MicroRNA-630 alleviates inflammatory reactions in diabetic kidney disease rats by targeting TLR4

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Abstract

Background Diabetic kidney disease (DKD) is a major complication of diabetes mellitus. Numerous studies have demonstrated that renal tubular epithelial cell (TEC) damage, which is strongly associated with the inflammatory response and mesenchymal transdifferentiation, plays a significant role in DKD, but the precise molecular mechanism is unknown. One of the recently identified microRNAs is called microRNA-630 (miR-630). It has been hypothesized that miR-630 has a close connection to cell migration, apoptosis, and autophagy. It is yet unknown how miR-630 and DKD are related, as well as how it works. As a result, the goal of this work was to investigate how miR-630 affects TEC injury and the inflammatory response in DKD rats.

Methods Six-week-old male rats were injected with streptozotocin (STZ) to establish a hyperglycemic diabetic model. Streptozotocin (STZ) was administered to six-week-old male rats to create a hyperglycemic diabetic model. After one and four weeks, the control and hyperglycemic diabetic mice were separated into four groups (control, DKD, DKD+NC agomir (LV-NC), DKD+ miR-630 agomir (LV-miR-630) by intraperitoneal injection of agomir NC, miR-630 agomir (50 nM). After eight weeks, urine and blood were collected for the kidney injury assay, and renal tissues were removed for further molecular assays such as real-time PCR, western blot, ELISA, and immunohistochemistry. Bioinformatics predicted miR-630’s target gene, and in vitro investigations, as well as double luciferase reporter gene assays, confirmed the targeting link
between miR-630 and TLR4. Then, in an in vivo test, overexpression of miR-630 changed the body weight, renal weight index, blood sugar, 24-hour urinary protein, urea nitrogen, serum creatinine, TLR4, α-SMA, IL-1 β and IL-6 in DKD rats, and histopathological changes were observed.

**Results** The expression level of miR-630 was found to be reduced in the kidney tissue of rats with Diabetic Kidney Disease (DKD) (p<0.05). In vitro experiments using quantitative reverse transcription polymerase chain reaction (qRT-PCR) were conducted to measure the levels of miR-630 and TLR4 expression in rat renal tubular epithelial cells (NRK-52E). The mRNA expression level of miR-630 was significantly lower in the High Glucose (HG) group and the HG + mimic Negative Control (NC) group compared to the NG group (p<0.05). In contrast, the mRNA expression level of TLR4 was significantly higher in these groups (p<0.05). However, when comparing the HG + miR-630 mimic group to the HG + mimic NC group, there was a substantial increase in miR-630 mRNA expression along with a significant decrease in TLR4 mRNA expression (p<0.05). Furthermore, the HG and HG + mimic NC groups exhibited significantly higher levels of TNF-α, IL-1, and IL-6 compared to the NG group (p<0.05). However, in the HG + miR-630 mimic group, the levels of these cytokines were significantly lower when compared to the HG + mimic NC group (p<0.05). Notably, there were also changes in protein expression. The HG and HG + mimic NC groups showed a significant decrease in E-cadherin protein expression, while TLR4, α-SMA, and collagen IV protein expression increased (p<0.05). Conversely, in the HG + miR-630 mimic group, there was a significant increase in E-cadherin protein expression and a notable decrease in TLR4, α-SMA, and collagen IV protein expression when compared to the HG + mimic NC group (p<0.05). It was observed that miR-630 targets the expression of the TLR4 gene. In vivo experiments demonstrated that DKD rats treated with a miR-630 agomir exhibited significantly higher miR-630 mRNA expression compared to DKD rats injected with an agomir NC. Additionally, rats treated with the miR-630 agomir showed significant reductions in urinary albumin, blood glucose, TLR4, and pro-inflammatory markers (TNF-α, IL-1, and IL-6) expression levels (p<0.05). Moreover, these rats exhibited fewer kidney lesions and reduced inflammatory cell infiltration.

**Conclusions** MiR-630 may inhibit the inflammatory reaction of DKD by targeting TLR4, and has a certain protective effect on DKD.

