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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].

Table 1. Baseline data of enrolled patients

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

Abbreviations: TMJOA (temporomandibular osteoarthritis), TMD (temporomandibular disorders).

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 (SH9H-2019-TK247-1). The sample collection was authorized by the patients' family and signed the informed consent form.

Table 2. Clinical symptoms of enrolled patients

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

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

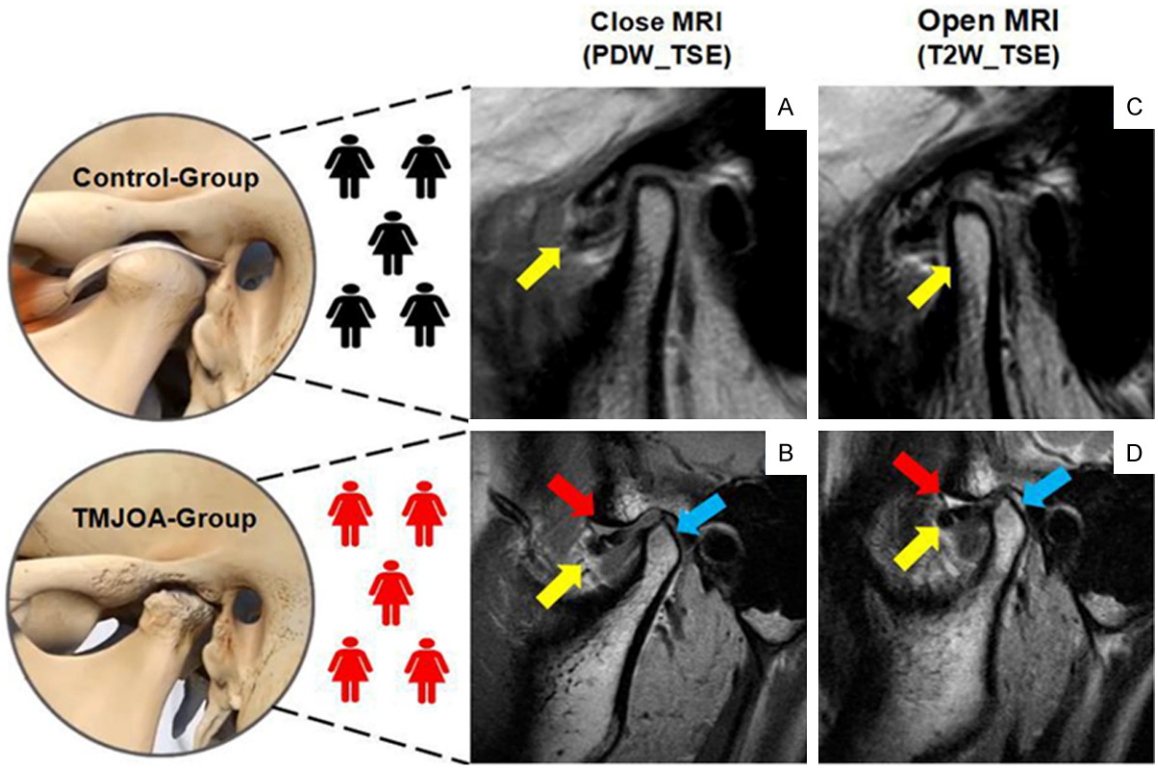

Figure 1. MRI images of control and TMJOA patients. A, B. Mouth opening positions of control and TMJOA patients. C, D. Mouth closing positions of control and TMJOA patients. (Yellow arrows: TMJ disc anterior displacement; Red arrows: TMJ hydrarthrosis in upper joint space; Blue arrows: bony changes/bone resorption at posterior slope of TMJ condyle).

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,

Table 3. Top 10 differentially-expressed CircRNAs for validation

CircRNAID	*P*-value	Fold Change	Regulation	circBase ID	Gene Name	Type	Predicted length
1. chr15:59204762-59209198-	0.004	162.17	Up	hsa_circ_0000605	SLTM	exonic	331 bp
2. chr12:120592774-120593523-	0.005	153.30	Up	hsa_circ_0000448	GCN1	exonic	389 bp
3. chr21:37711073-37717005+	0.005	104.71	Up	none	MORC3	sense-overlap	5933 bp
4. chr2:72945232-72960247-	0.001	425.31	Down	hsa_circ_0009043	EXOC6B	exonic	390 bp
5. chr5:134076753-134079742+	0.043	71.01	Down	hsa_circ_0003154	CAMLG	exonic	527 bp
6. chr12:12397196-12397589-	0.035	62.68	Down	hsa_circ_0000378	LRP6	exonic	394 bp
7. chr5:95091100-95099324+	0.014	55.09	Down	hsa_circ_0007444	RHOBTB3	exonic	479 bp
8. chr17:33495080-33495704+	0.039	47.18	Down	none	UNC45B	sense-overlap	625 bp
9. chr8:52773405-52773806-	0.030	46.20	Down	hsa_circ_0001801	PCMTD1	exonic	402 bp
10. chrX:139865340-139866824+	0.009	2.73	Down	hsa_circ_0001946	CDR1	antisense	1485 bp

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://gyanxet-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 ≥ 2.0 and *P*-value ≤ 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 ≤ 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 $^{2\text{-}\Delta\Delta}$Ct method. According to qPCR results, the circRNAs that consistent with RNA-Seq were chosen for enzyme tolerance test (RNase

