

We identified 4 miRNAs that were down-regulated in the glomeruli of losartan-treated KKAY mice compared to that of the untreated KKAY mice: miRNA-21, miRNA-196a, miRNA-503, and miRNA-181d (Table 4). The expression of miRNA-503 and miRNA-181d was significantly up-regulated in the glomeruli of KKAY mice and inhibited by losartan treatment.

Real-time reverse RT-PCR confirmed the miRNA array results

Consistent with the array results, real-time RT-PCR further validated the finding that miRNA-503 and miRNA-181d were

significantly up-regulated in the glomeruli of KKAY mice and inhibited by losartan treatment (Figure 3).

Discussion

Several miRNAs have been shown to be involved in diabetic nephropathy [16–18]. A series of our team's studies have found that inhibition of miR-21 expression can reduce podocyte and mesangial cell injury by increasing autophagy [19,20]. McClelland et al. found a unified model for a key role for miR-21 in the regulation of renal tubular extracellular matrix (ECM) synthesis and accumulation, which promotes renal fibrosis in diabetic nephropathy by targeting PTEN and SMAD7 [21]. MicroRNA-27a targets PPARγ to induce mesangial cell injury, and its knockdown *in vivo* prevents progression of diabetic nephropathy [22]. MicroRNA-23b alleviates fibrosis and albuminuria in diabetic nephropathy by targeting Ras GTPase-activating protein SH3 domain-binding protein 2 [23]. TGF-β-induced miR-192 reduced the expression of 2 E-box repressors (Zeb1 and Zeb2), which controlled collagen 1 alpha 2 gene activation, to increase the expression of collagen 1 alpha 2 [24]. The specific

