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Effects of miR-195 on diabetic nephropathy rats through targeting TLR4 and blocking NF-κB pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of micro ribonucleic acid (miR)-195 on diabetic nephropathy (DN) rats through targeting Toll-like receptor 4 (TLR4) and inhibiting the nuclear factor-κB (NF-κB) signaling pathway.

MATERIALS AND METHODS: The model of DN was first successfully established in rats. All rats were randomly divided into six groups, including control group (n=20), model group (n=20), 25 nM miR-195 mimics group (25 nM M group, n=20), 50 nM M group (n=20), 25 nM miR-195 inhibitor group (25 nM I group, n=20), and 50 nM I group (n=20). Urine volume, proteins and inflammatory factors were detected in each group, respectively. Subsequently, macrophages were cultured and transfected in vitro. The mRNA expressions of miR-195 and TLR4 in control group and model groups were determined using fluorescence quantitative polymerase chain reaction (qPCR). The protein expressions of TLR4 and NF-κB in macrophages were determined using Western blotting. Furthermore, the proliferation of macrophages was detected via cell counting kit-8 (CCK-8) assay.

RESULTS: Compared with model group, 24-h urine volume, urine protein, creatinine, urea nitrogen, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 levels declined significantly in 25 nM M group and 50 nM M group ($p<0.05$). However, they increased significantly in 25 nM I group and 50 nM I group ($p<0.05$). It could be suggested that miR-195 mimics might relieve the symptoms of DN rats. In kidney tissues in DN, miR-195 was lowly expressed, whereas TLR4 was highly expressed ($p<0.01$). This suggested that there was a negative correlation between the mRNA expressions of miR-195 and TLR4 ($r^2=0.4836$, $p=0.0007$). After overexpression of miR-195, the protein expression of TLR4 was significantly reduced ($p<0.01$), indicating that miR-195 could negatively regulate the protein expression of TLR4. Besides, the protein expressions of TLR4 and NF-κB in si-TLR4 group were evidently lower than those in NC group ($p<0.01$). Meanwhile, they also had significant differences in si-TLR4 group compared with si-TLR4 + miR-195 inhibitor group ($p<0.05$). The above results demonstrated that the protein expressions of TLR4 and NF-κB in macrophages could be markedly inhibited by si-TLR4, but be promoted by si-TLR4 + miR-195 inhibitor. CCK-8 assay demonstrated that the proliferation ability of macrophages was remarkably weaker in miR-195 mimics group than NC group ($p<0.001$). Furthermore, it was also significantly weaker in si-TLR4 + miR-195 inhibitor group than si-TLR4 group ($p<0.05$).

CONCLUSIONS: MiR-195 reduces the release of inflammatory factors and inhibits the proliferation of macrophages through targeting TLR4 and blocking the NF-κB pathway, thereby alleviating the symptoms of DN rats.

Key Words:
NF-κB pathway, MiR-195, Diabetic nephropathy (DN), Cell proliferation, TLR4.

Introduction

Diabetic nephropathy (DN) is a microvascular complication of diabetes, which is considered as the major cause of cardiovascular and chronic kidney diseases[1]. DN occurs in about 40% of diabetes mellitus (DM) patients, affecting 9% of adults around the world[2,3]. Currently, it has become one of the major threats to global public health. It is estimated that DM will be the seventh leading cause of death in the world by 2030. The life quality of patients is affected by DN and its complications, including cardiovascular diseases, retinopathy and diabetic neuropathy[4-7]. DN affects more than 40% of type 1 DM (T1DM) and T2DM[8]. In recent years, the treatment means for DN mainly include reducing blood lipids, blood glucose and blood pressure, and controlling the renin-angiotensin-aldosterone system[9]. However,

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the efficiency is far from satisfactory. Therefore, it is urgently needed to explore new therapeutic methods for DN.

Inflammation is considered as an important initiating factor in the pathophysiological process of DN. There is evidence that some cytokines and chemokines, including nuclear factor-κB (NF-κB), interleukin (IL), tumor necrosis factor (TNF) and transforming growth factor (TGF), play important roles in inflammation[10,11]. In the kidney of DN, macrophages can secrete and produce inflammatory factors (TNF-α, IL-1β and IL-6). Meanwhile, overexpression of these inflammatory factors in macrophages may aggravate the damage of kidney tissues[12,13]. Fibrosis-related factors matrix metalloproteinases (MMPs) and collagen also serve as important factors for DN. Therefore, reducing the release of inflammatory factors is the best treatment for DN.

