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MiR-146a alleviates inflammation of acute gouty arthritis rats through TLR4/MyD88 signal transduction pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of micro-ribonucleic acid (miR)-146a on acute gouty arthritis rats through Toll-like receptor-4/myeloid differentiation factor 88 (TLR4/MyD88) signal transduction pathway.

MATERIALS AND METHODS: A total of 30 clean-grade Sprague-Dawley rats were divided into three groups, including agomiR-146a group (n=10), antagomiR-146a group (n=10) and negative control group (NC, n=10). The model was successfully established *via* a one-time injection of sodium urate into ankle joint cavity. Subsequently, agomiR-146a (10 μL), antagomiR-146a (10 μL) and normal saline (10 μL) were intrathecally injected into rats in the three groups at 1 h before injection and 12 h, 24 h, 48 h and 72 h after injection, respectively. The ankle joint swelling index, joint dysfunction index and joint inflammation index of rats in the three groups were closely monitored. After 72 h of observation, the rats were euthanized, and synovial tissues were collected from the knee joint. The expression and distribution of nuclear factor-κB (NF-κB) in synovial tissues were detected using the immunohistochemical method. Meanwhile, the expression levels of inflammatory factors, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) were detected *via* enzyme-linked immunosorbent assay. Furthermore, the mRNA and protein expression levels of TLR4 and MyD88 were detected *via* quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blotting, respectively.

RESULTS: No statistically significant differences in the joint swelling index, joint dysfunction index, joint inflammation index, TLR4 and MyD88 and related inflammatory factors were found between the NC group and antagomiR-146a group. Compared with the NC group, agomiR-146a group showed markedly reduced ankle joint swelling index ($p<0.05$). Meanwhile, joint landing behavior and inflammatory swelling were significantly relieved in the agomiR-146a group ($p<0.05$). The mRNA and protein expression levels of TLR4 and MyD88 were remarkably decreased as well ($p<0.05$). Furthermore, the expression and distribution of NF-κB in synovial tissues of agomiR-146a group was markedly reduced when compared with the NC group ($p<0.05$). In addition, agomiR-146a group exhibited significantly lower expression levels of inflammatory factors (TNF-α, IL-1 and IL-6) in synovial tissues ($p<0.05$).

CONCLUSIONS: MiR-146a alleviates joint inflammation of acute arthritis in rats through the TLR4/MyD88/NF-κB signaling pathway, which may become a new therapeutic target.

Key Words:
MiR-146a, TLR4/MyD88, Acute gouty arthritis, Rats.

Introduction

Gouty arthritis, known as a metabolic disease, exerts higher morbidity rate among men and women currently. The specific pathogenic factor of gouty arthritis is declined uric acid excretion or metabolic abnormality of purine. With the improvement of living standards and the changes in lifestyle, the morbidity rate of gout has greatly increased in recent years. The main clinical features of gouty arthritis include urate crystal deposition and hyperuricemia. According to different types and severity, gouty arthritis is divided into acute arthritis, tophus and chronic tophaceous arthritis. Meanwhile, complications, such as uratic urinary calculi, joint disability and renal failure, may also occur in severe cases[1,2]. Failure to alleviate the hyperuricemia is the key leading to gout. In

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addition, inflammatory factors, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), have been indicated to play important roles in the occurrence and development of gout[3].

Membrane-type pattern recognition receptor can activate innate immune response to aggravate acute joint inflammation. Toll-like receptor-4 (TLR4) is one of the important members of innate immunity. TLR4 is also a kind of transmembrane protein receptor, which is significantly related to the recognition signaling pathway of urate crystal in the body. Its downstream signaling molecule, myeloid differentiation factor 88 (MyD88), nuclear factor-κB (NF-κB) and some inflammatory factors can eventually lead to a variety of inflammatory responses. Liu-Bryan et al[4] have demonstrated that they play extremely important roles in the regulation of gout immune and inflammatory responses. Therefore, further studying the mechanism of toll-like receptors (TLRs) and relevant signaling pathways makes it possible to find more effective therapeutic targets for gout[5]. Studies have demonstrated that micro ribonucleic acid (miR)-146a is involved in the progression of malignant tumors. Qing et al[6] have also shown that the role of miR-146a in the inflammatory response cannot be ignored. For example, miR-146a can regulate 1195 target genes in rats, including TNF-α and IL-6[7]. However, whether miR-146a affects the occurrence and development of arthritis by regulating the TLR4 signal transduction pathway in acute arthritis remains unclear.

