Original Article

Expression profile of circular RNA s in TMJ osteoarthritis synovial tissues and potential functions of hsa_circ_0000448 with specific back-spliced junction

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Abstract: Objective: As essential players in the occurrence and development of osteoarthritis, circular RNAs (circRNAs) have gradually received attention in recent years. However, their roles in temporomandibular joint osteoarthritis (TMJOA) featured with pain, restricted mouth opening and joint movement dysfunction, remains elusive. Methods: The expression profile of circRNAs in TMJ synovial tissues was analyzed by RNA-Seq. The differentially expressed circRNAs in the TMJOA were identified. The potential biological functions of these circRNAs were evaluated. Results: The expression profile of circRNAs in TMJ synovial tissues was stable and abundant, and most of which were newly discovered. A total of 58 differentially expressed circRNAs were identified in TMJOA, and four of which were identified by in vitro experiments. Among them, the up-regulated hsa_circ_0000448 with specific back-splice junction was involved in the TNF-α signaling pathway through CeRNA mechanism by targeting related microRNAs. Additionally, it was also predicted to bind several RNA binding proteins (RBPs), but almost had no protein-coding ability. Conclusion: circRNAs in TMJ synovial tissue participate in the progression of TMJOA and may become a potential therapeutic target. The highly up-regulated has_circ_0000448 probably promotes TNF-α secretion of synovium through CeRNA mechanism in TMJOA.

Keywords: Temporomandibular joint, osteoarthritis, circRNAs, synovial tissue, TNF-α

Introduction

Temporomandibular joint osteoarthritis (TMJOA) is a frequently-occurring disease with an incidence rate of 25%-70% [1]. It commonly occurs in women and its incidence rate increases over aging. TMJOA is mainly manifested with pain in the joint area, joint movement dysfunction, restricted mouth opening and joint noises [2]. As a chronic progressive disease, it affects the health and quality of life of patients. However, the specific pathogenesis is still unclear.

Synovial inflammation has been proven to play a key role in the incidence and development of TMJOA [3]. Vascular proliferation, fibrosis, and inflammatory cell infiltration of surrounding synovial tissue can destroy the TMJ condylar cartilage [4]. Due to the limited regenerative capacity, it eventually develops into TMJOA through an irreversible process. Previous studies mainly focus on the lesions of condylar cartilage and subchondral bone and the pathological mechanism of TMJOA. The changes in surrounding synovial tissues and the effect on TMJOA have been largely neglected. Thus, the inherent mechanism underlying the transcriptional regulation has been rarely reported, which might affect the progression of TMJOA.

CircRNAs have recently received widespread attention due to the influence on the transcriptional regulation, which is closely correlated with the occurrence, development, diagnosis and even treatment of certain diseases [5-7]. CircRNAs are a kind of non-coding circular RNAs widely present in eukaryotic cells, formed by the 3’ end link to the 5’ end through close covalent bonds [8]. It is characterized by abundant expression, high stability, a highly-conserved sequence, tissue specificity and specific expression at different developmental stages [9, 10].
Expression profile of circular RNAs in TMJ osteoarthritis synovial tissues

With the development of high-throughput sequencing technology (RNA-Seq) and bioinformatics, a large number of circRNAs have been identified. Several studies have found that circRNAs have multiple biological functions, such as acting as a miRNA sponge to regulate the post-transcriptional gene expression, binding to RNA-binding proteins (RBPs) to affect the mRNA transcription, translation proteins and a biomarker for the diagnosis and prognosis of certain diseases [11-13].

As for its role in osteoarthritis, circRNAs have been reported to exert significant impact on the limb and facet joint osteoarthritis. Liu et al. [14] have demonstrated that 71 circRNAs are differentially expressed in osteoarthritis articular cartilage compared with those in healthy controls. Among them, circRNA-100876 (circRNA-CER) is a chondrocyte extracellular matrix-related circRNA capable of regulating the degradation of cartilage extracellular matrix by absorbing miRNA-136 as a sponge mechanism.

Another circRNA (has_circ_0005105) reported by Wu et al. [15] is up-regulated in IL-1β-stimulated chondrocytes and participates in the development of OA through competitively binding to miRNA-26a, thus promoting the ECM degradation. In addition, some related circRNAs can also regulate the chondrocyte inflammatory responses, mediate cellular apoptosis and affect disease progression by participating in the TGF-β, JNK, ERK and other related signaling pathways [15, 16]. As for TMJOA, especially for synovial inflammation, the expression profile of circRNAs is still largely unknown. Besides, whether certain disease-related circRNAs that effect the occurrence and development of TMJOA exist remains elusive.

The purpose of this study was to explore the expression profile of circRNAs in TMJ synovial tissues by using the RNA-Seq. The differentially expressed circRNAs in the TMJOA were identified and the potential biological functions of these circRNAs were explored, aiming to provide evidence and insight into the therapeutic targets of TMJOA.

