Abstract. The differential microRNA (miRNA) omics of the synovial membrane were investigated using a rat model of knee osteoarthritis (KOA) induced by bilateral anterior cruciate ligament transection, which produced pathological biomarkers in KOA. Sprague-Dawley rats were randomly divided into two groups; Sham-operated and KOA-operated group. The KOA rats were subjected to bilateral anterior cruciate ligament transection. After 6 weeks, total RNA was extracted from the knee joint synovial membrane of the rats and a microRNA (miR) microarray was performed to identify differentially expressed miRs. Subsequently, the obtained differentially expressed miRs were validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. A total of 24 miRs were identified with alterations ≥1.5-fold in the synovial membrane in the KOA-operated group compared with the sham-operated group, of which 4 miRs (miR-532-5p, -200b-5p, -377-3p and -759-5p) were decreased and 20 miRs (miR-382-3p, -223-3p, -100-5p, -30d-5p, -183-5p, -130, -92b-3p, -125b-3p, -151-3p, -155-3p, 27a-3p, -146b-3p, -885-5p, -352, -184, -345-5p, -30a-5p and -9a-5p) were increased. Subsequently, RT-qPCR was used to validate the expressions of miR-223, -100, -345, -130, -382, -377, -352, -9a and -183, which were upregulated by a fold change of ≥1.5 in synovial membranes of KOA rats compared with shams. Furthermore, in vitro miR-223 mimic could suppress the luciferase activity of NACHT, LRR and PYD domains-containing protein 3 (NLRP3) 3’ untranslated region by detecting of dual luciferase reporter vector. Additionally, the expression of NLRP3, interleukin (IL)-1β and IL-18 significantly increased in the synovial membrane of KOA rats. A total of 24 different miRs were determined by comparing the miRNAomics in the synovial membrane of the KOA model rats. Furthermore, the miR-233-regulated NLRP3 inflammasome was implicated in synovial membrane injury, which may be an important mechanism of KOA pathogenesis.

Introduction

Knee osteoarthritis (KOA) is a common chronic osteoarthritis pain in people aged 60 years and over, characterized by progressive degeneration of the articular cartilage and synovial membrane, which seriously affects the daily activities of ~18.0% of women, and 9.6% of men worldwide (1,2). The occurrence of synovitis and synovial hyperplasia is severe around the cartilage in knee joints as diagnosed by magnetic resonance imaging when the suprapatellar bursa and posterior cruciate ligament of knee is damaged (3). The causes of pathological alterations in synovitis include the release of proinflammatory cytokines and oxidative stress damage. Although a number of inflammatory factors, including tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-18 and NACHT, LRR and PYD domains-containing protein 3 (NLRP3), are involved in the process of synovitis, there are few reports of small molecules that progressively degenerate articular synovial membrane in KOA (4,5).

Previous studies indicated that microRNAs (miRNAs/miRs), conservative endogenous non-coding RNAs of 18-25 nucleotides, are implicated in synovitis and synovial hyperplasia (6,7). More evidence suggests that miRs, which are altered in the...
KOA-operated rat model. Rats were subjected to bilateral knee anterior cruciate ligament transaction (ACLT) (4). Prior to ACLT, rats were sedated and anesthetized appropriately with sodium pentobarbital [0.1 ml/100 g intraperitonially (IP), 40 mg/kg]. The knee was shaved, then sterilized and draped in a sterile manner. A medial arthroscopy was performed. Then the patella was dislocated and the anterior cruciate liga-
mented was isolated and transected. The ACLT was confirmed with Lachman testing by the surgeon and an observer (15). Following irrigation with sterile saline solution, the wounds were closed in layers and antiseptically treated. Rats were given appropriate postoperative care and allowed free activities in individual cages. Animal grouping. All animals were divided into two groups according to a random number table. The KOA-operated group and the sham-operated group, each group included 9 rats according to SPSS13.0 statistics software (SPSS, Inc., Chicago, IL, USA).
GTAGGTTGCATAGTA-3', and Reverse, 5'-GCTGTCAAC
GATACGCTACCTA-3'), miR-200b (Forward 5'-CATCTT
ACTGGGCAGCATTGGA-3', and Reverse, 5'-GCTGTCAAC
GATACGCTACCTA-3'), miR-9a (Forward 5'-TCTTCTGTT
ATCTAAGCACTGA-3', and Reverse, 5'-GCTGTCAACGAT
ACCTCTACCTA-3'), miR-183 (Forward 5'-TATGGACTGT
AGAATTACT-3', and Reverse, 5'-GCTGTCAACGATAC
CTACCTA-3') and U6 (Forward 5'-CTCGTTCGCGACGA
CA-3', and Reverse, 5'-AAGCCTTCACAGTTGGCCT-3')
using the Plexor™ One-Step qRT-PCR System (Promega
Corporation, Madison, WI, USA) in the Bio-Rad CFX96
Detection System (Bio-Rad Laboratories, Inc., Hercules, CA,
USA). The primers: the fold-change in relative miR expres-
sion was determined using the 2^ΔΔCq method and U6 small
nuclear RNA as the internal control (16).

