Attenuation of the Activation of NLRP3 Inflammasome in Fibroblast Like Synoviocytes of Rheumatoid Arthritis by Baicalin through Regulating the Let-7i-3p/PI3K/Akt/NF-κB Signaling Axis

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Abstract [**Objectives**] To study the effect and mechanism of baicalin on the activation of NLRP3 inflammasome in human fibroblast like synoviocytes of rheumatoid arthritis (HFLS-RA). [**Methods**] To confirm that baicalin alleviated the activation of NLRP3 inflammasome in HFLS-RA, the expression of NLRP3 before and after baicalin treatment was observed by immunofluorescence. Western blot was used to detect the protein expression of p-PI3K, p-Akt, NF-κB p65, NLRP3, ASC and caspase-1 after baicalin treatment for 48 h, and the contents of IL-1 and IL-18 in the supernatents were detected by ELISA. In order to explore the mechanism of baicalin alleviating the activation of NLRP3 inflammasome, the corresponding relationship between let-7i-3p and PIK3CA was verified by double luciferin and Westen blot analysis. The expression of let-7i-3p and PI3K before and after baicalin intervention was detected by RT-qPCR. let-7i-3p interference was used to verify whether baicalin mitigated the activation of enhanced NLRP3 inflammasome. [**Results**] Baicalin (50 and 100 mg/L) significantly reduced the activation of NLRP3 inflammasome, inhibited the protein expressions of p-PI3K, p-Akt, NF-κB p65, NLRP3, ASC and caspase-1, and the secretion of IL-1 and IL-18. let-7i-3p and PIK3CA had a targeted correspondence, and baicalin up-regulated the expression of let-7i-3p and down-regulated the expression of PIK3CA. Baicalin attenuated the activation of NLRP3 inflammasome enhanced by let-7i-3p interference. [**Conclusions**] Baicalin can up-regulate let-7i-3p expression, inhibit PI3K/Akt/NF-κB signal transduction, and thus reduce the activation of NLRP3 inflammasome in HFLS-RA.

Key words Baicalin, Rheumatoid arthritis, Human fibroblast like synoviocytes of rheumatoid arthritis, NLRP3 inflammasome, miRNA, Dual-luciferase

1 Introduction

Rheumatoid arthritis (RA) is an autoimmune disease, and can cause swelling, pain, damage and even disability of joints throughout the body in severe cases[1]. In the pathologic progression of RA, various interactions between immune cells are involved, of which the abnormal activation of NLRP3 inflammasome in human fibroblast like synoviocytes of rheumatoid arthritis (HFLS-RA) plays an important role[2]. Therefore, it is of great significance to study the factors influencing the abnormal activation of NLRP3 inflammasome and develop new drugs for RA.

NLRP3 inflammasome is an intrinsic immune component composed of NLRP3, apoptotic speckle like protein (ASC) and caspase-1. ASC activates caspase-1 by binding to the precursor of caspase-1, and then promotes the secretion of IL-1β and IL-18, which directly affects the inflammatory state of RA[3]. At present, the main treatment strategy for RA is to inhibit the activation of NLRP3 inflammasome[4]. Previous studies have shown that baicalin has a good effect on alleviating the secretion of inflammatory cytokines by HFLS-RA[5] and alleviating joint lesions in rats with collagen-induced arthritis[6]. The anti-RA mechanism of baicalin should be further elucidated. In this study, taking HFLS-RA as the research object, whether baicalin can reduce the activation of NLRP3 inflammasome and its possible mechanism were discussed to provide a more solid theoretical basis for the clinical treatment of RA with baicalin.

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2 Materials and methods

2.1 Cell lines HFLS-RA (article No.: C1195) was purchased from Shanghai Guandao Biological Engineering Co., Ltd. 293T cells (article No.: CL0005) were purchased from Fenghui Biotechnology Co., Ltd.