Materials and methods

Patient selection and sample collection

Ten patients who underwent TMJ disc surgery in the Department of Oral Surgery of Shanghai Ninth People’s Hospital from December 2017 to February 2018 were enrolled (Table 1). All selected patients had no systematic inflammatory diseases. According to clinical symptoms (Table 2) and MRI images (Figure 1), all patients were divided into the experimental group with the diagnosis of TMJOA (Wilkes Stage 4 with bony changes and arthroedema; n = 5), and control group (Wilkes Stage 2-3 without bony changes and arthroedema but with anterior disc displacement; n = 5). Specimens in the experimental group were taken from synovial tissues on the affected side of TMJOA patients, and those in the control group were collected from the synovial tissues of the anterior disc displacement (ADD) patients’ surgery side. Intraoperatively, a 5-mm diameter specimen was collected. The specimen was repeatedly washed with 0.9% normal saline to remove impurities, such as blood cells and immediately placed in liquid nitrogen for storage.

Statement

All subjects gave their informed consents for inclusion before the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Shanghai Ninth People’s Hospital affiliated to Shanghai Jiaotong University (S9H-2019-TK247-1). The sample collection was authorized by the patients’ family and signed the informed consent form.

Table 1. Baseline data of enrolled patients

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Clinical Stage</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. OQY</td>
<td>Female</td>
<td>28 y</td>
<td>TMJOA</td>
<td>Wilkes Stage 4</td>
<td>Experiment</td>
</tr>
<tr>
<td>2. ZXR</td>
<td>Female</td>
<td>27 y</td>
<td>TMJOA</td>
<td>Wilkes Stage 4</td>
<td>Experiment</td>
</tr>
<tr>
<td>3. SQ</td>
<td>Female</td>
<td>57 y</td>
<td>TMJOA</td>
<td>Wilkes Stage 4</td>
<td>Experiment</td>
</tr>
<tr>
<td>4. LKK</td>
<td>Female</td>
<td>18 y</td>
<td>TMJOA</td>
<td>Wilkes Stage 4</td>
<td>Experiment</td>
</tr>
<tr>
<td>5. CWH</td>
<td>Female</td>
<td>45 y</td>
<td>TMJOA</td>
<td>Wilkes Stage 4</td>
<td>Experiment</td>
</tr>
<tr>
<td>6. XXF</td>
<td>Female</td>
<td>24 y</td>
<td>TMD</td>
<td>Wilkes Stage 2-3</td>
<td>Control</td>
</tr>
<tr>
<td>7. FJY</td>
<td>Female</td>
<td>39 y</td>
<td>TMD</td>
<td>Wilkes Stage 2-3</td>
<td>Control</td>
</tr>
<tr>
<td>8. SYL</td>
<td>Female</td>
<td>55 y</td>
<td>TMD</td>
<td>Wilkes Stage 2-3</td>
<td>Control</td>
</tr>
<tr>
<td>9. HYX</td>
<td>Female</td>
<td>26 y</td>
<td>TMD</td>
<td>Wilkes Stage 2-3</td>
<td>Control</td>
</tr>
<tr>
<td>10. CLF</td>
<td>Female</td>
<td>54 y</td>
<td>TMD</td>
<td>Wilkes Stage 2-3</td>
<td>Control</td>
</tr>
</tbody>
</table>

Abbreviations: TMJOA (temporomandibular osteoarthritis), TMD (temporomandibular disorders).
RNA extraction, library construction and high-throughput sequencing

Trizol was used to extract the total RNA and the concentration of each sample was detected by Nano Drop 2000 (Thermo Fisher Scientific, USA). RNA integrity and gDNA (genomic DNA) contamination were measured by denaturing agarose gel electrophoresis. The rRNAs (ribosomal RNA) were dropped by Ribo-Zero rRNA removal kit (Illumina, USA). Library construction using rRNA-depleted RNAs was finished by TruSeq stranded total RNA library prep kit (Illumina, USA). The quality and quantified controls of RNA library were detected by the BioAnalyzer 2100 system (Agilent Technologies, USA).

