Comprehensive Analysis of Aberrantly Expressed CircRNAs in the Ossification of the Posterior Longitudinal Ligament

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Research Article

Keywords: OPLL, microarray expression profiling dataset, circRNAs, miRNAs, Microarray analysis

Posted Date: December 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-150735/v2

Abstract

Introduction

With causing severe neurological deficits, ossification of the posterior longitudinal ligament (OPLL) has attracted attention in mounting numbers. Increasing evidence shows that circRNAs act as a vital regulatory role in the biological process of human disease development. However, the potential mechanism of circRNAs in OPLL was not fully revealed.

Methods

In this study, 3 OPLL patients and 3 controls were selected for Microarray analysis, and the expression profiles of circRNAs were established. TargetScan and Miranda's miRNA target prediction software were used to predict the circRNA-microRNA interaction. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to identify the biological process and key pathways. we used the STRING database to analyze and construct a Protein-Protein Interaction (PPI) network. Several circRNAs were randomly selected for quantitative real-time PCR (qRT-PCR) validation.

Results

Microarray data profiling indicated that 489 circRNAs (234 up and 255 down) were highly differentially expressed with setting screening criteria (FC $\geq$ 2.0 and p-value $\leq$ 0.05). GO enrichment and KEGG pathways analysis revealed some processes and pathways that might influence the progress of OPLL such as osteoclast differentiation, vasopressin-regulated water reabsorption, Wnt, MAPK, VEGF signaling pathway. We have obtained some significant genes such as AKT3, ACVR1, RBPJ, NFATC1, PTK2, SLC8A1. Extended sample verification results were consistent with sequencing data.

Conclusions

It is the first study for portraying circRNAs expression profiles of OPLL. This provides new ideas for the prevention and alleviation of OPLL. The specific functions and mechanisms of circRNAs in OPLL should be further verified to provide more clinical treatment options for OPLL patients.

1. Introduction

Ossification of the posterior longitudinal ligament (OPLL) is a progressive ossification of the spinal ligament[1]. The ossified tissue encroached on the spinal canal resulting in compression of the spinal cord and severe neurological deficits[2, 3]. The stiff ligament limits the normal mobility of the spine, which seriously impaired the patient's quality of life[4]. Compared with North America and Europe, East Asians have a higher prevalence of OPLL[5, 6]. Recent research on OPLL has shown that it is a multifactorial disease, in which both genetic and environmental factors play a vital role[7]. Cardiovascular disease, diabetes, cerebrovascular disease are significantly associated with OPLL[8]. Realizing the molecular mediator and its mechanism will help to expand our understanding of the pathogenesis of OPLL and provide a theoretical basis to prevent OPLL.

Studies have found that multiple signaling pathways were involved in the ossification process of posterior longitudinal ligament fibroblasts. Environmental factors such as stress stimulation can up-regulate the expression of fibroblast gap junction protein 43 (Cx43), activate extracellular regulated protein kinase 1/2 (ERK1/2), mitogen protein kinase (MAPK), and JNK pathways to make osteoblast differentiation[9]. The transcription factor osterix promotes the differentiation of fibroblasts into osteoblasts by activating the bone formation regulator Runx2 while inhibiting the β-catenin signaling pathway[10]. Transforming growth factor-β (TGF-β) and other cytokines are also involved in the process of fibroblast differentiation into osteoblasts[3]. Recent research found some non-coding RNA was involved in the occurrence of OPLL.

MicroRNAs (miRNAs) are small non-coding single-stranded RNAs with a length of about 18-22 nt, which bind to the 3'-UTR of the target gene to inhibit the translation of the target gene mRNA or directly degrade it. Abnormal expression of miRNAs is

closely related to a variety of orthopedic diseases, including osteoarthritis, osteosarcoma, osteoporosis, and lumbar degenerative disease.[11] For example, miR-122 inhibits the proliferation of osteoblasts in osteoporosis by activating the PCP4-mediated JNK pathway[12]. Long non-coding RNAs (LncRNAs), a kind of non-coding RNA with a length greater than 200 nucleotides, play a complex regulatory function in gene expression[13]. A previous study showed that Long non-coding RNA MALAT1 acts as a miR-1 sponge and regulates the OPLL mediated by Cx43 [14].

Circular RNAs(circRNAs), a class of non-coding RNAs, have a continuous closed loop structure formed by covalent bonds that are widely found in eukaryotic cells and do not contain 5'caps and 3'polyA tails. CircRNAs can bind to the corresponding miRNAs, suppressing the inhibition of the corresponding target gene, and play the role of competitive endogenous RNA(ceRNA) [15, 16]. For example, circRNA LARP4 regulates miRNA-424 through the ceRNA mechanism, which can regulate the occurrence and prognosis of osteosarcoma[17].