**Keywords** miR-630; Toll-like receptor 4; diabetic kidney disease; inflammatory reaction; renal tubular epithelial cells

**Introduction**

The chronic kidney condition known as diabetic kidney disease (DKD), which is caused by diabetes, is characterized by a steady decline in albuminuria and glomerular filtration rate. End-stage renal failure (ESRD) has been linked to DKD more frequently than chronic glomerulonephritis, according to previous studies [1-4]. The primary early morphological alterations in DKD include glomerular and tubular hypertrophy, thickening of the glomerular
basement membrane, fusion of the foot processes, growth of the mesangial matrix, etc. Later, it progressed to various degrees of tubulointerstitial fibrosis, which ultimately caused the loss of renal function [5]. However, the molecular mechanism of DKD is unclear.

Renal tubular epithelial cell (TEC) damage has been linked to DKD according to recent studies[6]. Recent years have seen a surge in interest in the TEC mesenchymal transition (EMT), which is a key mechanism of renal interstitial fibrosis[7]. EMT is caused by a complex interaction of several variables, including inflammatory response, hypoxia, oxidative stress, growth factors, signaling pathways, miRNA, and transcription factors SnailSlug and Twist, among others [8]. The molecular mechanisms underlying the damage to renal tubular epithelial cells in DKD may therefore be better understood by concentrating on these pathways.

MicroRNAs (miRNAs) are small noncoding RNAs with a length of 19~23 bp that negatively regulate gene expression posttranscriptionally by targeting the 3'-untranslated regions (3'UTR) of protein-coding messenger RNA (mRNA) transcripts; they play important roles in different physiological and pathological processes [9, 10]. MiRNAs may serve as diagnostic biomarkers and therapeutic targets for DKD because it has been demonstrated that miRNA imbalance is directly associated with pathological processes in the disease. For instance, DKD is promoted by increased Toll-Like Receptor 4 (TLR4) when miR-203 is expressed at low levels[11]. Previous research has demonstrated that overexpressing miR-92b can minimize renal fibrosis and restore miR-92b expression levels to normal levels in the kidneys of DKD mice [12].

A recently discovered miRNA is called miR-630. According to previous studies [13–14], miR-630 is intimately linked to tumor cell development and apoptosis and demonstrates aberrant expression in a variety of malignancies, including liver cancer, colon cancer, gastric cancer, and other tumor tissues. According to Liu et al.'s research[15] in the field of kidney, miR-630 targets TLR4 in IgA nephropathy to control the production of glycosylated IgA1 in tonsils. Research is still missing on the expression of miR-630 in DKD-related renal tissue as well as its function and mechanism in the disease's etiology.

In this study, we assessed the relative expression of miR-630 mRNA in the kidney tissue of DKD rats and discovered that it was considerably lower in comparison to normal rats. A target gene for miR-630 is Toll-like receptor 4 (TLR4), according to mechanistic studies. As a result, miR-630 can be thought of as a possible pharmacological target for DKD treatment and as a
noninvasive biomarker for diagnosing DKD and determining prognosis.

MATERIALS AND METHODS

MATERIALS

1.1 Animals and Cells

Sixty SPF male SD rats, 6 weeks old and weighing 200±20 g, were provided by the Experimental Animal Center of Jiangsu University. The temperature of the experimental animal feeding room was controlled at 21°C, the humidity was controlled at 55°C, and the light/dark period was 12/12 h. Rats were all fed common feed for one week before the experiment. The rat NRK-52E cell line was purchased from the culture treasure cell bank of the China Academy of Sciences.

1.2 Reagents Streptozotocin (STZ) was purchased from Shanghai Aiyan Biotechnology Co., Ltd. Citrate buffer was purchased from Beijing Noble Food Technology Co., Ltd. Lenti viral negative control (LV-NC), LV-miR630 and primers were all purchased from ABclonal. RIPA lysis buffer and BCA kits were purchased from Beyotime. The TLR4 antibody was purchased from Affinity. ELISA kits for interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) were purchased from Mibio.