### Table 2. Clinical symptoms of enrolled patients

<table>
<thead>
<tr>
<th>Name</th>
<th>Pain</th>
<th>Clicking</th>
<th>MIO</th>
<th>Hydrarthrosis</th>
<th>Articular Disc &amp; Bone Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. OQY</td>
<td>√</td>
<td>none</td>
<td>25 mm</td>
<td>√</td>
<td>R-ADDWo/R with bony changes</td>
</tr>
<tr>
<td>2. ZXR</td>
<td>√</td>
<td>none</td>
<td>26 mm</td>
<td>√</td>
<td>Bi-ADDWo/R with bony changes</td>
</tr>
<tr>
<td>3. SQ</td>
<td>√</td>
<td>none</td>
<td>30 mm</td>
<td>√</td>
<td>L-ADDWo/R with bony changes</td>
</tr>
<tr>
<td>4. LKK</td>
<td>√</td>
<td>none</td>
<td>24 mm</td>
<td>√</td>
<td>Bi-ADDWo/R with bony changes</td>
</tr>
<tr>
<td>5. CWH</td>
<td>√</td>
<td>none</td>
<td>17 mm</td>
<td>√</td>
<td>R-ADDWo/R with bony changes</td>
</tr>
<tr>
<td>6. XXF</td>
<td>none</td>
<td>√</td>
<td>30 mm</td>
<td>none</td>
<td>Bi-ADDWo/R without bony changes</td>
</tr>
<tr>
<td>7. FJY</td>
<td>none</td>
<td>√</td>
<td>35 mm</td>
<td>none</td>
<td>L-ADDWo/R without bony changes</td>
</tr>
<tr>
<td>8. SYL</td>
<td>none</td>
<td>√</td>
<td>25 mm</td>
<td>none</td>
<td>Bi-ADDWo/R without bony changes</td>
</tr>
<tr>
<td>9. HYX</td>
<td>none</td>
<td>√</td>
<td>35 mm</td>
<td>none</td>
<td>L-ADDWo/R without bony changes</td>
</tr>
<tr>
<td>10. CLF</td>
<td>none</td>
<td>√</td>
<td>25 mm</td>
<td>none</td>
<td>R-ADDWo/R without bony changes</td>
</tr>
</tbody>
</table>

Abbreviations: Bi (bilateral), R (right), L (left), MIO (maximal interincisal opening), ADDWo/R (anterior disc displacement without reduction), “√” = having related clinical symptoms.

### Figure 1. MRI images of control and TMJOA patients

A, B. Mouth opening positions of control and TMJOA patients. C, D. Mouth closing positions of control and TMJOA patients. (Yellow arrows: TMJ disc anterior displacement; Red arrows: TMJ hydrarthrosis in upper joint space; Blue arrows: bony changes/bone resorption at posterior slope of TMJ condyle).
USA). Denaturing 10 pM library as single strand DNA, capturing on Illumina flow cells, then amplifying in situ as clusters and finally sequencing for 150 cycles on Illumina HiSeq sequencer. The RNA-Seq service was provided by CloudSeq Biotech (Shanghai, China).

**CircRNAs standardization, annotation and expression profile**

The paired-end reads were collected from sequencer, and Q30 was used for quality control. Following with 3’ adaptor-trimming and low-quality reads excluding using Cutadapt software [17]. The circRNAs with high quality trimmed reads were analyzed and aligned to the reference human genome (GRCh37/hg19) with STAR software, thus using DCC software for circRNAs identification [18]. Raw junction-reads were normalized by matched reads number using log\(^2\) transformation. The number of junction reads of the circRNAs found in each sample was shown by the number of aligned spliced reads as the expression level of the circRNAs. The circRNAs were identified using the circBase (http://www.circbase.org/) based on the genomic location and linked to the circ2Trait circular RNA-disease database (http://gyanex-beta.com/circdb/). According to the alignment position of the two ends of the circular RNA, it was divided into exonic, intronic, intergenic, sense overlapping and antisense circRNAs. The newly-identified circular RNAs were labeled as novel RNAs.

**CircRNAs clustering, differential expression and bioinformatics analysis**

Using R’s heatmap2 package for circRNAs clustering analysis based on normalized reads, the differential expression of circRNAs between two groups were calculated using the number of normalized reads with fold change and \(P\)-value. The differential expression of circRNAs was defined as fold change \(\geq 2.0\) and \(P\)-value \(\leq 0.05\). The host genes of differentially-expressed circular RNA were enriched for Gene ontology (GO) and KEGG pathway analyses using Metascape (http://metascape.org) [19]. Using \(P\)-value \(\leq 0.05\) as a significant threshold for enrichment. And their expression conditions in different cells were also found based on human proteome. The protein-protein interaction (PPI) network was constructed by STRING database [20].

**Validation of selected circRNAs**

Parts of differentially-expressed circRNAs were selected for validation. Selection criteria were as below: (1) host genes located in exon regions; (2) the predicted lengths within the range of 300-3000 bp (base pair); (3) \(P\)-value < 0.05; (4) Fold change > 2; (5) individual extreme values were excluded within the group. Then top 10 differentially expressed circRNAs (3 up-regulated and 7 down-regulated) were selected (Table 3). The sample size of the control group (\(n = 10\)) and the experimental group (\(n = 10\)) were expanded for qPCR verification. Specific primers were designed according to the circRNAs' back-splice sites using the “out-facing” strategy, where circle templates were amplified (Table 4). The results were normalized with β-actin gene as an internal reference. All samples were analyzed with three independent tests. The relative expression of circRNAs was calculated using \(2^{-\Delta\Delta Ct}\) method. According to qPCR results, the circRNAs that consistent with RNA-Seq were chosen for enzyme tolerance test (RNase