Up to now, there is a little research report on how circRNAs play a role in OPLL. In this study, we performed a circRNAs microarray analysis for the first time to establish the expression profile of circRNAs in the posterior longitudinal ligament of OPLL patients. We used bioinformatics prediction methods to annotate the differentially expressed circRNAs. Our findings provide new evidence for exploring the molecular mechanism of CircRNAs in the pathogenesis of OPLL.

2. Materials And Methods

2.1. Sample collection and cell culture

The specimens of the posterior longitudinal ligament were provided by patients from the First Affiliated Hospital of Harbin Medical University. Every patient underwent computed tomography (CT) examination, and OPLL was diagnosed if the thickness of the ectopic OPLL was more than 2 mm [7]. We set patients with only cervical disc herniation as a control group. In all 6 patients, we used Anterior Cervical Discectomy and Fusion (ACDF) surgery. During the operation, the ligament tissue (OPLL tis/sue n=3, normal PLL tissue n=3) was obtained while spinal cord decompressing. (Figure 1. AB) The surgically resected tissue was stained with HE and Masson to confirm that the collected tissue was posterior longitudinal ligament tissue. (Figure 1. CDEF)

The study protocol was approved by the Institutional Review Broad of Harbin Medical University, Harbin, Heilongjiang Province, China. Informed consent was acquired from all study participants. All research methods are within relevant ethical principles.

Primary cell culture of the posterior longitudinal ligament: The tissue obtained during surgery was rinsed with PBS. Removal of ossified tissue under the microscope and cutting into 3 pieces about 0.5mm in size, after rinsing with PBS, pieces were inoculated in T25 culture flasks containing high glucose DMEM culture medium (20% FBS, 100U/ml penicillin, 0.1mg/ml streptomycin) in a 37°C, 5% CO_2, saturated humidity incubator for static culture. After 3-6 hours, the tissue blocks adhered to the wall, turning the culture flask over and immersing the tissue block in a DMEM medium. The Medium was changed twice a week and cell morphology was observed in the electron microscope. When a mass of cells sprouted around the tissue block, they were digested and passaged with 0.25% trypsin containing EDTA. Cells from the second passage were used in downstream experiments.

2.2 Sequencing of circRNAs and Microarray analysis

The purity and concentration of total RNA samples were determined with NanoDrop ND-1000. The total RNA of each sample was treated with RNase R to enrich circular RNA. The enriched circular RNA was amplified and transcribed into fluorescent cRNA utilizing random primer. After hybridizing, incubating and washing, raw data was extracted by Agilent Feature Extraction software.[18]

A series of data processing including quantile normalization were performed using the R software limma package. The circRNAs that at least 3 out of 6 samples have flags in "P" or "M" (defined by GeneSpring software) were retained for further differential analyses. [19]The microarray data have been uploaded in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) [GSE164546: GEO].

2.3 Differential expression analysis

Comparing two groups of profiles, the fold change (FC, the ratio of the group average) between each circRNA was calculated. The statistical significance of the difference may be conveniently estimated with the t-test. CircRNA with FC ≥ 2.0 and p-value ≤ 0.05 was selected as the significantly differentially expressed. Microsoft Excel's Data/Sort & Filter functionalities were used to filter the analysis output, and the differentially expressed circRNA was sorted according to the fold change, p-value, etc.

2.4 Annotation for CircRNAs binding site

TargetScan and miRanda's miRNA target prediction software were used to predict the circRNA-microRNA interaction, and the differentially expressed circRNAs within all the comparisons were annotated in detail with the circRNA-miRNA interaction information. The miRNA response elements (MRE) predicted by the two algorithms were retained. We predicted the top 5 miRNAs of each circRNA based on the pairing scores, and constructed a miRNA-circRNA network through cytoscape_v3.8.0 software (http://cytoscape.org/).

2.5 GO annotation and KEGG pathway enrichment analyses of the DEGs

Gene Ontology (GO), including biological processes (BPs), cellular components (CCs), molecular functions (MFs), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analyses of DEGs were performed using the Database for Annotation, Visualization, and Integrated Discovery ($P<0.05$ as the criteria for enrichment significance).[20]

2.6 Protein-Protein Interaction (PPI) Network construction

To further explore the interaction of different target genes and the molecular mechanism of OPLL, we used STRING (https://string-db.org/) to analyze and construct a PPI network. Then the interaction network and the top 50 hub genes were visualized by cytoHubba in Cytoscape. The nodes in the network were represented as target genes, and the lines between the two nodes were denoted interactions.