In the present work, the contents of serum IL-1, IL-8, and TNF-α in acute arthritis rats were detected through the intervention of miR-146a expression. Meanwhile, the influence of miR-146a on acute gouty arthritis rats and its possible mechanism were explored. Our findings might lay a solid foundation for subsequent in-depth research.

Materials and Methods

Research Animals

A total of 30 male clean-grade Sprague-Dawley rats weighing (200 ± 20) g were provided by the Nanjing Medical University Laboratory Animal Center. All rats were fed strictly in accordance with the standards of the animal laboratory with free access to food and water. All animal experiments were reviewed and approved by the Ethics Committee of Nanjing Medical University. The rat model of acute gouty arthritis was successfully established *via* injection of 200 μL of sodium urate solution (2.5 g/100 mL) into the ankle joint cavity. At the day of modeling and 3, 5, 7, and 10 d after modeling, agomiR-146a (10 μL) or antagomiR-146a (10 μL) was injected to intervene miR-146a *in vivo*, respectively. Meanwhile, an equal amount of normal saline was injected in rats of the NC group.

Reagents and Instruments

Sodium urate crystals (MSU, Sigma-Aldrich, St. Louis, MO, USA), SP-9001 immunohistochemical staining kit (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China), RNA extraction kit (Invitrogen, Carlsbad, CA, USA), quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) kit (Jiancheng, Nanjing, China), bicinchoninic acid (BCA) protein quantification kit and cell lysis buffer (Hanbio, Shanghai, China), TLR4, MyD88 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology Inc., Danvers, MA, USA), and IL-6, IL-1β, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits (Hanbio, Shanghai, China).

Swelling Index of Tested Joints

1 d before modeling, the day of modeling, and 3, 5, 7 and 10 d after modeling, the ankle joint of rats was measured, respectively. The circumference was recorded using a tape at the same position of modeled joints. The average value was calculated. Subsequently, the swelling index at different time points was calculated as follows: swelling index = joint circumference at the measurement time point- initial circumference (joint circumference measured at 1 d before modeling)/ initial circumference.

Dysfunction Index of Tested Joints

The joint dysfunction index was graded as follows: grade 0: normal landing and walking; grade 1: toe flexion, poor landing and slight claudication; grade 2: foot huddling, no landing or upper foot surface landing and significant claudication. The influence of miR-146a on acute arthritis in rats was detected according to different grades.

Inflammation Index of Tested Joint

The influence of miR-146a on acute arthritis in rats was evaluated using the following joint inflammation index (grading criteria: grade 0:

normal; grade 1: joint skin erythema, mild swelling and visible bone landmarks; grade 2: marked joint redness and swelling but restricted in local joint, and disappearance of bone landmarks; and grade 3: extra-articular limb swelling).

Expression of Inflammatory Cytokines in Synovial Tissues Via ELISA

Synovial tissues cryopreserved at -80°C were first collected and placed in an Eppendorf tube (EP; Eppendorf, Hamburg, Germany). An appropriate amount of tissue lysis buffer was added, followed by fully ground with a grinder. Then, the tissues were placed on ice for 15 min, followed by centrifugation at 12000 g and 4°C for 5 min. The supernatant was transferred into another new EP tube, and the concentration of the proteins in the above samples was determined using the BCA method. The expression levels of inflammatory factors (including TNF-α, IL-1, and IL-6) in synovial tissues were detected according to the instructions of ELISA kits.