Table 4. Primers of circRNAs expression for q-RCR validation

Number	CircRNAID/Gene Name	Primer Type	Primer Sequence
1	chr15:59204762-59209198-	1-Forward	GAGGACATCGAAAGTCAGGAA
		1-Reverse	CCATCTTGCTCATGTGCCT
2	chr12:120592774-120593523-	2-Forward	GGAGGTTATGGGCAGGCT
		2-Reverse	TTCCGGATCTCCTCCTCC
3	chr21:37711073-37717005+	3-Forward	CTTGCCTACATCGAACGTGA
		3-Reverse	AGCAAGGCTGGCTTTTGA
4	chr2:72945232-72960247-	4-Forward	TTTCTGGAGAGCATCCGC
		4-Reverse	GCTTCAGCTCTTCCATTGCT
5	chr5:134076753-134079742+	5-Forward	TGGGATGTGCTCTTCTTGC
		5-Reverse	CGCTTTGAAACGGAAGGA
6	chr12:12397196-12397589-	6-Forward	GCATGTGATTGGCTTGGA
		6-Reverse	CGCAAGTCCCGTCTGTTT
7	chr5:95091100-95099324+	7-Forward	CAGGTGCTTTTCAGTGGGA
		7-Reverse	TGGCAGCAGAACAGCAAG
8	chr17:33495080-33495704+	8-Forward	GACGTTCGTCTCCCTGCT
		8-Reverse	CTTGTCCATGTCCTGGGG
9	chr8:52773405-52773806-	9-Forward	AGCCTGGAAGCATGGAAA
		9-Reverse	GCACTCACAGCTCCTCCC
10	chrX:139865340-139866824+	10-Forward	CGTCTCCAGTGTGCTGATCT
		10-Reverse	GTCCGGAAGACATGGATTGT
11	ACTB (β-actin)	11-Forward	GTGGCCGAGGACTTTGATTG
		11-Reverse	CCTGTAACAACGCATCTCATATT

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

each sample was ≥ 0.5 μg, 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 cirRNAs 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 ≥ 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-

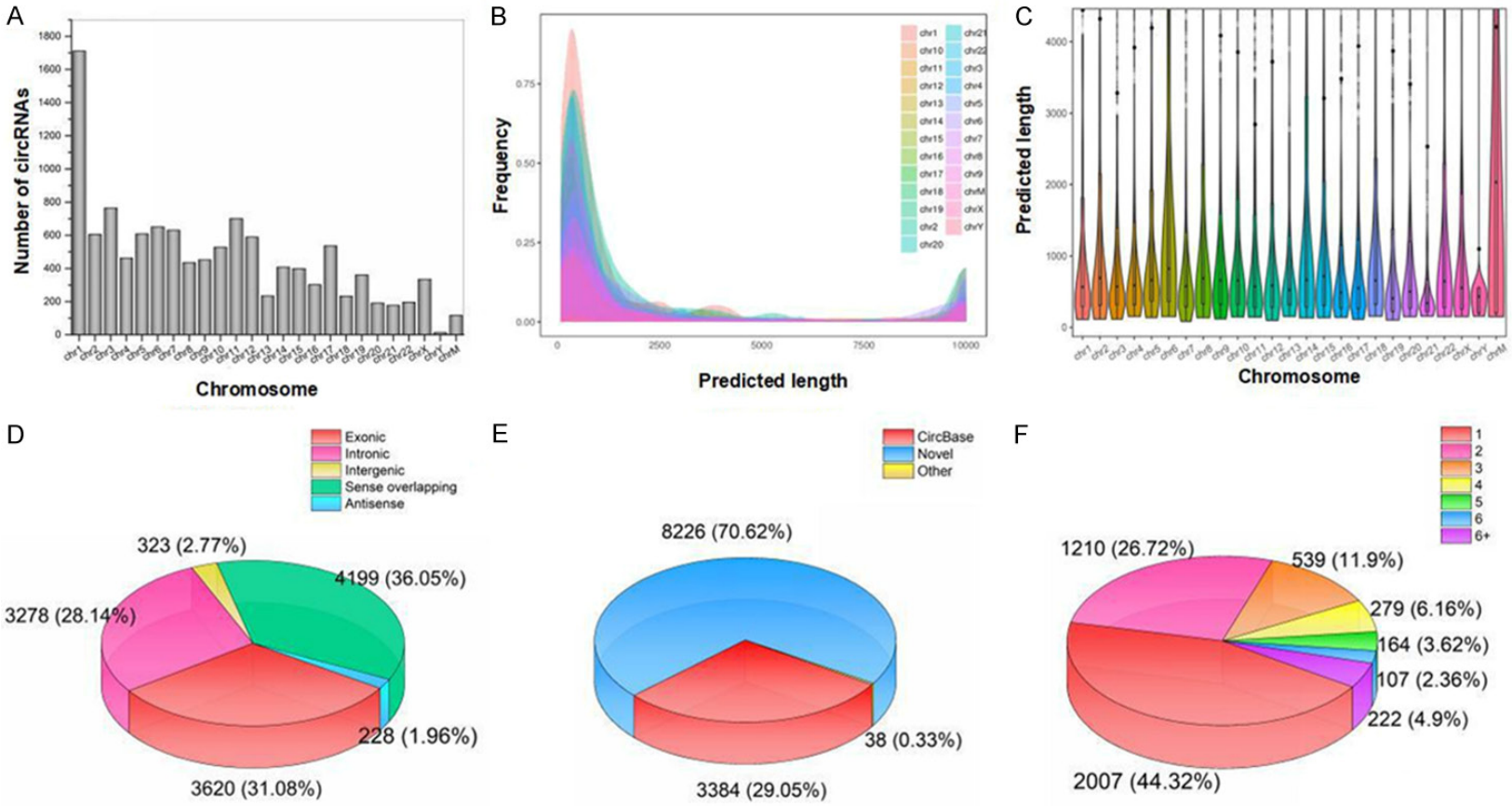

Figure 2. Expression of circRNAs in TMJ synoival tissues. A. Chromosome distribution of circRNAs ("ChrM": mitochondrial genome). B. Length distribution of circRNAs. Most of the circRNAs were ranged from 300-3000 bp in size. C. The violin pilot of circRNAs in each chromosome. And the average length of 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.