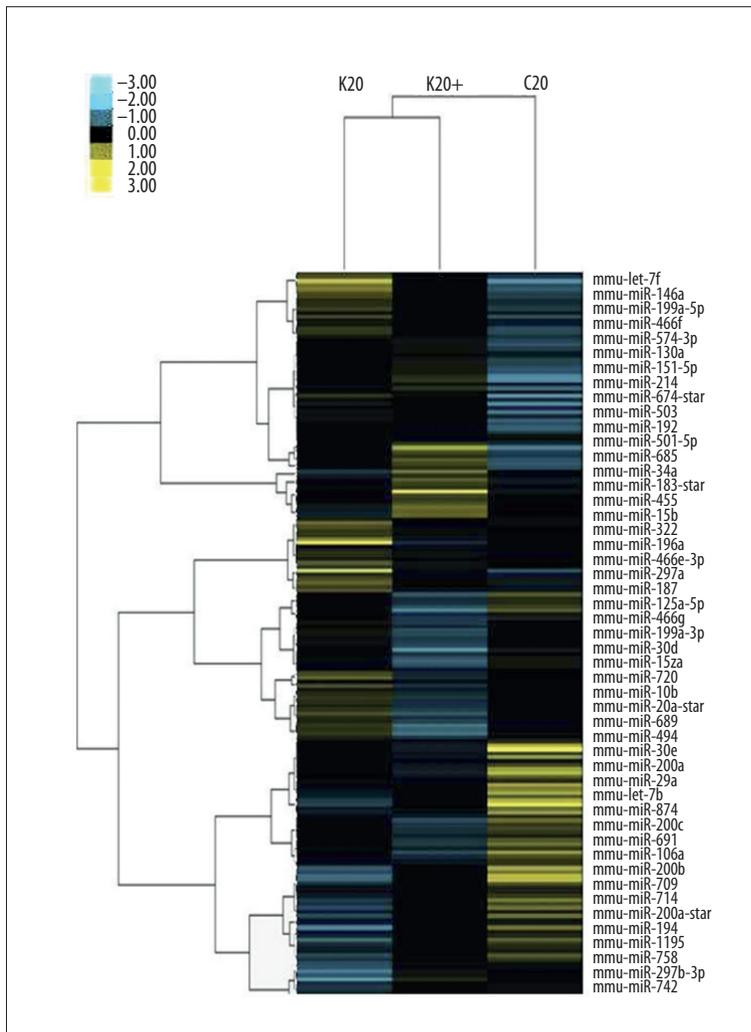


Figure 2. Unsupervised hierarchical clustering showed clustering of KKAY non-treated, KKAY losartan-treated and C57BL/6 mice glomeruli. We used the Cluster 3.0 software, with wxpression values set to the median, centered per gene and clustered using the Person correlation distance and average linkage. Yellow indicates highexpression, black indicates intermediate expression, blue indicates low expression and grey indicates no expression. K20 – the gloemruli of KKAY non-treated mice at 20 weeks of age; K20+ – the glomeruli of losartan-treated KKAY mice at 20 weeks of age; C20 – the glomeruli of C57BL/6 mice at 20 weeks of age.

Table 2. miRNAs up-regulated in the glomeruli of type 2 diabetic KKAY non-treated mice at 20 weeks of age.

miRNAs	KKAY glomeruli	p-value	Detection KKAY	C57BL/6 glomeruli	Detection C57BL/6	Fold change
mmu-miR-503	27.87	0.03290769	True	2.27	False	12.29
mmu-miR-669b	33.49	0.005930657	True	4.24	False	7.89
mmu-miR-181d	26.21	0.00326153	True	7.80	False	3.36
mmu-miR-146a	189.20	2.05282E-08	True	62.39	True	3.03
mmu-miR-615-3p	28.11	0.02023898	True	10.08	False	2.79
mmu-miR-669c	32.14	0.02833224	True	11.95	False	2.69
mmu-miR-297a	39.83	0.001769683	True	15.90	False	2.51
mmu-miR-133a	31.55	0.02165694	True	12.84	False	2.46
mmu-miR-29b	21.17	0.04727323	True	9.67	False	2.19
mmu-miR-574-3p	243.55	2.46828E-07	True	114.68	True	2.12

p-value – the reliability of detection; Detection – the state of miRNAs, True means present and False means absent; Fold change – KKAY non-treatment mice glomeruli vs. C57BL/6 mice glomeruli.

Table 3. miRNAs down-regulated in the glomeruli of type 2 diabetic KKAY non-treated mice.

miRNAs	KKAY glomeruli	Detection KKAY	C57BL/6 glomeruli	p-value	Detection C57BL/6	Fold change
mmu-miR-127	7.99	False	51.71	1.02866E-06	True	0.16
mmu-miR-379	10.45	False	36.22	0.003223371	True	0.29
mmu-miR-200c	67.63	True	167.86	2.05282E-08	True	0.40
mmu-miR-709	1996.23	True	4900.21	2.05282E-08	True	0.41
mmu-miR-714	95.39	True	227.38	5.47214E-08	True	0.42
mmu-miR-429	16.90	False	38.73	0.000208496	True	0.44
mmu-miR-194	134.08	True	305.91	2.05282E-08	True	0.44
mmu-miR-200b	46.32	True	100.62	2.05282E-08	True	0.46
mmu-miR-200a	33.05	True	68.59	3.54074E-07	True	0.48
mmu-miR-874	26.89	False	54.29	4.1393E-05	True	0.50
mmu-miR-16	23.37	False	46.94	7.89534E-05	True	0.50
mmu-miR-760	17.83	False	35.83	0.000882259	True	0.50

p-value – the reliability of detection; Detection – the state of miRNAs, True means present and False means absent; Fold change – KKAY non-treatment mice glomeruli versus C57BL/6 mice glomeruli.

Table 4. miRNAs down-regulated in type 2 diabetic KKAY mice treated with losartan.

miRNAs	losartan-treated KKAY glomeruli	Detection losartan-treated KKAY	KKAY non-treatment glomeruli	p-value	Detection non-treatment KKAY	Fold change
mmu-miR-181d	9.73	False	26.21	0.00326153	True	0.37
mmu-miR-503	11.29	False	27.87	0.03290769	True	0.41
mmu-miR-196a	19.70	False	44.17	0.000274579	True	0.45
mmu-miR-21	20.44	False	43.47	0.00318556	True	0.47

p-value – the reliability of detection; Detection – the state of miRNAs, True means present and False means absent; Fold change – losartan-treated KKAY mice glomeruli versus KKAY non-treatment mice glomeruli.

reduction of renal miR-192 alleviates renal fibrosis and albuminuria in diabetic nephropathy [25]. However, despite growing evidence of the regulatory effects of miRNAs in DN, limited information is available on the consequences of modulating renal miRNA expression *in vivo* [26].