The MRNA Expression Levels of TLR4 and MyD88 Via qRT-PCR

50 mg synovial tissues cryopreserved at -80°C were taken and placed into EP tubes. 200 μL of TRIzol (Invitrogen, Carlsbad, CA, USA) was added into each tube, and the tissues were ground with a grinder on ice. TRIzol reagent was added until the total volume was 1 mL. After incubation for 5 min, chloroform was added and violently shaken for 15 s. After incubation for 15 min, the aqueous layer was transferred into another new EP tube, followed by addition of isopropanol. Subsequently, the mixture was shaken up and down several times and centrifuged. The white precipitate at the bottom of the tube was RNA. Then, extracted RNA was washed and dissolved in water, the concentration and purity of RNAs were detected. The absorbance $(A)_{260}/A_{280}$ ratio of 1.8-2.0 indicated qualified RNA. RNA was reverse transcribed into cDNA, and the expression level of mRNA was detected *via* qRT-PCR. The primer sequences used in this study were shown in Table I. Specific reaction conditions were as follows: 94°C for 5 min, 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, for a total of 40 cycles, and 72°C for 5 min. Experimental data were processed using Microsoft Excel. The relative expression level of target genes was calculated by $2^{-\Delta\Delta Ct}$ with GAPDH as a control gene. ΔCt (target gene) = Ct (target gene) – Ct (control gene), ΔΔCt = ΔCt (target gene) – ΔCt (standard value).

Western Blotting

50 mg synovial tissues cryopreserved at -80°C were first collected and placed into EP tubes. 200 μL of radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was added in each tube, and the tissues were fully ground with a grinder on ice for 20 min, followed by shaken once every 5 min. After being fully lysed, the tissues were centrifuged at 12000 rpm and 4°C for 10 min. The supernatant was collected into another new EP tube. The protein concentration was determined according to the instructions of the BCA kit and quantified, and 30 μg proteins were taken for Western blotting. After separated by electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 5% skimmed milk at room temperature for 2 h, the membranes were washed on a shaker and incubated with primary antibodies (1:1000) at 4°C overnight. After washing with Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with corresponding secondary antibody (1:5000) at room temperature for 1 h. The color was developed with enhanced chemiluminescence (ECL;

Table I. qRT-PCR primer sequences.

Gene	Primer	Primer sequence
TLR4	Forward	5'-CTCATCGCAGATGCCTGGAA-3'
	Reverse	5'-TTCAGGTAATAGGCACCCTTGAAGA-3'
MyD88	Forward	5'-ACGCACGACGTCTTCCAGTA-3'
	Reverse	5'-CCACCTGGTTCAACTCACTCC-3'
GAPDH	Forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
	Reverse	5'-CGCTTCACGAATTTGCGTGTCAT-3'

Table II. Comparison of swelling index of tested joint.

Group	n	1 d before modeling	The day of modeling	3 d after modeling	5 d after modeling	7 d after modeling	10 d after modeling
NC	8	0.14 ± 0.10	0.18 ± 0.10	0.23 ± 0.13	0.27 ± 0.16	0.36 ± 0.19	0.38 ± 0.12
AntagomiR-146a	8	0.14 ± 0.09	0.17 ± 0.09	0.24 ± 0.09	0.29 ± 0.11	0.36 ± 0.17	0.39 ± 0.13
AgomiR-146a	8	0.15 ± 0.08	0.18 ± 0.13	0.19 ± 0.10*	0.20 ± 0.12*	0.25 ± 0.11**	0.27 ± 0.15**

Note: *p<0.05 & **p<0.05 *vs*. NC group.

Thermo Fisher Scientific, Waltham, MA, USA) in the dark, followed by scanning and data record using a gel imager (Bio-Rad Laboratories, Hercules, CA, USA). Finally, the gray level was analyzed and compared. GAPDH was used as an internal reference.