<table>
<thead>
<tr>
<th>CircRNAID</th>
<th>P-value</th>
<th>Fold Change</th>
<th>Regulation</th>
<th>circBase ID</th>
<th>Gene Name</th>
<th>Type</th>
<th>Predicted length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. chr15:59204762-59209198+</td>
<td>0.004</td>
<td>162.17</td>
<td>Up</td>
<td>hsa_circ_0000605</td>
<td>SLTM</td>
<td>exonic</td>
<td>331 bp</td>
</tr>
<tr>
<td>2. chr12:120592774-120593523+</td>
<td>0.005</td>
<td>153.30</td>
<td>Up</td>
<td>hsa_circ_0000448</td>
<td>GCN1</td>
<td>exonic</td>
<td>390 bp</td>
</tr>
<tr>
<td>3. chr21:37711073-37717005+</td>
<td>0.005</td>
<td>104.71</td>
<td>Up</td>
<td>none</td>
<td>MORC3</td>
<td>sense-overlap</td>
<td>5933 bp</td>
</tr>
<tr>
<td>4. chr7:27945327-27960247+</td>
<td>0.001</td>
<td>425.31</td>
<td>Down</td>
<td>hsa_circ_00009043</td>
<td>EXOC6B</td>
<td>exonic</td>
<td>527 bp</td>
</tr>
<tr>
<td>5. chr5:364076753-134079742+</td>
<td>0.043</td>
<td>71.01</td>
<td>Down</td>
<td>hsa_circ_0003154</td>
<td>CAMLG</td>
<td>exonic</td>
<td>527 bp</td>
</tr>
<tr>
<td>6. chr12:12397196-12397589+</td>
<td>0.035</td>
<td>62.68</td>
<td>Down</td>
<td>hsa_circ_0003378</td>
<td>LRPS6</td>
<td>exonic</td>
<td>394 bp</td>
</tr>
<tr>
<td>7. chr5:95091100-95099324+</td>
<td>0.014</td>
<td>55.09</td>
<td>Down</td>
<td>hsa_circ_0007444</td>
<td>RHOBTB3</td>
<td>exonic</td>
<td>479 bp</td>
</tr>
<tr>
<td>8. chr17:33495080-33495704+</td>
<td>0.039</td>
<td>47.18</td>
<td>Down</td>
<td>none</td>
<td>UNC45B</td>
<td>sense-overlap</td>
<td>625 bp</td>
</tr>
<tr>
<td>9. chr5:52773405-52773806+</td>
<td>0.030</td>
<td>46.20</td>
<td>Down</td>
<td>hsa_circ_0001801</td>
<td>PCMTD1</td>
<td>exonic</td>
<td>402 bp</td>
</tr>
<tr>
<td>10. chrX:139865340-139866824+</td>
<td>0.009</td>
<td>2.73</td>
<td>Down</td>
<td>hsa_circ_0001946</td>
<td>CDR1</td>
<td>antisense</td>
<td>1485 bp</td>
</tr>
</tbody>
</table>
Expression profile of circular RNAs in TMJ osteoarthritis synovial tissues

R treatment for qPCR) and Sanger sequencing to confirm the circRNAs.

CircRNA-miRNA-target gene network

The up-regulated circRNAs with significant differences were used for advanced analysis. The binding ability of circRNAs to miRNAs were predicted by StarBase (http://starbase.sysu.edu.cn) [21], circRNA Interactome (https://circinteractome.nia.nih.gov/) [22] and RegRNA2.0 (http://regrna2.mbc.nctu.edu.tw) [23]. The target genes of related miRNAs were predicted using Targetscan (http://www.targetscan.org/vert_72) [24] and miRanda (http://www.microrna.org/microrna/) [25]. And the circRNA-miRNA-target gene network was constructed using Cytoscape software [26].

Visualization of hsa_circ_0000448 and prediction of potential functions

The specific back-splice site of the circRNA targeted TNF-related miRNA was examined by Sanger sequencing of the qPCR product. Visualization of this circRNA and its conserved characteristic were shown by human genome browser. By combining the circBase information, genome browser results and Sanger sequencing, the formation mechanism of the target circRNA was predicted. Lastly, the RBP binding and protein-coding abilities of the circRNA were predicted using circRNA Interactome and circRNAdb database respectively.

Results

Baseline data and MRI imaging

Patients with systemic diseases were excluded from this study. All patients included in this study were female. In the experimental group, the mean age of patients was (35.0 ± 15.7) years and (39.6 ± 15.8) years in the control group. The baseline data and clinical symptoms of the patients are shown in Tables 1, 2. The typical MRI images of the two groups are shown in Figure 1.