Luciferase reporter assay. The interaction of miR-223
and NLRP3 was predicted in terms of Microcosm (www.
ebi.ac.uk/errors/failure.html) and Targetsan (www.
targetsanc.org/vert_72/) containing computationally predicted
targets for miRNAs and identify potential binding sites for a
given miRNA in genomic sequences by dynamic program-
ing alignment (17). The Luciferase reporter assay was used
to determine their interaction. The Luciferase reporter vectors
of psiCHECK2-NLRP3 and psiCHECK2-NLRP3-Mut
(GeneCopoeia, Inc., Rockville, MD, USA) were constructed,
which include the wild type or mutated putative NLRP3
3'-untranslated region (UTR) sequence that is targeted by
miR-223. The 3'-UTR of NLRP3 primers were designed for
RT-qPCR detection. NLRP3-forward 1: 5'-cccccaauaacugU
GACGUu-3'; NLRP3-reverse 1: 5'-UCUUGUCUUGUAAC
UGACC-3'; mutant (Mut) NLRP3-forward 1: 5'-cccccaauaacu
uAACUGuCa-3'; MutNLRP3-reverse 1: 5'-UCUUGUCUUG
UUAACUGACC-3'. Each amplified product was ~1,000 bp
including putative or mutated miR-223 recognition sequence
on NLRP3 3'-UTR. Clones were selected by restriction digestion
with XhoI and NotI. 293T cells (ATCC, Manassas, VA, USA)
cultured with DMEM (Gibco; Thermo Fisher Scientific, Inc.),
supplemented with 10% FBS (Gibco; Thermo Fisher Scientific,
Inc.), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific,
Inc.) and 100 units/ml penicillin/streptomycin (Gibco; Thermo
Fisher Scientific, Inc.) were seeded at a density of 3x10^4
cells per well in 96-well white assay plates 1 day before cell transfec-
tion was determined using the 2^ΔΔCq method and HRP was used
with enhanced chemiluminescence

Western blotting. The synovial membrane (n=3 each group)
was dissected and homogenized in radio-immunoprecipitation
assay lysis buffer (Nanjing Jiangcheng Bioengineering
Institute, Nanjing, China), and their protein concentrations
were assessed using the bicinchoninic acid protein assay
(Nanjing Jiangcheng Bioengineering Institute). A total of
50 µg of total protein each sample was separated by 10%
SDS/PAGE and transferred into nitrocellulose membrane blot
(Bio-Rad Laboratories, Inc.). The blots were blocked with 3%
BSA (Life Technologies, Carlsbad, CA, USA) and incubated
at room temperature (22˚C) for 2 h with primary antibodies,
including rabbit monoclonal anti-NLRP3 antibody (1:500; cat.
no. ab210491; Abcam, Cambridge, UK), mouse monoclonal
anti-IL-1β (1:1,000, cat. no. ab150777; Abcam) and IL-18 anti-
body (1:1,000; cat. no. ab191860; Abcam) and β-actin (1:600;
cat. no. sc-47778, Santa Cruz Biotechnology, Inc., Dallas, TX,
USA), and pre-adsorbed goat polyclonal secondary antibody to
rabbit IgG-(1:1,000; cat. no. ab6940; Abcam) or pre-adsorbed
goat polyclonal secondary antibody to mouse IgG (1:800;
cat. no. ab97035; Abcam). Protein bands were detected with a
secondary antibody conjugated to horseradish peroxidase
(HRP; Jackson Immunoresearch Europe, Ltd., Newmarket,
UK) and HRP was used with enhanced chemiluminescence
detection (UVP, LLC, Phoenix, AZ, USA), which was quanti-
fied using a Bio-Image analysis system version 6.0.1 (Bio-Rad
Laboratories, Hercules, CA, USA).