The Protein Expression of NF-κB in Pathological Tissues Via Immunohistochemistry

First, synovial tissues fixed were embedded in paraffin, sliced into sections and deparaffinized. Endogenous peroxidase was inactivated with 3% H_2O_2, followed by antigen retrieval *via* microwave. Then, the tissues were sealed with serum at an appropriate concentration, and marked with an immune-histochemical pen. Subsequently, the tissues were incubated with primary antibody in a wet box at 4°C overnight in the dark. On the next day, the tissues were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) 3 times. After incubation with corresponding secondary antibody for 30 min, the tissues were washed again with PBS 3 times. The color was developed with 3,3'-Diaminobenzidine tetrahydrochloride (DAB; Solarbio, Beijing, China). The tissues were added dropwise with resin glue, covered with cover glass and photographed under a microscope (TE2000-U, Nikon, Tokyo, Japan).

Statistical Analysis

Experimental data were expressed as mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. One-way analysis of variance was performed to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). p<0.05 was considered statistically significant.

Results

Comparison of Swelling Index of Tested Joint

No statistically significant difference in the swelling index of tested ankle joints was found between NC group and antagomiR-146a group. However, the swelling index of tested ankle joints was markedly declined in agomiR-146a group (p<0.05; Table II).

Comparison of Inflammation Index of Tested Joint

Compared with the NC group, agomir-146a group showed significantly alleviated skin erythema, bone landmarks and extra-articular limb swelling. However, no remarkable difference was found between NC group and antagomiR-146a group (Table III).

Table III. Comparison of inflammation index of tested joint.

Group	n	1 d before modeling	The day of modeling	3 d after modeling	5 d after modeling	7 d after modeling	10 d after modeling
NC	8	0	0	1	2	3	3
AntagomiR-146a	8	0	0	1	2	3	3
AgomiR-146a	8	0	0	1	1*	2	2

Note: Grading criteria for joint inflammation index: grade 0: normal, grade 1: joint skin erythema, mild swelling and visible bone landmarks, grade 2: obvious joint redness and swelling but restricted in local joint, and disappearance of bone landmarks, and grade 3: extra-articular limb swelling.

Table IV. Comparison of dysfunction index of tested joint.

Group	n	1 d before modeling	The day of modeling	3 d after modeling	5 d after modeling	7 d after modeling	10 d after modeling
NC	8	0	0	1	2	2	2
AntagomiR-146a	8	0	0	1	2	2	2
AgomiR-146a	8	0	0	0	1	1	1

Note: Grading criteria for joint dysfunction index: grade 0: normal gait and landing, grade 1: slight claudication, reduced landing and toe flexion, grade 2: foot flexion, upper foot surface landing and evident claudication.

Comparison of Dysfunction Index of Tested Joint

Compared with the NC group, rats in agomiR-146a group exhibited remarkably improved normal gait, landing, toe splaying and claudication. However, no marked difference was found between NC group and antagomiR-146a group (Table IV).

Influence of MiR-146a on the Expression of Related Inflammatory Factors

As shown in Figure 1, compared with the NC group, the expression levels of inflammatory factors (including TNF-α, IL-1 and IL-6) were remarkably decreased in the agomiR-146a group (p<0.05). However, they were not significantly altered in the antagomiR-146a group.

Influences of MiR-146a on the mRNA Expression Levels of TLR4 and MyD88

As shown in Figure 2, compared with the NC group, the mRNA expression levels of TLR4 and MyD88 were markedly decreased in the agomiR-146a group (p<0.05). However, they were not significantly changed in the antagomiR-146a group. This indicated that miR-146a could significantly affect the mRNA expression levels of TLR4 and MyD88.

Influence of MiR-146a on the Protein Expression Levels of TLR4 and MyD88

As shown in Figure 3, compared with the NC group, the protein expression levels of TLR4 and MyD88 were remarkably decreased in the agomir-146a group (p<0.05), whereas they exhibited no marked differences in the antagomir-146a group. The above findings suggested that miR-146a significantly affected the protein expression levels of TLR4 and MyD88.