2.7 Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA were extracted from OPLL tissues using Trizol reagent (Invitrogen, CA), and a reverse-transcription kit (Takara, Japan) was used to synthesize total RNA into cDNA. [21]The process of the reverse transcription reaction was as follows: 37°C for 15 minutes, 85°C for 5 seconds, and storage at 4˚C. To measure the expression of circular RNAs, linear RNA was degraded with RNase-R. Real-time PCR was performed with SYBR PreMix Ex Taq kit (Takara) and applied biological system (Foster City, CA). The thermal cycle conditions were set to 50˚C for 2 minutes, 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds, 60˚C for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control of circRNAs. [22]The fold change was calculated using the 2-ΔΔCT method. The primer sequences are listed in Table 1.

Table 1

Sequences of primers for Qrt-PCR

Name		Sequence
has_circRNA_101725	Forward	5'-TTCATCTCCTTCCTCAGC-3'
	Reverse	5'-GCTGTTGTGGTGCCTGCT-3'
has_circRNA_000950	Forward	5'-ACCTCTATAAAACGGGAA-3'
	Reverse	5'-AGGCAGAGCTGGGATTCA-3'
has_circRNA_405283	Forward	5'-TGTTACCAGCATGTTATC-3'
	Reverse	5'-TTTTGCAGCCTTGTGTTA-3'
has_circRNA_048764	Forward	5'-TGCCCTCTCCCTGAAATA-3'
	Reverse	5'-TCACGTTCTTGGTCACTT-3'
has_circRNA_406748	Forward	5'-TTTGTCCATCTCCCAAAC-3'
	Reverse	5'-TTTCCGACATAACATCCT-3'
has_circRNA_005411	Forward	5'-GTCTCCCACATCACTCGG-3'
	Reverse	5'-TGGAGTTGTAGAGCCAGG-3'

2.8 Statistical analyses

All statistical analyses were analyzed with SPSS 17.0(Chicago, IL, USA). The t-test was used for the comparison of samples between test and control groups and the Benjamini Hochberg FDR (the FDR cutoff was 0.05) was used for multiple-testing correction. $P<0.05$ was considered statistically significant.

3. Result

3.1 Microarray data

Comparing the results of OPLL groups and control groups, analyzing and normalizing the results, we found 13,617 circRNAs. Through the setting screening criteria (FC $\geq$ 2.0 and p-value $\leq$ 0.05), we identified 234 up-regulated circRNAs and 255 down-regulated circRNAs. Based on these data, a hierarchical cluster analysis was performed to generate a heat map of differentially expressed circRNAs. (Figure 2A) The top 10 up-regulated and top 10 down-regulated circRNAs were listed in Table 2.

Among the differentially expressed circRNAs, which derived from linear transcript exons accounted for the majority with 414 (84.7%) (Figure 2B, Table 3). We used a scatter plot to compare the distribution of differentially expressed circRNAs between samples. (Figure 2C) Based on fold change values and p values, we used a volcano plot to show the overall data differential expression distribution of circRNAs in ligament cells of OPPL patients and normal patients. (Figure 2D) After data standardization, the median of each chip were at the same level, and the probes distribution have been also relatively similar, which means that the chip data quality was reliable (Figure 2E). In summary, comparing the circRNAs expression data of OPLL and healthy ligament tissue cells, we found significant differences in circRNA expression between the two groups.

Table 2

The top 10 upregulated and downregulated circRNAs detected by microarray analysis in the OPLL group

circRNA ID	P-value	Fold change	Regulation	best_transcript	GeneSymbol	Location
Upregulated CircRNAs						
hsa_circRNA_406587	4.5024E-06	6.3609517	up	ENST00000344204	TRIO	Chromosome5
hsa_circRNA_400780	7.5409E-06	2.2160872	up	NM_002335	LRP5	Chromosome11
hsa_circRNA_000365	1.4614E-05	4.6482155	up	NM_152715	TBCEL	Chromosome11
hsa_circRNA_401977	9.3081E-05	5.1339392	up	NM_015277	NEDD4L	Chromosome18
hsa_circRNA_104519	9.4413E-05	2.3534214	up	NM_020781	ZNF398	Chromosome7
hsa_circRNA_403103	1.1663E-04	4.6116453	up	NM_203284	RBPJ	Chromosome4
hsa_circRNA_009054	1.1738E-04	3.1922117	up	NM_002387	MCC	Chromosome5
hsa_circRNA_102389	1.5944E-04	2.6813609	up	NM_007345	ZNF236	Chromosome18
hsa_circRNA_102850	1.9747E-04	3.3088343	up	NM_025000	DCAF17	Chromosome2
hsa_circRNA_101004	1.9897E-04	6.2469359	up	NM_080730	IFFO1	Chromosome12
Downregulated CircRNAs						
hsa_circRNA_102771	2.1603E-05	2.3520316	down	NM_004389	CTNNA2	Chromosome2
hsa_circRNA_104940	7.6180E-05	2.1316901	down	NM_005085	NUP214	Chromosome9
hsa_circRNA_026134	2.5427E-04	2.9170136	down	uc001rtt.1	TUBA1C	Chromosome12
hsa_circRNA_101967	3.1928E-04	2.3007214	down	NM_019013	FAM64A	Chromosome17
hsa_circRNA_001057	3.1944E-04	2.8446833	down	NM_017546	CNOT11	Chromosome2
hsa_circRNA_406019	3.3428E-04	2.3614312	down	NM_003879	CFLAR	Chromosome2
hsa_circRNA_045999	3.8292E-04	2.2134819	down	NM_173626	SLC26A11	Chromosome17
hsa_circRNA_050648	4.0077E-04	5.1294633	down	ENST00000004982	HSPB6	Chromosome19
hsa_circRNA_005411	4.0663E-04	2.2488033	down	NM_001440	EXTL3	Chromosome8
hsa_circRNA_050444	4.4410E-	2.0319829	down	NM_018025	GPATCH1	Chromosome19