Statistical analysis. All data are expressed as the mean ±
the standard error of mean and were analyzed using SPSS 13.0
statistical analysis software (SPSS, Inc., Chicago, IL, USA).
The data were subjected to a two-tailed Student's t-test analysis
for comparison between two groups of the sham group and

Figure 1. Identification of differentially expressed miRs in KOA rats. The differentially expressed miRs were detected in the synovial membrane of KOA rats. A total of 24 miRs exhibited ≥1.5 fold-change using cluster analysis (P<0.05 and false discovery rate ≤5%). KOA group vs. sham group. Green, downregulated miRNAs; red, upregulated miRNAs. n=3 each group. miR, microRNA; KOA, knee osteoarthritis.
the KOA group. All final results were analyzed by observers blinded to the experimental conditions. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Alterations in miR expression profiling in the synovial membrane of KOA rats.** To investigate the miRs differential expression in the synovial membrane of KOA rats, miR microarray profiling was performed and a total of 24 miRs were demonstrated to be altered by ≥1.5-fold, and P<0.05, FDR ≤5% in the KOA group compared with the Sham group, of which 4 miRs (miR-532-5p, -200b-5p, -377-3p and -759-5p) were decreased, whereas 20 miRs (miR-382-3p, -223-3p, -100-5p, -30d-5p, -183-5p, -130, -92b-3p, -125b-3p, -151-3p, -155-3p, 27a-3p, -146b-3p, -885-5p, -352, -184, -345-5p, -30a-5p, and -9a-5p) were increased (Fig. 1).

**Expression validation of miRs by RT-qPCR.** To confirm the miR expression results from the microarray, 10 miRs were randomly chosen and assessed by RT-qPCR. The expression of miR-223, -100, -345, -130, -382, -377, -352, -200b, -9a and -183 were upregulated with a fold-change of ≥1.5, similar to the microarray data (Fig. 2).

miR-233 negatively regulates the expression of NLRP3. The binding locus of the miR-223 and NLRP3 interaction was predicted in terms of Microcosm (www.ebi.ac.uk/ crispr/ failure .html) and Targetscan (www.targetscan.org/ vert_72/; Fig. 3). Subsequently, the interaction of miR-223 and NLRP3 was assessed in *vitro* using a luciferase reporter assay; the results suggested that the miR-223 mimic significantly inhibited the luciferase activity of NLRP3 3’UTR in 293T cells (P<0.05), whereas for the NLRP3 3’UTR mutant, luciferase activity was unaltered by miR-223 mimic (Fig. 3). These results suggested that miR-223 downregulated NLRP3.

Alteration of NLRP3 inflammasome-associated molecules in the synovial membrane of KOA rats. Furthermore, the expression of NLRP3 inflammasome-associated NLRP3, IL-1β and IL-18 proteins was measured in synovial membrane. The results demonstrated that the expression of NLRP3, IL-1β and IL-18 significantly increased in the synovial membrane in the KOA group compared with the Sham group (P<0.05; Fig. 4).

**Discussion**

The synovial membrane is located in the joint space and maintains normal joint function by secreting hyaluronan to lubricate the tissues of joints that provide nutrition for the cartilage. However, when the synovium function is abnormal, the joint fluid is not generated and absorbed normally. The morphological alterations of the synovial membrane can also affect the cartilage of the knee joint if not treated in time, and can lead to KOA. The synovial membrane releases inflammatory factors and cytokines that result in the cartilage damage and synovial hyperplasia in patients with KOA (18). Therefore, therapeutics focus on the treatment targeting the
cartilage in KOA may be altered and that this may allow for novel treatment modalities that target the synovial membrane to be developed. In order to identify diagnostic biomarkers and therapeutic targets in different diseases, numerous studies have used miRNAomics analysis. Serum miRNAomics have been used to identify specific biomarkers of cartilage degeneration from patients with OA (8,19).