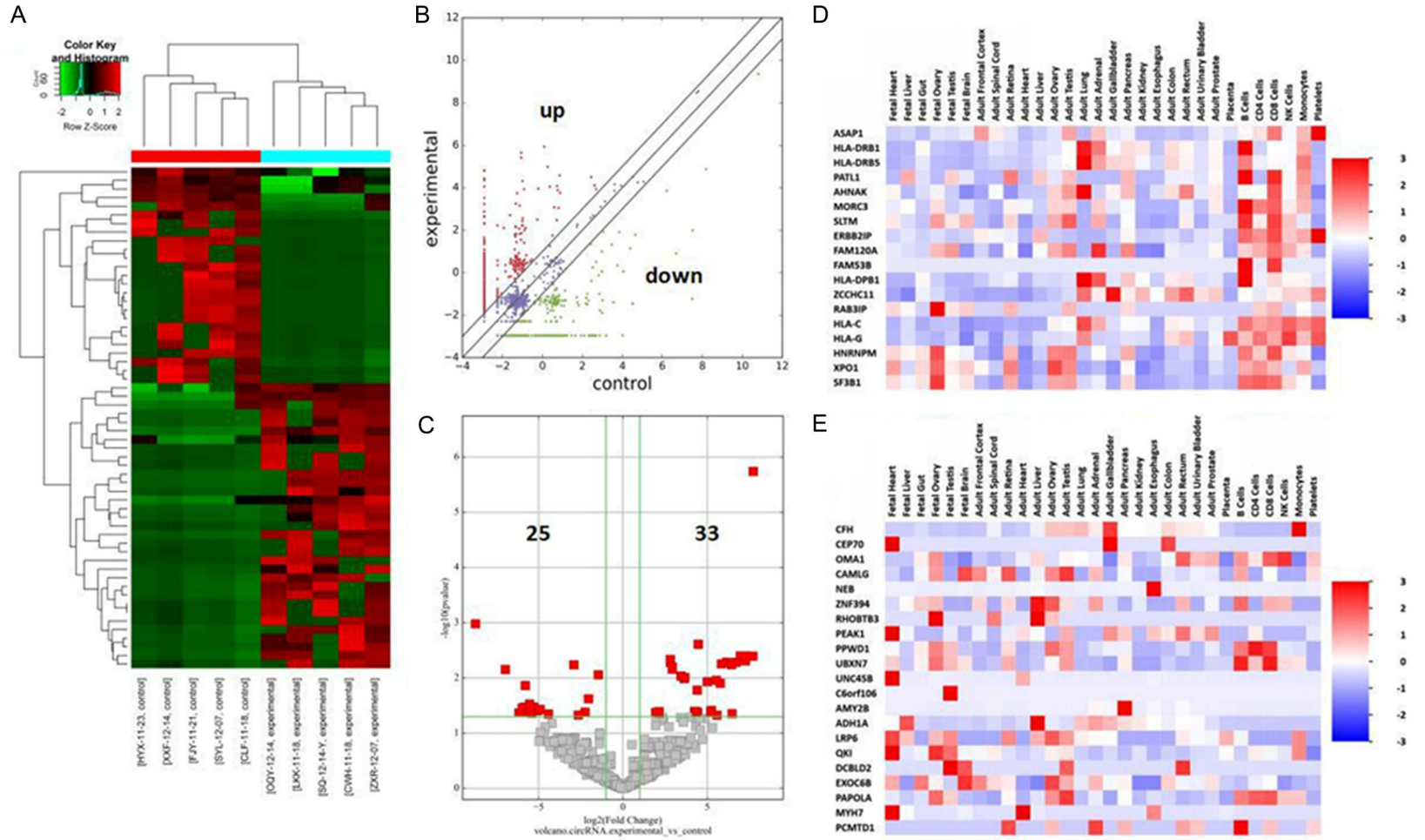

Figure 3. Clustering and definition of differentially-expressed circRNAs in TMJOA. A. Heatmap of differentially-expressed circRNAs clustering analysis. B. Scatter plot of circRNAs between experimental and control groups. C. Volcano plot defined up-regulated/down-regulated circRNAs. D, E. The translation conditions of host gene from each up-regulated/down regulated circRNA based on human proteome.

