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MiR-21 relieves rheumatoid arthritis in rats *via* targeting Wnt signaling pathway

X.-G. LIU[1], Y. ZHANG[2], W.-F. JU[1], C.-Y. LI[3], Y.-C. MU[4]

[1]Department of Rheumatology, 970 Hospital of the Chinese People's Liberation Army, Weihai, China
[2]Department of Anesthesiology, 970 Hospital of the Chinese People's Liberation Army, Weihai, China
[3]Department of Nephrology, 970 Hospital of the Chinese People's Liberation Army, Weihai, China
[4]Department of Neurology, 970 Hospital of the Chinese People's Liberation Army, Weihai, China

Xiaoguang Liu and *Yan Zhang* contributed equally to this work

Abstract. – OBJECTIVE: To investigate the influence of micro ribonucleic acid (miR)-21 on rats with rheumatoid arthritis (RA) through the Wnt signaling pathway.

MATERIALS AND METHODS: A total of 30 rats were divided into three groups: control group (healthy rats, n=10), model group (rat model of RA, n=10), and MiR group (rat model of RA injected with miR-21 lentivirus, n=10). The paw volume, arthritis indexes, and protein expression level in each group were analyzed by means of paw volume and arthritis index measurement, reverse transcription-polymerase chain reaction (RT-PCR) assay, and fluorescent Western blotting.

RESULTS: The expression levels of inflammatory factors declined in MiR group compared with those in model group, while they were higher in model group than those in control group and MiR group (p<0.05). At 15 d after transfection with lentivirus, the paw volume in MiR group was smaller than that in model group, which was decreased markedly with the extended time of transfection (p<0.05). On the 30th d, MiR group had a remarkably smaller paw volume than model group. In comparison with that in control group, the paw volume in model group was increased notably from the 7th d and displayed a significant difference in the 30th d (p<0.05). The arthritis indexes in MiR group were lower than those in model group; however, there were no apparent inflammations at the joints at 15 d after drug administration. Moreover, the longer the time of drug administration was, the less apparent the inflammations at the joints will be. The inflammations at the joints were ameliorated evidently on the 30th d in MiR group (p<0.05). Compared with those in control group, the inflammations in model group were increased significantly from the 7th d, with significant differences in the 30th d (p<0.05). The messenger RNA (mRNA) expression levels of interleukin-6 (IL-6), IL-8, and Wnt in MiR group were higher than those in control group, but lower than those in model group (p<0.05), while they were higher in model group than those in control group (p<0.05). The expression level of Wnt protein was decreased in MiR group compared with that in model group (p<0.05), and model group had a prominently elevated expression level of Wnt protein in comparison with control group (p<0.05).

CONCLUSIONS: MiR-21 overexpression can repress the expressions of IL-6 and IL-8 and relieve the symptoms of RA by down-regulating the Wnt signal.

Key Words:
MiR-21, Wnt signaling pathway, Arthritis, Rheumatoid, Inflammatory factors.

Introduction

Rheumatoid arthritis (RA) is a chronic disease with a relatively high incidence rate of about 0.35%[1]. It usually invades the synovial membrane of affected joints and causes synovitis and formation of massive pannus, triggering articular cartilage injury, and finally resulting in cartilage and bone destruction[2]. The clinical manifestations of RA include local pathological changes and degenerative changes in the articular cartilage, such as joint synovitis, osteoarticular injury, vascular inflammation, and blood stasis[3]. RA mostly occurs in multiple small joints of the hands and feet and affects the whole joints, thus leading to fibrosis and degeneration of the articular cartilage, as well as deformity and function loss of the joint[4]. Some studies[5] have manifested that Wnt is involved in the chronic inflammations of RA, and it plays roles in limb and embryonic

Corresponding Author: Yanchun Mu, BM; e-mail: mych968@163.com

development. Correlation research revealed that the Wnt signaling pathway-related proteins are decreased remarkably in the synovial cells of RA patients compared with those in normal people, and the major pathogenesis of RA is likely to be immune disorder[5]. Micro ribonucleic acids (miRNAs) exert immunoregulatory effects on the surfaces of various immune cells, including T cells and B cells, and are widely expressed, having close correlations with inflammations and autoimmune diseases[6]. According to reports[7], miR-21 is lowly expressed in the macrophages and peripheral blood mononuclear cells in the synovial membrane and fluid of RA patients. Also, it has protective effects on clinical and experimental arthritis. Angelotti et al[8] elaborated that miR-21 stimulates the abnormal activation of the immune system in RA and serves as a potential biomarker and therapeutic target of diseases, whose pathogeny may be associated with infection and heredity[9]. To deeply understand the mechanisms of the Wnt signaling pathway and miR-21 in RA, a rat model of RA was established to analyze the correlation of the Wnt signaling pathway with inflammatory factors during RA onset and the impact of miR-21 on the disease, providing a new basis for clinical treatment.