Numerous studies have suggested that renin-angiotensin-aldosterone system (RAAS) blocking agents are particularly useful in decreasing intraglomerular pressure and hyperfiltration and are known to decrease urine protein excretion in both type 1 and type 2 diabetes mellitus [27–30]. However, the effects of RAAS blocking agents on the glomeruli miRNA expression profile in DN remain unknown. Our miRNA array analysis showed that the expression of 10 miRNAs was up-regulated, and 12

miRNAs were down-regulated in the glomeruli of KKAY mice at 20 weeks of age compared to those of C57BL/6 mice of the same age. The expression of 4 miRNAs was down-regulated in the glomeruli of KKAY mice treated with losartan compared to that of untreated KKAY mice of the same age. The miR-29b and miR146a expression was up-regulated in the glomeruli of KKAY mice by 2.2 and 3.0 fold, respectively. The miR-194 and miR-200c expression was down-regulated in the glomeruli of KKAY mice by 2.3 and 2.5 fold, respectively. Losartan treatment inhibited glomerular miR-21 expression by 2.1 fold. miR-21, miR-29b, miR-146a, miR-194, and miR-200c are expressed in both mouse and human kidneys [31], and some reports have linked miR-29b to fibrosis, especially in renal disease [32]. Thus, these differentially expressed miRNAs may

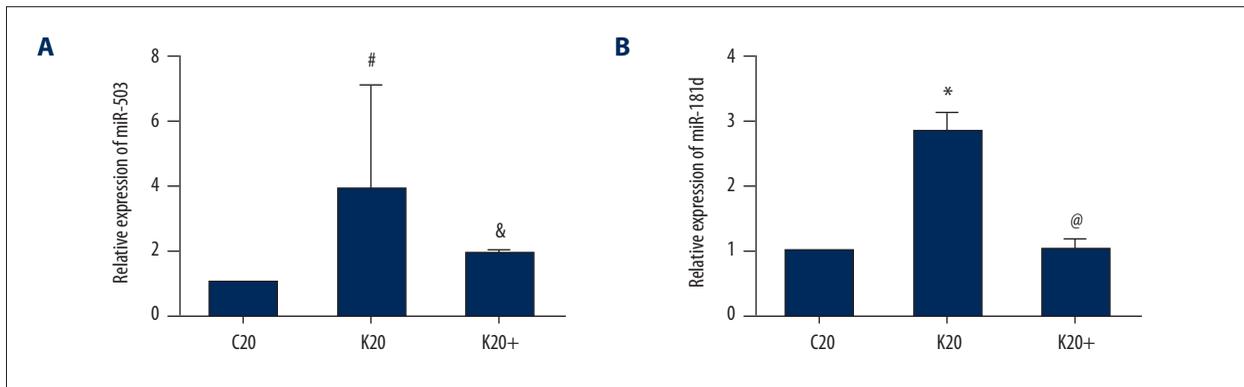


Figure 3. Expression of miR-503 and miR-181d by real-time RT-CPR. Relative fold changes of gene expression were calculated by the $\Delta\Delta CT$ method, and the values are expressed as $2^{-\Delta\Delta CT}$. C20 – the glomeruli of C57BL/6 mice at 20 weeks of age; K20 – the glomeruli of KKAY non-treated mice at 20 weeks of age; K20+ – the glomeruli of losartan-treated KKAY mice at 20 weeks of age. Data are presented as the expression level relative to the C57BL/6 control group. Results are the means of triplicate measures for each group (n=3). (A) C20: 1; K20: 4.03 ± 2.08 ; K20+: 1.92 ± 0.087 . (B) C20: 1; K20: 2.91 ± 0.206 ; K20+: 1.02 ± 0.123 . * $P < 0.01$ vs. C57BL/6 group; @ $P < 0.01$ vs. KKAY non-treatment group; # $P < 0.05$ vs. C57BL/6 group; & $P < 0.05$ vs. KKAY non-treatment group.

contribute to the pathogenesis and may be new therapeutic targets for DN in humans.

