European Review for Medical and Pharmacological Sciences 2019; 23: 10957-10963

MiR-133 inhibits kidney injury in rats with diabetic nephropathy via MAPK/ERK pathway

X. SHAO[1], W.-X. KONG[1], Y.-T. LI[2]

[1]Department of Nephrology, Suzhou Kowloon Hospital, Medical School of Shanghai Jiaotong University, Suzhou, China
[2]Department of Geriatrics, Suzhou Kowloon Hospital, Medical School of Shanghai Jiaotong University, Suzhou, China

Abstract. – OBJECTIVE: The aim of this study was to explore the effect of micro ribonucleic acid (miR)-133 on kidney injury in rats with diabetic nephropathy (DN) through the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway.

MATERIALS AND METHODS: The model of DN was first established in rats. Blood glucose, renal index, urinary micro-albumin (UMA), and creatinine clearance rate (CCr) were detected. Meanwhile, the protein expression levels of miR-133, kidney injury molecule-1 (KIM-1) and anti-inflammatory cytokine interleukin-8 (IL-8) were measured using Western blotting. Human renal proximal tubular epithelial cell line human kidney-2 (HK-2) was treated with high glucose to simulate DN cells *in vivo*. Subsequently, Western blotting was performed to detect the protein expression of KIM-1. After HK-2 cells were treated with high glucose and silenced miR-133 for 24 h, the expression changes in KIM-1 was evaluated.

RESULTS: In DN group, blood glucose, renal index, UMA, and CCr were all markedly higher than those of control group. This indicated the successful establishment of DN model in rats. The expression level of miR-133 was significantly up-regulated in DN model rats. Meanwhile, the downstream protein phosphorylated-EPK (p-EPK) showed a significantly increasing trend as well. Additionally, the protein expressions of KIM-1 and IL-8 were notably elevated. High-glucose-treated HK-2 cells showed significantly up-regulated expression levels of miR-133, KIM-1, and IL-8. After 24 h of combined treatment with high glucose and miR-133 silence, the expressions of KIM-1 and IL-8 were markedly down-regulated.

CONCLUSIONS: MiR-133 may be related to the occurrence and development of DN. The silence of miR-133 inhibits kidney injury in DN *via* the MAPK/ERK signaling pathway. Our findings suggest that miR-133 may be an effective target for the treatment of DN.

Key Words:
MiR-133, MAPK/ERK, Diabetic nephropathy (DN).

Introduction

Endogenous non-coding micro ribonucleic acids (miRNAs) are involved in numerous biological processes, including cell proliferation, apoptosis, and migration. Meanwhile, they play a crucial role in the growth and development of individuals[1]. Researches[2,3] have manifested that miRNAs can regulate epigenetic inheritance, stem cell self-renewal, and differentiation, as well as the occurrence and development of metabolic diseases. Diabetes mellitus is an extremely complicated metabolism disorder, whose incidence rate is increasing year by year. It seriously endangers the health and living quality of humans[4]. Diabetic nephropathy (DN) is a complication frequently occurring at the advanced stage. Currently, DN accounts for about 45% of all patients with diabetes, seriously threatening human health[5-7].

Xiang et al[8] have manifested that the expression of miR-133a, which modulates osteoblast differentiation, is significantly up-regulated in the femoral tissues of rats with diabetic osteoporosis. Meanwhile, the expression of Runx2/Osterix is up-regulated by inhibiting miR-133a, thereby improving bone microstructure and osteoporosis status. Kidney injury molecule-1 (KIM-1), a type 1 transmembrane protein, has been found significantly elevated in patients with kidney injury[9]. Therefore, it is of great guidance value to explore its role in renal function injury for early diagnosis and treatment.

In this study, KIM-1 was selected as the marker of kidney injury. The model of DN was successfully established in rats. Subsequently, we explored the effect of miR-133 on kidney injury in DN rats and preliminarily investigated its mechanism of action. Our findings might provide significant clinical guidance for early diagnosis and prevention of end-stage disease of DN.