Micro ribonucleic acid (miR)-195, a tumor suppressor gene, is lowly expressed in not only tissues and cell lines in colon cancer and breast cancer, but also in kidney tissues and cell lines in DN[14-16]. The Toll-like receptor 4 (TLR4)/NF-κB signaling pathway is an important pathway to regulate the expressions of inflammatory factors (such as TNF-α, IL-1β and IL-6) in cells. After activation, the TLR4-MD2-CD14 complex controls the MyD88 signaling pathway to activate a series of cascade reactions. Meanwhile, it can also activate the IκB kinase complex to control the phosphorylation of NF-κB p65 subunit[17]. This may eventually reduce the release of inflammatory factors. Currently, the effect of miR-195 on DN rats through targeting TLR4 and blocking the NF-κB pathway has not been fully elucidated. In this paper, therefore, the model of DN was established in rats to explore whether miR-195 targeted TLR4 and inhibited the NF-κB signaling pathway in DN.

Materials and Methods

Materials

Male SD rats weighing 200-220 g were obtained from the Liaocheng People's Hospital. This investigation was approved by the Animal Ethics Committee of Liaocheng People's Hospital Animal Center.

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA), mirVana miRNA Isolation kit and mirVana reverse transcription-quantitative polymerase chain reaction (RT-qPCR) miRNA Detection kit from Ambion (Waltham, MA, USA), miR-195 mimic, miR-95 inhibitor and miRNA control from Guangzhou Ribobio Co., Ltd. (Guangzhou, China), Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Rockville, MD, USA), fetal bovine serum (FBS) from Gibco (Rockville, MD, USA), penicillin-streptomycin solution from Ambion (Waltham, MA, USA), Western blotting goat anti-rabbit primary antibodies and horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG secondary antibodies from Abcam (Cambridge, MA, USA), propidium iodide (PI) and Annexin from ABM (Richmond, Canada), and enzyme-linked immunosorbent assay (ELISA) kits of TNF-α, IL-1β and IL-6 from Abcam (Cambridge, MA, USA).

Establishment of DN Rat Model and Grouping

After 120 rats were deprived of food and water for 12 h, STZ was injected (120 μg/g) into some rats *via* the caudal vein. After 72 h, fasting blood glucose was detected. Blood glucose level ≥11.1 mmol/L indicated successful modeling. All rats were randomly divided into six groups, including blank control group (normal rats intraperitoneally injected with normal saline, n=20), model group (DN rats intraperitoneally injected with normal saline, n=20), 25 nM miR-195 mimics group (25 nM M group, DN rats intraperitoneally injected with 25 nM miR-195 mimics, n=20), 50 nM M group (DN rats intraperitoneally injected with 50 nM miR-195 mimics, n=20), 25 nM miR-195 inhibitor group (25 nM I group, DN rats intraperitoneally injected with 25 nM miR-195 inhibitor, n=20), and 50 nM I group (n=20). Rats in each group were fed with an equal amount of high-fat diets every day.

Detection of Urine Volume, Proteins and Inflammatory Factors in Kidney Tissues in Each Group

At 4 weeks, 24 h-urine was fist collected. Urine volume and protein content were determined using a measuring cylinder and end-point method, respectively. Meanwhile, the levels of inflammatory factors (TNF-α, IL-1β and IL-6) in kidney tissues were detected according to the instructions of ELISA kits in each group.

Culture and Transfection of Macrophages

After diabetic rats were sacrificed, kidney tissues were taken, and macrophages were ex-

tracted. Macrophages were cultured in DMEM containing 11.0% FBS and 1.0% penicillin-streptomycin in an incubator with 5% CO_2 at 37°C. Cell passage was performed upon reaching 70-80% of confluence. According to the instructions of the TurboFect transfection reagent, macrophages were transfected with miR-195 mimic/inhibitor and TLR4-knockdown plasmids to up- or down-regulate miR-195 expression and down-regulate TLR4 protein expression, respectively. All macrophages were divided into four groups, including negative control group (NC group), miR-195 group (miR-195 mimic added), si-TLR4 group (TLR4 knockdown), and si-TLR4 + miR-195 inhibitor group (miR-195 inhibitor added and TLR4 knockdown).