1.3 Instruments The BK-200 automatic biochemical analyser was purchased from BIOBASE; the DR3518G enzyme-labelled instrument was purchased from Wuxi Hiwell Diatek; the FluorChem HD2 gel imaging system was purchased from Proteinsimple; and CytoFLEXS flow cytometry was purchased from Beckman.

2 Methods

Model construction and processing

The experimental rats were randomly divided into a control group, model (DKD) group, model+NC agomir (LV-NC) group and model+miR-630 agomir (LV-miR-630) group with 15 rats in each group. After fasting for 12 h, the model group and experimental group were injected with 60 mg/kg streptozotocin solution (STZ) intraperitoneally to establish the DKD model, while the normal group was injected with the same volume of sodium citrate buffer (0.1 mmol/L) [16]. After 72 hours, blood was collected from the tail vein to detect blood sugar, and the diabetic model was successfully established with blood sugar ≥ 16.7 mmol/L. Urine was collected for 24 h, and the urine protein content was more than 30 mg/24 h, which was considered successful DKD.
modelling [17]. At the 2nd and 5th weeks after STZ injection, rats in each experimental group were injected with 100 μL agomir NC and miR-630 agomir (50 nM) intravenously, while rats in the model group were injected with the same amount of normal saline intravenously. Eight weeks later, rats in each group were fasted for 12 h before blood collection, and blood samples were taken from the tail vein to detect fasting blood glucose. Urine was collected in a metabolic cage for 24 hours 1 day before execution. Rats were injected with 3.5% chloral hydrate intraperitoneally, blood was taken from the abdominal aorta and kidneys, and then the rats were killed. Renal tissue was collected for further analysis.

2.2 Indicator monitoring
The mental state, color change of hair nails, activity, urine volume and weight of rats were observed during administration. On the last day of the experiment, 24-hour urine samples of rats were collected in a metabolic cage. After mixing, they were centrifuged at 3000 r/min (centrifugal radius 16.5 cm) for 10 min, and the supernatant was taken to detect the 24-hour urine protein quantification. Random blood sugar was measured, and 10% chloral hydrate (350 mg/kg) was injected intraperitoneally for anaesthesia. Blood was taken from the abdominal aorta and then centrifuged at 4°C and 3500 r/min (centrifugal radius 16.5 cm) for 15 min. Serum BUN and SCr levels were detected by the kit method.

2.3 Calculation of Renal Weight Index Rat kidneys were washed with precooled normal saline, dried by filter paper and weighed, and the rat kidney index was calculated. Kidney index (%) = total weight of bilateral kidneys/weight of rats ×100%.

2.4 Quantitative reverse transcription polymerase chain reaction (qRT–PCR) detection of miR-630 and TLR4 mRNA expression The TRIzol method was used to extract each group. Total RNA woven. The expression of miR-630 and TLR4 mRNA was detected by a one-step reverse transcription fluorescence quantitative kit (see Table 1 for primers), and the reaction system and RT–qPCR procedure were performed according to the manufacturer’s instructions. \(2^{-\Delta\Delta C_t}\) was used for relative quantitative analysis, with U6 and GAPDH as internal references.

Tab. 1 Primers for RT–qPCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>sequence ( 5’⁻3’ )</th>
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<tbody>
<tr>
<td>TLR4 forward</td>
<td>TAGCCATTGCTGCCAACATC</td>
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</table>
TLR4 reverse ACACCAACGGCTCTGGATAA
miR-630 forward TTGAGCTGGATTGGCGGAT
miR-630 reverse TTGACGGATCGGGAGGCT
GAPDH forward TATGTCGTGGAGTCTACTGTGT
GAPDH reverse GAGTTGTCATATTTCTCGTGG
U6 forward CATCACCATCAGGAGAGTCG
U6 reverse TGACGCTTGCCCACAGCCTT

2.5 Luciferase Reporter Assay

Construction of wild-type and mutant TLR4 3’-UTR double fluorescent reporter plasmids. 293T cells in the logarithmic growth period were inoculated on a 12-well cell plate at a rate of 1×10^5 cells/well, and negative controls of TLR4-WT, TLR4-MUT and miR-630 mimics or mimics NC were transfected into 293T cells according to the instructions of LipofectamineTM2000. Three replicates were set for each group of experiments. After 48 hours of culture in the incubator, luciferase activity was detected according to the steps of the double luciferase reporter gene detection kit.