However, the miRNAomics of the synovial membrane has been rarely investigated; furthermore, to the best of our knowledge, there are no studies reporting the differences in synovial membrane miRNAomics in a KOA model induced by bilateral ACLT. Therefore, the different miRNAomics of the synovial membrane were investigated via microarray in a rat model of KOA. The results illustrated the miRNAomics of the synovial membrane were different, with a total of 24 miRs exhibiting >1.5 fold-change in KOA rats, of which miR-532-5p, -200b-5p, -377-3p and -759-5p were down-regulated, whereas miR-382-3p, -223-3p, -100-5p, -30d-5p, -183-5p, -130, -92b-3p, -125b-3p, -151-3p, -155-3p, 27a-3p, -146b-3p, -885-5p, -352, -184, -345-5p, -30a-5p and -9a-5p were upregulated in KOA rats compared with sham rats. Furthermore, 10 miR were randomly selected to validate the results of miR microarrays; RT-qPCR revealed that the expression of miR-223, -100, -345, -130, -382, -377, -352, -200b, -9a and -183 were downregulated by >1.5-fold in synovial membranes of KOA rat, which was similar to the microarray results. Using bioinformatics analysis with Microcosm and Targetscan, it was demonstrated that the rat miR-223 was implicated in inflammatory injury by interaction with the NLRP3 inflammasome. Notably, Bauernfeind et al (20) previous identified that NLRP3 inflammasome activity is negatively controlled by human miR-223. Furthermore, the miR-233 sequence is different in humans and animals: Rat, rno-miR-223-3p, 5'-UGU CAG UUU GUC AAA UAC CCC-3'; human, has-miR-223-3p, 5'-TGG GGT ATT TGA CAA ACT GAC A-3'. In addition, the locus of miR-233 binding NLRP3 in humans and rats is different. In animal experiments, it was reported that activation of Toll-like receptor 9 enhanced miR-223 expression during liver injury in vivo and in vitro (21). Nanoparticle-mediated overexpression of
miR-223 attenuated experimental colitis, reduced NLRP3 levels and reduced IL-1β release (22). Elevated levels of miR-146a, miR-155 and miR-223 were demonstrated in paraffin-embedded synovial tissue of patients with established rheumatoid arthritis (23). In addition, high levels of miR-223 were present in the peripheral blood of patients with OA (24). The relative expression levels of miR-223 in patients with OA were reported to be significantly increased compared with those demonstrated in healthy controls; and in the early stages of OA, miR-223 expression was significantly increased compared with later stages (24).

NLRP3 is a member of the NLR family characterized by binding with ribonucleotide-phosphates, which are important for self-oligomerization and are able to assemble and oligomerize into a common structure that collectively activates the caspase-1 cascade; thus leading to the production of pro-inflammatory cytokines, particularly IL-1β and IL-18 (25,26). Therefore, NALP3 activation leads to an inflammatory state in the osteoarthritic joint (27). In KOA models, the synovia promote the production of proinflammatory mediator that are released into the cartilage via the synovial fluid, where they activate the chondrocytes to produce more proinflammatory cytokines, including IL-1 (28). A previous study has demonstrated that IL-1β knockout mice were protected from surgically-induced instability OA damage (29); whereas, similar models also demonstrated that cartilage injury was exacerbated in caspase 1 and IL-1β knockout mice (30). Furthermore, IP injection of IL-1 in osteoarthritic mice did not relieve OA features. Additionally, the IL-1β level in the synovial membrane was positively correlated with OA grade, and joint space width was negatively correlated with joint activity (31,32). Additionally, IL-1β was detected in human OA cartilage, especially in early stage OA and the IL-1β level in the synovial fluid was correlated with synovial fluid uric acid level in patients with KOA; thus it was concluded that synovial fluid uric acid could be a danger signal that contributes to increasing KOA risk through NLRP3-mediated inflammasome (33,34).

In conclusion, a total of 24 differentially expressed miRs were identified by comparing the miRNAomics in the synovial membrane of the KOA model and sham rats. Furthermore, the miR-233 downregulated-NLRP3 inflammasome was implicated in synovial membrane injury, which may be involved in the pathogenesis of KOA. These results revealed that miR-233 regulated NLRP3 in synovial membrane injury. Therefore, in future studies miR-233 transgenic models or miR-233 mimic and inhibitors will be used to elucidate the mechanism of miR-233 regulating targets in pathogenesis of KOA.

Acknowledgements

Authors would like to thank Dr Liu Weilin, from the Department of Rehabilitation Medicine, Fujian University of Traditional Chinese Medicine, for his helpful discussion.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81774345).

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

XL and JZ designed experiments. JZ, YZ, GW, BL and ZL performed the experiments. JZ wrote the manuscript. All authors discussed the results and approved the final manuscript.

Ethics approval and consent to participate

All experimental rats and procedures were approved by Animal Care and Usage Committee of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

References