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Table 3

Proportion of differentially expressed circRNA types in OPLL

circRNA_type	circRNAs		Total
	Up Regulation	Down Regulation	
exonic	210	204	414
intronic	11	19	30
antisense	2	10	12
sense overlapping	11	17	28
intergenic	0	5	5
Total	234	255	489

3.2 CircRNAs binding site prediction

The differentially expressed circRNAs were predicted by TargetScan and miRanda targets. we retained the top 5 predicted miRNAs according to their scores. (Table 4) MiRNA-circRNA network was constructed using cytoscape_v3.8.0. (Figure 3) CircRNA/miRNA interaction information provided detailed annotations for all differentially expressed circRNAs in the comparison. (Figure 4) Using the circRNA-targeted miRNA-gene network, we chose four differentially expressed cirRNAs to show the relationship between circRNA/miRNA. (Figure 5, Table 5)

Table 4

The binding scores of the top five predicted miRNAs for the10 differentially expressed circRNAs in OPLL group

circRNA ID	miRNA 1	miRNA 2	miRNA 3	miRNA 4	miRNA 5	Regulation
hsa_circRNA_406587	hsa-miR-4700-5p	hsa-miR-371b-5p	hsa-miR-95-5p	hsa-miR-548n	hsa-miR-3662	up
hsa_circRNA_400780	hsa-miR-936	hsa-miR-4712-5p	hsa-miR-4640-5p	hsa-miR-1976	hsa-miR-4726-5p	up
hsa_circRNA_000365	hsa-miR-3606-3p	hsa-miR-335-3p	hsa-miR-4713-5p	hsa-miR-424-5p	hsa-miR-8068	up
hsa_circRNA_401977	hsa-miR-629-5p	hsa-miR-146a-3p	hsa-miR-6839-3p	hsa-miR-519d-5p	hsa-miR-26a-1-3p	up
hsa_circRNA_104519	hsa-miR-766-3p	hsa-miR-597-3p	hsa-miR-761	hsa-miR-214-3p	hsa-miR-657	up
hsa_circRNA_403103	hsa-miR-616-5p	hsa-miR-548ai	hsa-miR-570-5p	hsa-miR-218-1-3p	hsa-miR-2052	up
hsa_circRNA_009054	hsa-miR-33a-5p	hsa-miR-4685-5p	hsa-miR-6855-5p	hsa-miR-2467-5p	hsa-miR-4446-3p	up
hsa_circRNA_102389	hsa-miR-514a-5p	hsa-miR-218-5p	hsa-miR-589-3p	hsa-miR-23b-5p	hsa-miR-151a-5p	up
hsa_circRNA_102850	hsa-let-7g-5p	hsa-miR-18b-5p	hsa-let-7i-5p	hsa-miR-98-5p	hsa-miR-141-3p	up
hsa_circRNA_101004	hsa-miR-150-5p	hsa-miR-670-3p	hsa-miR-1301-3p	hsa-miR-762	hsa-miR-185-3p	up
hsa_circRNA_102771	hsa-miR-492	hsa-miR-1298-3p	hsa-miR-22-3p	hsa-miR-505-3p	hsa-miR-136-5p	down
hsa_circRNA_104940	hsa-miR-608	hsa-miR-185-3p	hsa-miR-653-3p	hsa-miR-377-5p	hsa-miR-298	down
hsa_circRNA_026134	hsa-miR-593-5p	hsa-miR-4739	hsa-miR-1293	hsa-miR-548al	hsa-miR-103a-3p	down
hsa_circRNA_101967	hsa-miR-412-3p	hsa-miR-18a-3p	hsa-miR-597-3p	hsa-miR-518c-5p	hsa-miR-661	down
hsa_circRNA_001057	hsa-miR-4700-5p	hsa-miR-193b-5p	hsa-miR-9-5p	hsa-miR-2467-5p	hsa-miR-3679-5p	down
hsa_circRNA_406019	hsa-miR-6805-3p	hsa-miR-5584-5p	hsa-miR-1914-5p	hsa-miR-6856-5p	hsa-miR-3929	down
hsa_circRNA_045999	hsa-miR-6720-5p	hsa-miR-1972	hsa-miR-4711-5p	hsa-miR-6838-5p	hsa-miR-6805-5p	down
hsa_circRNA_050648	hsa-miR-1182	hsa-miR-608	hsa-miR-6803-5p	hsa-miR-6756-5p	hsa-miR-6883-5p	down
hsa_circRNA_005411	hsa-miR-6834-3p	hsa-miR-4665-5p	hsa-miR-1244	hsa-miR-4525	hsa-miR-7153-3p	down
hsa_circRNA_050444	hsa-miR-6815-5p	hsa-miR-6882-3p	hsa-miR-766-5p	hsa-miR-6837-3p	hsa-miR-920	down