2.6 Western blotting was used to detect TLR4 expression in renal tissue.

RIPA lysate was added to the tissues of each group, placed on ice for 20 min, and centrifuged at 4°C and 13000 r/min at 4°C for 20 min, and the protein content in the supernatant was determined by a BCA kit. SDS–PAGE of 35 µg protein solution, PVDF membrane transfer, sealing with 2% BSA sealing solution, adding primary antibody at 4°C overnight, taking GAPDH antibody as reference, adding secondary antibody and incubating for 1 h at room temperature. ECL exposure imaging was performed using an Alpha Imager HP gel imaging system to analyse the results.

2.7 HE staining was used to observe renal injury.

The kidney was fixed with 4% paraformaldehyde solution, dehydrated routinely and made into paraffin sections. Then, after dewaxing, hematoxylin and eosin staining, 1% hydrochloric acid ethanol differentiation, 0.6% ammonia returning to blue, and 0.5% eosin staining, conventional dehydration, xylene transparency and neutral gum sealing were performed, and renal injury was observed under a 400-fold microscope.

2.8 Masson staining was used to observe the histological changes in the kidney.

Slices were routinely dewaxed in water. The sections were stained with the prepared Weigert
hematoxylin staining solution for 5-10 min, differentiated with acidic ethanol differentiation solution for 5-15 s, and washed with water. Masson bluing solution returns to blue for 3-5 min and is washed with water. Distilled water washing for 1 min. Staining with ponceau magenta dye solution for 5-10 min. In the above operation, the weak acid working solution is prepared according to the ratio of distilled water to weak acid solution =2:1, and washed with weak acid working solution for 1 min; Wash with phosphomolybdic acid solution for 1-2 min. Wash 1 min with prepared weak acid working solution. The sample was directly placed into aniline blue dyeing solution for dyeing for 1-2 min. The sample was washed with prepared weak acid working solution for 1 min. The sample was dehydrated quickly with 95% ethanol for 2-3 s and dehydrated with anhydrous ethanol 3 times for 5-10 s each time. 10. The sections were transparentized with xylene 3 times for 1-2 min each time and sealed with neutral gum.

2.9 ELISA was used to detect the levels of IL-6, IL-1β and TNF-α in renal tissue. Renal tissue was ground on ice and made into 10% tissue homogenate. The levels of IL-6, IL-1β and TNF-α in renal tissue were detected according to the instructions of the TNF-α ELISA kit.

3. Statistical Analysis
Data were statistically analysed using SPSS 17.0 and GraphPad Prism 9.0 software, and the results are expressed as the mean ± standard error (mean ± SEM) or mean ± standard deviation (mean ± SD). The T test, one-way ANOVA, and SNK-q tests were used to compare the means of each pair of groups, as well as the means within each pair of groups. The difference was statistically significant with a P value of 0.05 when Pearson correlation analysis and linear regression were utilized in the correlation analysis.

RESULTS

2.1 General observation
In the normal group, rats were in a good mental state, lively and active, with bright eyes, sensitive reaction, white and shiny fur. The model rats were depressed; their fur was yellow, dry and dirty; their movements were slow; they had symptoms such as excessive drinking, excessive eating, excessive urination; and their feces became thin. As time went on, some DN rats also showed abdominal distension to different degrees. The general situation of rats in the miR-630 ago-mir group was improved compared with that in the model group.