Materials and Methods

Materials

Collagenase type I reagent (Sigma-Aldrich, St. Louis, MO, USA), goat-anti-mouse IgG antibody (Shandong Changjian Biological Pharmaceutical Co., Ltd. Changjian, China), interleukin-6 (IL-6) and IL-8 biclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR; Thermo Fisher Scientific, Waltham, MA, USA).

Grouping of Laboratory Animals and Drug Administration

A total of 40 clean female rats weighing (200 ± 18) g were selected. Among them, 10 were enrolled into control group, and the remaining 30 were used to establish the arthritis model. The model was successfully established among 22 rats, with an arthritis index >3 points. Then, 20 out of the 22 rats were randomly selected and divided into model group (n=10) and MiR group (n=10). The rats in control group and model group were intraperitoneally injected with 1.2 mL of normal saline every day, while those in MiR group received a daily intrathecal injection of miR-21 lentivirus (5 μmol) for 7 d. This reseatch was approved by the Animal Ethics Committee of 970 Hospital of the Chinese People's Liberation Animal Center.

Model Establishment

The collagenase type I dissolved in acetic acid (0.12 mol/L) was mixed with CFA in an equal volume to prepare emulsion with a final concentration of 0.6 g/L. Next, the rats were anesthetized by chloral hydrate (0.4 mL/50 g). In model group, multi-point subcutaneous injection of the prepared collagenase type I solution (1.5 mL) was performed on the back of the rats. 5 d later, the injection was conducted once again at the same site using the same dose and method. Moreover, rats in control group were injected with normal saline.

Pathological Sections of Synovial Membrane

The ankle joint synovial tissues collected from the rats under sterile conditions were fixed with 11% paraformaldehyde solution, soaked for 40 h, washed with sterile water for 20 h, and put into 6% nitric acid solution for decalcification for 12 d. The solution was changed every 3 d. After complete decalcification, the tissues were placed in PA solution for later fixation, embedded in paraffin, and stained.

Hematoxylin and Eosin (HE) Staining

The principle of HE staining is that the alkaline hematoxylin can stain the cells blue, and the acidic eosin can stain the cells red, with a final result of the blue nucleus and red cytoplasm. The HE staining also possesses the physical effect of adsorption. Therefore, it can offer a preferable dyeing effect of nucleus and cytoplasm contrast. The adherent cells were prepared and digested using trypsin, and the concentration was adjusted to about 1×10^5/mL. Then, the cells were added on to glass slides in drops and cultured for 7 d. After that, the coverslips were taken out and washed with phosphate-buffered saline (PBS). Next, the samples were fixed in 95% ethanol for 14 min and washed with PBS for 3 times (2 min/time), followed by staining with hematoxylin for 1-3 min and washing in clear water. If the nucleus was stained deep color observed under a microscope, 1% hydrochloric acid solution was utilized for the color separation. The cytoplasm was immersed in eosin for staining, followed by washing with clear water, air drying, and mounting.

Observation of Indicators of Paw Volume and Arthritis

1. Indicator of paw volume: the changes in the paw swelling volume at 0, 15, 22, and 30 d after drug administration in each group were observed and recorded, and the blood was drawn from the abdominal aorta after the last drug administration. Next, the rats were sacrificed, and the inflammatory ankle joints of both hind paws were taken and fixed with neutral formalin solution, followed by decalcification, dehydration in ethanol, and clearing with xylene. After the joints were embedded in paraffin, deparaffinized, and dehydrated in sequence, the nuclei were stained with hematoxylin, and the sections were mounted and observed in high-power fields under a light microscope. Proliferation of synovial cells: no abnormality, mild abnormality (dense and swelling monolayers), moderate abnormality (synovial cells proliferating as two layers), and severe abnormality (synovial cells proliferating as three or more than three layers). Infiltration of inflammatory cells: no abnormality, mild abnormality (a small amount of sparse and scattered inflammatory cells), moderate abnormality (a moderate amount of relatively dense inflammatory cells), and severe abnormality (a large amount of dense inflammatory cells).
2. Indicator of arthritis: arthritis index: 0 point (no apparent inflammations at the joints), 1 point (mild inflammation at the joint of little toe), 2 points (apparent inflammations at the toe joints and toes), 3 points (apparent inflammations in the paws below the ankle joints), and 4 points (apparent inflammations in the whole paws including the ankle joints) (Table I).