Table 5

circRNA-miRNA-mRNA interaction network

circRNA	miRNA	target genes
hsa_circRNA_001588	hsa-miR-4786-5p	ACVR1 DCAF17 GNAI2 MCM2 RBPJ APBB2 ANKRD17 BRD9 MCC SEC24A ATXN1 FKBP5 EPB41L2 DAGLB MPP6 BAZ1B GTF2IRD1 MTPN AFF2 DYRK1A PLEKHA2 ASPH UBAP2 MASTL NAP1L4 ANO5 IFFO1 ATF7IP USP8 RNF111 DPP8 LMF1 BFAR ZNF720 NFATC3 PKD1L2 GSE1 RPH3AL ZNF652 USP36 NEDD4L ERGIC3 DYRK1A MAN1A2 ELK4 AKT3 STRN
	hsa-miR-514a-3p	DCAF17 RICTOR SERINC5 DAGLB ANK1 ZNF652
	hsa-miR-3121-5p	MAN1A2 NID1 SLC8A1 GTDC1 PHC3 TRIO AP3S1 C6orf106 NR4A3 PARD3 STIM1 WNK1 IFFO1 NALCN ZNF720 NFATC3 DHX33 SS18 AFF2
hsa_circRNA_002082	hsa-miR-6887-3p	PDE4DIP NID1 STRN SETD2 SMARCC1 LRIG1 PHC3 EMB ARL15 PAPD4 MCTP1 SEC24A RANBP9 ATXN1 DDR1 FKBP5 EPB41L2 MTPN XPO7 PLEKHA2 PCMTD1 PTK2 UBAP2 WNK1 IFFO1 ATF7IP DPP8 WWP2 DHX33 SPECC1 NF1 MED1 ZNF652 SS18 NFATC1 DYRK1A NONO FLNA
	hsa-miR-4773	RASAL2 ELK4 STRN ARL6IP6 ACVR1 DCAF17 SMARCC1 GNAI2 PHC3 VPS8 APBB2 MCTP1 ATXN1 C6orf106 ZNF398 ASPH PTBP3 CAPRIN1 ATF7IP RNF111 DPP8 ABCC1 PKD1L2 DHX33 SPECC1
	hsa-miR-512-5p	TCEA3 AKT3 STRN SMARCC1 PHC3 RBPJ APBB2 AFF1 ARL15 SERINC5 MCC ATXN1 MTPN ZNF398 PLEKHA2 ASPH PTK2 UBAP2 PTBP3 PARD3 CHST15 TMEM120B DPP8 ZNF720 NFATC3 SPECC1 USP36 NFATC1 UBA52 AFF2
hsa_circRNA_103730	hsa-miR-581	OSBPL9 AKT3 SLC8A1 PAX3 ATXN1 DPP8 AFF2
	hsa-miR-501-5p	OSBPL9 MAN1A2 RASAL2 AKT3 SLC8A1 GTDC1 ACVR1 PAX3 SETD2 PHC3 APBB2 FRYL SERINC5 MCTP1 MCC AP3S1 ATXN1 C6orf106 EPB41L2 XPO7 PLEKHA2 ANK1 ASPH EYA1 NR4A3 PTBP3 CHST15 CAPRIN1 IFFO1 ATF7IP TMEM120B BFAR ABCC1 ZNF720 NF1 ZNF652 TLK2 FOXK2 NEDD4L NFATC1 NONO AFF2
hsa_circRNA_103224	hsa-miR-605-5p	TCEA3 ELK4 GNAI2 SLC25A26 LRIG1 PHC3 RBPJ AFF1 EMB ARL15 ATXN1 C6orf106 EPB41L2 MPP6 ZNF398 PTK2 NR4A3 PTBP3 ANO5 PICALM DPP8 ABCC1 WWP2 NF1 NEDD4L DNM2
	hsa-miR-889-5p	GNB1 EIF4G3 RASAL2 TBC1D23 PHC3 MCC ATXN1 FKBP5 MPP6 PLEKHA2 ASPH ANO5 TBCEL ATF7IP GPATCH8 ZNF652 ANKRD12 NFATC1 UBA2
	hsa-miR-876-3p	RASAL2 PHC3 FKBP5 EPB41L2 PCMTD1 ASPH ASAP1 PTK2 MASTL TMEM120B GSE1 RPH3AL ZNF652