2.2 The expression of miR-630 mRNA in kidney tissue of DKD rats and the pathological
changes in kidney tissue.

As demonstrated in Figure 1-a, the miR-630 mRNA expression in the DKD group declined dramatically compared to the control group, as did body weight, while blood sugar levels and the kidney weight index increased significantly, all P<0.01. The level of miR-630 in rats was positively correlated with body weight and albumin (ALB) according to Pearson correlation analysis but negatively correlated with renal weight index, urea nitrogen, serum creatinine (Scr), 24-hour urine protein quantification, blood sugar, and other variables (Figure 1-B). These findings imply that miR-630 expression in renal tissue is associated with clinical variables and may be linked to DKD. The HE test results are shown in Figure 1-C. The renal tissue cell structure remained unaltered in the control group, and no overt pathological alterations were discovered. Renal tubular epithelial cells enlarged or detached, renal interstitial cells infiltrated, mesangial hyperplasia and interstitial fibrosis were detected, and glomerular edema in the DKD group.
FIGURE 1 | Differential expression of miR-630, renal weight index, and blood glucose in DKD rats (A). The correlations between the expression of miR-630 and clinical parameters, including body weight, serum albumin (ALB), renal weight index, BUN, Scr and proteinuria, were analysed by Pearson correlation analysis and linear regression analysis (B). N=15. The data are expressed as the mean ± SEM, **p < 0.01. Pathological changes in renal tissue in rats under a high glucose environment (HE, *400) (C)

2.3 Overexpression of miR-630 inhibits TEC damage induced by high glucose in vitro.

qRT–PCR was used to determine the expression levels of mir-630 and TLR4 in rat renal tubular epithelial cells (NRK-52E), and the findings are displayed in Figure 2-A. TLR4 and miR-630 mRNA expression levels in the NG group and HM group were not significantly different. MiR-630 and TLR4 mRNA expression levels significantly decreased in the HG group and HG+mimic NC group compared to the ng group; however, tlr4 mRNA expression levels dramatically increased. When compared to the HG+miR-630 mimic NC group, the mRNA expression levels of miR-630 and TLR4 dramatically increased and decreased, respectively.

Figure 2 Overexpression of miR-630 inhibits TEC damage induced by high glucose in vitro.

qRT–PCR detection of miR-630 and TLR4 expression in rat renal tubular epithelial cells in a high glucose environment (NRK-52E) (A). Detection of TNF-α, IL-1β and IL-6 expression in rat renal
tubular epithelial cells (NRK-52e) under a high glucose environment by ELISA (B). Western blot detection of NRK-52E TLR4 in rats under a high glucose environment α-SMA and collagen IV protein expression (C).

Note: NG (Normal glucose, 5.6 mmol/L), HG (high glucose, 20 mmol/L) and HM (high mannitol) (5.6 mmol/L glucose + 14.4 mmol/L mannitol), * P<0.01 vs NG, and # # P<0.01 vs HG+mimic NC.

ELISA was used to determine the levels of TNF-, IL-1, and IL-6 in each group. The results are displayed in Figure 2-B. TNF-, IL-1, and IL-6 levels in the NG and HM groups were not altered considerably, but they did increase significantly in the HG and HG+mimic NC groups compared to the NG group. The levels of TNF-, IL-1, and IL-6 in the HG+miR-630 mimic group were considerably lower than those in the HG+mimic NC group.

Figure 2-C displays the findings of the Western blot analysis used to determine the protein expression levels of TLR4, E-cadherin, E-SMA, and collagen IV in each group. TLR4, E-cadherin, E-SMA, and collagen IV expression levels in the NG group and HM group were not significantly altered. E-cadherin expression levels in the HG group and HG+mimic NC group were significantly decreased compared to those in the NG group, although TLR4, -SMA, and collagen IV expression levels were dramatically increased. In the HG+miR-630 mimic group, compared to the HG+mimic NC group, the expression levels of E-cadherin protein increased dramatically, whereas those of TLR4, -SMA, and collagen IV protein fell significantly.

2.4 miR-630 targeted the downregulation of TLR4

TargetScan and other databases predicted that miR-630 has a binding site in the 3'-untranslated region (3'-UTR) of TLR4 (see Supplementary Materials Table 1 and Supplementary Figure 1 for further information) (Figure 3-A). According to the experimental findings using a double luciferase reporter gene, high levels of miR-630 dramatically reduced the luciferase activity of a TLR4 plasmid that was wild-type (P 0.01) but had no effect on a mutant TLR4 plasmid (Figure 3-B).

qRT–PCR was used to determine the levels of miR-630 and TLR4 mRNA expression, and the findings are displayed in Figure 3-C. The mRNA expression of miR-630 increased dramatically in the HG+miR-630 mimic group compared to the HG+mimic NC group, while the mRNA expression of TLR4 significantly decreased. The mRNA expression of miR-630 in the
HG+miR-630 mimic+oe-TLR4 group was considerably lower than that in the HG+miR-630 mimic+oe-NC group, but the mRNA expression of TLR4 was dramatically increased.