RT-PCR Assay

The rat's articular cartilage tissues were digested with trypsin, flushed with phosphate-buffered saline (PBS), and added dropwise with 0.9% Nacl solution. Then, the tissues were added with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) reagent and 2 mL of RNA reagent to extract the RNA. Next, 20 mg of tissue cells were placed into the Eppendorf (EP) tube, and the RNA extracted was reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using A3500 reverse transcription (RT) kit. After that, the product was applied to determine the expressions of target genes *via* nucleic acid gel stain. The related primers utilized were designed using NCBl/Primer. The reaction conditions were as follows: at 98°C for 6 min, at 8°C for 28 s, 75°C for 30 s, and 80°C for 4 min in the solution for 55 cycles in total. Effective denaturation could be realized in each cycle. The primer sequences of genes are shown in Table II.

Detection of Wnt Protein Expression Via Fluorescent Western Blotting Assay

The synovial tissues of the joint were taken from the rats, washed with PBS by shaking, and added with mouse anti-human Survivin dual antibodies (diluted at 1:24) in drops, followed by incubation in a wet box at 36°C for 25 min. Then, the tissues were taken out and cooled to room temperature, and fluorescence-labeled horse anti-mouse secondary antibodies (diluted at 1:50) were added dropwise, followed by mounting with mounting medium containing 40% glycerol and PBS. Subsequently, the expression results were immediately observed under a fluorescence microscope. The ratio of the area of fluorescence color in the cytoplasm to the total area of cytoplasm >5% suggested the negative expression. Both positive and negative controls were set for each batch of fluorescent Western blotting.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA)) software was used for data analysis. The *t*-test was adopted for evaluation of paw volume, measurement of arthritis index, and comparison of

Table I. Measurement of arthritis indexes.

Location	Status	Index
Joint	No redness and swelling	0 point
Joint of little toe	Mild swelling	1 point
Toe joints and toes	Swelling	2 points
Paws below the ankle joints	Swelling	3 points
Whole paws including the ankle joints	Swelling	4 points

Table II. Primer sequences of genes.

Protein	Gene	Primer sequence
IL-6	Forward	5'-CCGGAGCTGAATGACGCTCTCAGG-3'
	Reverse	5'-TACTGTAATGTTTTGATTTCATTG-3'
IL-8	Forward	5'-CTTAGGGCTGAGCTGGTGGACGCT-3'
	Reverse	5'-ATTTCATTGAATGTTTACTTGTGT-3'
Wnt	Forward	5'-GAGCTGGCTGAGGTTGTGGACGCT-3'
	Reverse	5'-TGTGCTTTGAATGTTTACTTGTTG-3'
β-actin	Forward	5'-CTCCTCTTGCTCGATAGTCTCTCG-3'
	Reverse	5'-TCGATGTCGAAGACCTTCTTCAAC-3'

protein expression among control group, model group, and MiR group. The count data were expressed as mean ± standard deviation, test criterion $p<0.05$ suggested significant differences, and univariate analysis was used for comparisons within groups.

Results

Observation Results of Pathological Sections of Ankle Joint Synovial Membrane of Rats

The ankle joint capsule and the synovial membrane were in uniform shapes in control group. In model group, there were increased the synovial membranes in the joint, extensive infiltration of inflammatory cells in the synovial tissues and visible cystic hyperplasia in the joint capsule. Vasodilatation was observed and *pannus* was formed in the synovial membrane. The intracapsular ligament was injured, while hyperplasia of synovial membrane and fibrous tissue was detected. The expression levels of inflammatory factors declined in MiR group compared with those in model group, while they were higher in model group than those in control group and MiR group ($p<0.05$) (Figure 1).