Influence of MiR-146a on NF-κB Expression

As shown in Figure 4, compared with the NC group, agomiR-146a group showed remarkably decreased expression level of NF-κB protein. However, no significant difference was found in the antagomiR-146a group. These results demonstrated that miR-146a significantly affected the expression level of NF-κB protein.

Discussion

In recent years, the incidence rate of gout has been greatly increased. It has become the most common inflammatory joint disease in

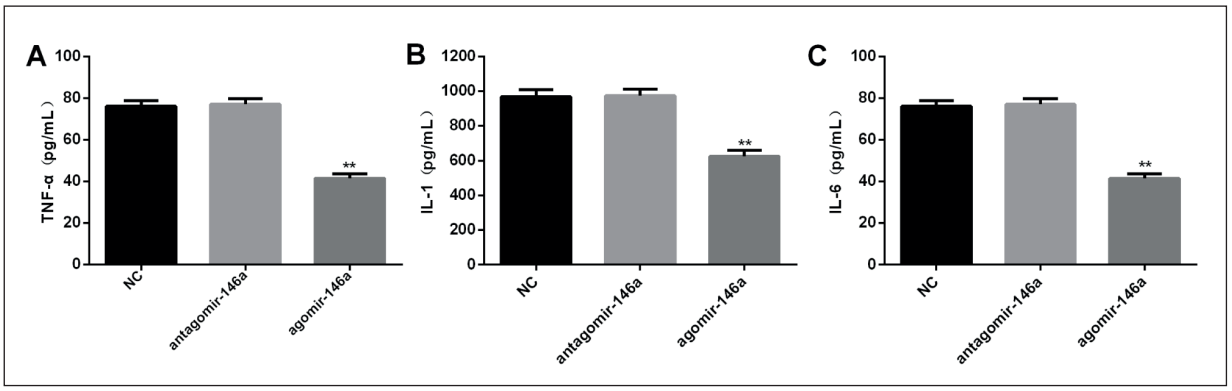

Figure 1. Influence of miR-146a on the expression of related inflammatory factors detected *via* ELISA. **p<0.01 *vs.* NC group.

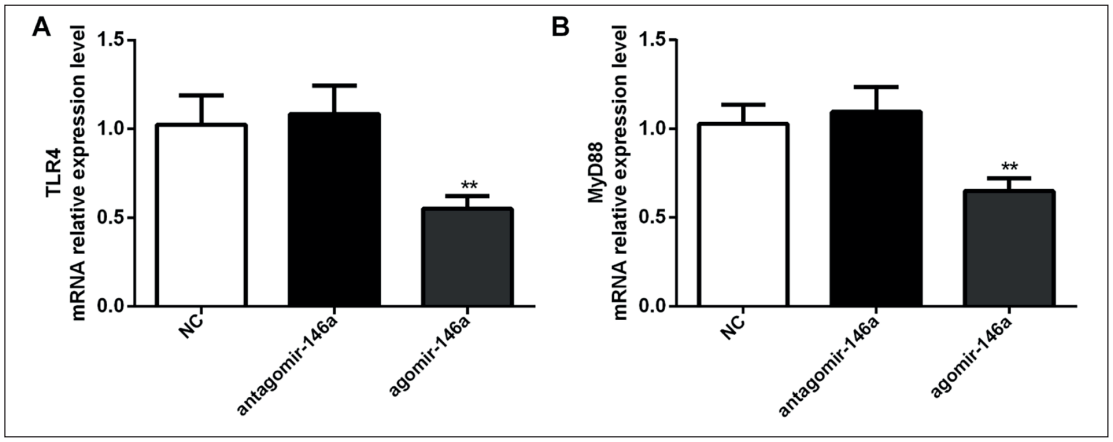

Figure 2. Influence of miR-146a on the mRNA expression levels of TLR4 and MyD88 detected *via* qRT-PCR. ***p*<0.01 *vs.* NC group.