Corresponding Author: Xiang Shao, BM; e-mail: 385534405@qq.com

Materials and Methods

A total of 42 healthy male Sprague Dawley (SD) rats weighing 160-180 g were provided by the Laboratory Animal Center of Medical School of Shanghai Jiaotong University. All rats were adaptively fed for 1 week before experiments. This research was approved by the Animal Ethics Committee of Medical School of Shanghai Jiaotong University Animal Center. Materials used in this study included: human renal proximal tubular epithelial cell line human kidney-2 (HK-2; Yanjing, Shanghai, China), miR-133, KIM-1, and interleukin-8 (IL-8) antibodies (Abcam, Cambridge, MA, USA), streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum (FBS), Opti-MEM, and Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), scale, cell incubator, Sorvall Evolution RC high-speed refrigerated centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Also, Roche blood glucose meter (Roche Diagnostics, Basel, Switzerland) and Beckman AU480 automatic biochemistry analyzer (Beckman, Miami, FL, USA).

Establishment of DN Model in Rats and Grouping

The modeling method was based on the literature of Hu et al[10]. After 1 week of adaptive feeding, 6 rats were randomly selected as control group and fed with a normal diet. Meanwhile, the remaining rats were fed with a high-fat and high-glucose diet for 4 weeks and fasted for 12 h. Subsequently, these rats were intraperitoneally injected with STZ, while those in control group were injected with the same dose of citrate buffer solution. Fasting blood glucose was extracted from the tail tip and detected 72 h later. Blood glucose value ≥16.7 mmol /L indicated successful the establishment of the DN model in rats. After successful modeling, the rats were continued to be observed and fed for 4 weeks. During this process, the following indicators were monitored, including blood glucose, urine volume, and weight. Once the indicators were stable, DN model rats were applied in the experiments.

Sampling and Detection

After fasting for 12 h, DN model rats were weighed and blood and urine samples were collected. Subsequently, the kidneys were taken *via* anatomy and weighed, followed by calculation of the renal index [kidney weight/body weight (KW/BW)]. Next, the kidneys were fixed in 10% formaldehyde solution, embedded in paraffin, stained by hematoxylin-eosin (HE), and sliced for observation. Additionally, changes in islet β cells were observed under an electron microscope. Blood samples were extracted from the tail tip and the content of blood glucose was determined using the blood glucose meter. Furthermore, the expressions of miR-133, KIM-1, and IL-8 were detected *via* Western blotting.

Establishment of Cell Model

Human kidney proximal tubular epithelial cell line HK-2 was cultured and treated with high glucose according to the methods of Chen et al[11]. Then, the cells were cultured for another 24 h. All cells were divided into high glucose (HG) group and normal glucose (NG) group, respectively. Finally, the cells in each group were collected for Western blotting.

Cell Transfection

An appropriate number of HK-2 cells in logarithmic growth phase were obtained in an aseptic operation room and inoculated into 6-well plates. When the conference of cells reached about 60%, they were transfected with scrambled small interfering RNA (siRNA) and simiR-133 at a concentration of 55 nmol/L according to the instructions of Lipofectamine 2000. 6.5 h after transfection, the fresh culture medium was replaced, followed by culture for 24 h. Next, the cells were collected for detection *via* Western blotting.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation. Independent-sample *t*-test was used to compare the difference between the two groups. Bonferroni's corrected *t*-test was adopted for the inter-group mean comparison. $p<0.05$ was considered statistically significant.

Results

Establishment of DN Model in Rats

Compared with control group, the rats in DN group exhibited significantly reduced weight, increased individual water intake and urine volume (Table I), and up-regulated blood glucose ($p<0.05$). Moreover, significantly elevated renal index, urinary micro-albumin (UMA), and creatinine clearance rate (CCr) ($p<0.05$) (Table II), as well as hypertrophy were observed in renal tissues of DN group (Figure 1).

Table I. Average individual weight, water intake and urine volume in DN rats.