The expression levels of miRNA-503 and miRNA-181d were significantly up-regulated in the glomeruli of KKAY mice and inhibited by losartan treatment, which suggests that these miRNAs could be new therapeutic targets for DN. By searching online for “miRNA targets” using Pictar, TargetScan, and MiRanda and through a function and pathway analysis using the KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) databases, we found that microRNA-503 can regulate the expression of genes involved in the cell cycle, Wnt signaling pathway, focal adhesion, mTOR signaling pathway, VEGF signaling pathway, and regulation of the actin cytoskeleton [33]. These pathways play important roles in podocyte injury, proteinuria, mesangial proliferation, extracellular matrix accumulation, and endothelial cell injury in DN [34,35]. Recently, Caporali et al. reported the importance of miR-503 in diabetes mellitus-associated ischemic disease [33]. The combination of high glucose and starvation remarkably enhances the *in vitro* expression of miR-503 in human endothelial cells, as does diabetes mellitus in endothelial cells extracted from murine ischemic limb muscles [33]. Lentivirus-mediated miR-503 forced expression inhibited EC proliferation, migration, and network formation. In a diabetic mouse model of limb ischemia, local inhibition of miR-503 activity accelerated vascular healing and blood flow recovery [33]. In fact, miR-503 was found to be up-regulated in muscular biopsies and peripheral blood-derived plasma of diabetic patients with critical limb ischemia [33]. Further studies of Caporali et al. show that modulation of p75^{NTR} expression in ECs exposed to high glucose activates transcription of miR-503, which negatively affects pericyte function [35]. p75^{NTR} activates NF- κ B to bind the miR-503 promoter and upregulate miR-503 expression in ECs.

NF- κ B further induces activation of Rho kinase and shedding of endothelial microparticles carrying miR-503, which transfer miR-503 from ECs to vascular pericytes. The integrin-mediated uptake of miR-503 in the recipient pericytes reduces expression of EFNB2 and VEGFA, resulting in impaired migration and proliferation [36]. Collectively, their data demonstrate that miR-503 regulates pericyte-endothelial cross-talk in microvascular diabetic complications [36]. From a therapeutic perspective, manipulation of miR-503 may represent a novel molecular means for promoting reparative angiogenesis in diabetic patients. In the diagnostic context, more studies are necessary to determine whether miR-503 could be exploited as a biomarker of progressive vascular disease. Differential expression and function of miR-503 in diabetic nephropathy has not been reported.

Studies have shown that microRNA-181d may regulate the expression of matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase (TIMP), heat shock protein 70, Bcl-2 family members, mitogen-activated protein kinase (MAPK), and the Notch signaling pathway, among others [34,35,37–39], and miR-181d-5p is the key regulating miRNA of expression of O6-methylguanine-DNA methyltransferase (MGMT) [40,41]. Our previous proteomics study also found differential expression of the predicted target genes of miR-181d, such as heat shock protein 75, GRP75, and GRP78, in the glomeruli of diabetic KKAY mice (in press). Interestingly, the TGF β -Smad and Wnt/ β -catenin pathways may upregulate miRNA-181d expression [34,42,43], and the expression of Wnt3a is regulated by miRNA-503 [34]. Thus, there may be cross-talk between the miRNA-181d and miRNA-503 regulatory networks that contributes to the pathogenesis of DN.

Conclusions

The expression levels of miR-503 and miR-181d were significantly up-regulated in the glomeruli of type 2 diabetic KKAY mice at 20 weeks of age, and this expression was inhibited by losartan treatment. The protective effect of losartan on DN may be achieved by affecting the regulatory network miR-503 and

miR-181d. We plan to perform an *in vivo* microRNA interference study to investigate the network of targets regulated by miR-503 and miR-181d and their contribution to the pathogenesis of DN.

Conflicts of interest

The authors declare that they have no conflicts of interest.