TaqMan qRT-PCR

After urine and inflammatory factors were detected at 4 weeks, kidney tissues were collected in control group and model group. Total RNA in kidney tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantitatively analyzed. (1) The qRT-PCR system included forward primers, reverse primers, TaqMan probe primers, complementary deoxyribose nucleic acid (cDNA) template and ddH_2O. Primer sequences used in the study were shown in Table I. (2) The reaction conditions included pre-denaturation, denaturation, annealing, extension and full extension. (3) Experimental data were analyzed and standardized with β-actin. The relationship between the mRNA expressions of miR-195 and TLR4 in DN kidney tissues and normal kidney tissues was plotted into a scatter diagram and analyzed.

Determination of Protein Expressions of TLR4 and NF-κB Using Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was first prepared, and protein concentration in each group was detected using the bicinchoninic acid (BCA) method. Protein samples were added with 1/4 of 5×loading buffer, stirred evenly and heated at 100°C for 5 min, followed by electrophoresis and membrane transfer assay. Subsequently, the membranes were incubated with primary antibodies at an appropriate concentration for 12-24 h and added with 1× phosphate buffered saline with Tween-20 (PBST) on a shaker. After washing for 3 times, the membranes were incubated again with corresponding secondary antibodies on the shaker. Immunoreactive bands were finally exposed by enhanced chemiluminescence (ECL) apparatus.

Table I. TaqMan primer sequences.

Primer	Sequence
β-actin	F: 5'-GCAAGTTCAACGGCACAG-3'
	R: 5'-GCCAGTAGACTCCACGACAT-3'
miR-195	F: 5'-GATAGCAGCACAGAAATATTGGC-3'
	R: 5'-CAGTGCGTGTCGTGGAGT-3'
TLR4	F: 5'-ACCTCCCCTTCTCAACCAAG-3'
	R: 5'-GGCTCTGATATGCCCCATCT-3'

Detection of Proliferation of Macrophages Using Cell Counting Kit-8 (CCK-8) Assay

Macrophages in each group were first diluted into cell suspension at a density of 1×10^6 cells/mL. Subsequently, 10% CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added, followed by incubation in an incubator with 5% CO_2 at 37°C for 4 h. Optical density at 490 nm was detected by a micro-plate reader. Cell proliferation multiple was calculated, and cell growth curves were finally plotted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. All experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.01$ was considered statistically significant.

Results

Effects of MiR-195 on 24 h Urine Volume, Urine Protein, Creatinine and Urea Nitrogen In DN Rats

As shown in Table II, 24 h-urine volume, urine protein, creatinine and urea nitrogen content increased significantly in model group compared with control group ($p<0.01$). Compared with model group, 24 h-urine volume, urine protein, creatinine and urea nitrogen content declined sig-

Table II. Effects of miR-195 on 24 h urine volume, urine protein, creatinine and urea nitrogen in DN rats (n=20, $\bar{x} \pm s$).

Group	Dose (nM)	24 h urine volume (mL)	Urine protein (mg/d)	Creatinine (mg/dL)	Urea nitrogen (mg/dL)
Control	/	0.61 ± 0.02	2.01 ± 0.28	0.22 ± 0.07	15.36 ± 1.20
Model	/	3.15 ± 0.28^{x}	9.42 ± 1.06^{x}	0.72 ± 0.13^{x}	38.78 ± 4.52^{x}
M	25	2.16 ± 0.19^{xy}	4.20 ± 0.75^{xy}	0.45 ± 0.09^{xy}	28.31± 2.51^{xy}
M	50	1.36 ± 0.21^{xyz}	2.78 ± 0.35^{xyz}	0.34 ± 0.08^{xyz}	20.23 ± 2.21^{xyz}
I	25	3.32 ± 0.32^{xyz}	10.35 ± 1.54^{xyz}	0.82 ± 0.21^{xyz}	39.45 ± 5.12^{xyz}
I	50	3.98 ± 0.52^{xyz}	14.37 ± 2.18^{xyz}	1.02 ± 0.28^{xyz}	46.25 ± 4.56^{xyz}

Note: $^{x}p<0.01$ *vs*. control group, $^{y}p<0.05$ *vs*. model group, $^{z}p<0.05$ *vs*. 25 nM M group.