Group	Average weight (g)	Average daily water intake (mL)	Average urine volume (mL)
Control	263.5±9.7	40.2±5.6	20.2±3.1
DN	201.4±7.8[a]	68.2±4.1[a]	32.4±4.1[a]

Note: [a]$p<0.05$, *vs.* control group.

Table II. Individual blood glucose, renal index, UMA and CCr in DN rats.

Group	Control	DN
Average blood glucose (mmol/L)	6.9±1.2	19.2±2.3[a]
Average KW/BW	0.014±0.01	0.017±0.02[a]
Average UMA (mg/L)	7.2±1.4	16.3±3.1[a]
Average CCr (μL/min/g)	4.8±0.8	14.1±1.42[a]

Note: [a]$p<0.05$, *vs.* control group.

Expression of MiR-133 in Renal Tissues of DN Model Rats

A total of 3 renal tissues were randomly selected from the rats of DN model group. Western blotting indicated that DN group exhibited significantly up-regulated miR-133 expression in comparison of control group ($p<0.05$). These results indicated that miR-133 might be associated with the occurrence of DN (Figures 2A and 2B)

Expressions of Phosphorylated Extracellular Signal-Regulated Kinase (p-ERK), KIM-1, and IL-8 in DN Model Rats

Western blotting results showed that compared with control group, the protein expressions of miR-133 and its downstream protein p-ERK were remarkably up-regulated in DN group ($p<0.05$). Meanwhile, the expressions of KIM-1 and IL-8 increased markedly as well ($p<0.05$). This suggested that kidney injury and related inflammatory responses occurred in rats of DN model group, which might be related to miR-133 (Figures 3A and 3B).

Expressions of p-ERK, KIM-1 and IL-8 in High-Glucose-Treated HK-2 Cells

Western blotting results revealed that the expression levels of miR-133 and its downstream protein p-ERK, KIM-1, and IL-8 were significantly up-regulated in HK-2 cells treated with high glucose ($p<0.05$). The secretion of inflammatory cytokine IL-8 in HK-2 cells increased significantly ($p<0.05$), further verifying our *in vivo* results (Figures 4A and 4B).

Establishment of HK-2 Cells with Silenced MiR-133

According to the results of Western blotting, HK-2 cells transfected with simiR-133 showed substantially decreased miR-133 expression when compared with those transfected with scrambled siRNA ($p<0.05$). This suggested that the cells were successfully established (Figures 5A and 5B).

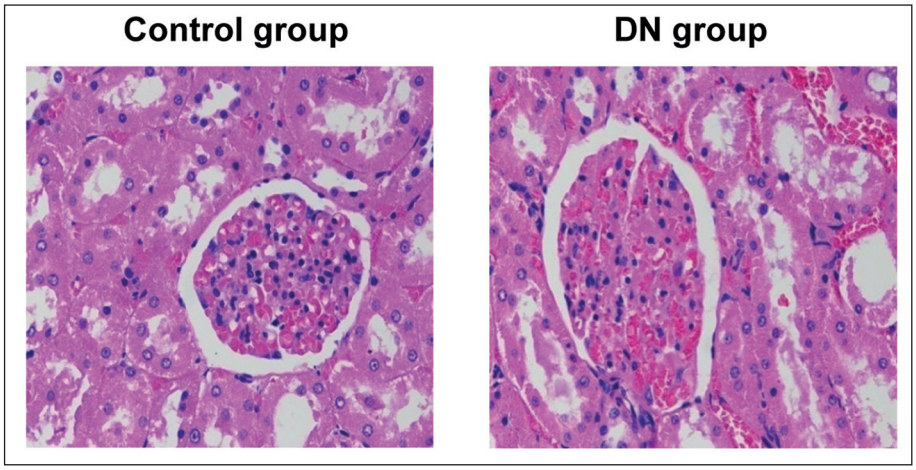

Figure 1. HE staining of renal tissues in DN rats (HE ×400).

