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MiR-146a relieves kidney injury in mice with systemic lupus erythematosus through regulating NF-κB pathway

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Abstract. – OBJECTIVE: To explore the effect of micro ribonucleic acid (miRNA)-146a on kidney injury in mice with systemic lupus erythematosus (SLE), and to investigate its possible mechanism.

MATERIALS AND METHODS: A total of 45 female MRL/lpr mice were randomly divided into control group, miR-146a mimic group and miR-146a inhibitor group. Urine protein level was measured every 2 weeks. Meanwhile, the levels of serum anti-dsdeoxyribonucleic acid (anti-dsDNA), anti-ssDNA, antinuclear antibody (ANA) and anti-chromatin were measured using enzyme-linked immunosorbent assay (ELISA). At 2 weeks after drug treatment, the effects of miR-146a mimic and inhibitor on kidney tissues of MRL/lpr mice were detected and analyzed by gene chip and gene set enrichment analysis, respectively. The mice were executed at the age of 24 weeks, and the blood samples were collected. Subsequently, the level of blood urea nitrogen (BUN) was measured using the BUN analyzer. After that, kidney tissues were taken, and the effect of drug treatment on the morphology of kidney tissues was detected via hematoxylin-eosin (HE) staining. Moreover, the effects of drug treatment on the mRNA levels of inflammatory factors and the nuclear factor-κB (NF-κB) signaling pathway in kidney tissues were detected via quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting, respectively.

RESULTS: MiR-146a mimic significantly reduced urine protein in a time-dependent manner, which also significantly reduced BUN level at 24 weeks. The results of HE staining showed that both glomerular injury and renal vascular injury in miR-146a mimic group were significantly alleviated. In miR-146a mimic group, serum autoantibodies of anti-dsDNA, anti-ssDNA, anti-chromatin and ANA decreased significantly. However, the survival time of mice was significantly prolonged. High-throughput gene expression chip technique elucidated that in miR-146a mimic group, the expression of positive regulatory gene of NF-κB showed a decreasing trend. However, the expression of negative regulatory gene of NF-κB showed an increasing trend. MiR-146a mimic remarkably down-regulated the expression levels of RELA, IRAK1, interleukin-1B (IL1B) and IL-10 in kidney tissues. Furthermore, the results of Western blotting showed that miR-146a mimic inhibited both the classical and non-classical NF-κB signaling pathways.

CONCLUSIONS: MiR-146a reduces SLE-induced kidney injury in MRL/lpr mice through regulating classical and non-classical NF-κB signaling pathways.

Key Words:
MiR-146a, NF-κB signaling pathway, MRL/lpr mice, Kidney injury.

Introduction

Systemic lupus erythematosus (SLE) is a chronic multi-system autoimmune disease caused by massive production of autoantibodies and deposition of immune complexes[1]. According to epidemiological studies, 30-60% of adults and 70% of children with SLE suffer from nephritis and nephritis-induced kidney injury[2]. With the improvement of immunosuppressive regimens and general medical care in the last 2 decades, partial differences in the long-term therapeutic outcome between proliferative and membranous lupus nephritis have been eliminated. However, the complete remission rate of nephritis in SLE patients remains less than 12%. Moreover, up to 40% of patients with stage III-V lupus nephritis still have the impairment of renal function in a certain degree[3]. In addition, the therapeutic effect of immune-suppressors on SLE-induced

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nephritis is still far from satisfactory even under the good clinical test conditions. Therefore, it is necessary to search for new drug targets for SLE.

Wang G. et al[4] have pointed out that micro ribonucleic acids (miRNAs) and long non-coding RNAs (lncRNAs) play important roles in the pathogenesis of SLE. Some studies have revealed the potential mechanisms of miRNAs and lncRNAs in the pathogenesis of SLE. Meanwhile, their potential as biomarkers for the diagnosis of SLE has also been elucidated. Among all miRNAs, miR-146a has been widely recognized as a key miRNA in immune-regulation. Moreover, the research results of Brightbill *et al*[5] have manifested that the expression level of miR-146a is significantly up-regulated in peripheral blood mononuclear cells of SLE patients. Meanwhile, its expression level is significantly correlated with the activity of SLE in patients. Therefore, miR-146a may play a key role in SLE. However, the specific mechanism of miR-146a in regulating the pathological process of SLE remains unknown.