Comparison of Paw Volume of Arthritis Rats Among Groups

At 15 d after transfection with lentivirus, the paw volume in MiR group was smaller than that in model group, which was decreased markedly with the extended time of transfection ($p<0.05$). On the 30th d, MiR group had a remarkably smaller paw volume than model group. In comparison with that in control group, the paw volume in model group was increased notably from the 7th d and displayed a significant difference in the 30th d ($p<0.05$) (Table III).

Comparison of Arthritis Indexes of Rats with Arthritis Among Groups

The arthritis indexes in MiR group were lower than those in model group; however, there were no apparent inflammations at the joints at 15 d after drug administration. Moreover, the longer the drug administration was, the less apparent the in-

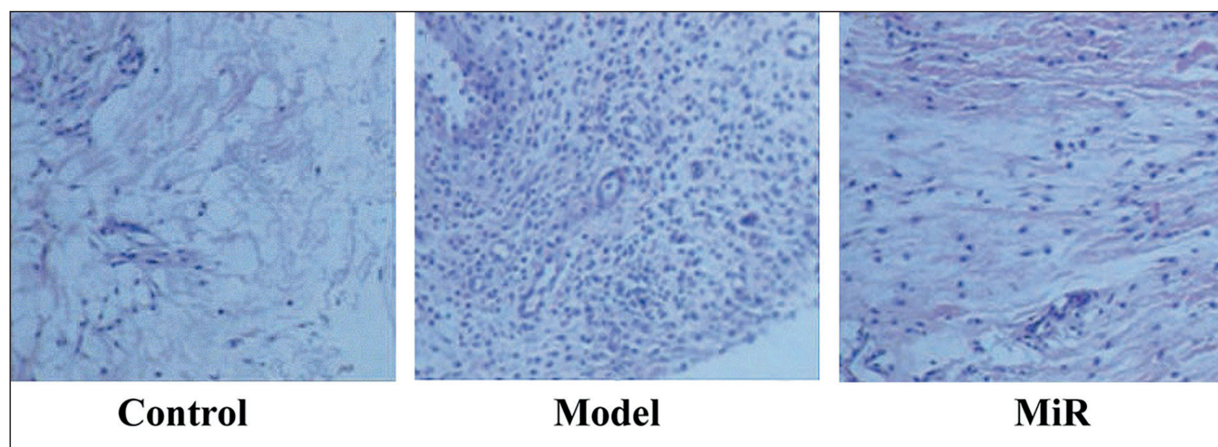

Figure 1. HE staining for ankle joint synovial tissues of each group of rats (magnification ×20).

Table III. arison of paw volume among groups (mL).

Group	0 d	7 d	15 d	22 d	30 d
Control group	1.25 ± 0.02	1.22 ± 0.03	1.23 ± 0.01	1.26 ± 0.02	1.25 ± 0.03
Model group	1.25 ± 0.02	2.26 ± 0.01	3.43 ± 0.03*	3.05 ± 0.01*	3.20 ± 0.02*
MiR group	1.19 ± 0.02	1.83 ± 0.03	2.34 ± 0.19*#	1.72 ± 0.12*#	1.65 ± 0.04*#

Note: *$p < 0.05$ *vs*. control group, #$p < 0.05$ *vs*. model group.

Table IV. Comparison of arthritis indexes among groups.

Group	0 d	7 d	15 d	22 d	30 d
Control group	0	0	0	0	0
Model group	0	1.25 ± 0.01*	2.67 ± 0.03*	3.05 ± 0.01*	3.10 ± 0.02*
MiR group	0	1.02 ± 0.03*#	1.35 ± 0.04*#	1.62 ± 0.12*#	1.5 ± 0.19*#

Note: *$p < 0.05$ *vs*. control group, #$p < 0.05$ *vs*. model group.

flammations at the joints will be. The inflammations at the joints were ameliorated evidently on the 30th d in MiR group (p<0.05). In comparison with that in control group, the arthritis indexes in model group were raised notably from the 7th d and manifested significant differences in the 30th d (p<0.05) (Table IV).

Messenger RNA (mRNA) Expressions of IL-6, IL-8, and Wnt in Synovial Tissues in Each Group of Rats

According to the results of RT-PCR, the mRNA expression levels of IL-6, IL-8, and Wnt in the synovial tissues in model group were higher than those in control group. After treatment with miR-21 lentivirus, the mRNA expression levels of IL-6, IL-8, and Wnt in the synovial tissues were down-regulated evidently in MiR group in comparison with those in model group (Figure 2A). The same results for the protein levels of IL-6, IL-8, and Wnt were obtained from the Western blotting assay (Figure 2B).