References:

- Li L, Cheng WY, Glicksberg BS et al: Identification of type 2 diabetes subgroups through topological analysis of patient similarity. *Sci Transl Med*, 2015; 7: 311ra174
- Gray SP, Cooper ME: Diabetic nephropathy in 2010: Alleviating the burden of diabetic nephropathy. *Nat Rev Nephrol*, 2011;7(2): 71–73
- Nawroth PP, Isermann B: Mechanisms of diabetic nephropathy – old buddies and newcomers part 1. *Exp Clin Endocrinol Diabetes*, 2010; 118(9): 571–76
- Lee RC, Feinbaum RL, Ambros V: The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, 1993; 75(5): 843–54
- Karolina DS, Wintour EM, Bertram J, Jayaseelan K: Riboregulators in kidney development and function. *Biochimie*, 2010; 92(3): 217–25
- Koga K, Yokoi H, Mori K et al: MicroRNA-26a inhibits TGF- β -induced extracellular matrix protein expression in podocytes by targeting CTGF and is downregulated in diabetic nephropathy. *Diabetologia*, 2015; 58: 2169–80
- Kasinath BS, Feliers D: The complex world of kidney microRNAs. *Kidney Int*, 2011; 80(4): 334–37
- Badal SS, Danesh FR: Diabetic nephropathy: Emerging biomarkers for risk assessment. *Diabetes*, 2015; 64: 3063–65
- Najafian B, Alpers CE, Fogo AB: Pathology of human diabetic nephropathy. *Contrib Nephrol*, 2011; 170: 36–47
- Najafian B, Fogo AB, Lusco MA, Alpers CE: *AJKD atlas of renal pathology: Diabetic nephropathy*. *Am J Kidney Dis*, 2015; 66(5): e37–38
- Diani AR, Sawada GA, Zhang NY et al: The KKAY mouse: A model for the rapid development of glomerular capillary basement membrane thickening. *Blood Vessels*, 1987; 24: 297–303
- Sasaki M, Uehara S, Ohta H et al: Losartan ameliorates progression of glomerular structural changes in diabetic KKAY mice. *Life Sci*, 2004; 75(7): 869–80
- Sitek B, Potthoff S, Schulenburg T et al: Novel approaches to analyse glomerular proteins from smallest scale murine and human samples using DIGE saturation labelling. *Proteomics*, 2006; 6: 4337–45
- de Hoon MJ, Imoto S, Nolan J, Miyano S: Open source clustering software. *Bioinformatics*, 2004; 20: 1453–54
- Saldanha AJ: Java Treeview-extensible visualization of microarray data. *Bioinformatics*, 2004; 20: 3246–48
- Chandrasekaran K, Karolina DS, Sepramaniam S et al: Role of microRNAs in kidney homeostasis and disease. *Kidney Int*, 2012; 81(7): 617–27
- Trionfini P, Benigni A, Remuzzi G: MicroRNAs in kidney physiology and disease. *Nat Rev Nephrol*, 2015; 11(1): 23–33
- Schena FP, Salustio F, Serino G: MicroRNAs in glomerular diseases from pathophysiology to potential treatment target. *Clin Sci (Lond)*, 2015; 128(11): 775–88
- Xu L, Fan Q, Wang X et al: Ursolic acid improves podocyte injury caused by high glucose. *Nephrol Dial Transplant*, 2015; 12. pii: gfv382
- Lu X, Fan Q, Xu L et al: Ursolic acid attenuates diabetic mesangial cell injury through the up-regulation of autophagy via miRNA-21/PTEN/Akt/mTOR suppression. *PLoS One*, 2015; 10: e0117400
- McClelland AD, Herman-Edelstein M, Komers R et al: MiR-21 promotes renal fibrosis in diabetic nephropathy by targeting PTEN and SMAD7. *Clin Sci (Lond)*, 2015; 129(12): 1237–49
- Wu L, Wang Q, Guo F et al: MicroRNA-27a induces mesangial cell injury by targeting of PPARgamma, and its *in vivo* knockdown prevents progression of diabetic nephropathy. *Sci Rep*, 2016; 6: 26072
- Zhao B, Li H, Liu J et al: MicroRNA-23b targets ras GTPase-activating protein SH3 domain-binding protein 2 to alleviate fibrosis and albuminuria in diabetic nephropathy. *J Am Soc Nephrol*, 2016; 27(9): 2597–608