3.3 Enrichment analyses of the target genes

We performed GO enrichment and KEGG pathway analysis of the 489 differentially expressed circRNA target genes to further clarify their functional characteristics. (Figure 6) In our research, we found that it is involved in biological processes, cellular components, and molecular functions such as ossification, tissue morphogenesis, site of polarized growth, protein kinase activity, ubiquitin-like protein binding, etc. Several significant pathways were also discovered, including osteoclast differentiation, Wnt signaling pathway, vasopressin-regulated water reabsorption, MAPK signaling pathway, VEGF signaling pathway, etc. (Table 6)

Table 6

Functional annotation of target genes of differentially expressed circRNA in OPLL

GO	biological process	ossification, tissue morphogenesis, striated muscle tissue development, stem cell developement, regulation of ossification, regulation of cell migration, osteoblast differentiation, organ morphogenesis, negative regulation of cellular protein metabolic process, negative regulation of cell differentiation, muscle tissue development, muscle structure development
	cellular component	site of polarized growth, extrinsic component of plasma membrane, extrinsic component of cytoplasmic side of plasma membrane
	molecular function	ubiquitin-like protein binding, protein kinase activity, RNA polymerase II distal enhancer sequence-specific DNA binding, transcription factor activity, RNA polymerase II distal enhancer sequence-specific binding
KEGG		Wnt signaling pathway,Osteoclast differentiation, VEGF signaling pathway, Vasopressin-regulated water reabsorption, MAPK signaling pathway, Tight junction, ErbB signaling pathway, T cell receptor signaling pathway, Chemokine signaling pathway, Ribosome, Protein processing in endoplasmic reticulum, Progesterone-mediated oocyte maturation, Viral myocarditis, mTOR signaling pathway, Ubiquitin mediated proteolysis, Long-term depression, Leukocyte transendothelial migration, Hypertrophic cardiomyopathy (HCM), Fc gamma R-mediated phagocytosis, Taste transduction

3.4 PPI Network Analysis

The STRING online database was used to distinguish the connections between the target genes, and a network composed of 206 nodes and 707 edges was obtained, with a confidence score > 0.4 as significant. We used ClusterOne in Cytoscape for gene clustering. The top 50 scoring genes were represented by orange circles, and the color shades were used to correlate significance. (Figure 7)

3.5 qRT-PCR validation of sequencing data

Six differently expressed circRNAs were randomly selected for qRT-PCR experiments in OPLL patients and control samples, including three up-regulated circRNAs (hsa_circRNA_101725, hsa_circRNA_000950, hsa_circRNA_405283) and three down-regulated cirRNAs (hsa_circRNA_048764, hsa_circRNA_406748, hsa_circRNA_005411). The results of qRT-PCR were consistent with the results of Microarray analysis data, indicating relative reliability of the sequencing data. (Figure 8)

4. Discussion

Because of the increasing incidence of ossification of the posterior longitudinal ligament and poor prognosis, OPLL needed to be dealt with urgently[23]. Due to regional differences and ethnic characteristics, OPLL has been restricted in epidemiology and pathogenesis research[24]. At present, surgery is widely adopted as the main treatment method for myelopathy caused by OPLL, but it does not fundamentally solve the problem of heterotopic ossification[25, 26]. The development of proteomics and high-throughput sequencing tools greatly expanded our understanding of the molecular mechanism of OPLL. In previous studies, it was revealed that some non-coding RNAs play an important role in ossification[14, 27]

Up to now, there is no report on the role of circRNAs in OPLL. It was not until the derivation of new sequencing and transcript calculation methods that more functions of circRNAs were revealed[28]. To this end, we collected samples from OPLL patients for high-throughput sequencing and performed bioinformatics analysis. We found that many circRNAs were abnormally expressed during OPLL, which suggests that circRNAs play an important role in the occurrence and development of OPLL. Further research found that most of the differentially expressed circRNAs are derived from exons, which indicate

that circRNAs mostly play a regulatory role in the cytoplasm. However, the specific shear mode and function retention of circRNAs still needed to be explored[29, 30].