Table III. Effects of miR-195 on inflammatory factors in kidney tissues in DN rats (n = 20, $\bar{x} \pm s$).

Group	Dose (nM)	TNF-α (ng/L)	IL-1β (ng/L)	IL-6 (ng/L)
Control	/	69.51 ± 7.98	7.12 ± 1.08	11.38 ± 1.27
Model	/	137.25 ± 8.79^{x}	19.75 ± 2.06^{x}	60.15 ± 7.13^{x}
M	25	100.12 ± 7.56^{xy}	11.03 ± 1.75^{xy}	32.12 ± 3.09^{xy}
M	50	82.13 ± 7.21^{xyz}	8.78 ± 1.35^{xyz}	18.19 ± 1.78^{xyz}
I	25	145.25 ± 8.32^{xyz}	22.35 ± 2.34^{xyz}	70.13 ± 7.61^{xyz}
I	50	158.08 ± 8.92^{xyz}	26.32 ± 2.87^{xyz}	80.15 ± 9.16^{xyz}

Note: $^{x}p<0.01$ *vs*. control group, $^{y}p<0.05$ *vs*. model group, $^{z}p<0.05$ *vs*. 25 nM M group.

nificantly in 25 nM M group and 50 nM M group ($p<0.05$). However, they increased remarkably in 25 nM I group and 50 nM I group ($p<0.05$). It could be seen that miR-195 mimics could relieve the symptoms of DN rats.

Effects of MiR-195 on Inflammatory Factors In Kidney Tissues In DN Rats

As shown in Table III, the content of TNF-α, IL-1β and IL-6 increased remarkably in model group compared with control group ($p<0.01$). Compared with model group, the content of TNF-α, IL-1β and IL-6 significantly declined in 25 nM M group and 50 nM M group ($p<0.05$). However, it rose obviously in 25 nM I group and 50 nM I group ($p<0.05$). These results suggested that miR-195 mimics could reduce the content of inflammatory factors (TNF-α, IL-1β and IL-6) in DN rats.

MiR-195 Was Lowly Expressed In DN Kidney Tissues

The expression of miR-195 in normal kidney tissues (1.02±0.13) was significantly higher than that in DN kidney tissues (0.43±0.09) ($p<0.01$) (Figure 1).

TLR4 Was Highly Expressed In DN Kidney Tissues

The expression of TLR4 in normal kidney tissues (1.04±0.15) was significantly lower than that in DN kidney tissues (1.59±0.12) ($p<0.01$) (Figure 2).

There Was a Negative Correlation Between Expressions of MiR-195 and TLR4 In DN Kidney Tissues

Statistical analysis indicated that there was a negative correlation between the mRNA expres-

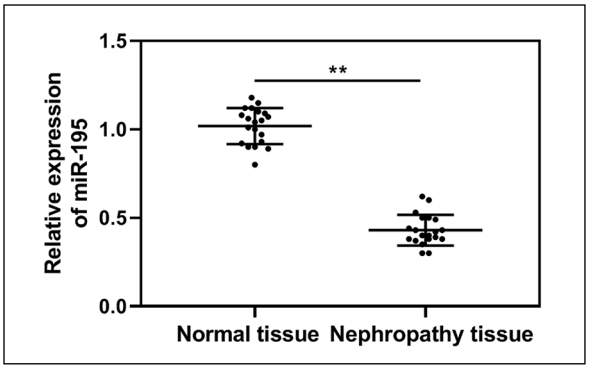

Figure 1. Expression of miR-195 in kidney tissues. The expression of miR-195 was markedly higher in normal tissues than that in DN tissues ($p<0.01$).