Nuclear factor-κB (NF-κB) signaling pathway regulates multiple biological processes, such as production of inflammatory factors, proliferation and survival of cells, differentiation of effector T cells and regulatory T cells, and maturation of dendritic cells[6]. Therefore, it is beyond doubt that the dysregulation of NF-κB signaling pathway plays a key role in various autoimmune diseases (including SLE) and inflammatory diseases. NF-κB signal transduction is realized in two different pathways. In classical NF-κB signal transduction, the activation of receptors leads to the degradation of downstream inhibitor of NF-κB (I-κB) of the inhibitory kappa B kinase (IKK) α/β/γ complex. This may cause the translocation of classical NF-κB subunit (such as p65/p50) into the nucleus, eventually triggering immune gene expression in the nucleus[7]. Moreover, non-classical NF-κB signal transduction is strictly dependent on NF-κB-inducing kinase (NIK) (MAP3K14). Meanwhile, this pathway is weakened by continuous degradation of NIK protein through binding to the TNF receptor-associated factor 2/3-ubiquitin ligase complex. The tumor necrosis factor receptor superfamily (TNFRSF) signal transduction dissociates NIK from the complex, which can accumulate and phosphorylate IKKα. Subsequently, IKKα phosphorylates NF-κB p100 in turn, and p100 is cleaved to release mature transcription factor p52[8]. After that, p52 dimerizes with RelB, translocates to the nucleus and triggers the transcription of target genes. A previous animal model has suggested that inhibiting classical and non-classical NF-κB signaling pathways can significantly improve SLE-induced kidney injury. Current studies have demonstrated that there is a negative feedback regulatory effect between miR-146a and NF-κB signaling pathway. For example, LPS induces miR-146a expression in a NF-κB-dependent manner in human monocytes. Moreover, miR-146a down-regulates the signaling proteins of TRAF6 and IRAK1, thereby inhibiting NF-κB signaling pathway[9]. In the present study, therefore, it is theoretically assumed that miR-146a relieves SLE-induced kidney injury through regulating the NF-κB signaling pathway.

Materials and Methods

Animal Model

A total of 45 female MRL/lpr mice aged 12-week-old were fed in the specific pathogen-free animal room under the temperature of 25°C, humidity of 45% and light cycle of 12/12 h. They were given free access to food and water. All mice were randomly divided into three groups, including: control group, miR-146a mimic group and miR-146a inhibitor group. Meanwhile, 15 C57BL/6J mice were taken as C57BL/6J control group. MiR-146a mimic and miR-146a inhibitor were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). This study was approved by the Animal Ethics Committee of Shandong University Animal Center.

Detection of Renal Function

Urine protein level was measured every 2 weeks. Mice were first fed separately in metabolic cages for 24 h. Subsequently, the urine was collected, and urine protein was measured using Multistix 10SG reagent strips (Siemens Healthineers, Erlangen, Germany). Urine protein level was graded (grade 0-4): grade 0: no, grade 1: 30-100 mg/dL, grade 2: 100-300 mg/dL, grade 3: 300-2000 mg/dL and grade 4: >2000 mg/dL. All mice were executed at the age of 24 weeks. Blood samples were then collected, followed by detection of blood urea nitrogen (BUN) level using the BUN analyzer (Beckman Coulter Inc., Brea, CA, USA).

Hematoxylin-Eosin (HE) Staining

The mice to be detected were executed *via* dislocation at one time. Kidney tissues were

taken and treated with 4% paraformaldehyde/PBS (phosphate-buffered saline) (pH 7.4) at 4°C for 48 h. The tissues were washed with running water, dehydrated with 70%, 80% and 95% ethanol and treated with 100% ethanol. After that, the ethanol was removed with xylene. Next, the tissues were embedded into 2-μm paraffin sections, followed by staining in strict accordance with HE staining kit (Beyotime, Shanghai, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of anti-dsDNA, anti-ssDNA, antinuclear antibody (ANA) and anti-chromatin were measured according to the manufacturer's instructions of ELISA kit (Jianglai, Shanghai, China).

Detection of Changes in Inflammatory Factors in Kidney Tissues via Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The mRNA expressions of inflammatory factors in kidney tissues of the three groups were detected *via* qRT-PCR. In RT, 500 ng of ribonucleic acid (RNA) samples (1:10) in each group were divided into 3 pieces, and 3 μL miR-106b was taken for PCR amplification. The amplification levels of target genes were verified using 5% agarose gel electrophoresis. Experimental data were quantified and processed using LabWorks 4.0 image acquisition and analysis software (UVP Inc., Upland, CA, USA). This experiment was repeated for 3 times in each group[11]. In the present study, the changes in relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were shown in Table I[10].

Table I. Primer sequences.