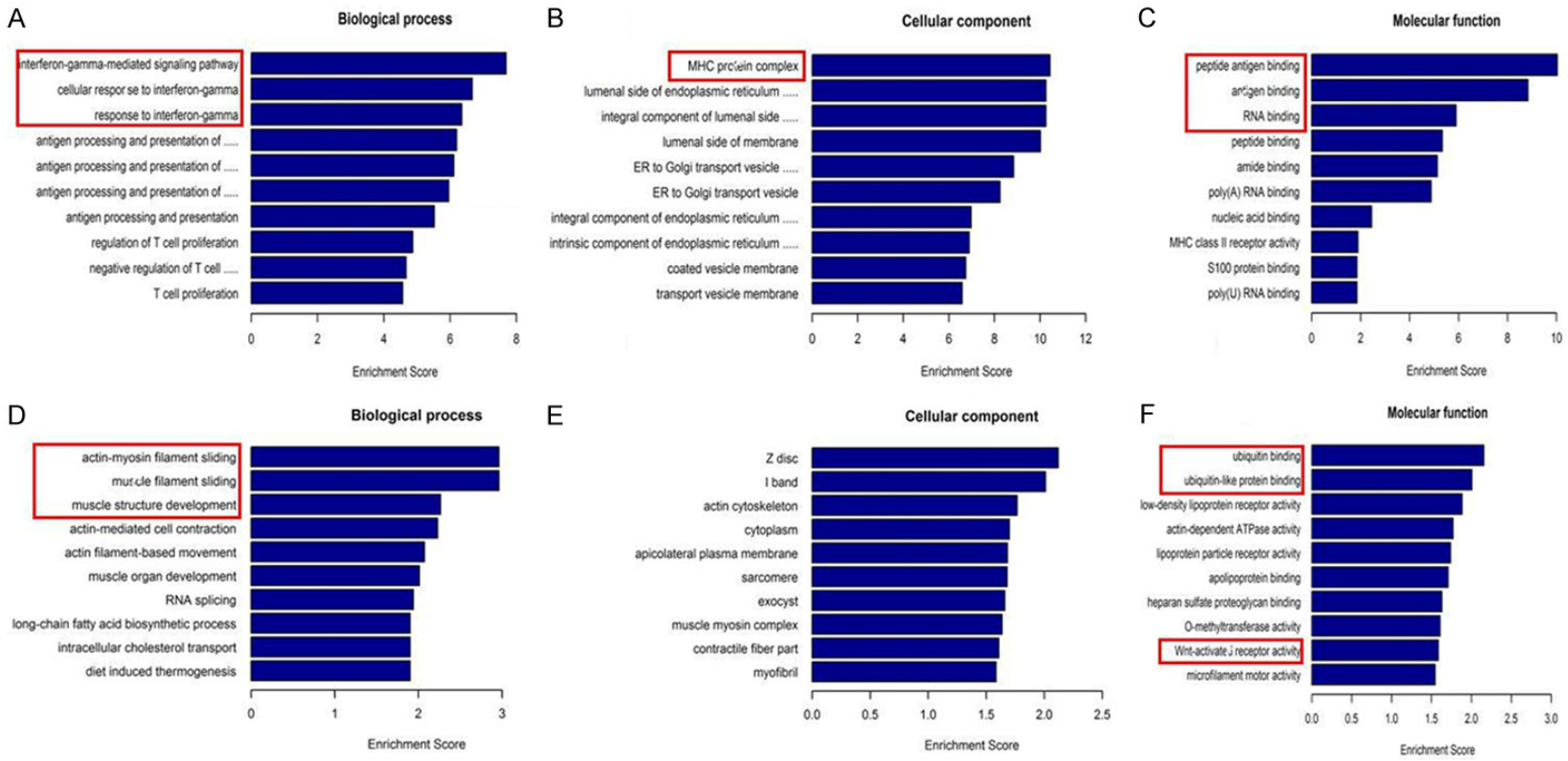

Figure 4. Gene ontology analysis of differentially-expressed circRNAs. A-C. The biological process, cellular component and molecular function of up-regulated circRNAs based on enrichment score. D-F. The biological process, cellular component and molecular function of down-regulated circRNAs. (Red boxes indicated the terms were disease related).

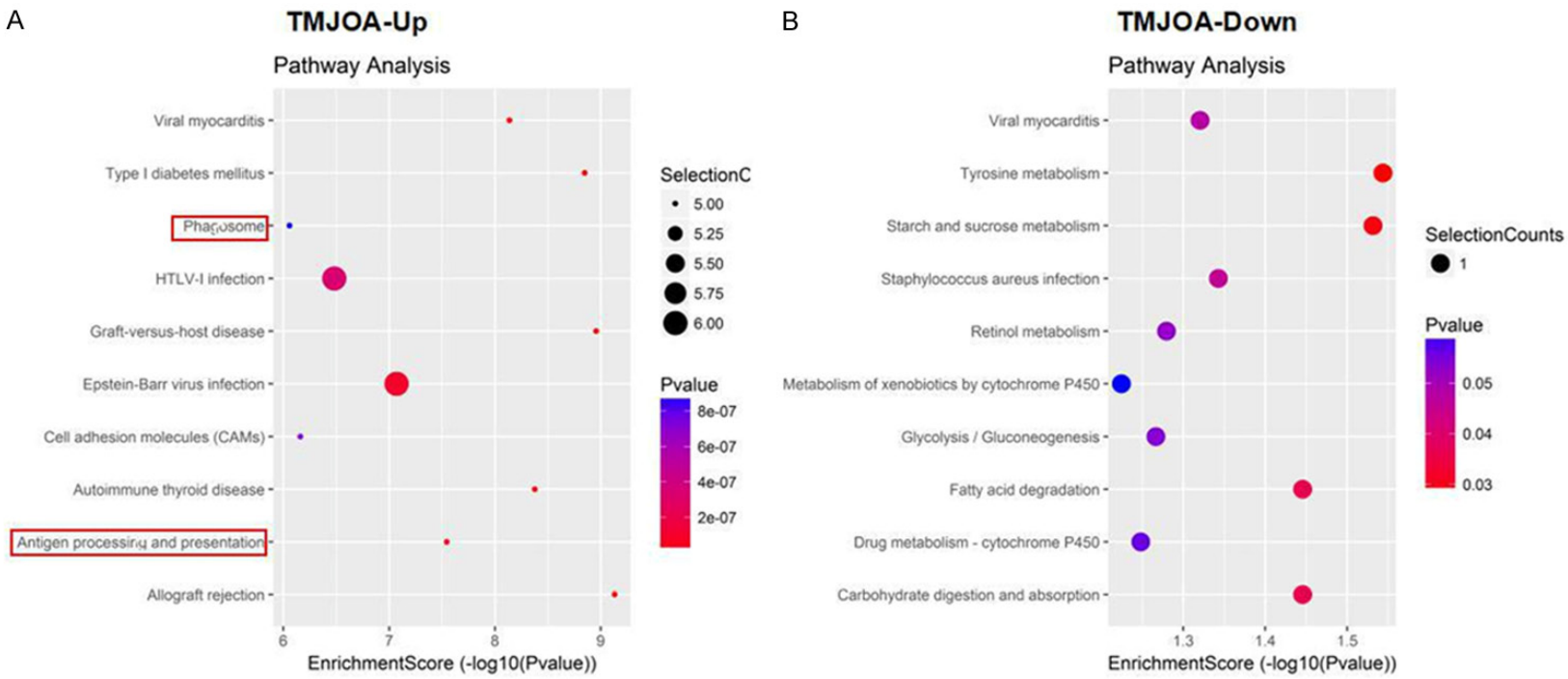

Figure 5. KEGG pathway analysis of differentially-expressed circRNAs. A. Pathway enrichment of up-regulated circRNAs. B. Pathway enrichment of down-regulated circRNAs. (Red boxes indicated the terms were disease related).