- Lorenzen JM, Haller H, Thum T: MicroRNAs as mediators and therapeutic targets in chronic kidney disease. *Nat Rev Nephrol*, 2011; 7(5): 286–94
- Putta S, Lanting L, Sun G et al: Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy. *J Am Soc Nephrol*, 2012; 23: 458–69
- Long J, Wang Y, Wang W et al: MicroRNA-29c is a signature microRNA under high glucose conditions that targets sprouty Homolog 1, and its *in vivo* knockdown prevents progression of diabetic nephropathy. *J Biol Chem*, 2011; 286(13): 11837–48
- Mogensen CE, Neldam S, Tikkanen I et al: Randomised controlled trial of dual blockade of renin-angiotensin system in patients with hypertension, microalbuminuria, and non-insulin dependent diabetes: The candesartan and lisinopril microalbuminuria (CALM) study. *BMJ*, 2000; 321(7274): 1440–44
- Schjoedt KJ, Jacobsen P, Rossing K et al: Dual blockade of the renin-angiotensin-aldosterone system in diabetic nephropathy: The role of aldosterone. *Horm Metab Res*, 2005; 37(Suppl. 1): 4–8
- Schjoedt KJ, Rossing K, Juhl TR et al: Beneficial impact of spironolactone in diabetic nephropathy. *Kidney Int*, 2005; 68(6): 2829–36
- Parving HH, Persson F, Lewis JB et al: Aliskiren combined with losartan in type 2 diabetes and nephropathy. *N Engl J Med*, 2008; 358(23): 2433–46
- Saal S, Harvey SJ: MicroRNAs and the kidney: Coming of age. *Curr Opin Nephrol Hypertens*, 2009; 18(4): 317–23
- Liu Y, Taylor NE, Lu L et al: Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. *Hypertension*, 2010; 55(4): 974–82
- Caporali A, Meloni M, Völlenkle C et al: Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. *Circulation*, 2011; 123(3): 282–91
- Wang B, Hsu SH, Majumder S et al: TGF beta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. *Oncogene*, 2010; 29(12): 1787–97
- Ouyang YB, Lu Y, Yue S et al: MiR-181 regulates GRP78 and influences outcome from cerebral ischemia *in vitro* and *in vivo*. *Neurobiol Dis*, 2012; 45(1): 555–63
- Caporali A, Meloni M, Nailor A et al: p75(NTR)-dependent activation of NF-kappaB regulates microRNA-503 transcription and pericyte-endothelial crosstalk in diabetes after limb ischaemia. *Nat Commun*, 2015; 6: 8024
- Cichocki F, Felices M, McCullar V et al: Cutting edge. MicroRNA-181 promotes human NK cell development by regulating Notch signaling. *J Immunol*, 2011; 187(12): 6171–75
- Ouyang YB, Lu Y, Yue S, Giffard RG: MiR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes. *Mitochondrion*, 2012; 12(2): 213–19
- Yuan Y, Kang R, Yu Y et al: Crosstalk between miRNAs and their regulated genes network in stroke. *Sci Rep*, 2016; 6: 20429
- Khalil S, Fabbri E, Santangelo A et al: miRNA array screening reveals cooperative MGMT-regulation between miR-181d-5p and miR-409-3p in glioblastoma. *Oncotarget*, 2016; 7(19): 28195–206
- Kreth S, Limbeck E, Hinske LC et al: In human glioblastomas transcript elongation by alternative polyadenylation and miRNA targeting is a potent mechanism of MGMT silencing. *Acta Neuropathol*, 2013; 125(5): 671–81
- Wang Y, Yu Y, Tsuyada A et al: Transforming growth factor- β regulates the sphere-initiating stem cell-like feature in breast cancer through miRNA-181 and ATM. *Oncogene*, 2011; 30(12): 1470–80
- Ji J, Yamashita T, Wang XW: Wnt/beta-catenin signaling activates microRNA-181 expression in hepatocellular carcinoma. *Cell Biosci*, 2011; 1(1): 4