Identification of inflammation related miRNA, lncRNA and mRNA in synovial tissue of knee osteoarthritis patients

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Research Article

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Abstract

Osteoarthritis is one of the most common chronic diseases in the elderly, which has caused a heavy burden to the family and society. At present, the clinical treatment can only be total joint replacement, but is hard to prevent the degeneration of cartilage tissue. The molecular mechanism of OA, especially the role of inflammation in disease progression, has been reported, but it is still unclear.

Here, we first collected synovial tissues of 8 OA patients and 2 RA patients as controls, and detected the expression profiles of lncRNA, miRNA and mRNA in tissues by RNA-seq technology, and tried to find out the differentially expressed genes and key pathways in OA patients.

Compared with the control group, 343 mRNAs, 270 lncRNAs and 247 miRNAs were significantly up-regulated, 232 mRNAs, 109 lncRNAs and 157 miRNAs were significantly down regulated. Then we predicted the targeted mRNAs of dysregulated lncRNAs. Through pathway enrichment and functional annotation of these target genes, we identified 12 differentially expressed transcripts related to inflammation, including CHST11, ALDH1A2, TREM1, IL-1 β , IL-8, CCL5, LIF and miR-146a-5p, miR-335-5p, as well as lncRNA GAS5, LINC02288 and LOC101928134.

In this study, we identified the differential expression of inflammation related genes and ncRNA in synovial tissue. These differentially expressed transcripts may indicate the network of ceRNA net in synovial tissue and its effect on OA progression. Our research will help to explore the pathogenesis and key therapeutic targets of OA.

Introduction

Osteoarthritis (OA), characterized by articular cartilage degeneration, subchondral osteosclerosis, osteophyte formation and synovitis, is the most common chronic joint disease in the elderly [1–3]. Globally, 250 million people are estimated to have knee osteoarthritis, and the incidence rate is increasing [4,5]. Osteoarthritis has become a huge economic burden for families and society all over the world, but the current treatment methods cannot effectively prevent the progress of OA. The pathogenesis of OA is complex and multifactorial. Recent studies have shown an association between synovial tissue and osteoarthritis.

Many patients with osteoarthritis will also develop low-grade synovitis. More and more evidence show that synovitis can affect the symptoms and cartilage degeneration rate of OA patients. At different stages of OA, the synovium was infiltrated by fibroblast like synovial cells and other immune cells [6]. Synovial tissue obtained by closed needle or arthroscopic biopsy can help to diagnose osteoarthritis [7]. Interestingly, similar expression patterns were detected in synovial fluid miRNA and synovial tissue miRNA [8]. Therefore, we studied synovial tissue, which is the main site of OA.

Studies have shown that there is an association between epigenetics and osteoarthritis, including lncRNA and miRNA [9,10]. Long ncRNAs (lncRNAs), composed of 200 nucleotides, are widely

transcribed in the human genome. Studies have also shown that LncRNA s are abnormally expressed in various human diseases and affect the disease process [11]. For example, the abnormal expression of LncRNA HOTAIR and LncRNA CIR in OA cartilage suggests that they may have an important impact on the diagnosis and prognosis of OA, and can be used as individual biomarkers for the treatment of OA progression [12–14]

miRNAs are small, evolutionarily highly conserved noncoding sequences consisting of 19 to 25 base pairs that act as post transcriptional inhibitors of gene expression. These molecules bind to target mRNA to form miRNA mRNA complexes, leading to degradation of target transcripts, translation inhibition or both [15]. Specific miRNAs may target multiple transcripts. Similarly, a single mRNA may be regulated by multiple miRNAs, thus forming a complex regulatory network [16,17]. Some lncRNAs also have binding sites with miRNA, which act as miRNA sponges in cells, and then release the inhibition of miRNA on its target mRNA, increase the expression level of target genes, and construct more complex ceRNA networks (ceRNets).

This study aims to analyze the differences of LncRNA, miRNA and mRNA expression patterns in fresh synovial tissues of OA patients by next generation sequencing technology, which may help to identify OA related ncRNAs and provide potential therapeutic biomarkers for clinical treatment. The most obvious inflammation was found in synovial tissue of OA. Compared with rheumatoid arthritis (RA), OA synovial inflammation is less obvious, but associated with pain and structural decline [18,19]. Here, we pay more attention to the expression of inflammatory related genes, hoping to find the key signal network of OA pathogenesis.