Figure 3-D displays the results of an ELISA that showed that miR-630 downregulated the levels of INF-α, IL-1β, and IL-6 in TLR4. The levels of INF-α, IL-1β, and IL-6 in the HG+miR-630 mimic group were considerably lower than those in the HG+mimic NC group. The INF-α, IL-1β, and IL-6 levels were significantly increased in the HG+miR-630 mimic+oe-TLR4 group compared to the HG+ miR-630 mimic+oe-NC group.

Western blotting was used to detect the protein expression levels of TLR4, α-SMA, collagen IV and E-cadherin downregulated by miR-630. The results are shown in Figure 3-E. Compared with the HG+mimic NC group, the protein expression levels of TLR4, α-SMA and collagen IV in the HG+miR-630 mimic group decreased significantly, while the protein expression level of E-cadherin increased significantly. Compared with the HG+ miR-630 mimic+oe-NC group, the expression levels of TLR4, α-SMA and collagen IV proteins in the HG+miR-630 mimic+oe-TLR4 group were significantly increased, while the expression level of E-cadherin protein was significantly decreased.
FIGURE 3 | TLR4 is the target gene of miR-630. (A) Bioinformatic analysis showed the putative miR-630 target sites in the TLR4 3′-UTR. The mutated nucleotides are underlined. (B) The WT-TLR4 3′-UTR and the MUT-TLR4 3′-UTR reporters were cotransfected with miR-630 mimic or negative control into NRK-52Es. Forty-eight hours after transfection, the luciferase activities were measured. (C) qRT–PCR detection of miR-630-targeted downregulation of TLR4 mRNA expression levels. * * P<0.01 vs HG+mimic NC, # # P<0.01 vs HG+miR-630 mimic+oe NC. (D) ELISA detection of TNF in miR-630-targeted downregulation of TLR4, α-SMA, IL-1β, and IL-6 content. **P<0.01 vs HG+mimic NC, # # P<0.01 vs HG+miR-630 mimic+oe NC. (E) Western blotting was used to detect the protein expression of TLR4, α-SMA, collagen IV and E-cadherin downregulated by miR-630.

Note: NG (Normal glucose, 5.6 mmol/L), HG (high glucose, 20 mmol/L) and HM (high mannitol) (5.6 mmol/L glucose + 14.4 mmol/L mannitol), **p<0.01 vs HG+Mimic NC, ##p<0.01 vs HG+Mir-630mimic+OE-NC. The data are expressed as the mean ± SEM, NC, negative control.

2.5 Overexpression of miR-630 improves the biochemical changes in DKD model rats.

The test results of rat body weight, renal weight index, blood sugar, 24-hour urinary protein, blood urea nitrogen and serum creatinine are shown in Figure 4-A. There were no significant differences in body weight, renal weight index, blood sugar, 24-hour urinary protein, blood urea nitrogen or serum creatinine between the DKD group and the DKD+NC agomir group. Compared with the DKD+NC agomir group, the weight of the DKD+miR-630 agomir group increased significantly, and the renal index decreased significantly. The blood sugar test results of the rats are shown in Figure 4-A. There was no significant difference in blood sugar between the DKD group and the DKD+Ncadomir group. Compared with the DKD+NC agomir group, the blood sugar in the DKD+miR-630 agomir group decreased significantly. The results of the automatic biochemical analyser are shown in Figure 4-C. There was no significant difference in 24-hour urine protein, blood urea nitrogen and serum creatinine between the DKD group and the DKD+Ncadomir group. Compared with the DKD+NC agomir group, the contents of 24-hour urine protein, blood urea nitrogen and serum creatinine in the DKD+miR-630 agomir group decreased significantly.
FIGURE 4 | Overexpression of miR-630 improves the biochemical changes in DKD model rats. (A) Effects of miR-630 overexpression on body weight, renal weight index, blood glucose, 24-hour urinary protein, blood urea nitrogen and serum creatinine in rats. (B) Effect of overexpression of miR-630 on the expression of TLR4 mRNA in DKD rats. (C) MiR-630 was overexpressed, and the levels of IL-6, IL-1β and TNF-α were detected by ELISA. (D) MiR-630 was overexpressed, and the expression levels of TLR4, E-cadherin, α-SMA and collagen IV were detected by Western blotting. Note: **P<0.01 vs DKD+NC agomir.