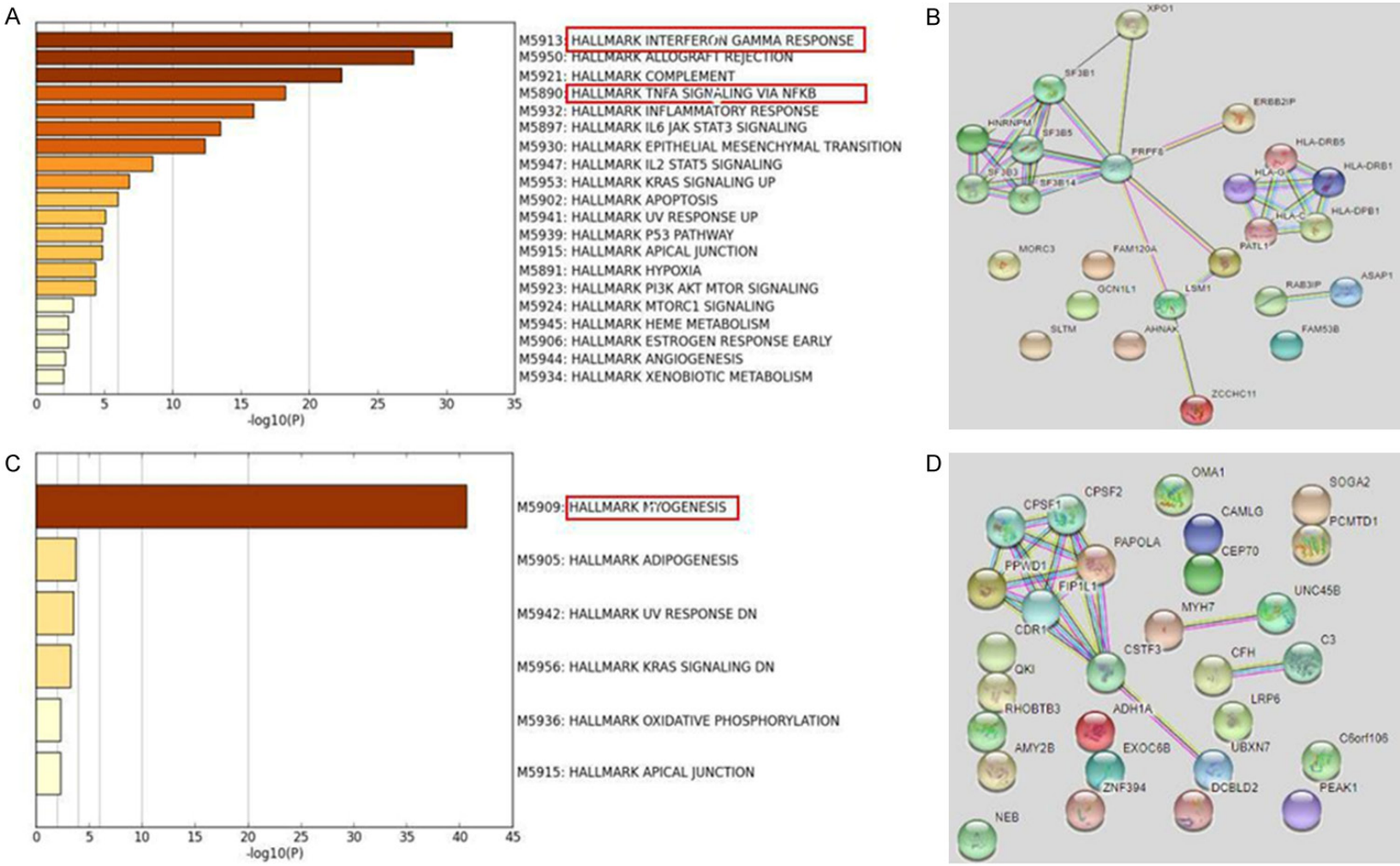

Figure 6. Hallmark gene set enrichment and protein-protein interaction (PPI) network. A, B. Hallmark gene set enrichment and PPI network of up-regulated circRNAs. C, D. Hallmark gene set enrichment and PPI network of down-regulated circRNAs. (Red boxes indicated the terms were disease related).