Expression of Wnt Protein in Synovial Tissues in Each Group of Rats Detected Via Fluorescent Western Blotting Assay

The expression level of Wnt protein in the synovial tissues was decreased in MiR group compared with that in model group (p<0.05). Also, model group had a prominently elevated expression level of Wnt protein compared with control group (p<0.05) (Figure 3).

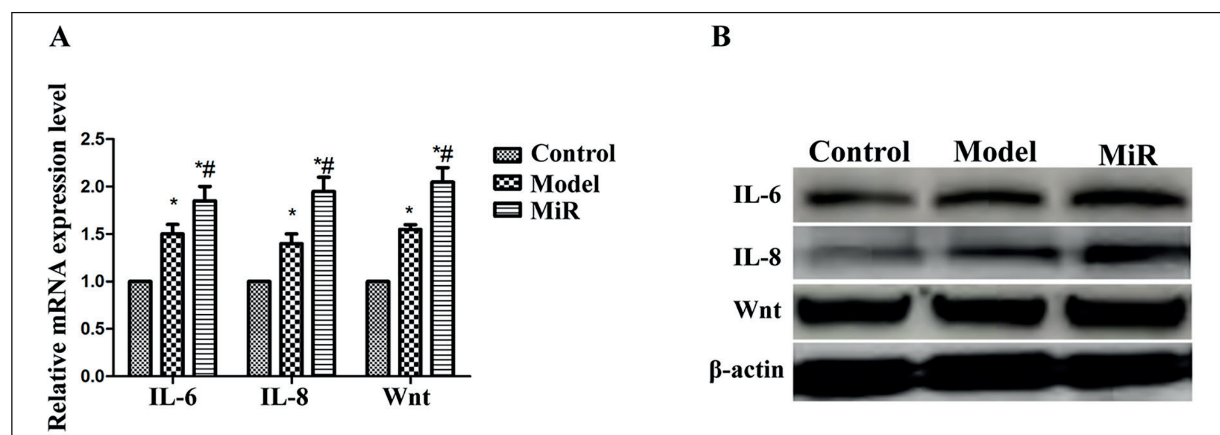

Figure 2. Expressions of IL-6, IL-8, and Wnt in synovial tissues in each group of rats. ***A,*** Relative mRNA expression levels of IL-6, IL-8, and Wnt in osteocytes in each group of rats, ***B,*** Relative protein expression levels of IL-6, IL-8, and Wnt in osteocytes in each group of rats.

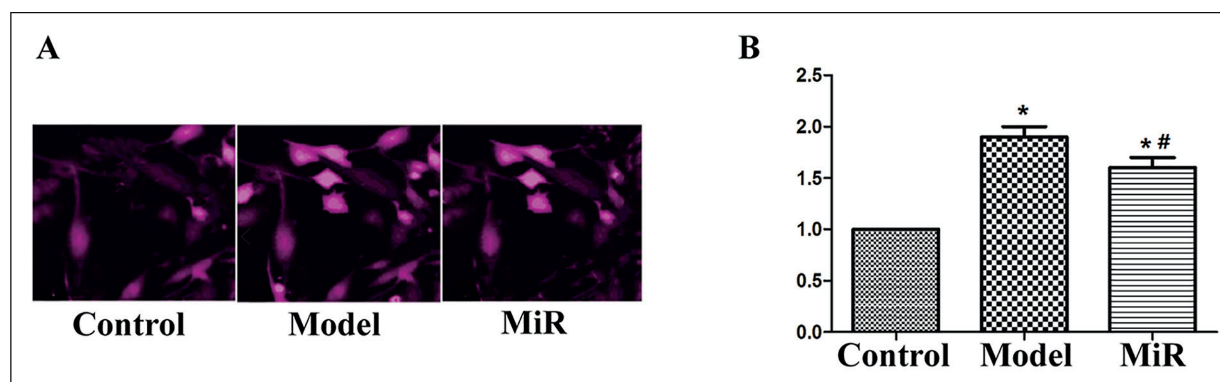

Figure 3. Relative expression of Wnt protein in synovial tissues in each group of rats (magnification ×100).