Methods

Patients and samples

Synovial tissue samples from all patients were obtained during joint surgery at the Wuxi No.2 People's Hospital in 2020. Fresh materials were immediately transferred to liquid nitrogen for preservation. All patients obtained written informed consent and were treated according to the regulations of the local ethics committee (Ethics Committee of Wuxi No.2 People's Hospital). The whole transcriptome analysis (RA = 2, OA = 8) was performed in 10 patients. The diagnosis was made according to ACR / EULAR standard 2010. 8 samples of OA patients served as OA group and 2 samples of RA patients served as control group, because synovial tissue of healthy people could not be obtained by surgery.

RNA library construction and sequencing

Total sample RNA was isolated and purified using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. The RNA amount and purity of each sample was quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed by Agilent 2100 with RIN number > 7.0. Approximately 5 ug of total RNA was used to deplete ribosomal RNA according to the manuscript of the Ribo-Zero™ rRNA Removal Kit (Illumina, San Diego, USA). After removing

ribosomal RNAs, the left RNAs were fragmented into small pieces using divalent cations under high temperature. Then the cleaved RNA fragments were reverse-transcribed to create the cDNA, which were next used to synthesize U-labeled second-stranded DNAs with E. coli DNA polymerase I, RNase H and dUTP. An A-base is then added to the blunt ends of each strand, preparing them for ligation to the indexed adapters. Each adapter contains a T-base overhang for ligating the adapter to the A-tailed fragmented DNA. Single- or dual-index adapters are ligated to the fragments, and size selection was performed with AMPure XP beads. After the heat-labile UDG enzyme treatment of the U-labeled second-stranded DNAs. The ligated products are amplified with PCR by the following conditions: initial denaturation at 95°C for 3 min; 8 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 30 sec; and then final extension at 72°C for 5 min. The average insert size for the final cDNA library was 300 bp ($\pm$ 50 bp). At last, we performed the paired-end sequencing on an Illumina HiSeq 4000 (LC Bio, China) following the vendor's recommended protocol.

Bioinformatics analysis of miRNA Library

Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18 ~ 26 nucleotide were mapped to specific species precursors in miRBase 22.0 by BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. Length variation at both 3' and 5' ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to specific species mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known specific species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p derived miRNA candidates. The remaining sequences were mapped to other selected species precursors (with the exclusion of specific species) in miRBase 22.0 by BLAST search, and the mapped pre-miRNAs were further BLASTed against the specific species genomes to determine their genomic locations. The above two we defined as known miRNAs. The unmapped sequences were BLASTed against the specific genomes, and the hairpin RNA structures containing sequences were predicted from the flank 80nt sequences using RNAfold software (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>).

The criteria for secondary structure prediction were:

- (1) number of nucleotides in one bulge in stem (≤ 12)
- (2) number of base pairs in the stem region of the predicted hairpin (≥ 16)
- (3) cutoff of free energy (kCal/mol ≤ -15)
- (4) length of hairpin (up and down stems + terminal loop ≥ 50)
- (5) length of hairpin loop (≤ 20).

- (6) number of nucleotides in one bulge in mature region (≤ 8)
- (7) number of biased errors in one bulge in mature region (≤ 4)
- (8) number of biased bulges in mature region (≤ 2)
- (9) number of errors in mature region (≤ 7)
- (10) number of base pairs in the mature region of the predicted hairpin (≥ 12)
- (11) percent of mature in stem (≥ 80).

Analysis of Differential expressed miRNAs (DEmiRNAs) and target genes prediction

Differential expression of miRNAs based on normalized deep-sequencing counts was analyzed by selectively using Fisher's exact test, Chi-squared 2X2 test, Chi-squared nXn test, Student t test, or ANOVA based on the experiment's design. The significance threshold was set to be 0.01 and 0.05 in each test.

In order to predict the most abundant miRNA target genes, we used two computational target gene prediction algorithms (TargetScan, V5.0 and Miranda, v3.3a) to identify miRNA binding sites. The data predicted by the two algorithms were combined to calculate the overlaps. The GO terms and KEGG Pathway of these most abundant miRNAs, miRNA targets were also annotated.