2.2 Drugs and reagents Baicalin (purity 98%) was purchased from Lanzhou Waterles Biotechnology Co., Ltd. let-7i-3p NC, let-7i-3p mimics, let-7i-3p inhibitor, and let-7i-3p inhibitor NC were synthesized by Suzhou Hongxun Biotechnology Co., Ltd. TRIzol (article No.: 15596-026) and RT-qPCR kit (article No.: AM1200) were bought from Invitrogen. M-MLV reverse transcription kits (article No.: RR036A) were purchased from TaKaRa. Primary antibody PI3K p110α (article No.: AG2867), p-Akt (Ser473, article No.: AF5740), NLRP3 (article No.: AF2155), ASC/TMS1 (article No.: AF6234), and caspase-1 (article No.: AF1681) were all purchased from Shanghai Beyotime Biotechnology Co., Ltd. NF-κB p65 antibody (article No.: 4764) was bought from Cell Signaling Technology. GAPDH primary antibody (article No.: 60004-1-Ig) and FITC labeled goat anti-rabbit IgG (article No.: SA00003-2) were bought from ProteintechGroup Inc. HRP tagged goat anti-rabbit IgG secondary antibody (article No.:

ZB-2301) was purchased from Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd. Human IL-1β (article No.: RX106152H) and IL-18 (article No.: RX106154H) ELISA kits were bought from Ruixin Biotechnology Co., Ltd.

2.3 Instruments Main instruments include PCR instrument (qTOWER3G, German Jena Analytical Instruments Co., Ltd.), Westen blot electrophoresis apparatus (DYY-300B, Beijing Dongfang Rayleigh Electrophoresis Equipment Co., Ltd.), Westen blot multi-functional imaging system (SH-523, Hangzhou Shenhua Technology Co., Ltd.), and fluorescence microscope (DMI3000 + DFC310FX, German Leica Company).

2.4 Cell culture HFLS-RA was cultured in DMEM medium containing 10% fetal bovine serum, and after 3 – 4 generations, the follow-up experiment was carried out.

2.5 Effects of baicalin on the activation of NLRP3 inflammasome

2.5.1 Fluorescence intensity of NLRP3 detected by immunofluorescence. There were 4 groups in this experiment, including control group and baicalin (25, 50 and 100 mg/L) intervention groups, with 3 compound pores in each group. HFLS-RA was inoculated on a 24-well plate covered with cell slivers with the density of 2×10^4 cells/well, and cultured for 24 h. After baicalin intervention for 48 h, the cell slivers were taken out, and the cells were fixed with 4% paraformaldehyde for 15 min. They were treated with 0.25% Triton X – 10 010 min. Afterwards, they were sealed with goat serum at 37 ℃ for 30 min and incubated at 37 ℃ for 1 h after the addition of NLRP3 antibody. After the addition of FITC fluorescent secondary antibody, they were incubated for 30 min away from light. Finally, DAPI was added to seal them, and they were observed by fluorescence microscope and photographed.

2.5.2 Detection of p-PI3K, p-Akt, NF-κB p65, NLRP3, ASC and caspase-1 protein expression by Western blot. The experimental grouping was the same as that in Section **2.5.1**. HFLS-RA was inoculated into a culture dish with a diameter of 35 mm with the density of 6×10^5 cells/dish. After cell adhesion and baicalin intervention for 48 h, total protein of cells in each group was extracted and quantified by BCA method. After SDS-PAGE electrophoresis and film transfer, and 5% skim milk sealing, the corresponding primary antibody was added to them for incubation at 4 ℃ overnight. TBST bleach was used to wash film 3 times, 10 min each time. HRP-labeled secondary antibody was added to them for incubation at 37 ℃ for 1 h. After ECL luminescent solution was added, and exposure, development, and fixing were carried out by X-ray. Finally, Image-Pro Plus software was used to analyze the gray level of the target bands, and the gray ratio of each target band to GAPDH was the relative expression level of the target protein.

2.5.3 Detection of inflammatory factor content in the cell supernatant by ELISA. The content of IL-1β and IL-18 in the cell supernatant of each group was determined according to the instructions of IL-1β and IL-18 kits.