Primer	Primer sequence (5'-3')
RELA	5'-TGCGATTCCGCTATAAATGCG-3' 5'-ACAAGTTCATGTGGATGAGGC-3'
IRAK1	5'-CCACCCTGGGTTATGTGCC-3' 5'-GAGGATGTGAACGAGGTCAGC-3'
IL10	5'-GAGGATGTGAACGAGGTCAGC-3' 5'-GCAGCTCTAGGAGCATGTGG-3'
IL1B	5'-GAAATGCCACCTTTTGACAGTG-3' 5'-TGGATGCTCTCATCAGGACA-3'
GAPDH	5'-AATGACCCCTTCATTGAC-3' 5'-TCCACGACGTACTCAGCGC-3'

Transcriptome Analysis

In this study, the difference in gene expression induced by injection of miR-146a mimic and inhibitor for 2 weeks was explored using high-throughput expression profile chip. After 2 weeks, total RNA was extracted from kidney tissues using TRIzol kit (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted total RNA was quantified using the NanoDrop kit (Thermo Fisher Scientific, Waltham, MA, USA), and its integrity was evaluated using Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). 100 mg total RNA was prepared into cRNA using according to the instructions of Affymetrix 3' IVT Express kit. After that, hybridization was performed on an Affymetrix Primeview Human array at 45°C for 16 h according to the instructions of GeneChip 3' Array (Affymetrix, Silicon Valley, CA, USA). The array was then washed and stained on the Affymetrix FS-450 fluid station, followed by scanned on the Affymetrix GeneChip scanner. Raw data of CEL file were imported into Partek Genomics Suite 6.6 software Sigma-Aldrich (St. Louis, MO, USA). The probe set was normalized using the Robust Multiarray Average method. Finally, differentially expressed genes were determined using one-way analysis of variance, and the *p*-value was corrected using false discovery rate (FDR)[11].

Gene Set Enrichment Analysis (GSEA)

GSEA was performed for differentially expressed genes using GSEA software[12] (Broad Institute, http://www.broadinstitute.org/gsea/index.jsp). The genome items were obtained from Molecular Signature Database v4 .0 (Broad Institute, http://www.broadinstitute.org/gsea/index.jsp). Differentially expressed genes in the gene microarray were screened using R-3.5.2 software based on the following screening criteria: $p<0.05$ and $|logFC| >2.0$. Finally, the differential expression heat map was plotted using the LDheatmap software (Burnaby, Canada) package in R language[13].

Western Blotting

An appropriate amount of radio immune-precipitation assay (RIPA: Beyotime, Shanghai, China) lysis buffer and protease inhibitor phenylmethanesulfonyl fluoride (PMSF: Beyotime, Shanghai, China) (RIPA: PMSF = 100:1) was first added in cells and mixed evenly. After the cells were digested with trypsin, lysis buffer was added. Subsequently, the lysate was col-

lected and transferred into an Eppendorf (EP) tube, followed by centrifugation at 14000 rpm and 4°C for 30 min using refrigerated high-speed centrifuge. Next, protein supernatant was collected and subjected to heating bath at 95°C for 10 min for protein denaturation. Extracted protein samples were placed in a refrigerator at -80°C for use. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA: Beyotime, Shanghai, China) method. After that, protein samples were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) under the constant pressure of 80 V for 2.5 h, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer method. After that, PVDF membranes were immersed in Tris-buffered saline and Tween 20 (TBST) containing 5% skim milk powder and shaken slowly for 1 h on a shaking table to be sealed. After incubation with primary antibodies (Abcam, Cambridge, MA, USA) diluted with 5% skim milk powder, the membranes were rinsed with TBST for 3 times (10 min/time). Subsequently, the membranes were incubated again with corresponding secondary antibody at room temperature for 2 h, followed by washing again with TBST twice and TBS once (10 min/time). Immune-reactive bands were detected using the enhanced chemiluminescence (ECL) reagent, followed by exposure in dark. The relative expression level of proteins was analyzed using Image-Pro Plus v6 (Media Cybernetics, Silver Spring, MD, USA).

Statistical Analysis

Experimental data were expressed as mean ± standard deviation (SD). Student's *t*-test was used for statistical analysis of intergroup differences. Kaplan-Meier and log-rank analysis were used to evaluate survival rate. $p<0.05$ was considered statistically significant.

Results

MiR-146a Mimic Significantly Reduced Kidney Injury in MRL/lpr Mice

MiR-146a mimic significantly reduced urine protein in a time-dependent manner, which also significantly decreased BUN level at 24 weeks in MRL/lpr mice compared with mice in control group ($p<0.05$). However, miR-146a inhibitor significantly increased urine protein in a time-dependent manner and obviously up-regulated BUN level at 24 weeks in MRL/lpr mice ($p<0.05$). Moreover, the results of HE staining showed that both glomerular injury and renal vascular injury in miR-146a mimic group were significantly alleviated when compared with those in control group (Figure 1).

MiR-146a Mimic Reduced Production of Autoantibodies

In miR-146a mimic group, serum autoantibodies of anti-dsDNA, anti-ssDNA, anti-chromatin and ANA decreased obviously when compared with those in control group, showing statistically significant differences ($p<0.05$). In addition, the serum levels of anti-dsDNA, anti-ssDNA and ANA increased remarkably at 16, 20 and 24 weeks in miR-146a inhibitor group when compared with those in control group, and the differences were statistically significant ($p<0.05$) (Figure 2).