This study used bioinformatics methods to explore cell functions and biological pathways related to OPLL. We found that circRNAs are involved in osteoblast differentiation, regulation of ossification, tissue morphogenesis, and cellular protein metabolic process, which indicates that circRNAs may regulate the abnormal ossification of posterior longitudinal ligament through molecular function and cellular component. Such as the Wnt/β-Catenin signaling pathway is involved in the occurrence of osteoarthritis and myeloma[31]. The MAPK/SPARC/ERK signaling pathway regulates heterotopic ossification[32], and the YAP/TAZ signaling pathway plays a role in enhancing bone growth and angiogenesis[33]. The ErbB signaling pathway promotes the development of cartilage and bone[34]. In vitro culture of fibroblasts under the action of BMP-2 combined with a certain concentration of tumor necrosis factor (TNF-α) can detect the secretion of ALP and OC and the formation of bone nodules[35]. In the process of osteoporosis, inhibiting the expression of miR-185 can activate the BMP/Smad signaling pathway to increase bone formation[36]. Our result of KEGG pathway enrichment also verified that the mentioned pathways may also participate in the occurrence and development of OPLL. We have obtained some significant genes such as AKT3, ACVR1, RBPJ, NFATC1, PTK2, SLC8A1 and etc, which have also been confirmed to be specifically expressed in other related diseases. AKT3 can regulate cartilage ossification In mouse chondrocytes, and NF-κB/MAPK-mediated osteoarthritis is further promoted by ACVR1[37-39].

Previously, most of the research on OPLL started from the direction of bone ectopic hyperplasia. lncRNA XIST aggravated the ossification of ligaments through the miR-17-5p/BMP2 signaling pathway[40], and miR-10a promoted the bone formation of mouse ligament cells through the ID3/RUNX2 pathway[41]. However, our research found that the biological process of osteoclasts also seems to be involved in the development of OPLL. Previous studies have proved that the targeted knockout of AKT1 promoted the differentiation of osteoblasts, but knocking out AKT2 has reversed the differentiation of osteoblasts and restored normal osteoclast production[42]. Through functional analysis of the differentially expressed circRNAs in OPLL tissues, we found that some circRNAs seem to be involved in this biological process. It seems that promoting the local osteoclasts process can be a new way to treat OPLL. But circRNAs regulate the osteogenic and osteoclast activity in OPLL needs to be further verified.

The tissue specificity of circRNAs makes it possible to become a biomarker of OPLL.[43] At present, in addition to radiographic results, OPLL does not have other diagnostic criteria, which greatly delayed the detection and treatment of the disease.[44] The relevance of the expression trend of circRNAs in the serum and tissues in OPLL patients needs to be further verified. However, recent studies have shown that a multitude of circRNAs is also present in exosomes, and exo-circRNA are extremely stable in serum which provides a new direction for the diagnosis of OPLL.[45]

This study also has several limitations: 1. We select a scarce number of samples for microarray analysis, which may affect the validity of the sequencing results. 2. Due to the limitations of research methods, we only predicted the function of differentially expressed circRNAs, but could not clarify the detailed mechanism of action in these circRNAs. Selectively up-regulating or down-regulating the expression of circRNAs in cell culture and in vivo will lead to changes in various phenotypes and gene expression. Subsequently, the functional mechanism of circRNAs can be explored by RIP-QCR, RNA pulls down, Luciferase assay, etc methods[46, 47].

5. Conclusions

It is the first study for portraying circRNA expression profiles of OPLL. This provides new ideas for the prevention and alleviation of OPLL. The specific functions and mechanisms of circRNAs in OPLL should be further verified to provide more clinical treatment options for OPLL patients.

Abbreviations

OPLL: ossification of the posterior longitudinal ligament; **Cx43**: fibroblast gap junction protein 43; **ERK1/2:** extracellular regulated protein kinase 1/2; **MAPK**: mitogen-activated protein kinase; **TGF-β:** transforming growth factor-β; **ACVR1**: activin A receptor type I; **RBPJ:** recombination signal binding protein for immunoglobulin kappa J region; **NFATc1:** Nuclear factor of activated T cell cytoplasmic 1; **SLC8A1**: solute carrier family 8 member A1; **GO**: Gene Ontology; **KEGG**: Kyoto Encyclopedia of Genes and Genomes; **PPI**: Protein-Protein Interaction

Declarations

Ethical Approval and Consent to participate

The study protocol was approved by the Institutional Review Broad of Harbin Medical University, Harbin, Heilongjiang Province, China. Informed consent was acquired from the all study participants. All research methods are within relevant ethical principles.

Consent for publication

All patients or relatives of the organization donors were fully understood the purpose and plan of the study, and agreed to publish the results of the study. Subsequently, a written informed consent was obtained.