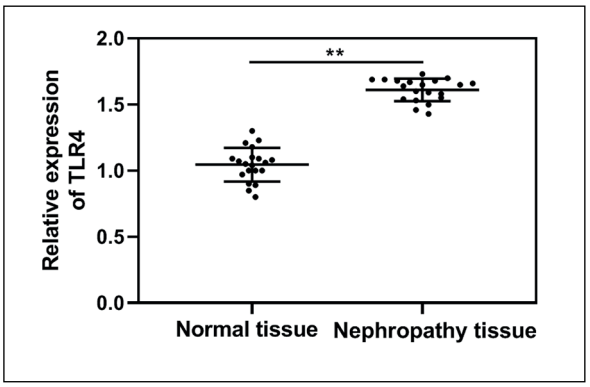

Figure 2. Expression of TLR4 in kidney tissues. The expression of TLR4 was markedly lower in normal tissues than that in DN tissues (p<0.01).

sions of miR-195 and TLR4 in DN kidney tissues (r^2=0.4836, p=0.0007) (Figure 3). The results suggested that miR-195 might negatively regulate the expression of TLR4.

Protein Expressions in Cells After Overexpression of MiR-195 Detected Using Western Blotting

The results of Western blotting showed that after overexpression of miR-195, the protein expression of TLR4 was significantly reduced (p<0.01) (Figure 4). This indicated that miR-195 could negatively regulate the protein expression of TLR4.

Protein Expressions In Cells After Knockdown of TLR4 and MiR-195 Detected Using Western Blotting

The results of Western blotting manifested that the protein expressions of TLR4 and NF-κB

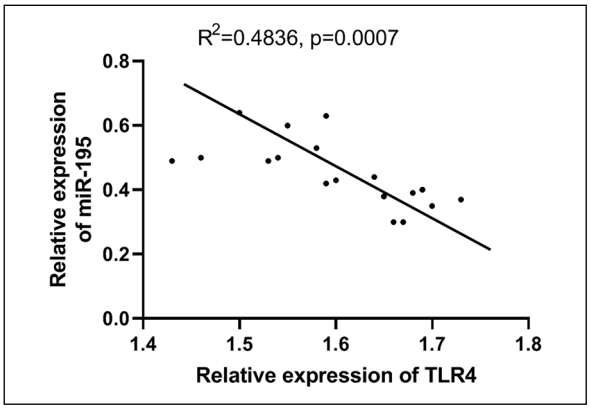

Figure 3. There was a significant negative correlation between mRNA expressions of miR-195 (low) and TLR4 (high) in DN kidney tissues.

were remarkably lower in si-TLR4 group than those in NC group (p<0.01). Meanwhile, they had evident differences in si-TLR4 group compared with those in si-TLR4 + miR-195 inhibitor group (p<0.05) (Figures 5). The above results demonstrated that the protein expressions of TLR4 and NF-κB in macrophages could be markedly inhibited by knockdown of TLR4, but could be promoted by knockdown of TLR4 and miR-195.

Knockdown of TLR4 In Macrophages Could Remarkably Enhance Cell Proliferation

TLR4-knockdown macrophages were constructed *via* lentiviral transfection, and cell proliferation ability was detected using MTT assay. The results revealed that the proliferation ability of macrophages was significantly weaker in miR-195 mimics group than NC group (p<0.001). Meanwhile, it was also weaker in si-TLR4 + miR-195 inhibitor group than that in si-TLR4 group (p<0.05) (Figure 6). The above findings demonstrated that miR-195 could inhibit the proliferation and promote the apoptosis of macrophages through targeting TLR4 and blocking the NF-κB