MiR-146a Mimic Significantly Increased Survival Rate of MRL/lpr Mice

Compared with control group, the survival time in miR-146a mimic group was significantly prolonged. However, it was significantly shortened in miR-146a inhibitor group than that of control group ($p<0.01$) (Figure 3).

Effect of miR-146a on Kidney Tissues in MRL/lpr Mice Analyzed Using Gene Expression Chip

High-throughput gene expression chip technique indicated that in miR-146a inhibitor group, the expression of positive regulatory gene of NF-κB showed an increasing trend. However, the expression of negative regulatory gene of NF-κB showed a decreasing trend. In miR-146a mimic group, opposite results were observed. The results of GSEA revealed that significantly enriched gene sets included Positive_Regulation_of_Nf_Kappab (NS=0.52, $p=6.9\times10^{-11}$) and Negative_Regulation_of_Nf_Kappab (NS=0.47, $p=5.5\times10^{-10}$) in miR-146a inhibitor group and miR-146a mimic group, respectively (Figure 4).

MiR-146a Mimic Significantly Inhibited Inflammation of Kidney Tissues

Compared with control group, miR-146a inhibitor remarkably up-regulated the expressions of RELA, IRAK1, IL1B and IL-10 in kidney tissues, displaying statistically significant differences ($p<0.05$). However, miR-146a mimic

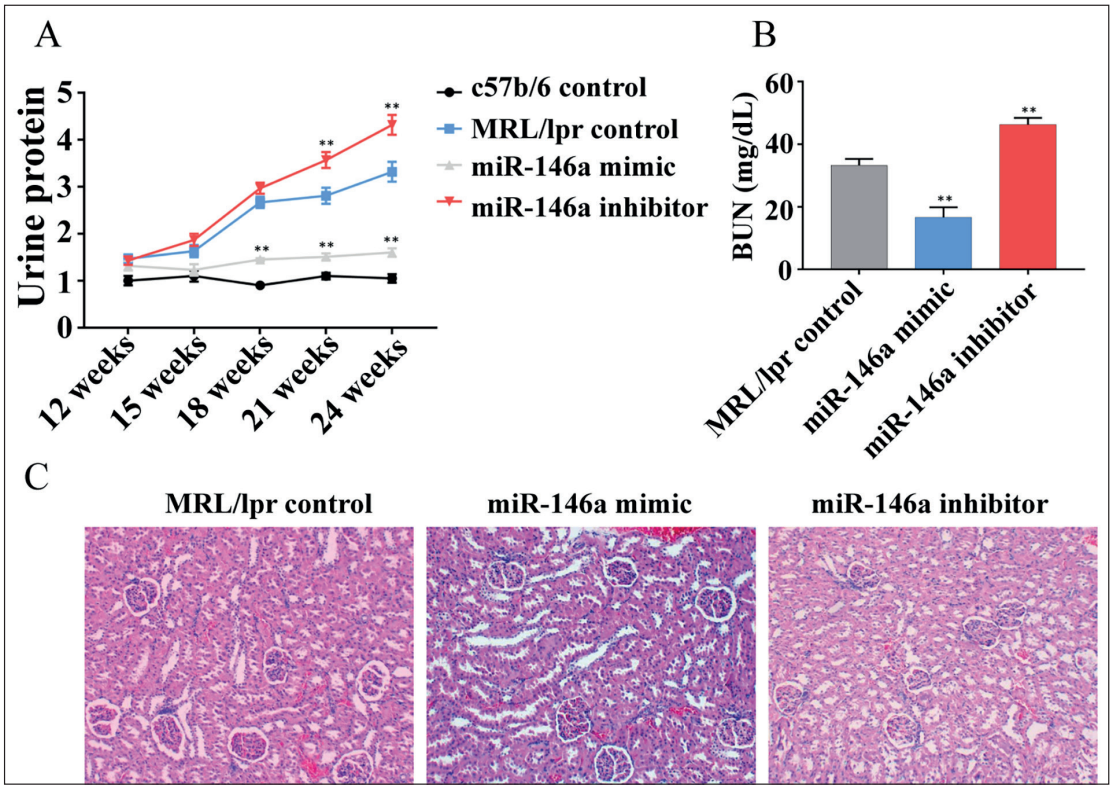

Figure 1. Effect of miR-146a on kidney injury in MRL/lpr mice. ***A,*** Changes in urine protein level at 12-24 weeks in different groups. ***B,*** Serum BUN content at 24 weeks in different groups. ***C,*** Results of HE staining in different groups (magnification: 400×) $^{**}p<0.01$.