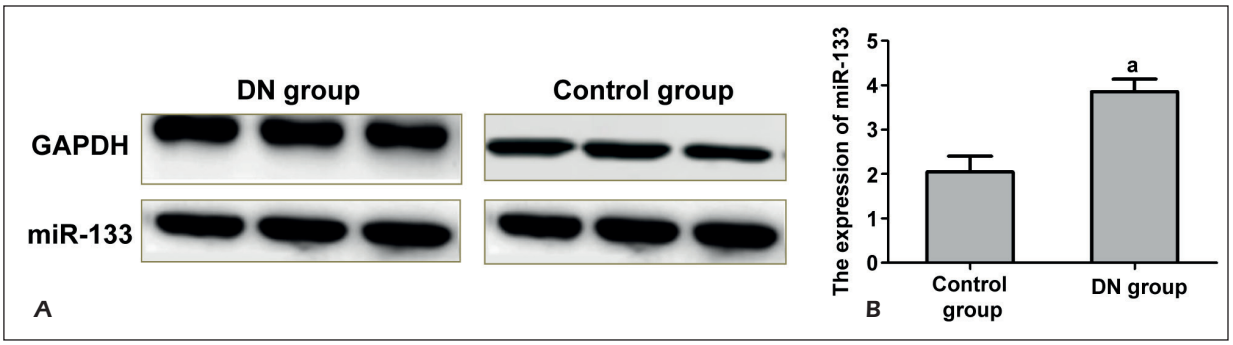

Figure 2. Expression of miR-133 in renal tissues of DN rats ($^{a}p<0.05$, *vs.* control group).

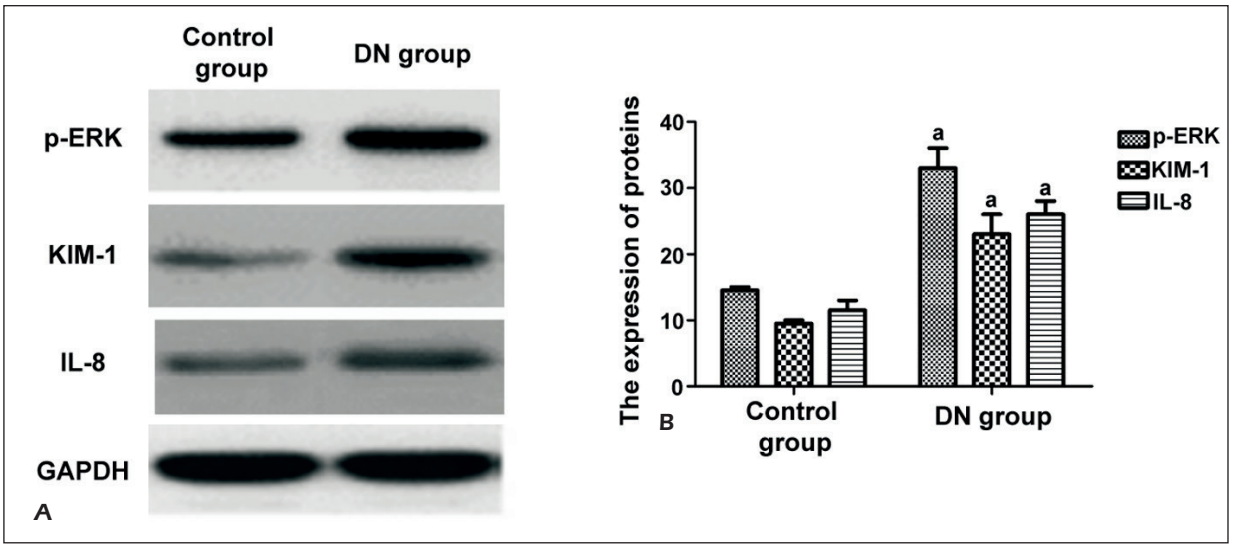

Figure 3. Levels of kidney inflammatory factors and KIM-1 proteins in DN rats ($^{a}p<0.05$, *vs.* control group).