RNA and library quality

The A260/280 value of each sample was 1.8 to 2.0, indicating that the total RNA had high purity. The total amount of RNA extracted from

<table>
<thead>
<tr>
<th>Number</th>
<th>CircRNA ID/Gene Name</th>
<th>Primer Type</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>chr15:59204762-59209198-1-Forward</td>
<td>1-Forward</td>
<td>GAGGACATCGAAGCTCAGGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-Reverse</td>
<td>CCATCTTGCTCATGTGCTCCT</td>
</tr>
<tr>
<td>2</td>
<td>chr12:120592774-120593523-2-Forward</td>
<td>2-Forward</td>
<td>GGAGGTTATGGGCAGGGCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-Reverse</td>
<td>TTCCGGGTACTCTCTCTCTC</td>
</tr>
<tr>
<td>3</td>
<td>chr21:37711073-37717005+3-Forward</td>
<td>3-Forward</td>
<td>CGCAAGCCCAGAAGCTGTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-Reverse</td>
<td>AGCAAAGCTGAGCTGTTTT</td>
</tr>
<tr>
<td>4</td>
<td>chr2:72945232-72960247-4-Forward</td>
<td>4-Forward</td>
<td>TTTCGGAGAGCAGACCCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-Reverse</td>
<td>GCTTCAGCTCTCCATGCTC</td>
</tr>
<tr>
<td>5</td>
<td>chr5:134076753-134079742+5-Forward</td>
<td>5-Forward</td>
<td>TGGGATGTCCTCTCCTGCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-Reverse</td>
<td>CGCTTGGAAAGCCAGAAG</td>
</tr>
<tr>
<td>6</td>
<td>chr12:12397196-12397589-6-Forward</td>
<td>6-Forward</td>
<td>GCATGTCATGGGCTGTTT</td>
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<tr>
<td></td>
<td></td>
<td>6-Reverse</td>
<td>CCGAAGTCCGTCCTGTTT</td>
</tr>
<tr>
<td>7</td>
<td>chr5:95091100-95099324+7-Forward</td>
<td>7-Forward</td>
<td>CAGGTCCTTTCAGGTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-Reverse</td>
<td>TGGGACGAGCACAGCAG</td>
</tr>
<tr>
<td>8</td>
<td>chr17:33495080-33495704+8-Forward</td>
<td>8-Forward</td>
<td>GACGTTCGTCTCCCTGCT</td>
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<tr>
<td></td>
<td></td>
<td>8-Reverse</td>
<td>CTGGTCCAGCTCTCTG</td>
</tr>
<tr>
<td>9</td>
<td>chr8:52773405-52773806-9-Forward</td>
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<td>AGCCTGGAGAAGCTGTTT</td>
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<tr>
<td></td>
<td></td>
<td>9-Reverse</td>
<td>GCACTACAGCCTCTCCT</td>
</tr>
<tr>
<td>10</td>
<td>chrX:139865340-139866824+10-Forward</td>
<td>10-Forward</td>
<td>CGTCTCCAGTGCTGCTGATCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10-Reverse</td>
<td>GTCGGGAAGACATGGTGT</td>
</tr>
<tr>
<td>11</td>
<td>ACTB (β-actin)</td>
<td>11-Forward</td>
<td>GTGCCGGAGACCTTTGATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-Reverse</td>
<td>CCTGTAACAAAGCAGCATCATT</td>
</tr>
</tbody>
</table>
each sample was $\geq 0.5 \mu$g, which met the quality inspection standard and sequencing requirement. Gel electrophoresis showed that the 28S and 18S rRNA bands were sharp and strong and the intensity of 28S was approximately twice the intensity of 18S, suggesting that the extracted RNA was intact without gRNA contamination. Library quality control results also met the sequencing requirement.

Expression profile of circRNAs in TMJ synovial tissues

A total of 11,648 circRNAs were expressed in TMJ synovial tissues, of which 8,226 (70.62%) were newly-discovered circRNA, 3,384 (29.1%) matched with CircBase, and 38 (0.28%) from other sequencing sources provided by CloudSeq Biotech (Shanghai, China) (Figure 2E). The location of these circRNAs was across all chromosomes, among which chromosome 1 contained the most circRNAs (1,711), whereas the chromosomes X and M contained only 335 and 117 circRNAs, significantly fewer than the others (Figure 2A). Based on genomic origin and sequence types, the number and composition ratio of each type of circRNAs were: Sense-overlapping (n = 4199, 36%), exonic (n = 3620, 31.1%), intronic (n = 3278, 28.1%), intergenic (n = 323, 2.8%), and antisense (n = 228, 2.0%) (Figure 2D). The predicted length of circRNAs mainly ranged from 300 to 3,000 bp (Figure 2B), and the average length was approximately 500 bp (Figure 2C). Although most of the genes (82.94%) generated 1-3 circRNAs, 772 genes (17.06%) generated more than three circRNAs. Among them, some genes (4.9%) generated > 6 circRNAs (Figure 2F).