2.6 Mechanism of inhibition of baicalin on the activation of NLRP3 inflammasome

2.6.1 Verification of the targeting effect of let-7i-3p on PIK3CA by the double luciferase report experiment. By using bioinformatics TargetScan 7.0 software analysis, PIK3CA was found to be one of the targets of let-7i-3p. In order to further verify the targeting relation between PIK3CA and let-7i-3p. In this experiment, luciferase reporter plasmid wild-type 3'-UTR (GV272-WT-PIK3CA-3'-UTR), mutant 3'-UTR (GV272-MUT-PIK3CA-3'-UTR) and let-7i-3p expression plasmids (GV251-let-7i-3p) were constructed. There were 6 groups in this experiment: group 1 (let-7i-3p-NC + 3'-UTR-NC), group 2 (let-7i-3p + 3'-UTR-NC), group 3 (Let-7i-3p-NC + 3'-UTR-WT), group 4 (let-7i-3p + 3'-UTR-WT), group 5 (let-7i-3p-NC + 3'-UTR-MUT), and group 6 (let-7i-3p + 3'-UTR-MUT). The 293T cells were co-transfected with X-tremegene HP transfection agent. After transfection for 48 h, the activities of firefly luciferase and Renilla luciferase were detected, and 0.5 μg of GFP plasmid was transfected separately when the target plasmid was transfected. The plasmid construction was completed by Shanghai Jikai Gene Technology Co., Ltd.

2.6.2 Verification of the targeting effect of let-7i-3p on PIK3CA by Western blot. There were four groups in this experiment: let-7i-3p mimics group, let-7i-3p NC group, let-7i-3p inhibitor group, and let-7i-3p inhibitor NC group. HFLS-RA was inoculated into a dish (35 mm in diameter) with the density of 2×10^5 cells/well, and there were 3 replicates in each group. The cells were cultured with DMEM medium containing 10% fetal bovine serum. When the cells grew to 80% – 90%, the supernatant was discarded, and they were washed twice with PBS. According to the transfection instructions of Lipofectamine 3000, let-7i-3p mimics, let-7i-3p NC, let-7i-3p inhibitor NC and let-7i-3p inhibitor NC were transfected into corresponding groups. After they were cultured at 37 ℃ in 5% CO_2 incubator for 5 h, new DMEM containing 10% fetal bovine serum was used in the culture for 48 h. Protein extraction and Western blot were similar to that in Section **2.5.2**.

2.6.3 Detection of mRNA expression of let-7i-3p and PIK3CA before and after baicalin intervention by RT-qPCR. In the experiment, there were two groups: control group and 100 mg/L baicalin intervention group, with 3 pores in each group. HFLS-RA was inoculated into a 6-well plate with the density of 6×10^5 cells/well. After baicalin treatment for 48 h, total RNA was extracted and quantitized by ultraviolet spectrophotometer. cDNA was synthesized by reverse transcription using 3 μg RNA as template, and then amplified with 2 μL cDNA as template. The total volume of PCR was 20 μL. The amplification conditions are as follows: predenaturation was conducted at 94 ℃ for 15 min; denaturation was carried out at 95 ℃ for 20 s; annealing was conducted at 60 ℃ for 35 s; extending was carried out at 72 ℃ for 20 s; there were a total of 35 cycles; after extending at 72 ℃ for 10 min, the reaction was stopped at 4 ℃. $2^{-\Delta\Delta CT}$ was used to calculate the relative gene expression, and GAPDH was the internal reference of PIK3CA,

while U6 was the internal reference of miRNA. The primers were synthesized by Shanghai Sangon Bioengineering Co., Ltd., and the sequences are shown in Table 1.

Table 1 Primers for RT-PCR

Items	Primers
PIK3CA	F: 5'-CGGTGACTGTGTGGGACTTATTG-3'
	R: 5'-TGATGTAGTGTGTGGCTGTTGAAC-3'
let-7i-3p	F: 5'-CGCTGCGCAAGCTACTGC-3'
	R: 5'-AGTGCAGGGTCCGAGGTATT-3'
Stem ring	5'-GTCGTATCCAGTGCAGGGTCCG
	AGGTATTCGCACTGGATACGAC-3'
GAPDH	F: 5'-GGAGCGAGATCCCTCCAAAAT-3'
	R: 5'-GGCTGTTGTCATACTTCTCATGG-3'
U6	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'
	R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'