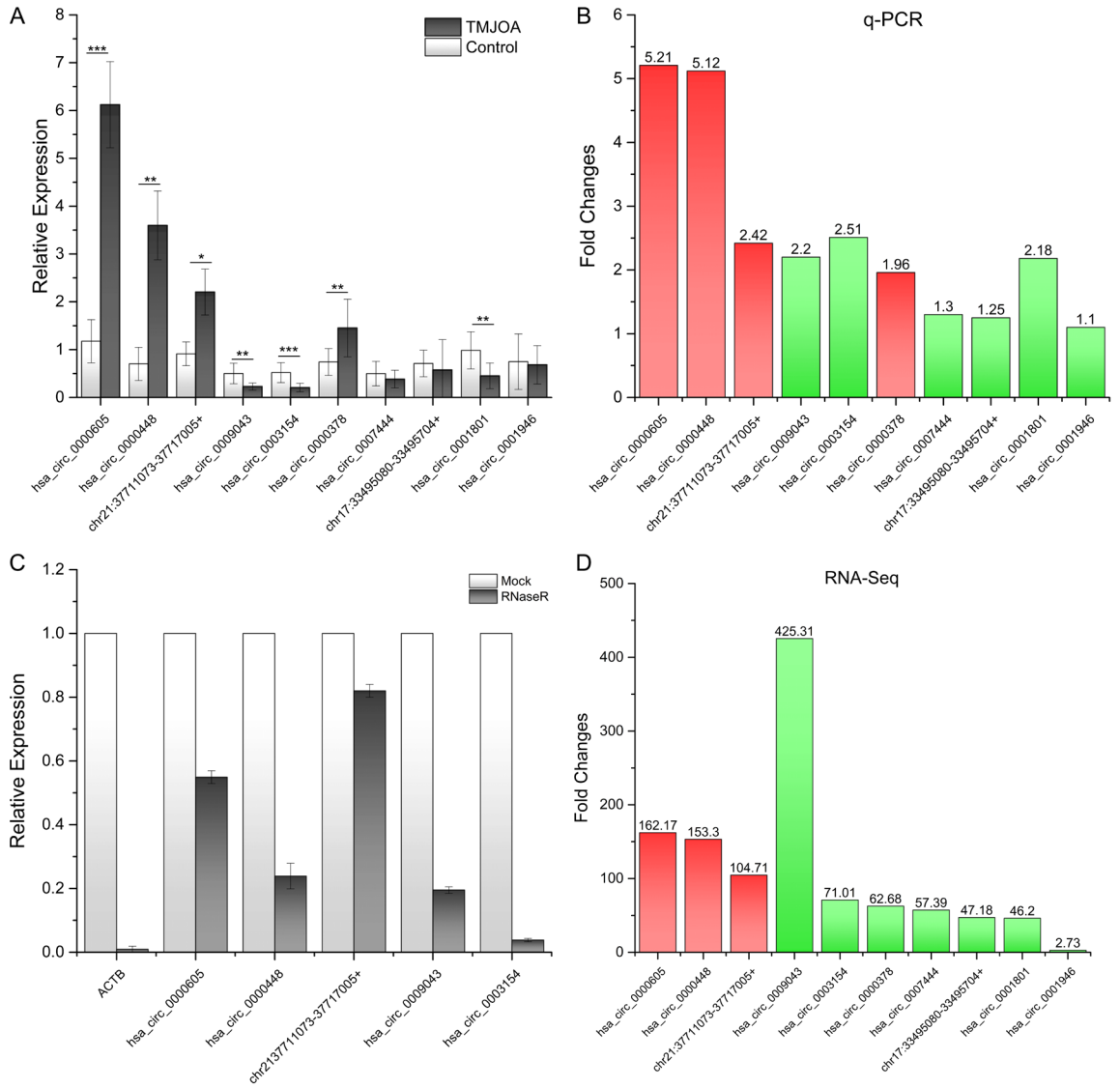

Figure 7. Validation of selected circRNAs. A. Relative expression of selected circRNAs by q-PCR between TMJOA and control groups. B and D. Fold changes and trends of selected circRNAs based on RNA-seq (high-throughout) and q-PCR (low-throughout) results. C. Enzyme tolerance tests of selected circRNAs by RNase R treatment. (Bars represent mean ± SD (n = 10); ***: $P < 0.001$; **: $P < 0.005$; *: $P < 0.05$).

ing of interested circRNA (hsa_circ_0000448) showed it had specific back-splice junction site, which was consistent with the information found in CircBase (**Figure 9**).

CircRNA-miRNA-target gene network

The differentially-expressed circRNAs verified above underwent prediction of miRNA binding by StarBase, circRNA Interactome and Reg-RNA2.0 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_

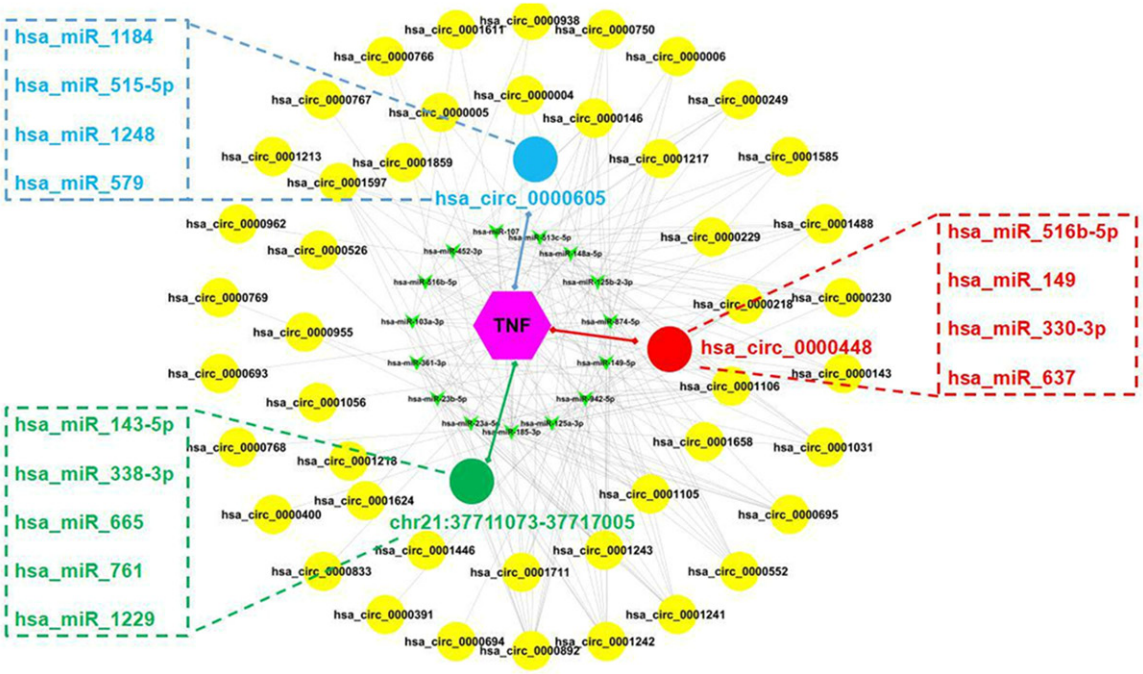

Figure 8. The CeRNA network (circRNA-miRNA-mRNA) of up-regulated circRNAs (hsa-circ-0000605, hsa-circ-0000448, chr21:37711073-37717005+).

circ_0000448-hsa_miR_516b-5p-TNF, hsa_circ_0000448-hsa_miR_149-TNF, hsa_circ_0000448-hsa_miR_330-3p-TNF, hsa_circ_0000448-hsa_miR_637-TNF. The newly discovered MORC3 gene-derived circRNA (chr21:37711073-37717005+) interacted with hsa-miR-143-5p, hsa-miR-338-3p, hsa-miR-761, hsa-miR-665 and hsa-miR-1229 miRNA, *etc*. In the down-regulated circRNA, hsa_circ_0009043 interacted with hsa-miR-7, hsa-miR-1264, hsa-miR-421 and other 15 miRNAs. Three up-regulated circRNAs (hsa-circ-0000605, hsa-circ-0000448, chr21:37711073-37717005+) with targeted gene of TNF were used to construct the circRNA-miRNA-target gene network (**Figure 8**). This network was highly complex, where one circRNA interacted with multiple miRNAs and one miRNA could inhibit multiple target genes. Moreover, *in vivo* functions of circRNAs detected above and their endogenous competing mechanism through "miRNAs sponge" influencing target gene post-transcription required additional studies.