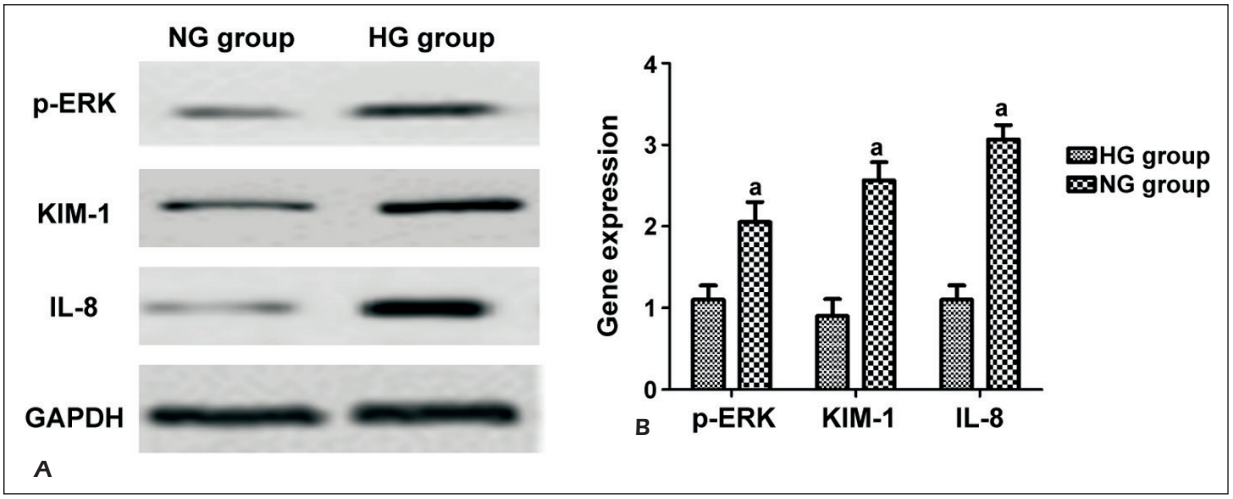

Figure 4. Expressions of p-ERK, KIM-1 and IL-8 in high-glucose-treated HK-2 cells ($^{a}p<0.05$, *vs.* NG group).

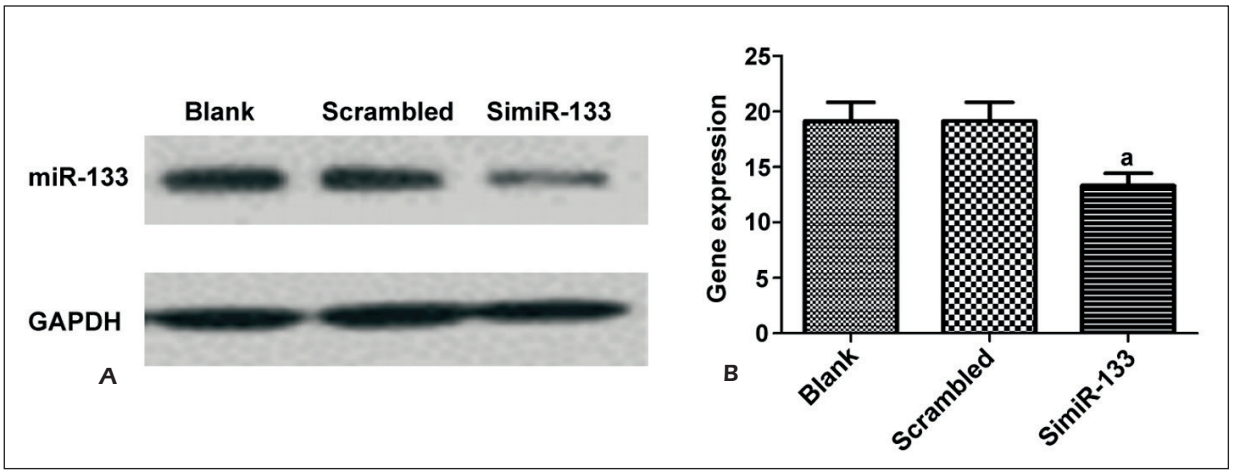

Figure 5. Silence of miR-133 in HK-2 cells ($^{a}p<0.05$, *vs.* miR-133 expression in cells transfected with scrambled siRNA).