Differential expression of circRNAs and bioinformatic analyses

By comparing the expression levels of circRNAs between TMJOA and control groups, the clustering map of differentially-expressed circRNAs was obtained by hierarchical clustering analysis using R software (Figure 3A). The expression pattern of circRNAs between groups was significantly different and the differential expression of circRNAs could be well classified. There were 58 differentially-expressed circRNAs defined as fold changes $\geq 2$ ($P < 0.05$, Figure 3B). Among them, 33 circRNAs were up-regulated and 25 were down-regulated (Figure 3C). Based on the expression levels of each sample, the extreme value interference was excluded, and then the top 10 circRNAs which were significantly up-regulated and down-regulated are shown in Table 3. Chr14:106090742-106109468-(circRNA ID) had the maximum fold changes (212.26 times) in the up-regulated circRNAs and hsa_circ_0009043 with 425.31 fold changes among the down-regulated circRNAs. The host gene expression levels of up-regulated circRNAs are illustrated in Figure 3D and those of the down-regulated in Figure 3E. Most of the up-regulated circRNAs of the host genes were highly activated in immune cells.

GO and KEGG pathway analyses of differentially-expressed circRNAs revealed that most of the differential circRNAs were localized in the cytoplasm. The up-regulated circRNAs were closely related to the TNF-α and IFN-γ signaling pathways, IL-10 secretion and regulation, RNA and peptide antigen binding and MHC protein complex (Figures 4, 5). Those down-regulated circRNAs were closely related to muscle fiber actin contraction, ubiquitin and ubiquitin-like proteins and activation of the Wnt signaling pathway receptors (Figures 4, 5). The hallmark gene set enrichment also indicated the up-regulated circRNAs were intimately correlated with the IFN-γ responses and TNF-α signaling via NF-κB (Figure 6A). However, the down-regulated circRNAs were correlated with myogenesis (Figure 6C). The PPI network is illustrated in Figure 6B-D.

Validation of the top 10 differentially-expressed circRNAs

The top 10 differentially-expressed circRNAs were chosen for validation. In vitro qPCR showed that 5 circRNAs were consistent with high-throughput sequencing trends and were statistically significant (all $P < 0.05$) (Figure 7). Three circRNAs of them were up-regulated: hsa_circ_0000605 (5.206 times), hsa_circ_0000448 (5.124 times), chr21:37711073-37717005+ (2.418 times). The other two circRNAs were down-regulated: hsa_circ_0009043 (2.2 times) and hsa_circ_0003154 (2.5 times). Further enzyme tolerance test verified hsa_circ_0000605, hsa_circ_0000448, chr21:37711073-37717005+ and hsa_circ_0009043 were circRNAs (Figure 7). Sanger sequenc-
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Figure 2. Expression of circRNAs in TMJ synovial tissues. A. Chromosome distribution of circRNAs (“ChrM”: mitochondrial genome). B. Length distribution of circRNAs. Most of the circRNAs were ranged from 300-3000 bp in size. C. The violin pilot of circRNAs in each chromosome. And the average length of circRNAs was approximately 500 bp. D. Classification of circRNAs. E. The proportion of identified circRNAs and newly discovered circRNAs. F. Distribution of circRNAs per gene, where most gene (82.94%) contain 1-3 circRNAs.
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Figure 3. Clustering and definition of differentially-expressed circRNAs in TMJOA. A. Heatmap of differentially-expressed circRNAs clustering analysis. B. Scatter plot of circRNAs between experimental and control groups. C. Volcano plot defined up-regulated/down-regulated circRNAs. D, E. The translation conditions of host gene from each up-regulated/down regulated circRNA based on human proteome.
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Figure 4. Gene ontology analysis of differentially-expressed circRNAs. A-C. The biological process, cellular component and molecular function of up-regulated circRNAs based on enrichment score. D-F. The biological process, cellular component and molecular function of down-regulated circRNAs. (Red boxes indicated the terms were disease related).
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**Figure 5.** KEGG pathway analysis of differentially-expressed circRNAs. A. Pathway enrichment of up-regulated circRNAs. B. Pathway enrichment of down-regulated circRNAs. (Red boxes indicated the terms were disease related).
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Figure 6. Hallmark gene set enrichment and protein-protein interaction (PPI) network. A, B. Hallmark gene set enrichment and PPI network of up-regulated circRNAs. C, D. Hallmark gene set enrichment and PPI network of down-regulated circRNAs. (Red boxes indicated the terms were disease related).
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Figure 7. Validation of selected circRNAs. A. Relative expression of selected circRNAs by q-PCR between TMJOA and control groups. B and D. Fold changes and trends of selected circRNAs based on RNA-seq (high-throughput) and q-PCR (low-throughput) results. C. Enzyme tolerance tests of selected circRNAs by RNase R treatment. (Bars represent mean ± SD (n = 10); ***: P < 0.001; **: P < 0.005; *: P < 0.05).