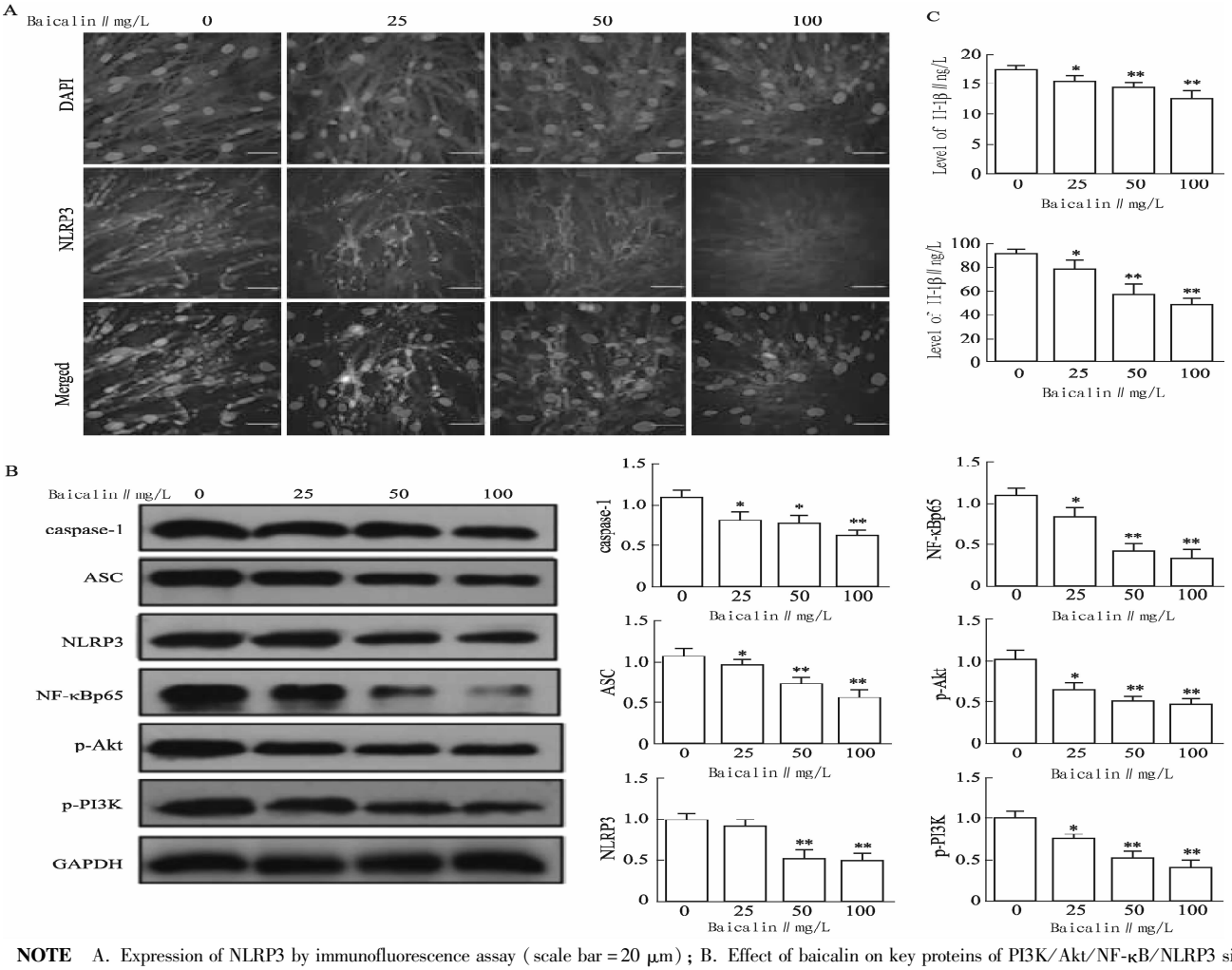
2.6.4 Verification of inhibition of baicalin on the activation of NLRP3 inflammasome by regulating let-7i-3p/PI3K by miRNA interference. Four groups were set up in this experiment, including control group, 100 mg/L baicalin intervention group, let-7i-3p inhibitor group, and let-7i-3p inhibitor + 100 mg/L baicalin intervention group, with 3 pores in each group. HFLS-RA was inoculated on a 24-well plate covered with cell slivers with the density of 2×10^4 cells/well, and cultured for 24 h. After culture for 24 h, miRNA interference and baicalin intervention were performed. The transfection mode of let-7i-3p inhibitor and the intervention mode of baicalin were similar to that in Section **2.6.2** and Section **2.6.3**, respectively.