Discussion

Rheumatoid diseases are common clinical disorders that are often accompanied with the involvement of extra-articular organs and positive rheumatoid factors in the serum, which is inconspicuous in the early stage in most cases, with a slow course. RA can result in disability. Therefore, identifying the pathogenesis of RA and exploring highly efficient therapeutic methods are urgent problems to be solved at present.

In this experiment, the pathological sections of the ankle joint synovial membrane were tested. It was found that the expression levels of inflammatory factors were reduced in MiR group in comparison with those in model group, while they were relatively higher in model group than those in control group and MiR group ($p<0.05$). The high expression of miR-21 is capable of promoting the differentiation of cartilage tissues and protecting the osteocytes. Besides, miR-21 can improve the balance between osteoclasts and osteocytes. With the progression of disease and activities, the bone formation is increased, thus stimulating the proliferation of osteocytes. A study[10] has indicated that miR-21 plays a crucial role in biological equilibrium related to the synovial tissues in RA. The abnormal activation of high miR-21 expression, abnormality of blood coagulation indexes, and imbalance of the cytokine network are closely associated with the bone growth and development.

By comparing the paw volume among the groups in this experiment, at 15 d after transfection with lentivirus, MiR group had a smaller paw volume than model group, and the paw volume was decreased markedly with the prolonged time of transfection ($p<0.05$). On the 30th d, MiR group displayed a notably smaller paw volume than model group. In comparison with that in control group, the paw volume in model group was increased markedly from the 7th d, with a significant difference in the 30th d ($p<0.05$). The comparison of arthritis indexes among groups indicated that the arthritis indexes in MiR group were lower than those in model group. There were no apparent inflammations at the joints at 15 d after drug administration, and the inflammations at the joints became less apparent as the time of drug administration was increased, which were ameliorated evidently on the 30th d in MiR group ($p<0.05$). Compared with those in control group, the inflammations in model group were increased significantly from the 7th d, and there were significant differences in the 30th d ($p<0.05$). Reports[11] have revealed that miR-21 can inhibit target proteases (MMPs). Moreover, miR-21 silencing can stimulate the rearrangement of chondrocytes in osteoarthritis, thereby leading to the development of osteoarthritis[12]. The research on miR-21 in RA has confirmed that miR-21 is able to repress the inflammatory factors in the synovial tissues.

According to the results in this experiment, the mRNA expression levels of IL-6, IL-8, and Wnt in MiR group were higher than those in control group but lower than those in model group ($p<0.05$), while they were higher in model group than those in control group ($p<0.05$), suggesting that Wnt is positively correlated with IL-6 and IL-8. The results of fluorescent Western blotting assay showed that the expression level of Wnt protein in the osteocytes was lowered in MiR group compared with that in model group ($p<0.05$), and it was prominently elevated in model group compared with that in control group

($p<0.05$). Reports[13] have illustrated that the Wnt signaling pathway plays a role in the pathogenesis of RA. It is discovered[14] that Wnt exerts effects in osteocyte differentiation but plays more important roles in bone destruction and inflammation. According to investigations[15], Wnt also performs vital functions in the bone destruction induced by arthritis and other chronic inflammations. The expressions of inflammatory factors IL-6 and IL-8 in the synovial cells can be raised by Wnt[16]. Studies[17] have manifested that Wnt is highly expressed in the synovial tissues in arthritis, which can enhance the binding of osteoclasts to RANKL and induce the differentiation and activation of osteoclasts into mature cells. The generation of osteoclasts can be reduced by blocking the Wnt signaling pathway. The surface layer of osteoclast precursors is decreased, thus inhibiting the bone destruction in CIA. In the synovial tissues, the expressions of IL-8 and IL-6, which play crucial roles in RA onset, are increased evidently[18]. Moreover, the Wnt signaling pathway can promote cell survival and adhesion, in which the overexpression of Wnt3 can stimulate the production of proteases in RA patients[11,19]. As inflammatory mediators during RA onset, IL-6 and IL-8 are highly expressed in the process of arthritis attack, and their protein expression levels are higher than those in normal control group[12-14]. Research results[15] have indicated that the expressions of inflammatory mediators are raised in RA, which is consistent with the results in this research.

Conclusions

We found that miR-21 overexpression can repress the expressions of IL-6 and IL-8 and relieve the symptoms of RA by down-regulating the Wnt signal.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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