CircRNA-miRNA-target gene network

The differentially-expressed circRNAs verified above underwent prediction of miRNA binding by StarBase, circRNA Interactome and RegRNA2.0 databases and the target genes of related miRNAs consistent with GO analysis were also predicted. The visualization of circRNA-miRNA and miRNA-mRNA binding is shown in Figure 8. Each circRNA could bind to multiple miRNAs and acted as “miRNAs sponge”. In the up-regulated circRNAs, hsa-circ-0000605 had potential binding sites with hsa-miR-6852-3p, hsa-miR-3909, hsa-miR-556-5p, hsa-miR-455-5p, hsa-miR-515-5p and other 15 miRNAs. Among them, hsa-miR-1184 and hsa-miR-515-5p were predicted to bind hsa-circ-0000605 in two databases (StarBase, circRNA Interactome), and they could also bind to TNF (mRNA), potentially forming a regulatory network of hsa_circ_0000605-hsa_miR_515-5p-TNF and hsa_circ_0000605-hsa_miR_1184-TNF. In addition, hsa_circ_0000448 could also form a regulatory network of hsa_...
The visualization of hsa_circ_0000448 originated from GCN1L1 gene is illustrated in Figure 9. After gene transcription, the pre-mRNA with the length of 750 bp composed of exon29, exon30, exon31, intron29 and intron30. Then, alternative splicing of pre-mRNA could form a circularRNA containing only exon29, exon30 and exon31 with the length of 389 bp. The specific junction sequence was verified by Sanger sequencing and the characteristics of GCN1L1 gene were expressed by GC percent and 100 vertebrate conservation. The bioinformatic predictions indicated hsa_circ_0000448 had miRNA sponge or/and RBP binding abilities rather than the protein-coding ability, which could affect the level of TNF-α through pre- and post-transcriptional regulation. The changes of TNF-α in the synovial tissues eventually affected the condylar cartilage and subchondral bone.

Discussion

TMJOA is a common disorder. ADD may be the main cause of osteoarthritis. In ADD patients without reduction, the joints bear unbalanced stress during movements, resulting in the compression of the bilaminar zone, vascular proliferation and inflammatory exudation [27]. Long-term chronic joint synovial inflammation can lead to abnormal secretion of synovial fluid and cytokines. In early stage, medical treatment is primarily adopted. Further development of TMJOA can cause more destruction of cartilage.

Figure 8. The CeRNA network (circRNA-miRNA-mRNA) of up-regulated circRNAs (hsa-circ-0000605, hsa-circ-0000448, chr21:37711073-37717005+).
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and subchondral bone, resulting in end-stage osteoarthrosis. Total TMJ joint replacement may be required if conservative treatment is ineffective [28]. At present, the pathogenesis and developmental mechanism of TMJOA remain unclear. Therefore, the treatment strategies and protocols are not perfect and need further improvements. Identifying biomarkers for the disease would be a significant advance to discover new therapeutic targets and make early and rapid diagnosis.

The occurrence and development of circRNAs in osteoarthritis have been rarely reported, but studies have confirmed that circRNAs are differentially expressed in knee OA cartilage [29-31]. Differentially-expressed circRNAs are involved in the regulation of ECM degradation, inflammatory response and apoptosis in chondrocytes, which are closely related to the development of osteoarthrosis. These findings suggest that circRNAs are closely correlated with OA. However, its expression in TMJOA is not yet clear. In this study, high-throughput transcriptome sequencing was used to comprehensively detect the expression profile of circRNAs in TMJ synovial tissues. In addition, four differentially-expressed circRNAs of great importance were found and validated, all of which were newly discovered and different from circRNAs reported in large joints.

Previous studies have focused on the knee joint and cartilage tissues. However, there are many differences between the knee joint and the temporomandibular joint: (1) Although both are synovial joints, the TMJ is covered with fibrocartilage whereas the knee joint is covered with hyaline cartilage; (2) From the functional point of view, the knee joint is an important load-bearing joint and the TMJ is involved in chewing and swallowing and other physiological processes, and the stress is relatively small; (3) As for the pathogenesis, the incidence of TMJOA is closely associated with the displacement of the articular disc [32], suggesting that circRNAs play different roles in two types of diseases, and may be involved in different signaling pathways and biological processes affecting the progression of disease. Our results also indicated that differentially up-regulated genes are more frequent than down-regulated genes, which is contrary to the differential gene expression pattern of the large joints of the knee joint suggesting that the transcriptional expression pattern of TMJOA has its specificity. Further research is required to validate this conclusion.

Most of the up-regulated circRNAs of the host genes were highly activated in immune cells, indicating more chance for alternative splicing and circRNA forming in these immune cells,

Figure 9. The characteristics and ring formation mechanism of hsa_circ_0000448. Box.1 showed the characteristics of host gene (GCN1L1) with GC percent and DNA conservation. Box.2 showed the Sanger sequencing results of hsa_circ_0000448 with specific back splice sites and consistent with previous researches in CircBase database.
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which could play important roles in TMJ OA. The gene enrichment analysis of circRNAs-derived host genes showed that the differentially-expressed circRNAs were closely related to the production and secretion of cytokines and inflammatory responses. Moreover, these genes were highly transcribed in immune cells, which was likely to cause alternative splicing resulting in circRNA formations. The differentially-expressed up-regulated circRNA-derived gene sets are mainly involved in TNF-α, IFN-γ and its mediated signaling pathway, IL-10 secretion and regulation, MHC protein complex, and RNA and peptide antigen binding. TNF-α and IFN-γ are prominently secreted cytokines in TMJ OA joint fluid, which synergistically promote the inflammatory response in joints [33].