Transcripts Assembly for LncRNA

Firstly, Cutadapt was used to remove the reads that contained adaptor contamination, low quality bases and undetermined bases. Then sequence quality was verified using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). We used Bowtie2 and Hisat2 to map reads to the genome of Homo sapiens. The mapped reads of each sample were assembled using StringTie. Then, all transcriptomes from synovial tissue samples were merged to reconstruct a comprehensive transcriptome using perl scripts. After the final transcriptome was generated, StringTie and edgeR was used to estimate the expression levels of all transcripts.

LncRNA identification, Different expression analysis of mRNAs and lncRNAs and Target gene prediction

First of all, transcripts that overlapped with known mRNAs and transcripts shorter than 200 bp were discarded. Then we utilized CPC [6] and CNCI [7] to predict transcripts with coding potential. All transcripts with CPC score < -1 and CNCI score < 0 were removed. The remaining transcripts were considered as lncRNAs.

StringTie was used to perform expression level for mRNAs and lncRNAs by calculating FPKM. The differentially expressed mRNAs and lncRNAs were selected with $\log_2$ (fold change) > 1 or $\log_2$ (fold change) < -1 and with statistical significance (p value < 0.05) by R package – edgeR.

To explore the function of lncRNAs, we predicted the cis-target genes of lncRNAs. lncRNAs may play a cis role acting on neighboring target genes. In this study, coding genes in 100,000 upstream and downstream were selected by python script. Then, we showed functional analysis of the target genes for lncRNAs by using the BLAST2GO. Significance was expressed as a p value < 0.05

Results

lncRNA and miRNA sequencing profile

A total of 77821 mRNAs, 890 miRNAs and 47522 lncRNAs (known and novel) were identified in 10 synovial tissues. As shown in Fig. 1, the detected lncRNAs were widely distributed on all human chromosomes. By comparing the lncRNAs and mRNA expression profiles between OA group and control group, 343 mRNAs were significantly up-regulated and 232 mRNAs were significantly down regulated. For lncRNAs, 270 was significantly up-regulated and 109 was significantly down regulated. For miRNAs, 247 was significantly up-regulated and 157 was significantly down regulated ($P < 0.05$, fold change > 2).

The most up-regulated lncRNA was NEAT1, with a 210-fold change; the most down regulated lncRNA was FP671120, with a 0.047-fold change. The most up-regulated mRNA was LEP, with a 57-fold change; the most down regulated known mRNA was LAMP5, with a 0.079 fold change. Table 1 show the top 5 up and down regulated lncRNAs and mRNAs. The volcanic plots and thermal map of lncRNAs and mRNAs were displayed in Fig. 2.

Table 1
Top 5 Significantly Up- and Down-regulated mRNAs, lncRNAs and miRNAs.

Gene name	log2(fold change)	Regulation	RNA sort
LAMP5	-3.65	Down	mRNA
HJV	-3.64	Down	mRNA
CXCL8	-2.67	Down	mRNA
DMKN	-2.62	Down	mRNA
PLA2G7	-2.36	Down	mRNA
NAT8L	5.09	Up	mRNA
ADH1B	5.44	Up	mRNA
CYP4Z1	5.78	Up	mRNA
AL845331	5.78	Up	mRNA
LEP	5.83	Up	mRNA
ARRDC3-AS1	-3.88	Down	lncRNAs
GUSBP11	-3.88	Down	lncRNAs
MIR4435-2HG	-4.02	Down	lncRNAs
CKMT2-AS1	-4.32	Down	lncRNAs
FP671120	-4.39	Down	lncRNAs
NEAT1	7.72	Up	lncRNAs
AP000757	6.86	Up	lncRNAs
XIST	6.84	Up	lncRNAs
AC060780	6.35	Up	lncRNAs
AC008691	5.45	Up	lncRNAs

Table 2
Top 10 pairs of differential lncRNA-mRNA identified in OA

mRNA	lncRNA	cis location	P value
SLX1A	SLX1A-SULT1A3	1K	-0.70
BTK	ARMCX4	100K	-0.57
SYNPO2	SEC24D	100K	-0.50
NBR1	AC060780	10K	-0.42
REV3L	MFSD4B	100K	-0.35
CSNK1A1	CARMN	100K	-0.34
ZFP62	LINC00847	100K	-0.31
CRIP2	MIR8071-1	100K	-0.27
FKBP15	FAM225A	100K	-0.23
DDIT3	MBD6	100K	-0.22

GO and KEGG Enrichment analysis of lncRNAs/miRNA target mRNAs

In order to further study the regulation of lncRNAs on gene expression through a possible ceRNA network during OA, we predicted the interaction between the upstream and downstream of differentially expressed lncRNAs within 100k bp. 55 pairs of differential lncRNA-mRNA were identified. These differentially expressed lncRNAs may affect OA process through cis regulation of mRNAs, which were differential expressed. The results are shown in supplementary Table 2 (TOP10).