Changes in the Levels of ERK Signaling Protein and IL-8 in HK-2 Cells Treated with High Glucose and Silenced MiR-133

HK-2 cells treated with silenced miR-133 and ERK inhibitors were cultured using high glucose. The results of Western blotting revealed that the levels of miR-133 downstream proteins of p-ERK, KIM-1, and IL-8 were significantly down-regulated ($p<0.05$). However, no significant changes were observed in the protein level of total ERK. This suggested that the expression of silenced miR-133 could inhibit the secretion of cytokines by renal tubular epithelial cells and damaging capacity of cytokines *via* blocking ERK signaling (Figures 6A and 6B).

Discussion

The miR-133 family mainly consists of three members, namely, miR-133a-1, miR-133a-2, and miR-133b[12]. MiR-133a-1 and miR-133a-2 have the same sequences, while the sequence of miR-133b differs slightly. The miR-133 family has been found closely correlated with the occurrence, differentiation, proliferation, and migration of tumors[13-15]. Scholars cloned and identified miR-133 genes from human and mouse musculature in 2003. Their expressions exhibited time-space specificity. The expression level of miR-133 is relatively low in the myocardium and skeletal muscle in the embryonic phase. It gradually increases after birth and reaches a peak in adulthood. How-

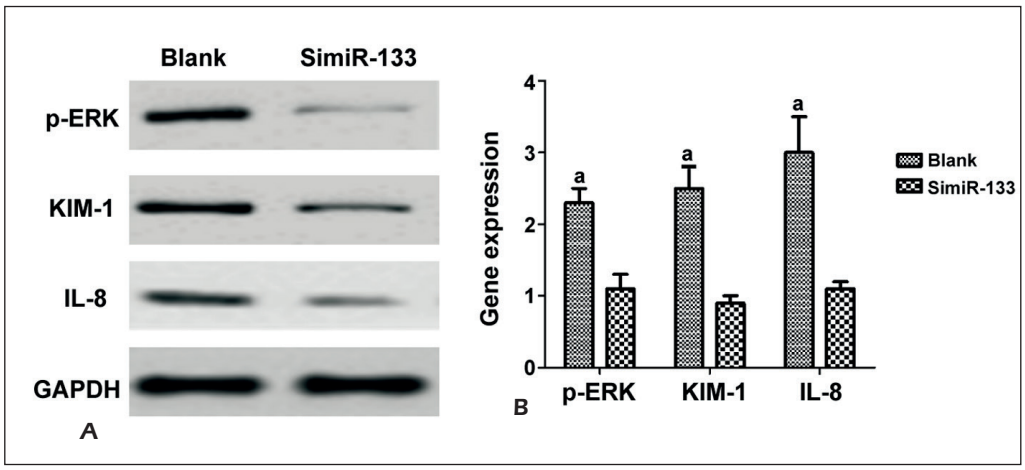

Figure 6. Expressions of p-ERK, KIM-1 and IL-8 in cells with miR-133 silence ($^{a}p<0.05$, *vs.* simiR-133 group, with significant differences).

ever, it suppresses cell differentiation to promote the proliferation of myocytes[16,17]. MiR-133 participates in the atrial fibrosis process. Transfection of atrial fibroblasts with miR-133 can reduce the expression of TGF-1 and the synthesis of extracellular matrices[18]. Moreover, miR-133 modulates the synthesis of type I collagen through regulating COLA1 expression, which is the target gene of miR-133, thereby taking part in atrial fibrosis[19]. MiR-133b can repress epithelial-mesenchymal transition (EMT) and metastasis of colorectal cancer cells through the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathways. Furthermore, it can regulate malignant behaviors of tumor cells *via* the PI3K/AKT signaling pathway as well. Additionally, miR-133b plays a regulatory role in colorectal cancer. It affects the proliferation and apoptosis of tumor cells by regulating the MET receptor tyrosine kinase[19,20].