Visualization of hsa_circ_0000448 and potential functions prediction

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 (hsa_circ_0000448) containing only exon29, exon30 and exon31 with the length of 389 bp. The specific junction sequence was verified by Sanger sequencing (**Figure 9** Box.1) and the characteristics of GCN1L1 gene were expressed by GC percent and 100 vertebrate conservation (**Figure 9** Box.2). 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 synoival 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

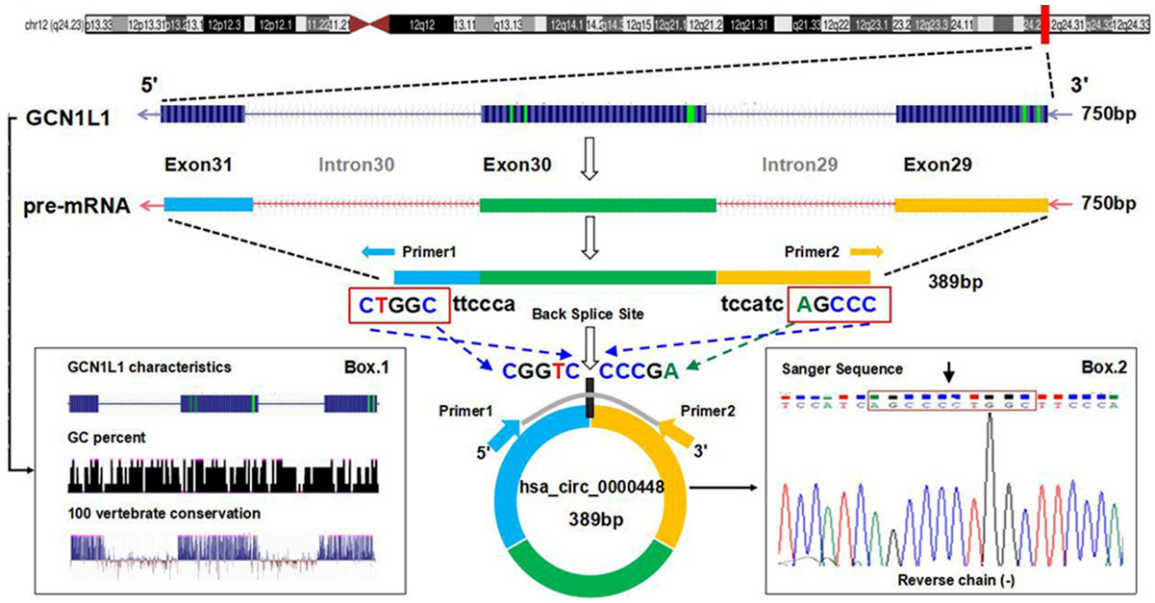

Figure 9. The characteristics and ring formation mechanism of hsa_circ_0000448. Box.1 showed the characteristics of host gene (GCN1L1) with GC percent and DNA conservation. Box.2 showed the Sanger sequencing results of hsa_circ_0000448 with specific back splice sites and consistent with previous researches in CircBase database.

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 raid 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 osteoarthritis. 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 TMJAO 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,

which could play important roles in TMJOA. 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 transcripted 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 complexes and RNA and peptide antigen binding. TNF-α and IFN-γ are prominently secreted cytokines in TMJOA 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 TMJOA 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 TMJOA patients was significantly weakened, which was closely related to the decline of muscle function. The down-regulation of related circRNAs in TMJOA 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 TMJOA.

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_0000605, hsa_circ_0000448, chr21:37711073-37717005+, hsa_circ_0009043) with multiple miRNA binding sites may regulate the development of TMJOA 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, lncRNA and circRNA, may compete with these miRNAs through the CeRNA mechanism that influences transcriptional or post-transcriptional regulation.

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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References

[1] Das SK. TMJ osteoarthritis and early diagnosis. J Oral Biol Craniofac Res 2013; 3: 109-110.

[2] Cui D, Li H, Xu X, Ye L, Zhou X, Zheng L, Zhou Y. Mesenchymal stem cells for cartilage regeneration of TMJ osteoarthritis. Stem Cells Int 2017; 2017: 5979741.

[3] Okamoto K, Kiga N, Shinohara Y, Tojyo I, Fujita S. Effect of Interleukin-1beta and dehydroepiandrosterone on the expression of lumican and fibromodulin in fibroblast-like synovial cells of the human temporomandibular joint. Eur J Histochem 2015; 59: 2440.

[4] Wang XD, Zhang JN, Gan YH, Zhou YH. Current understanding of pathogenesis and treatment of TMJ osteoarthritis. J Dent Res 2015; 94: 666-73.

[5] Yang Z, Xie L, Han L, Qu X, Yang Y, Zhang Y, He Z, Wang Y, Li J. Circular RNAs: regulators of cancer-related signaling pathways and potential diagnostic biomarkers for human cancers. Theranostics 2017; 7: 3106-3117.

[6] Chen LL. The biogenesis and emerging roles of circular RNAs. Nat Rev Mol Cell Biol 2016; 17: 205-211.

[7] Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K, Li H. Circular RNA: a new star of noncoding RNAs. Cancer Lett 2015; 365: 141-148.

[8] Szabo L, Salzman J. Detecting circular RNAs: bioinformatic and experimental challenges. Nat Rev Genet 2016; 17: 679-692.

[9] Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 2013; 495: 333-338.

[10] Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol 2014; 32: 453-461.

[11] Kulcheski FR, Christoff AP, Margis R. Circular RNAs are miRNA sponges and can be used as a new class of biomarker. J Biotechnol 2016; 238: 42-51.

[12] van Rossum D, Verheijen BM, Pasterkamp RJ. Circular RNAs: novel regulators of neuronal development. Front Mol Neurosci 2016; 9: 74.

[13] Du WW, Yang W, Liu E, Yang Z, Dhaliwal P, Yang BB. Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. Nucleic Acids Res 2016; 44: 2846-2858.

[14] Liu Q, Zhang X, Hu X, Dai L, Fu X, Zhang J, Ao Y. Circular RNA related to the chondrocyte ECM regulates MMP13 expression by functioning as a miR-136 'Sponge' in human cartilage degradation. Sci Rep 2016; 6: 22572.