GO analysis was performed on all the target mRNAs expressing significantly different lncRNAs. As shown in Fig. 3A, negative regulation of receptor-mediated endocytosis, ribosomal large subunit binding, filopodium membrane signaling pathway are the main functions associated with dysregulated lncRNAs.

We performed KEGG pathway analysis on the target mRNA of these dysregulated lncRNAs. The main enrichment pathways include ABC transporters, EGFR tyrosine kinase inhibitor resistance, allograft rejection (Fig. 3C).

We also predicted the possible target genes of differential miRNA. Go enrichment analysis showed that thromboxane-a synthase activity, B cell apoptotic process and fibrin odium membrane were the main enriched GO terms (Fig. 3B). Pathway in cancer was the main enriched KEGG signaling pathway (Fig. 3D).

OA related lncRNAs, miRNA and mRNAs enriched in synovial tissue samples

In this study, we focus on the expression of OA related genes and inflammatory markers. Part of those genes, lncRNA and miRNAs were dysregulated in OA tissues. OA related genes CHST11, ALDH1A2 and inflammatory genes TREM1, IL-1 β , IL-8, CCL5, LIF were dysregulated in our OA group. miR-146a-5p, miR-335-5p (target with IL-1 β) and lncRNA GAS5 (miR-34a-Bcl2 axis), LINC02288 (miR-374a-3p-RTN3 axis) and LOC101928134 (Target with IFNA1) and were also identified to be differentially expressed. More details were shown in Fig. 4.

Discussion

Osteoarthritis mainly affects the joint parts, including knee, hand, hip and spine, which is the main cause of elderly people's mobility [20,21]. Although genetic susceptibility, aging, obesity and malalignment of joints have been regarded as risk factors for OA, the pathogenesis of OA is still unclear [22,23]. Therefore, in addition to total joint replacement, there is no effective intervention to slow down the progress of OA or delay the irreversible degeneration of cartilage [24]. In the past decade, with the application of genome-wide association scanning (GWAS) technology, the amount of data of osteoarthritis genome analysis has increased exponentially. In this study, RNA-seq technique was used to study the whole transcriptome changes of synovial tissue in OA patients and control group. By comparing the transcriptome of control group, we identified 1358 differentially expressed transcripts (mRNA, lncRNA and miRNA) between the control group and OA group, including 860 up-regulated transcripts and 498 down-regulated transcripts. Some of these genes have been confirmed to be involved in the progression of inflammation in OA.

CHST11 protein belongs to the thiotransferase 2 family, which catalyzes sulfate transfer to the fourth position of N-acetylgalactosamine (GalNAc) residue of chondroitin. Chondroitin sulfate is the main proteoglycan in cartilage, which is distributed on the surface of many cells and extracellular matrix. Therefore, the abnormal expression of this gene is associated with osteochondral dysplasia, short finger deformity and other diseases. In 2012, a GWAS study on knee osteoarthritis in European population found that CHST11 was associated with OA ($P = 1.64 \times 10^{-8}$) [25]. In our study, CHST11 was down-regulated in OA group ($P < 0.05$).

ALDH1A2 belongs to the aldehyde dehydrogenase family. As an enzyme, it catalyzes the synthesis of retinoic acid from retinoic aldehyde. Retinoic acid is an active derivative of vitamin A (retinol), which plays a role in development and adult tissues. Studies of mouse like genes have shown that this enzyme, with the help of cytochrome CYP26A1, maintains retinoic acid levels, promotes bone development and prevents spina bifida. The expression level of ALDH1A2 is closely related to the pathogenesis of hand OA in Icelandic patients [26]. In this study, we also found that the expression of ALDH1A2 was significantly down regulated in OA patients.