2.7 Data analysis The experimental data were shown as $\bar{x} \pm s$ and analyzed by SPSS 20.0 software. One-way ANOVA was used for comparison between groups, and $P < 0.05$ meant that the difference was statistically significant.

3 Results and analysis

3.1 Inhibition of baicalin on the activation of NLRP3 inflammasome

3.1.1 Effects on the activation of NLRP3 inflammasome. As shown in Fig. 1A, NLRP3 fluorescence intensity significantly reduced in the 50 and 100 mg/L baicalin intervention groups compared with the control group. These results indicated that baicalin could inhibit the activation of NLRP3 inflammasome.



NOTE A. Expression of NLRP3 by immunofluorescence assay (scale bar = 20 μm); B. Effect of baicalin on key proteins of PI3K/Akt/NF-κB/NLRP3 signal axis; C. Effects of baicalin on IL-1β and IL-18. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

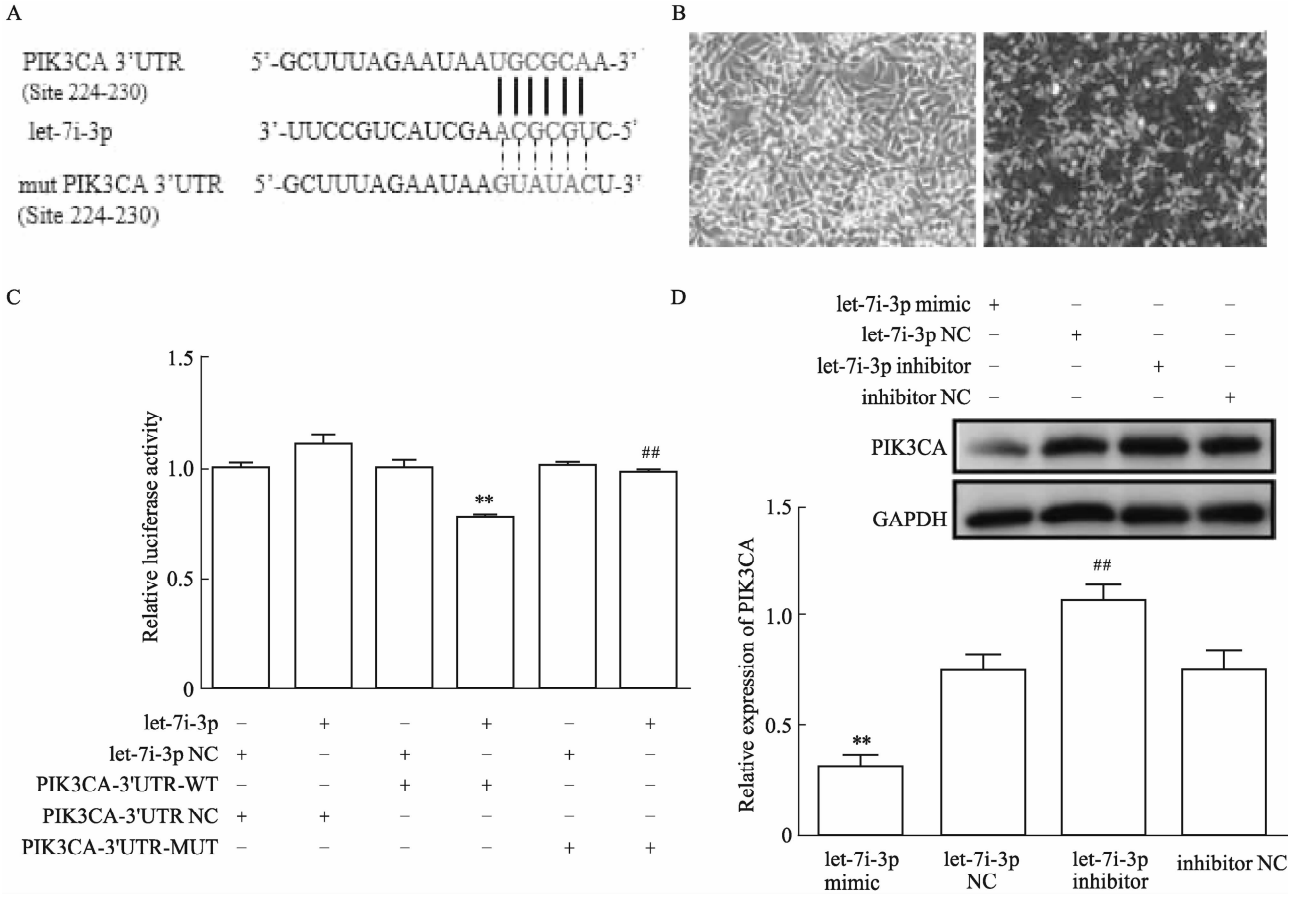
Fig. 1 Effects of baicalin on the activation of NLRP3 inflammasome in HFLS-RA ($\bar{x} \pm s$, $n = 3$)