[15] Wu Y, Zhang Y, Zhang Y. CircRNA hsa_circ_0005105 upregulates NAMPT expression and promotes chondrocyte extracellular matrix degradation by sponging miR-26a. Cell Biol Int 2017; 41: 1283-1289.

[16] Liu Q, Zhang X, Hu X. Emerging roles of circRNA related to the mechanical stress in human cartilage degradation of osteoarthritis. Mol Ther Nucleic Acids 2017; 7: 223-230.

[17] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. Embnet Journal 2011; 17: 10-12.

[18] Cheng J, Metge F, Dieterich C. Specific identification and quantification of circular RNAs from sequencing data. Bioinformatics 2016; 32: 1094-1096.
[19] Tripathi S, Pohl MO, Zhou Y, Rodriguez-Frandsen A, Wang G, Stein DA. Meta- and orthogonal integration of influenza "OMICs" data defines a role for UBR4 in virus budding. Cell Host Microbe 2015; 18: 723-735.
[20] Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 2017; 45: D362-368.
[21] Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res 2014; 42: D92-D97.
[22] Dudekula DB, Panda AC, Grammatikakis I, De S, Abdelmohsen K and Gorospe M. CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs. RNA Biology 2016; 13: 34-42.
[23] Chang TH, Huang HY, Hsu JB, Weng SL, Horng JT, Huang HD. An enhanced computational platform for investigating the roles of regulatory RNA and for identifying functional RNA motifs. BMC Bioinformatics 2013; 14 Suppl 2: S4.
[24] Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are MicroRNA targets. Cell 2005; 120: 15-20.
[25] Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in drosophila. Genome Biol 2004; 5: R1.
[26] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003; 13: 2498-2504.
[27] Yang C. The relationship between temporomandibular joint disc displacement and condylar resorption and the comprehensive treatment protocol. Zhonghua Kou Qiang Yi Xue Za Zhi 2017; 52: 157-160.
[28] Zhang LZ, Meng SS, He DM, Fu YZ, Liu T, Wang FY. Three-dimensional measurement and cluster analysis for determining the size ranges of Chinese temporomandibular joint replacement prosthesis. Medicine (Baltimore) 2016; 95: e2897.
[29] Yu CX, Sun S. An emerging role for circular RNAs in osteoarthritis. Yonsei Med J 2018; 59: 349-355.
[30] Etich J, Holzer T, Pitzler L, Bluhm B, Brachvogel B. MiR-26a modulates extracellular matrix homeostasis in cartilage. Matrix Biol 2015; 43: 27-34.
[31] Rasheed Z, Al-Shobaili HA, Rasheed N, Mahmood A, Khan MI. MicroRNA-26a-5p regulates the expression of inducible nitric oxide synthase via activation of NF-κB pathway in human osteoarthritis chondrocytes. Arch Biochem Biophys 2016; 594: 61-67.
[32] Lowe J, Almarza AJ. A review of in-vitro fibrocartilage tissue engineered therapies with a focus on the temporomandibular joint. Arch Oral Biol 2017; 83: 193-201.
[33] Kellesarian SV, Al-Kheraif AA, Vohra F, Ghanem A, Malmstrom H, Romanos GE, Javed F. Cytokine profile in the synovial fluid of patients with temporomandibular joint disorders: a systematic review. Cytokine 2016; 77: 98-106.
[34] Donlin LT, Jayatilleke A, Giannopoulou EG, Kalliolias GD, Ivashkiv LB. Modulation of TNF-induced macrophage polarization by synovial fibroblasts. J Immunol 2014; 193: 2373-2383.
[35] Kelly A, Robinson MW, Roche G, Biron CA, O'Farrelly C, Ryan EJ. Immune cell profiling of IFN-λ response shows pDCs express highest level of IFN-λR1 and are directly responsive via the JAK-STAT pathway. J Interferon Cytokine Res 2016; 36: 671-680.
[36] Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature 2000; 408: 600-605.
[37] Ohta K, Naruse T, Kato H, Ishida Y, Nakagawa T, Ono S. Differential regulation by IFN-γ on TNF-α-induced chemokine expression in synovial fibroblasts from temporomandibular joint. Mol Med Rep 2017; 16: 6850-6857.
[38] Cheng X, Zhang L, Zhang K, Zhang G, Hu Y, Sun X. Circular RNA VMA21 Protects Circular RNA VMA21 protects against intervertebral disc degeneration through targeting miR-200c and X linked inhibitor-of-apoptosis protein. Ann Rheum Dis 2018; 77: 770-779.
[39] Danza K, De Summa S, Pinto R, Pilato B, Palumbo O, Carella M, Popescu O, Digennaro M, Lacalamita R, Tommasi S. TGFbeta and miRNA regulation in familial and sporadic breast cancer. Oncotarget 2017; 8: 50715-50723.
[40] Li J, Tang Z, Wang H, Wu W, Zhou F, Ke H, Lu W, Zhang S, Zhang Y, Yang S, Ni S, Huang J. CXCL6 promotes non-small cell lung cancer cell survival and metastasis via down-regulation of miR-515-5p. Biomed Pharmacother 2018; 97: 1182-1188.
[41] Lee KH, Chen YL, Yeh SD, Hsiao M, Lin JT, Goan YG, Lu PJ. MicroRNA-330 acts as tumor suppressor and induces apoptosis of prostate cancer cells through E2F1-mediated suppression of Akt phosphorylation. Oncogene 2009; 28: 3360-3370.

[42] Ren J, Ma R, Zhang ZB, Li Y, Lei P, Men JL. Effects of microRNA-330 on vulnerable atherosclerotic plaques formation and vascular endothelial cell proliferation through the WNT signaling pathway in acute coronary syndrome. J Cell Biochem 2018; 119: 4514-4527.

[43] Liu J, Huang GQ, Ke ZP. Silence of long intergenic noncoding RNA HOTAIR ameliorates oxidative stress and inflammation response in ox-LDL-treated human macrophages by upregulating miR-330-5p. J Cell Physiol 2018; 234: 5134-5142.