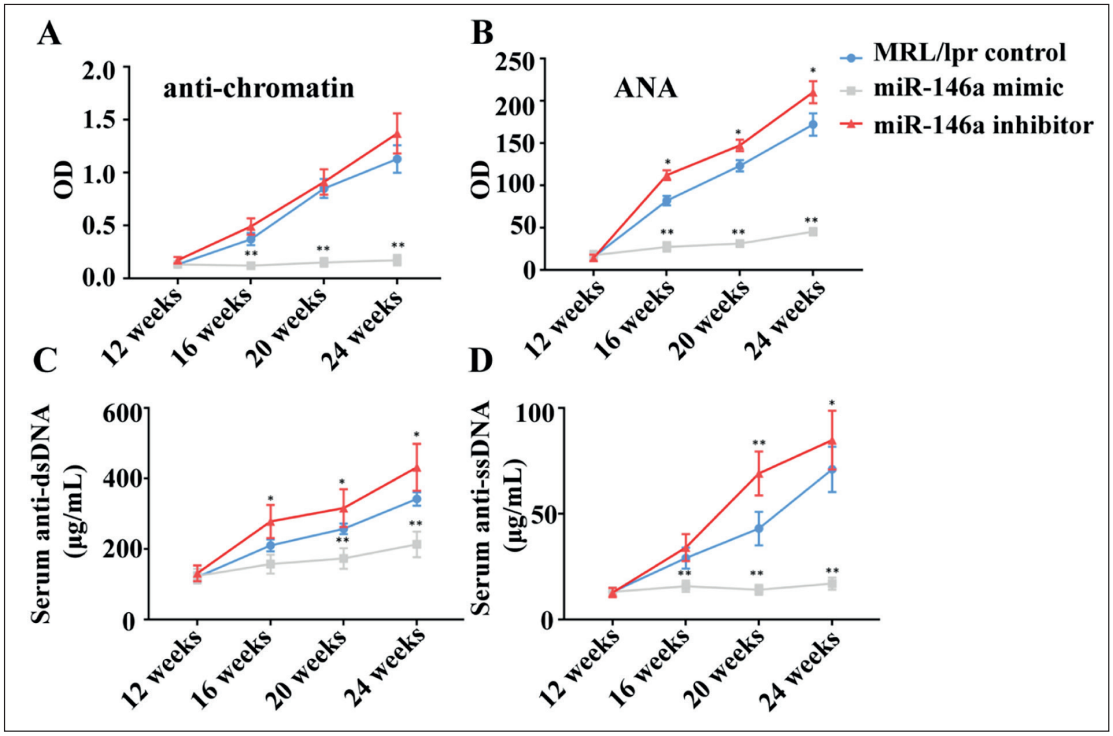

Figure 2. Effect of miR-146a on production of autoantibodies. ***A,*** Serum anti-chromatin content in different groups. ***B,*** Serum ANA content in different groups. ***C,*** Serum anti-dsDNA content in different groups. ***D,*** Serum anti-ssDNA content in different groups.

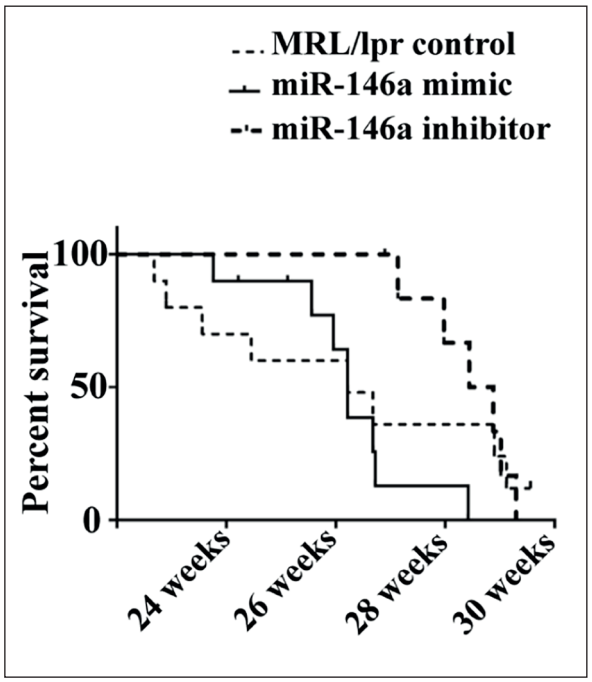

Figure 3. Effect of miR-146a on survival rate of MRL/lpr mice.

significantly lowered the expressions of RELA, IRAK1, IL1B and IL-10 in kidney tissues (p<0.01) (Figure 5).

Effect of miR-146a Mimic on Classical NF-κB Signaling Pathway

Western blotting demonstrated that miR-146a mimic remarkably reduced the protein expression levels of p-IKKα and p-IκBα. However, miR-146a inhibitor obviously up-regulated the protein expression levels of p-IKKα and p-IκBα when compared with control group, and there were statistically significant differences (p<0.05) (Figure 6).