3.1.2 Effects on the expression of key proteins of PI3K/Akt/NF-B/NLRP3 signal axis. Seen from Fig. 1B, compared with the control group, the protein expression levels of p-PI3K, p-Akt, NF-κB p65, NLRP3, ASC and caspase-1 in the 50 and 100 mg/L baicalin treatment groups significantly decreased. These results indicated that the anti-inflammatory effect of baicalin was related to the down-regulation of key protein expression of PI3K/Akt/NF-κB/NLRP3 signal axis.

3.1.3 Effects on the protein expression of IL-1β and IL-18 secreted by HFLS-RA. As shown in Fig. 1C, compared with the control group, the contents of IL-1β and IL-18 in the 50 and 100 mg/L baicalin treatment groups significantly dropped. It indicated that baicalin inhibited the secretion of IL-1β and IL-18.

3.2 Mechanism of inhibition of baicalin on the activation of NLRP3 inflammasome

3.2.1 Targeting relationship between let-7i-3p and PIK3CA. Prediction results of TargetScan 7.0 showed that let-7i-3p targeted the 3'-UTR of the PIK3CA gene (Fig. 2A). As shown in Fig. 2B, there were no abnormalities in the plasmid transfection system. Compared with the control group, let-7i-3p significantly reduced the activity of the wild plasmid but did not affect the activity of the mutant plasmid (Fig. 2C). As shown in Fig. 2D, after transfecting let-7i-3p mimic, let-7i-3p NC, let-7i-3p inhibitor and let-7i-3p inhibitor NC for 48 h, the expression of PIK3CA protein in the let-7i-3p mimic group significantly declined ($P < 0.01$) compared with let-7i-3p NC group. Compared with the let-7i-3p inhibitor NC group, the expression of PIK3CA protein in the let-7i-3p inhibitor group significantly increased ($P < 0.01$). It showed that there was a targeting relation between let-7i-3p and PIK3CA.



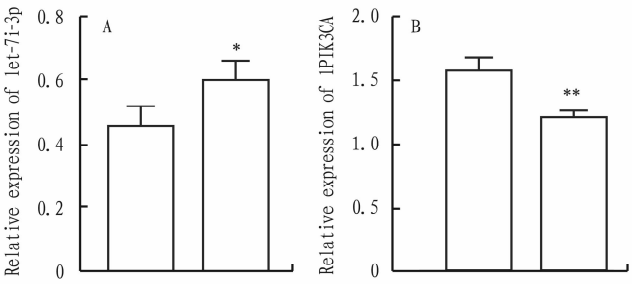
NOTE A. Let-7i-3p targeted the 3'-UTR of PIK3CA; B. The plasmid transfection system was normal; C. Dual-luciferase assay confirmed that let-7i-3p targeted PIK3CA 3'-UTR. $^{**}P < 0.01$ compared with PIK3CA-3'UTR NC; $^{\#\#}P < 0.01$ compared with PIK3CA-3'UTR-WT; D. Westen-blot confirmed that let-7i-3p targeted PIK3CA, $^{**}P < 0.01$ compared with let-7i-3p NC; $^{\#\#}P < 0.01$ compared with inhibitor NC.