2.6 Effects of miR-630 overexpression on TLR4, TNF-α, IL-1β and IL-6 in DKD rats

The mRNA expression levels of miR-630 and TLR4 were measured by qRT–PCR. As shown in Fig. 4-B, there was no significant difference in the mRNA expression of miR-630 and TLR4 between the DKD group and the DKD+NC agomir group. Compared with the DKD+NC agomir group, the mRNA expression of miR-630 in the DKD+miR-630 agomir group increased significantly, while the mRNA expression of TLR4 decreased significantly. The protein levels of IL-6, IL-1β and TNF-α were detected by ELISA. As shown in Figure 4-C, there was no significant difference in the contents of IL-6, IL-1β and TNF-α between the DKD group and the DKD+Ngomir group. Compared with the DKD+NC agomir group, the contents of IL-6, IL-1β and TNF-α in the DKD+miR-630 agomir group decreased significantly.
TLR4, E-cadherin, α-SMA and collagen IV were detected by Western blotting. As shown in Figure 4-D, there was no significant difference in the expression levels of TLR4, E-cadherin, α-SMA and collagen IV between the DKD group and the DKD+Ncadomir group. Compared with the DKD+NC agomir group, the expression of E-cadherin protein in the DKD+miR-630 agomir group was significantly increased, while the expression of TLR4, α-SMA and collagen IV protein was significantly decreased.

2.7 Effects of overexpression of miR-630 on glomerular morphology in DKD rats

The HE test results are shown in Figure 5-A. In the DKD group and DKD+N-cadherin group, glomerular swelling, renal tubular epithelial cells swelled or fell off, renal interstitial cells infiltrated inflammatory cells, and some mesangial hyperplasia and interstitial fibrosis were observed. In the DKD+miR-630 agomir group, renal pathological changes were obviously alleviated, accompanied by a small amount of inflammatory cell infiltration.

The Masson test results are shown in Figure 5-B, and a large number of blue-stained collagen fibres appeared in the renal glomeruli of rats in the DKD group and DKD+Ncadomir group. In the DKD+miR-630 agomir group, there were a few blue-stained collagen fibres in the kidney tissue of rats.

FIGURE 5 | Effects of overexpression of miR-630 on glomerular morphology in DKD rats. (A) Effect of overexpression of miR-630 on glomerular morphology in DKD rats (HE, *200). (B) Effect of overexpression of miR-630 on glomerular morphology in DKD rats (Masson, *200).

DISCUSSION

DKD is becoming increasingly common worldwide and poses a severe threat to people's lives and health. DKD has a very complex pathophysiology that has not been fully understood to date. Numerous studies have now established the role of miRNA in the occurrence and progression of diabetic nephropathy [18]. MiR-21, miR-146a-5p, miR-10a-5p, miR-874, and miR-192 were shown to have significant levels of expression in diabetic nephropathy, while miR-26a-5p, miR-451, and miR-155 had low levels [17, 19–21]. One of these, miR-21, works on the PTEN gene to stimulate the Akt kinase signal pathway, which in turn causes an increase in the production
of the renal fibrosis proteins type I collagen a2 and mucin and glomerular hypertrophy [17]. By targeting the ZEB1/2 gene, MiR-192 activates the TGF- signal pathway, increasing the transcription of the renal fibrosis protein Coll2 and the amount of albumin in the urine [21].