In recent years, studies[20-24] have found that miR-133b mediates the occurrence and development of DN by regulating the renal interstitial fibrosis, renal tubular EMT, and related processes. The detection of miRNA is of great significance for early diagnosis, development degree, efficacy judgment, prognosis evaluation, and specific drug design of DN. Meanwhile, miRNA-targeting drugs to be developed are expected to be applied to DN treatment in the near future. Bhatt et al[25] have discovered that the expression levels of miRNAs related to renal tubulointerstitial injury are significantly up-regulated. Coincidentally, researches have suggested that renal tubulointerstitial injury is correlated with the poor prognosis of DN patients to a certain degree. Eissa et al[26] have indicated that the expressions of miR-30a, miR-133b, and miR-342 in the urine of DN patients were evidently higher than those of controls. Similarly, Huang et al[13] have found that in DN patients' serum and renal tissues and diabetic mice, miR-135a is highly expressed. Meanwhile, it prevents Ca^{2+} from entering cells through the inhibition of the transient receptor potential cation channel C_1. This may deposit fibronectin and collagen, eventually promoting extracellular matrix deposition.

In the present work, the following experimental results were obtained, including the establishment of DN model in rats, high-glucose treatment of human renal tubular epithelial cell line HK-2 for simulating DN cells *in vivo*, 24 h of treatment with high glucose and silenced miR-133, and measurement of changes in the expression of KIM-1. Results demonstrated that blood glucose, renal index, UAM, and CCr were significantly higher in DN rats than controls. This indicated the successful establishment of DN model in rats. The expression levels of miR-133 and its downstream protein p-EPK were significantly up-regulated in DN model rats. Meanwhile, the expressions of KIM-1 and IL-8 were notably elevated as well. High-glucose-treated HK-2 cells exhibited remarkably increased expression levels of miR-133, KIM-1, and IL-8. After 24 h of combined treatment with high glucose and silenced miR-133, the expressions of KIM-1 and IL-8 were markedly down-regulated in HK-2 cells.

Conclusions

MiR-133 may be related to the occurrence and development of DN. The silence of miR-133 inhibits kidney injury in DN *via* the MAPK/ERK signaling pathway. Furthermore, miR-133 may be an effective target for the treatment of DN.

Conflict of Interests

The Authors declared that they have no conflict of interests.

References

1) Zhang D, Li X, Chen C, Li Y, Zhao L, Jing Y, Liu W, Wang X, Zhang Y, Xia H, Chang Y, Gao X, Yan J, Ying H. Attenuation of p38-mediated miR-1/133 expression facilitates myoblast proliferation during the early stage of muscle regeneration. PLoS One 2012; 7: e41478.
2) Jiang YR, Du JY, Wang DD, Yang X. MiRNA-130a improves cardiac function by down-regulating TNF-alpha expression in a rat model of heart failure. Eur Rev Med Pharmacol Sci 2018; 22: 8454-8461.
3) Sun C, Li J. Expression of miRNA-137 in oral squamous cell carcinoma and its clinical significance. J BUON 2018; 23: 167-172.
4) Hwang S, Park J, Kim J, Jang HR, Kwon GY, Huh W, Kim YG, Kim DJ, Oh HY, Lee JE. Tissue expression of tubular injury markers is associated with renal function decline in diabetic nephropathy. J Diabetes Complications 2017; 31: 1704-1709.
5) Lim AKH. Diabetic nephropathy - complications and treatment. Int J Nephrol Renovasc Dis 2014; 7: 361-381.
6) Ni WJ, Tang LQ, Wei W. Research progress in signalling pathway in diabetic nephropathy. Diabetes Metab Res Rev 2015; 31: 221-233.
7) Flyvbjerg A. The role of the complement system in diabetic nephropathy. Nat Rev Nephrol 2017; 13: 311-318.