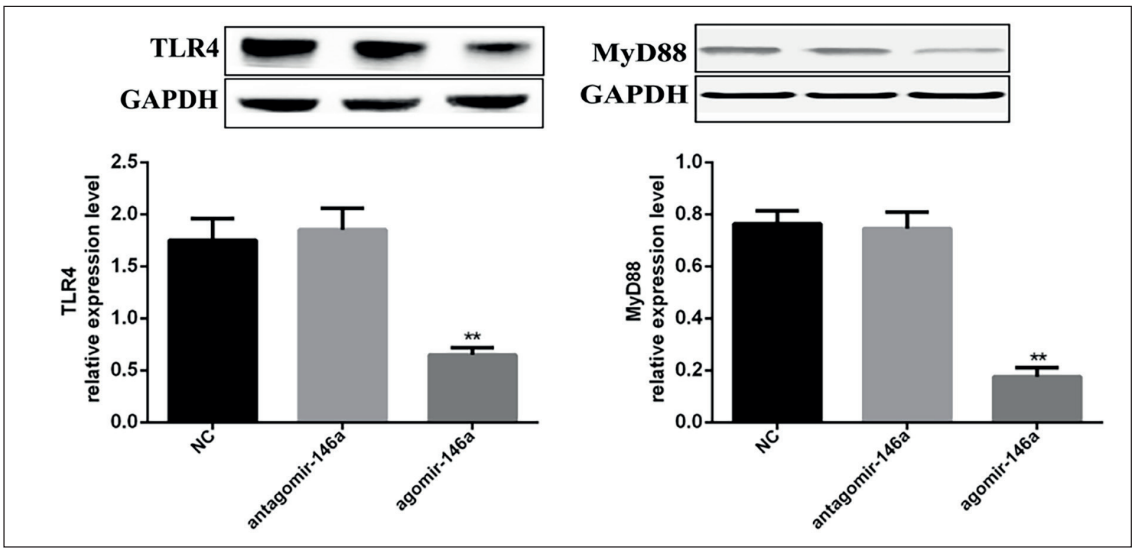

Figure 3. Influence of miR-146a on the protein expression levels of TLR4 and MyD88 detected *via* Western blotting. ***p*<0.01 *vs.* NC group.

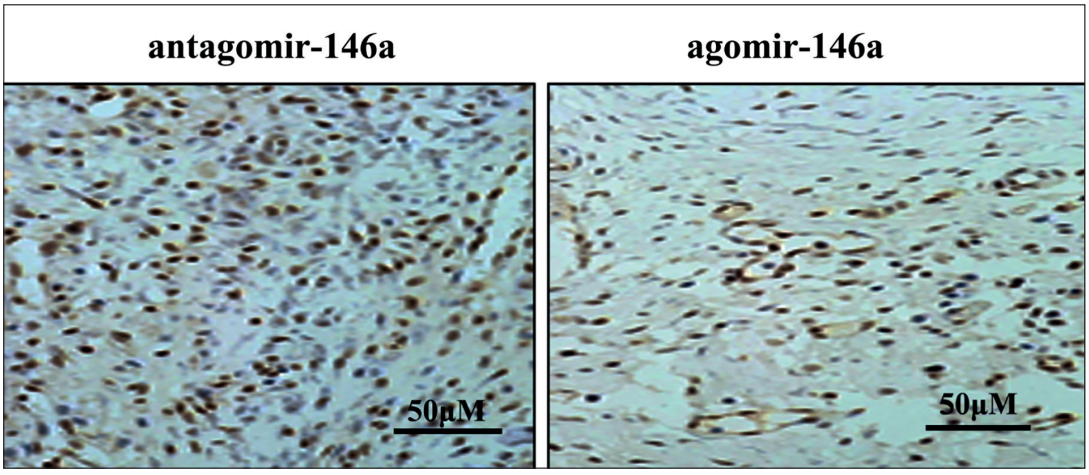

Figure 4. Influence of miR-146a on the protein expression level of NF-κB detected *via* Western blotting.

men, whose morbidity rate has far surpassed that of rheumatoid arthritis in elderly women[8]. Therefore, it is significant to search for new effective therapeutic targets for gout.