Fig. 2 Let-7i-3p targeting PIK3CA ($\bar{x} \pm s$, $n = 3$)

3.2.2 Effects on the mRNA expression of let-7i-3p and PIK3CA in HFLS-RA. As shown in Fig. 3, compared with the control group, the expression of let-7i-3p in the baicalin intervention group significantly increased ($P < 0.05$), while the expression of PIK3CA mRNA significantly decreased ($P < 0.01$).

3.2.3 Alleviation of the activation of NLRP3 inflammasome by targeting let-7i-3p/PIK3CA axis. As shown in Fig. 4, compared with the control group, baicalin significantly inhibited the activation of NLRP3 inflammasome after intervention. The activation of NLRP3 inflammasome enhanced after let-7i-3p inhibitor interfered with let-7i-3p. Baicalin significantly inhibited the activation of NLRP3 inflammasome enhanced by let-7i-3p inhibitor interfer-

ence. The experiment further demonstrated that baicalin mitigated the activation of NLRP3 inflammasome by targeting let-7i-3p/PIK3CA axis.



NOTE $^{*}P<0.05$, $^{**}P<0.01$ compared with control.

Fig. 3 Effects of baicalin on the mRNA expression of let-7i-3p (A) and PIK3CA (B) ($\bar{x}\pm s$, $n=3$)

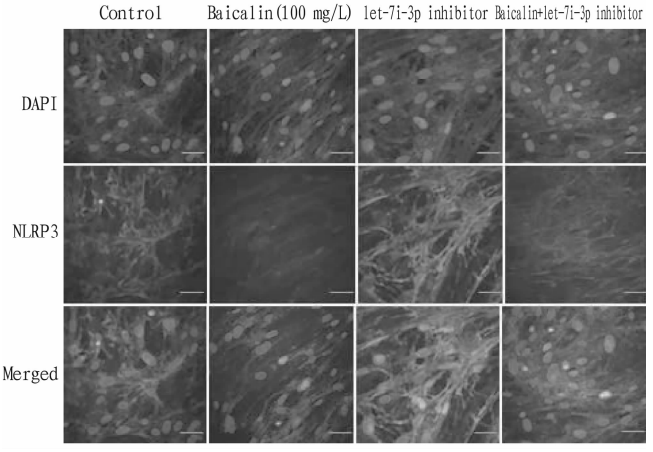


Fig. 4 Baicalin alleviated NLRP3 by targeting let-7i-3p/PI3K axis (scale bar =20 μm)

4 Discussion

The main pathological features of RA are the proliferation of synovium of the diseased joint and the continuous secretion of high levels of pro-inflammatory cytokines, leading to cartilage injury and bone erosion[7]. HFLS-RA is a major effector cell mediating RA joint destruction, and the high activation of NLRP3 inflammasome and the secretion of inflammatory mediators are associated with the sustained activation of PI3K-Akt signaling pathway[8].

The PI3K-Akt signaling pathway regulates the release of inflammatory factors and the formation of inflammation-related enzymes, and is involved in the pathological process of RA. It is widely present in HFLS-RA and is abnormally activated[9]. Depending on the structure and specific substrate, PI3K is divided into classes I, II and III, of which class-I PI3K is mainly studied. Mammals express four class-I catalytic subunits (p110α, β, δ, and γ) that are encoded by the *PIK3CA*, *PIK3CB*, *PIK3CD*, and *PIK3CG* genes, respectively. P13K is activated and catalyzes the formation of the cell's second messenger PIP3. Conversely, PIP3 also recruits Akt kinase and activates other downstream molecules[10]. Akt, the main mediator of PI3K signaling pathway, is located in the downstream of PI3K signaling pathway. As a serine/threonine kinase, Akt is divided into Akt1, Akt2 and Akt3. Akt1 is expressed in various human tissues[11]. Once being activated, Akt phosphorylates other molecules downstream, thereby activating the downstream pathway[12]. Phosphorylation of Akt activates IκB kinase, leading to degradation of NF-κB inhibitor IκB, thereby translocation of NF-κB nuclear upstream of NLRP3 inflammasome, promoting the expression of multiple inflammatory factors, and further participating in the occurrence of inflammatory diseases[13].