Effect of miR-146a Mimic on Non-Classical NF-κB Signaling Pathway

Western blotting also revealed that miR-146a mimic significantly reduced the protein expression levels of p52 and RelB in the nucleus, increased the protein expression level of p100 in the cytoplasm, and decreased the protein expression

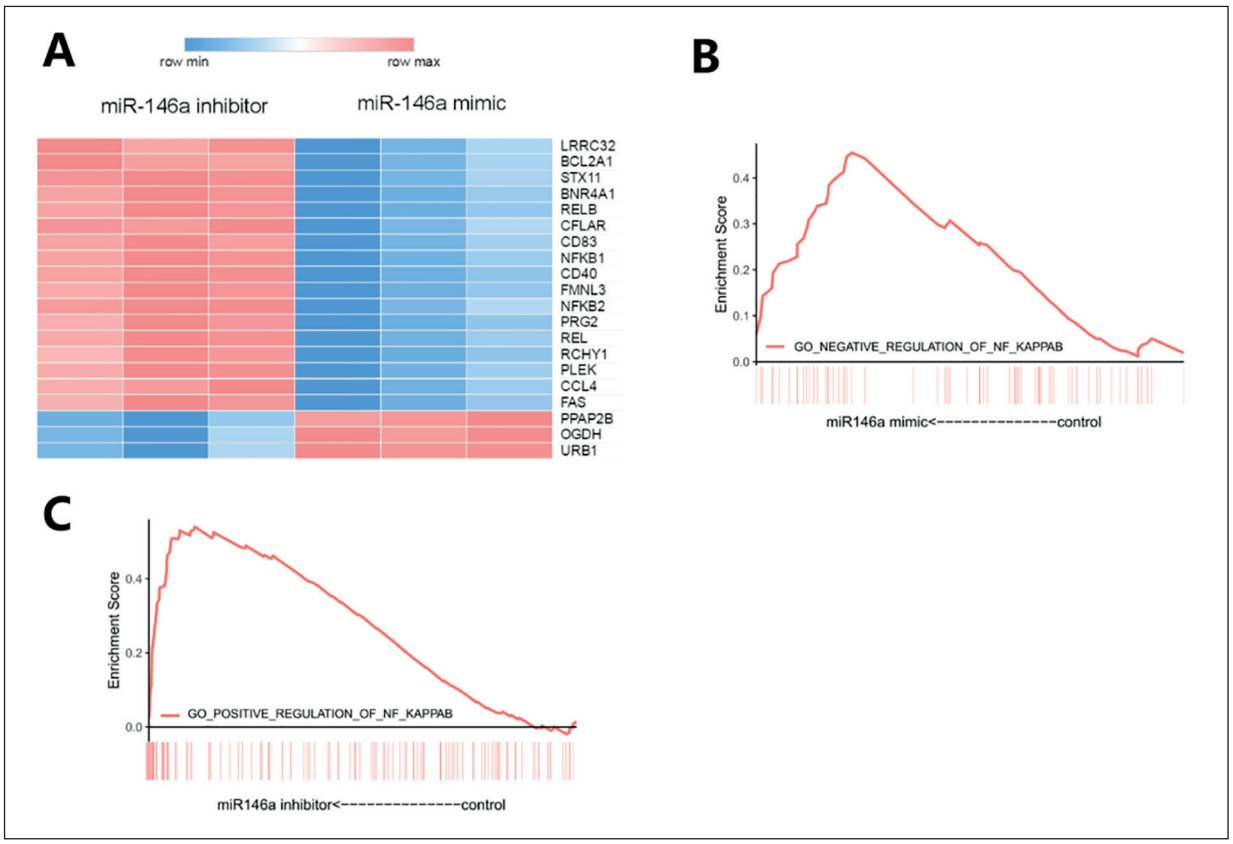

Figure 4. Effect of miR-146a on kidney tissues in MRL/lpr mice analyzed using gene expression chip. ***A,*** Heat map of NF-κB regulatory genes, ***B, C,*** GSEA results analysis.

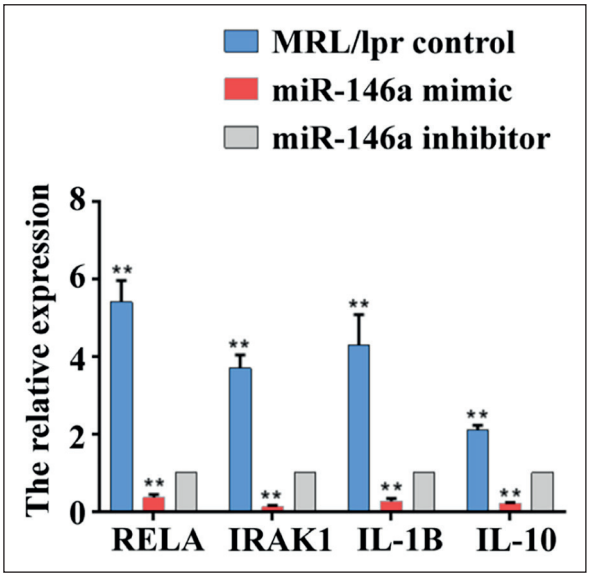

Figure 5. MiR-146a mimic significantly inhibited the inflammation of kidney tissues. **$p<0.01$.

level of NIK in the cytoplasm when compared with control group ($p<0.05$). In contrast, miR-146a inhibitor significantly increased the protein expression levels of p52 and RelB in the nucleus, reduced the protein expression level of p100 in the cytoplasm, and increased the protein expression level of NIK in the cytoplasm compared with control group ($p<0.05$) (Figure 7).