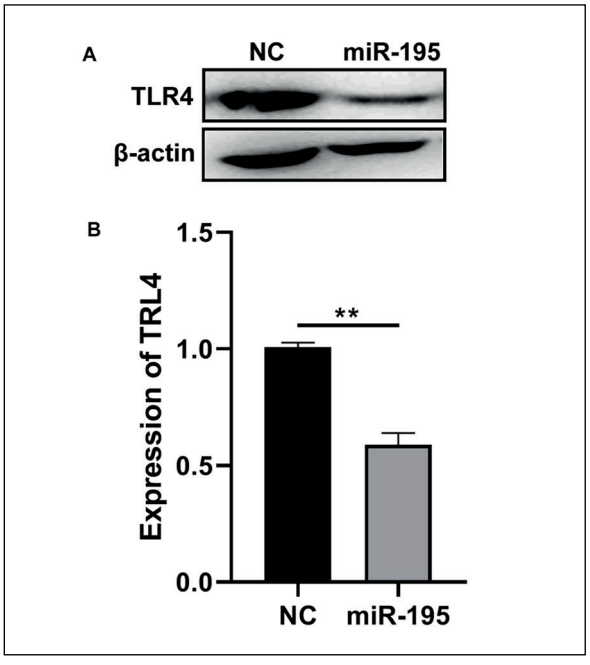

Figure 4. Effect of miR-195 mimics on TLR4 protein expression in macrophages. **A,** TLR4 protein expression detected using Western blotting. **B,** TLR4 protein expression in each group. The protein expression of TLR4 declined significantly in miR-195 group compared with that in NC group (p<0.01).

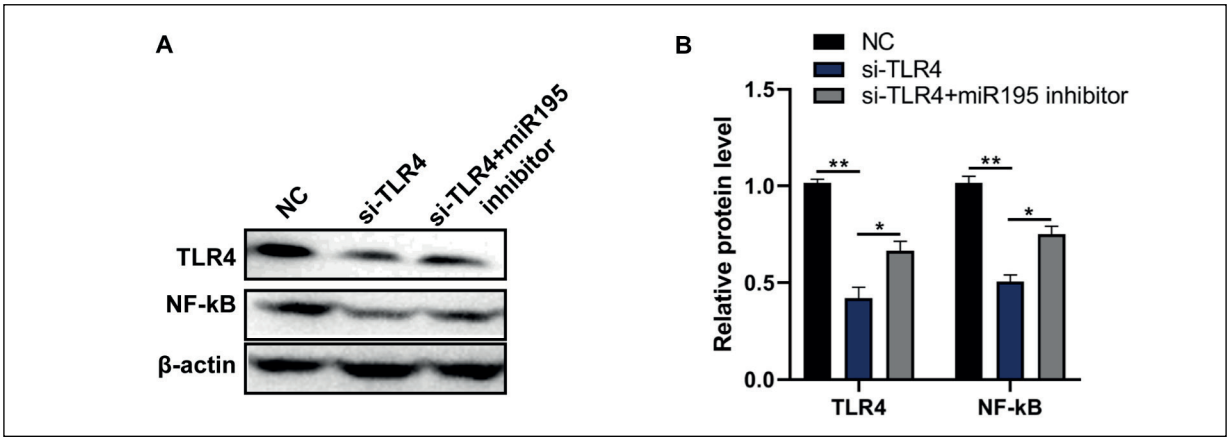

Figure 5. Effects of knockdown of TLR4 and miR-195 on protein expressions. **A,** TLR4 and NF-κB protein expressions detected using Western blotting. **B,** TLR4 and NF-κB protein expressions in each group. The protein expressions of TLR4 and NF-κB were evidently lower in si-TLR4 group than those in NC group ($p<0.01$). Meanwhile, they also had evident differences in si-TLR4 group compared with those in si-TLR4 + miR-195 inhibitor group ($p<0.05$).

pathway. Moreover, miR-195 reduced the release of inflammatory factors, thereby alleviating the symptoms of DN rats.

Discussion

DN is considered as a microvascular complication of diabetes, which is also the major cause of cardiovascular and chronic kidney diseases[1]. Inflammation plays an important role in the pathophysiological process of DN. In the kidney of DN, macrophages can secrete and produce inflammatory factors (TNF-α, IL-1β and IL-6). Meanwhile, overexpression of these inflammatory factors in macrophages can aggravate the damage of kidney tissues[12,13]. MiR-195, a tumor suppressor gene, is lowly expressed not only in tissues and cell lines in colon cancer and breast cancer, but also in kidney tissues and cell lines in DN[14-16]. Wang et al[18] have found that miR-195 promotes the apoptosis of oral squamous cell carcinoma cells through targeting the TRIM14 signaling pathway. The expressions of inflammatory factors (TNF-α, IL-1β and IL-6) are regulated by the TLR4/NF-κB pathway. After activation, the TLR4-MD2-CD14 complex controls the MyD88 signaling pathway to activate a series of cascade reactions. Meanwhile, it can also activate the IκB kinase complex to control the phosphorylation of NF-κB p65 subunit[16,17], thus reducing the release of inflammatory factors.