miRNA is a class of endogenous non-coding RNA with regulatory functions in eukaryotes. Non-coding small RNA with a length of 18 – 25 nucleotides can bind to the 3'-UTR region of target genes to inhibit the translation of target genes or cause the degradation of target genes, thus playing a negative regulatory role. The abnormal expression of miRNA is closely related to the occurrence and development of many diseases such as cancer and RA[14]. Studies have shown that miRNA plays an important role in PI3K signal transduction[15]. let-7 is one of the most widely studied miRNA[16], and its expression is down-regulated in many tumors[17]. As a member of the let-7 family, let-7i-3p is abnormally expressed in cancer[18].

In order to explore the anti-inflammatory mechanism of baicalin, through the immunofluorescence experiment was first conducted in this study, and it was found that 50 and 100 mg/L baicalin could significantly inhibit the activation of NLRP3 inflammasome in HFLS-RA. The study on the mechanism showed that 50 and 100 mg/L baicalin could significantly inhibit the protein expression of p-PI3K, p-Akt, NF-κB p65, NLRP3, ASC and caspase-1 in HFLS-RA, showing that the anti-inflammatory effect of baicalin was related to the down-regulation of PI3K/AKT/NF-κB/NLRP3 signal transduction. To further explore the molecular regulatory mechanism by which baicalin alleviated the activation of NLRP3 inflammasome, the miRNA acting on PI3K was predicted by using the miRNA targeting prediction software Targetscan, and it was found that let-7i-3p was one of miRNA regulating the PIK3CA subunit. Double luciferase experiment further confirmed the targeting relation between let-7i-3p and PIK3CA. Baicalin intervention experiment showed that baicalin up-regulated the expression of let-7i-3p and down-regulated the expression of PIK3CA in HFLS-RA. In order to investigate the relationship between baicalin, let-7i-3p and the activation of NLRP3 inflammasome, the let-7i-3p interference experiment was conducted. The results showed that baicalin significantly reduced the activation of NLRP3 inflammasome enhanced by let-7i-3p interference. The final results revealed that baicalin weakened the PI3K/Akt/NF-κB signal transduction by promoting the expression of let-7i-3p and inhibiting the expression of PIK3CA subunit of PI3K, and finally reduced the activation of NLRP3 inflammasome.

In conclusion, the anti-RA effect of baicalin is related to the up-regulation of let-7i-3p expression, the targeted inhibition of PI3K/Akt/NF-κB signal transduction, and then reducing the activation of NLRP3 inflammasome. This study deepens the anti-RA mechanism of baicalin and provides a more specific theoretical basis for the clinical treatment of RA.

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medicine characteristic diet therapy and medicated diet culture and carrying forward traditional advantages have broad prospects. Mongolian medicine diet therapy is the first of the four clinical treatments of Mongolian medicine, which holds that diet can cure diseases and cause diseases[12]. The diet therapy in Mongolian medicine is usually a diet therapy with Mongolian characteristics gradually formed by gradually accumulating experience in daily life and constantly groping for diet therapy. Malnutrition is very common in patients, and malnutrition will directly threaten the life safety of patients. Ancient Mongolian people invented a simple method to treat some diseases with diet. For example, they mastered the fermentation technology during milking, and used koumiss as a nourishing drink to preserve health and cure diseases. As a precious cultural heritage of Mongolian nationality, diet therapy in Mongolian medicine has distinct national characteristics[13]. This method of treating diseases was gradually explored and formed in the nomadic hunting life of Mongolian people. Standardizing the implementation of nutritional support therapy is beneficial to the improvement of patients' clinical outcomes.

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