Discussion

MiRNAs are a kind of non-coding nucleotide sequences with 20-25 bp in length. Current studies have indicated that miRNAs regulate the expression of multiple target genes through non-specific binding to the 3'-UTR of messenger RNAs (mRNAs). MiRNAs either block the translation of mRNA or directly lead to the degradation of target mRNAs[15]. The accurate complementation is not required for target mRNA during the binding process of miRNAs. Therefore, a single miRNA may be able to regulate multiple mRNAs. Although the effect of miRNAs on individual mRNA is slight, the combined effect is very significant. Moreover, miRNAs play important roles in various biological processes, such as immune-regulation, inflammation, cell cycle and stem cell differentiation. Most miRNAs are conserved in multiple species, indicating their importance in evolution as key biological processes and pathways[16]. With the discovery of miRNAs and their importance as key regulators in diseases, it is of great significance to explore the potentials of miRNAs as therapeutic drugs.

In the present study, the effect of miR-146a on SLE model mice was explored. Currently, female MRL/lpr mice are internationally recognized as the most classical SLE model animals. The symptoms of autoimmune diseases are extremely sim-

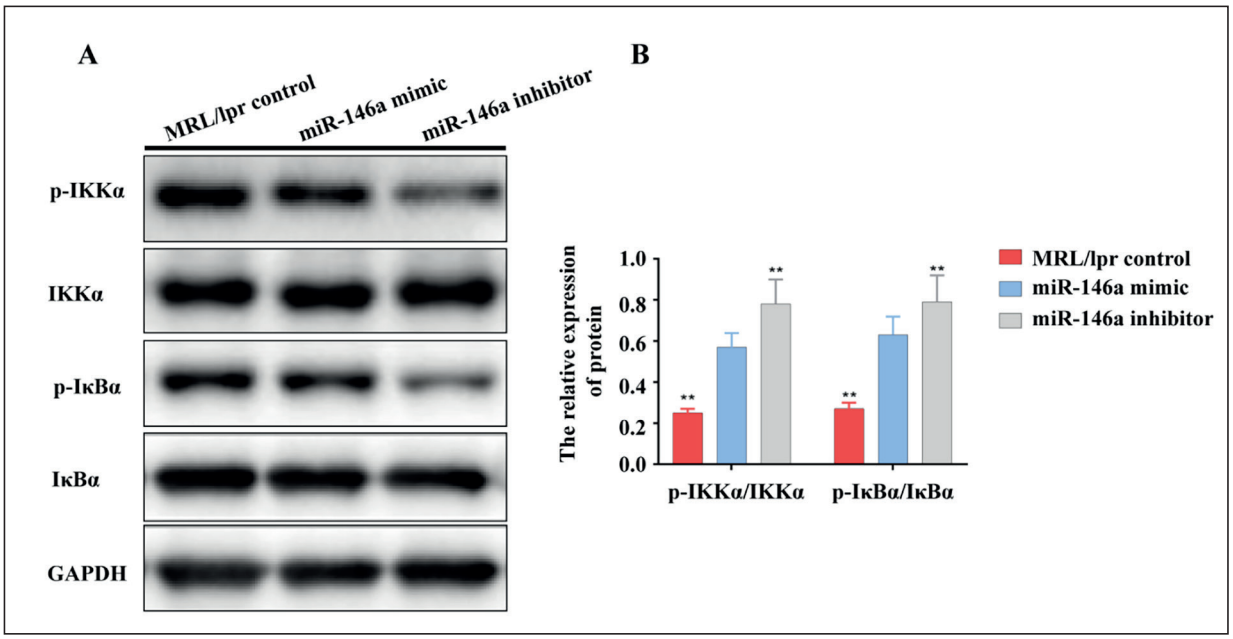

Figure 6. Effect of miR-146a mimic on classical NF-κB signaling pathway. **$p<0.01$.