8) Xiang KM, Li XR. MiR-133b acts as a tumor suppressor and negatively regulates TBPL1 in colorec-tal cancer cells. Asian Pac J Cancer Prev 2014; 15: 3767-3772.
9) Chen XN, Wang KF, Xu ZQ, Li SJ, Liu Q, Fu DH, Wang X, Wu B. MiR-133b regulates bladder cancer cell proliferation and apoptosis by targeting Bcl-w and Akt1. Cancer Cell Int 2014; 14: 70.
10) Hu G, Chen D, Li X, Yang K, Wang H, Wu W. MiR-133b regulates the MET proto-oncogene and inhibits the growth of colorectal cancer cells in vitro and in vivo. Cancer Biol Ther 2010; 10: 190-197.
11) Chen G, Peng J, Zhu W, Tao G, Song Y, Zhou X, Wang W. Combined downregulation of mi-croRNA-133a and microRNA-133b predicts chemosensitivity of patients with esophageal squamous cell carcinoma undergoing paclitaxel-based chemotherapy. Med Oncol 2014; 31: 263.
12) Yu H, Lu Y, Li Z, Wang Q. MicroRNA-133: expression, function and therapeutic potential in mus-cle diseases and cancer. Curr Drug Targets 2014; 15: 817-828.
13) Huang TH, Zhu MJ, Li XY, Zhao SH. Discovery of porcine microRNAs and profiling from skeletal muscle tissues during development. PLoS One 2008; 3: e3225.
14) Moridikia A, Mirzaei H, Sahebkar A, Salimian J. MicroRNAs: potential candidates for diagnosis and treatment of colorectal cancer. J Cell Physiol 2018; 233: 901-913.
15) Globinska A, Pawelczyk M, Kowalski ML. MicroRNAs and the immune response to respiratory vi-rus infections. Expert Rev Clin Immunol 2014; 10: 963-971.
16) Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 2006; 38: 228-233.
17) Torella D, Iaconetti C, Catalucci D, Ellison GM, Leone A, Waring CD, Bochicchio A, Vicinanza C, Aquila I, Curcio A, Condorelli G, Indolfi C. MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo. Circ Res 2011; 109: 880-893.
18) Duan LJ, Qi J, Kong XJ, Huang T, Qian XQ, Xu D, Liang JH, Kang J. MiR-133 modulates TGF-beta1-induced bladder smooth muscle cell hypertrophic and fibrotic response: implication for a role of microRNA in bladder wall remodeling caused by bladder outlet obstruction. Cell Signal 2015; 27: 215-227.
19) Castoldi G, Di Gioia CR, Bombardi C, Catalucci D, Corradi B, Gualazzi MG, Leopizzi M, Mancini M, Zerbini G, Condorelli G, Stella A. MiR-133a regulates collagen 1A1: potential role of miR-133a in myocardial fibrosis in angiotensin II-dependent hypertension. J Cell Physiol 2012; 227: 850-856.
20) Zhou W, Bi X, Gao G, Sun L. MiRNA-133b and miRNA-135a induce apoptosis via the JAK2/STAT3 signaling pathway in human renal carcinoma cells. Biomed Pharmacother 2016; 84: 722-729.
21) Al-Kafaji G, Al-Mahroos G, Al-Muhtaresh HA, Skrypnyk C, Sabry MA, Ramadan AR. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: a potential blood-based biomarker. Exp Ther Med 2016; 12: 815-822.
22) Kantharidis P, Hagiwara S, Brennan E, McClelland AD. Study of microRNA in diabetic nephropa-thy: isolation, quantification and biological function. Nephrology (Carlton) 2015; 20: 132-139.
23) Rossi G. Diagnosis and classification of diabetes mellitus. Recenti Prog Med 2010; 101: 274-276.
24) Gupta V, Reiser J. MicroRNAs: a macroview into focal segmental glomerulosclerosis. Am J Kidney Dis 2015; 65: 206-208.
25) Bhatt K, Kato M, Natarajan R. Mini-review: emerging roles of microRNAs in the pathophysiology of renal diseases. Am J Physiol Renal Physiol 2016; 310: F109-F118.
26) Eissa S, Matboli M, Bekhet MM. Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis. Biomed Pharmacother 2016; 83: 92-99.