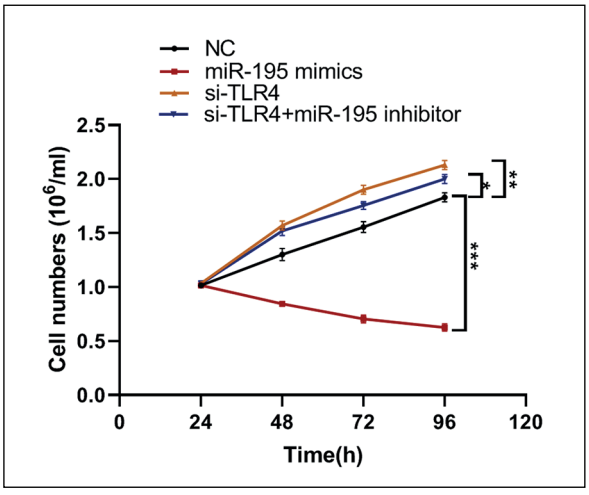

Figure 6. Knockdown of TLR4 in macrophages enhanced cell proliferation. The proliferation ability of macrophages was remarkably weaker in miR-195 mimics group than that in NC group ($p<0.001$), while it was also weaker in si-TLR4 + miR-195 inhibitor group than that in si-TLR4 group ($p<0.05$).

In the present research, the rat model of DN was successfully established to explore whether miR-195 targeted TLR4 and inhibited the NF-κB signaling pathway, as well as its effect on DN rats. 24 h-urine volume, urine protein, creatinine and urea nitrogen content, and the levels of TNF-α, IL-1β and IL-6 were determined in each group, respectively. The results showed that compared with model group, 24 h-urine volume, urine protein, creatinine and urea nitrogen content significantly declined in 25 nM M group and 50 nM M group ($p<0.05$). However, they significantly rose in 25 nM I group and 50 nM I group ($p<0.05$). It could be seen that miR-

195 mimics could relieve the symptoms of DN rats. Compared with model group, the content of TNF-α, IL-1β and IL-6 significantly declined in 25 nM M group and 50 nM M group ($p<0.05$). However, it increased remarkably in 25 nM I group and 50 nM I group ($p<0.05$), suggesting that miR-195 mimics could reduce the content of inflammatory factors (TNF-α, IL-1β and IL-6) in DN rats. MiR-195 expression in normal tissues was markedly higher than that in DN tissues ($p<0.01$), while TLR4 expression in normal tissues was markedly lower than that in DN tissues ($p<0.01$). This indicated that there was a negative correlation between the expressions of miR-195 and TLR4 ($r^2=0.4836$, $p=0.0007$). After overexpression of miR-195, the protein expression of TLR4 was markedly reduced ($p<0.01$), indicating that miR-195 could negatively regulate the protein expression of TLR4. The results of Western blotting also manifested that the protein expressions of TLR4 and NF-κB were evidently lower in si-TLR4 group than those in NC group ($p<0.01$). Meanwhile, they showed evident differences in si-TLR4 group compared with those in si-TLR4 + miR-195 inhibitor group ($p<0.05$). The above results demonstrated that the protein expressions of TLR4 and NF-κB in macrophages could be markedly inhibited by knockdown of TLR4, but be promoted by knockdown of TLR4 and miR-195. Finally, it was confirmed that the proliferation ability of macrophages was remarkably weaker in miR-195 mimics group than that in NC group ($p<0.001$). Furthermore, it was also significantly weaker in si-TLR4 + miR-195 inhibitor group than that in si-TLR4 group ($p<0.05$).

To sum up, miR-195 inhibits proliferation and promotes apoptosis of macrophages through targeting TLR4 and blocking the NF-κB pathway. Moreover, miR-195 reduces the release of inflammatory factors, thereby alleviating the symptoms of DN rats.

Conclusions

MiR-195 alleviates the symptoms of DN rats through targeting TLR4 and blocking the NF-κB pathway. All our findings may provide a theoretical basis for the treatment of DN.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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