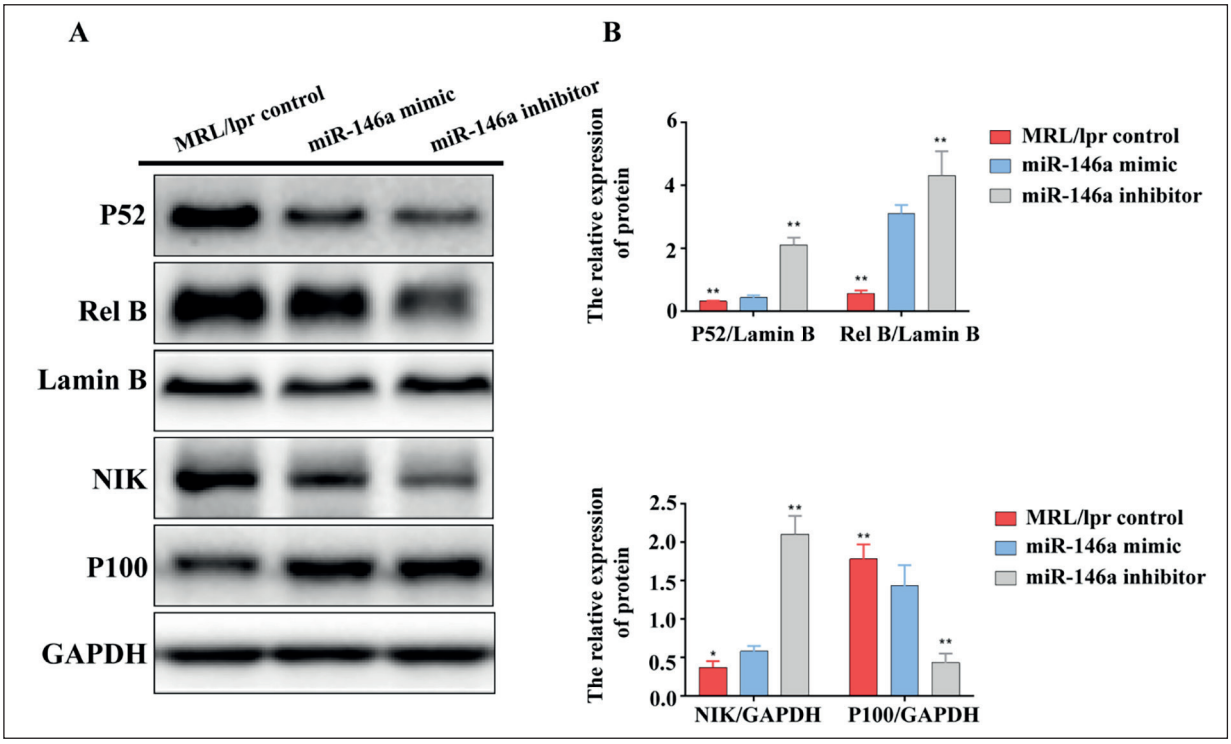

Figure 7. Effect of miR-146a mimic on non-classical NF-κB signaling pathway. **$p<0.01$.

ilar to those of humans. For example, due to immune dysfunction during growth, MRL/lpr mice suffer from glomerulonephritis and other kidney injury symptoms. Meanwhile, urine protein and BUN content increase significantly, and a large number of autoantibodies such as anti-dsDNA, anti-ssDNA, ANA and anti-chromatin are produced. The results of the present study revealed that miR-146a could significantly reduce kidney injury in MRL/lpr mice, down-regulate urine protein and BUN content, and decrease serum autoantibodies of anti-dsDNA, anti-ssDNA, ANA and anti-chromatin. In addition, miR-146a could also significantly reduce the expressions of inflammatory factors (RELA, IRAK1, IL1B and IL10) in kidney tissues of MRL/lpr mice. Furthermore, it markedly prolonged the survival time of MRL/lpr mice. The above findings indicated that miR-146a played a beneficial role in SLE-induced kidney injury.

Subsequently, the potential mechanism of miR-146a in improving kidney injury in MRL/lpr mice was further explored. The results of gene expression chip technique showed that NF-κB regulatory genes exhibited significant changes in the mRNA level in miR-146a mimic group. Moreover, the results of GSEA pointed out that differentially expressed genes in miR-146a mimic group were significantly enriched in the NEGATIVE_REGULATION_OF_NF_KAPPAB expression tag (NS=0.47, $p=5.5\times10^{-10}$). These results suggested that miR-146a mimic might improve SLE-induced kidney injury through regulating the NF-κB signaling pathway. Besides, the effects of miR-146a mimic on classical and non-classical NF-κB signaling pathways were investigated *via* Western blotting. It was found that miR-146a mimic could simultaneously inhibit classical and non-classical NF-κB signaling pathways. SLE is characterized by the production of a large number of autoantibodies by B cells. This may eventually lead to the formation and deposition of immune complexes and even tissue damage. Current research evidence has demonstrated that B cells play a key role in the pathological process of SLE. Moreover, it is well-known that the activation of non-classical NF-κB signaling pathway plays an important role in the massive production of autoantibodies by B cells[17].

Conclusions

We observed that miR-146a reduces SLE-induced kidney injury in MRL/lpr mice through

regulating the classical and non-classical NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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