Synovial macrophages are the main source of TNF-α, secreted by exosomes, exocytosis, and vesicle transport, which can induce chondrocyte necrosis and apoptosis, and cartilage extracellular matrix degradation [34]. In addition, IFN-γ activates related receptors, regulates the JAK-STAT signaling pathway, exerts immunomodulatory effects, and inhibits osteoclast formation by rapidly degrading TRAF6 in the RANK-RANKL signaling pathway [35, 36].

The circRNAs associated with the two are significantly up-regulated in the TMJ OA synovium, suggesting that it may affect the progression of disease by regulating the secretion of both. TNF and IFN are a pair of synergistic cytokines that promote inflammation, whereas IL-10 is an inhibitor of inflammation [37]. Therefore, we speculate that up-regulated circRNAs may regulate the balance of inflammation-promoting factors (TNF-α and IFN-γ) and inflammation inhibitors (IL-10) affecting the progression of disease.

Down-regulated circRNA-derived gene sets are closely related to muscle fiber actin contraction, ubiquitin and ubiquitin-like protein binding, and the activation of the Wnt signaling pathway receptor. Among them, the joint activity of TMJ OA patients was significantly weakened, which was closely related to the decline of muscle function. The down-regulation of related circRNAs in TMJ OA may affect the surrounding muscle function, suggesting that circRNA is equally important for the maintenance and stability of muscle function of the jaw. In summary, we speculate that circRNAs may affect the progression of disease by regulating the development of inflammatory and muscle function in TMJ OA.

At present, most studies focus on the endogenous RNA competition mechanism related to the functions of circRNAs, which interact with miRNAs to cause the dysregulation of miRNA and its target genes, thereby participating in the progression of disease. The cerebellar degeneration-related protein 1 (CDR1), also known as ciRS-7, is the first reported circRNA that acts as “miRNAs sponge”, containing 63 binding sites for miR-7 and acting as a miR-7 “sponge”, thus negatively regulating miR-7 activity [9]. In addition, the circRNA VMA21 prevents the disc degeneration by targeting miRNA-200C&X to associate apoptosis inhibitory proteins [38]. Additionally, four verified differentially-expressed circRNAs (hsa_circ_0000-605, hsa_circ_0000448, chr21:37711073-37717005+, hsa_circ_0009043) with multiple miRNA binding sites may regulate the development of TMJ OA through the mechanism described above. Among them, circRNAs that have been verified to be up-regulated (hsa_circ_0000605, hsa_circ_0000448) were predicted to bind to hsa-miR-1184, hsa-miR-515-5p and hsa-miR-330-3p based on different databases. Moreover, the related miRNAs target gene was TNF, which is consistent with the results of bioinformatics analyses.

MiRNAs that could bind to TNF (miR-1184, miR-515-5p and miR-330) and circRNAs in this study have been reported in breast cancer, small cell lung cancer and prostate cancer [39-41]. Up-regulation of these miRNAs can promote the proliferation, migration and differentiation of tumor cells. Besides, miR-330 regulates plaque formation and vascular endothelial cell proliferation through the WNT signaling pathway affecting acute coronary syndrome [42]. The expression level is negatively correlated with TNF-α. In addition, long-chain non-coding RNA HOTAIR can improve the inflammatory response of human macrophages after oxidative stress treatment by up-regulating miR-330-5p [43]. These findings indicate that these miRNAs may be involved in the regulation of inflammatory diseases. Consequently, the non-coding RNAs, such as snRNA, IncRNA and circRNA, may compete with these miRNAs through the CeRNA mechanism that influences transcriptional or post-transcriptional regulation.
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Conclusion

Taken together, the expression of circRNAs in TMJ synovial tissue is stable and abundant. Compared with normal synovial tissues, the expression profile of circRNAs of TMJOA is changed significantly. Four TMJOA-related circRNAs (circRNA-TMJOA) have been identified and validated (hsa_circ_0000605, hsa_circ_0000448, chr21:37711073-37717005+, hsa_circ_0009043). Bioinformatic analyses of the above circRNAs highly suggest that circRNAs (hsa_circ_0000605, hsa_circ_0000448, chr21:37711073-37717005+) may competitively bind to specific miRNAs (miR-1184, miR-515-5p and miR-330) and indirectly inhibit related mRNA (TNF-α) transcription.

Among them, hsa_circ_0000448 is further verified its specific back-splice junction sequence and has both miRNA sponge and RBP binding potentialities, suggesting that circRNA as a new type of non-coding RNA could play an important role in the development of TMJOA. Further research of whether and how these four circRNAs, especially hsa_circ_0000448, participate in the TNF signaling pathway in vitro and in vivo remain to be validated.

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Disclosure of conflict of interest

None.

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