Toll like receptor 4 (TLR4) is activated in acute inflammatory response, which induces up regulation of TREM1 expression. The latter results in the expression of proinflammatory cytokines (including TNF- α and IL-1 β) [27]. Western blot analysis confirmed that TREM1 was highly expressed in inflamed synovium

[28]. We also found that TREM1 was significantly up-regulated in the damaged cartilage [29]. These results are consistent with our results. The expression of TREM1 in OA patients is higher than that in RA control group, suggesting that there is a higher level of inflammation in OA cartilage. The high expression of downstream IL-1 β also confirmed this. IL-1 β is related to many pathological features of OA [30]. IL-1 β signaling has been shown to regulate the expression of 909 of 3459 genes in primary human articular chondrocytes, including significant induction of a large number of chemokines and inflammatory mediators, such as genes IL-11 and CCL5 [31]. Other OA related inflammatory factors include IL-8 and LIF [32].

The differentially expressed ncRNA identified in synovial samples were also been studied in previous studies. miR-146a-5p of OA patients was significantly over-expressed both in the articular cartilage tissue and serum [33]. miR-335-5p attenuates chondrocyte inflammation by activating autophagy in osteoarthritis [34]. Overexpression of miR-335-5p down regulates the expression of inflammatory mediators IL-1 β , IL-6 and TNF- α , and increases the expression of autophagy markers beclin-1, autophagy related protein 5 (ATG5) and ATG7. Studies have shown that lncRNA GAS5 is highly expressed in OA cartilage and increases with the progress of OA. GAS5 inhibits autophagy and promotes apoptosis of chondrocytes, which may be related to miR-34a / Bcl-2 axis or mTOR [35,36]. Similarly, LINC02288 expression was significantly up-regulated in OA rat model. Down regulation of LINC02288 can significantly reduce the apoptosis and pro-inflammatory cytokine production of OA chondrocytes induced by IL-1 β [37]. The study of lncRNA expression profile in synovial tissue showed that LOC101928134 was highly expressed in synovial tissue of OA rats, which regulated IFNA1 gene expression and inhibited JAK/STAT signaling pathway. The down-regulation of loc101928134 resulted in the reduction of synovitis, inflammatory injury and cartilage injury in OA rats. In addition, the down-regulation of LOC101928134 can enhance the apoptosis of synoviocytes and inhibit the apoptosis of chondrocytes in OA rats [38].

In conclusion, we identified the differential expression of lncRNA, mRNA and miRNA in synovial tissue of osteoarthritis by RNA-seq. Although the analysis of ceRNA network was not carried out in this study, 12 transcripts related to OA and its inflammatory progression were chosen. These differentially expressed transcripts may indicate the ceRNA network in synovial tissue during osteoarthritis and its effect on OA progression. Our study will contribute to the exploration of OA mechanism and key therapeutic targets.

Declarations

Ethics approval and consent to participate

Sample collection and experimental procedures were approved by the Ethics Review Committee of Nanjing Medical University (no. (2021)220). Inform consents were obtained from all the participants.

Consent for publication

All authors agree to publish this paper.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All the authors have no conflict of interests in relation to this study.

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Author contributions

Yanglin Gu designed all the experiments; Linghui Qiao, Guancheng Wang, Jie Han, and Yang Liu performed the experiments. Linghui Qiao wrote the manuscript. All authors reviewed the manuscript.

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Figures

Figure 1

Long noncoding RNAs (lncRNA) mapping shows lncRNA distribution in each chromosome. The outer 8 rings are the OA group, and the inner 2 are the control group.

Figure 2

The expression profiling in the radiation-induced lung injury group and control group. A total of upregulated lncRNAs and downregulated lncRNAs are displayed in (A) the heat map and (C) the volcano plot; upregulated mRNAs and downregulated mRNAs are displayed in (B) the heat map and (D) the volcano plot. Red represents upregulation and blue represents downregulation.

Figure 3

GO and KEGG enrichment analysis (A) Gene Ontology (GO) annotations and (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for the mRNAs regulated by lncRNA. (B) Gene Ontology (GO) annotations and (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for the miRNAs. The top 20 according to P value of each analysis are displayed in KEGG pathways.

Figure 4

12 OA related lncRNAs, miRNA and mRNAs enriched in synovial tissue samples.