The most important pathophysiological basis of gout is an acute inflammatory response of joints. Membrane-type pattern recognition receptor can activate the innate immune response to aggravating acute joint inflammation. TLR4, known as one of the important members of innate immunity, is also a kind of transmembrane protein receptor. It has been confirmed to be related to recognition signaling pathway of urate crystal in the body. The downstream signaling molecule, MyD88, NF-κB and some inflammatory factors can eventually lead to a variety of inflammatory responses[9]. The MyD88-dependent signal transduction pathway is dominated in the TLR4 signaling pathway. Meanwhile, the adaptor protein MyD88 acts as a bridge in the TLR signaling pathway as well. Appropriately inhibiting the expressions of TLR4 and MyD88 can significantly inhibit the occurrence and development of autoimmune and inflammatory diseases[10].

In the MyD88-dependent signal transduction pathway, TNF receptor-associated factor 6 (TRAF6) acts as key transduction and transcription protein. Once activated, TRAF6 can activate different key signaling pathways, including the NF-κB pathway. Meanwhile, it can promote the expression of different pro-inflammatory cytokines. More importantly, after the activation of NF-κB, the transcription of different genes is initiated. This may eventually lead to the release of a large number of inflammatory factors. Furthermore, these inflammatory factors can promote the activation of NF-κB as feedback regulators. Therefore, the pro-inflammatory signaling pathway, as a positive feedback regulator, amplifies inflammatory response[11-13]. MiR-146a is not only involved in innate immune-regulation, but also in tumorigenesis and inflammatory development. Studies[14,15] have demonstrated that IRAKI and TRAF6 are important target genes of miR-146a. Meanwhile, they play key roles in activating downstream signaling pathways as key members in TLR4 signal transduction pathway[16,17]. MyD88 can form the complex with IRAKI to activate downstream proteins and release the transcription factor of NF-κB. After that, the transcription of different genes is initiated, and the expression of some cytokines is significantly up-regulated[18,19]. However, whether miR-146a directly inhibits the TLR4 signal transduction pathway remains unclear. Furthermore, whether *in vivo* intervention of miR-146a expression can improve the progression of acute arthritis in rats has not been fully elucidated.

In the present work, the rat model of acute gouty arthritis was successfully established *via* a one-time injection of sodium urate into the ankle joint cavity. Subsequently, the intervention of miR-146a expression was performed *in vivo*. The ankle joint swelling index, joint dysfunction index and joint inflammation index of rats in each group were regularly observed. The mRNA and protein expression levels of TLR4 and MyD88 were detected *via* qRT-PCR and Western blotting, respectively. Meanwhile, the expression levels of inflammatory factors (including TNF-α, IL-1 and IL-6) were detected *via* ELISA. The expression of NF-κB in synovial tissues was detected using the immunohistochemical method. The results revealed that compared with the NC group, highly expressed miR-146a could significantly improve the joint swelling index, joint dysfunction index and joint inflammation index. Meanwhile, high expression of miR-146a markedly inhibited the expressions of TLR4, MyD88, related inflammatory factors and NF-κB in synovial tissues. Similarly, Wang et al[20] have found that inhibiting the expression of TLR4/NF-κB and MyD88 can significantly inhibit joint inflammation in arthritic rats. Qing et al[21] have demonstrated that the expression of TLR4 and NF-κB in patients with acute gouty arthritis is significantly higher than that of normal healthy people. The above findings all indicate that TLR4 plays an important role in the acute inflammatory response in gout patients.

Conclusions

We showed that miR-146a can alleviate joint inflammation of acute arthritis rats. The possible underlying mechanism may be related to the inhibition of the TLR4/MyD88/NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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