One of the recently identified microRNAs is called miR-630. A noncoding single-stranded RNA fragment called a miRNA (microRNA) with a length of 21–23 nucleotides regulates gene expression at the translational level and participates in a number of pathophysiological processes, including cell proliferation and differentiation [22], apoptosis [23], and immune response [24]. Numerous malignancies, such as liver cancer, colon cancer, gastric cancer, and other tumor tissues, have aberrant expression of the miR-630 gene [14,25]. Additionally, current research has revealed that miR-630 is strongly linked to autophagy as well as cell proliferation, migration, and apoptosis [26–28]. However, it is unknown whether miR-630 is expressed or how it works in the renal tissue of people with DKD. By injecting a significant dosage of STZ intraperitoneally, a DKD rat model was created for this study. The 24-hour UTP, SCr, and BUN levels rose, and the model rats manifested clear signs of diabetes. The model was successful since it revealed the characteristic renal pathological abnormalities of DKD when stained with Masson and HE. The expression of miR-630 was also investigated, and it was discovered that it was much lower in DKD renal tissue than in normal renal tissue, indicating that miR-630 may play a role in the pathogenesis of DKD.

Inflammation is crucial in the pathogenesis of DKD, according to mounting evidence [29]. The first confirmed member of the Toll-like family in humans is TLR4. It can activate signaling pathways, such as the NF-B pathway and the mitogen-activated protein kinase family pathway, after interacting with ligands in vivo, which increases the production of inflammatory markers and activates an inflammatory response [30]. Activation of the TLR4 signaling pathway is currently thought to be closely related to the pathogenesis of diabetic nephropathy, according to studies that have shown that the expression of TLR4 and related inflammatory factors TNF-, IL-6, and IL-1 increases during the occurrence and development of diabetic nephropathy [31–32]. This work used a bioinformatics website to predict that miR-630 may combine with the 3'-UTR of TLR4 and a twofold luciferase assay to confirm that TLR4 was the direct target of miR-630. Then, it was discovered that overexpressing miR-630 in DKD rats caused a drop in TLR4 expression as well as levels of the proinflammatory molecules TNF-, IL-6, and IL-1, demonstrating a negative regulatory link between miR-630 and TLR4. To prevent TLR4 from being translated and
transcribed, miR-630 attaches to its 3' UTR on the mRNA. This prevents the proinflammatory protein TNF-α from being produced. This study also discovered that overexpression of miR-630 in DKD rats led to an improvement in general rat health, an increase in weight, a drop in renal index, an improvement in urine protein and renal function, and a reduction in renal pathological damage. As a result, it was further confirmed that overexpression of miR-630 could lessen the inflammatory response and mesenchymal transdifferentiation of diabetic nephropathy. At the same time, the contents of TNF-α, IL-6, and IL-1 significantly decreased, and the expression of the renal tubular epithelial marker protein E-cadherin increased, while the expression of the mesenchymal marker proteins α-SMA and collagen IV decreased.

In addition, in this study, as shown in Figure 4-A, over-expression of mir-630 can significantly reduce blood sugar, which may be the protective factor of mir-630 to prevent the progress of DKD, or the function of mir-630 to promote insulin secretion, and its mechanism has not been reported in the literature at present, which needs further study and exploration.

In conclusion, this work initially showed that miR-630 targets TLR4 and inhibits the inflammatory response that results in DKD, as well as having some protective effects on the kidney under diabetic circumstances.

**CONCLUSIONS**

In summary, our results show for the first time, to the best of our knowledge, that the expression of miR-630 in renal tissue is significantly lower in DKD rats than in normal rats. The results reveal the underlying mechanism by which microRNA-630 alleviates renal injury and inflammatory reactions in diabetic kidney disease rats by targeting TLR4. Taken together, our findings provide new insights into the pathogenesis of DKD and show that miR-630 may be a noninvasive biomarker used to diagnose and predict the prognosis of DKD.

**Supplementary Information**

Additional file 1: Supplemental Table 1. Part of predicted target genes of miR-630
Additional file 2: Figure S1. Bioinformatics analysis of target genes of miR-630.

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AUTHOR CONTRIBUTIONS

Q-SW conceived and designed the experiments. Q-SW and D-NZ performed the experiments, analysed the data, and prepared all the figures. C J, H Q, J J, and Q H provided technical support. Q-SW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

REFERENCES


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