Availability of supporting data

The microarray data have been uploaded in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) [GSE164546: GEO]

Competing interests

The authors declare that they have no competing interests.

Funding

Acknowledgments This work was supported by Harbin Science and Technology Bureau (Grant NO.; 2017RAXXJ046)

Authors' contributions

JAG and QCG conceived the study; ZZZ, QCG, TS, WLT, ZGY, YHH, FCL, and HTS obtained the samples and carried out experiments; and QCG, JAG, FCL, and ZGY analyzed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Acknowledgements

This study was approved by the Institutional Review Broad of Harbin Medical University, Heilongjiang, China. We thank KangChen Bio-tech, P.R. China for their technical support for the microarray work and qRT-PCR experimentation.

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Figures

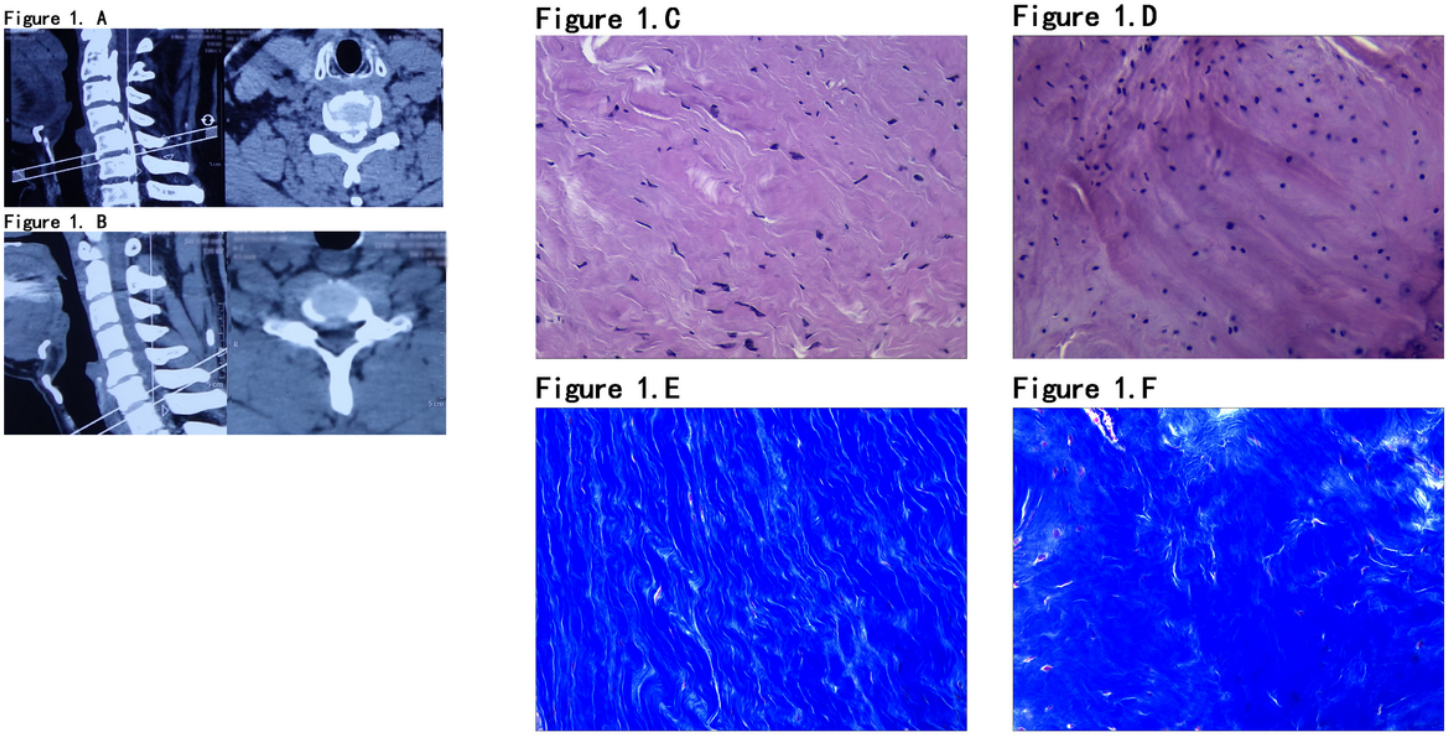


Figure 1

A.B Computed Tomography images of the patients before surgery. A. Sagittal and transverse section images of OPLL patients. Diagnosis: C2-C7 posterior longitudinal ligament ossification, cervical spinal stenosis. B. Sagittal and transverse section images of patients with cervical disc herniation. C. HE staining of normal ligament tissue. D. HE staining of OPLL ligament tissue. E. Masson staining of normal ligament tissue. F. Masson staining of OPLL ligament tissue.

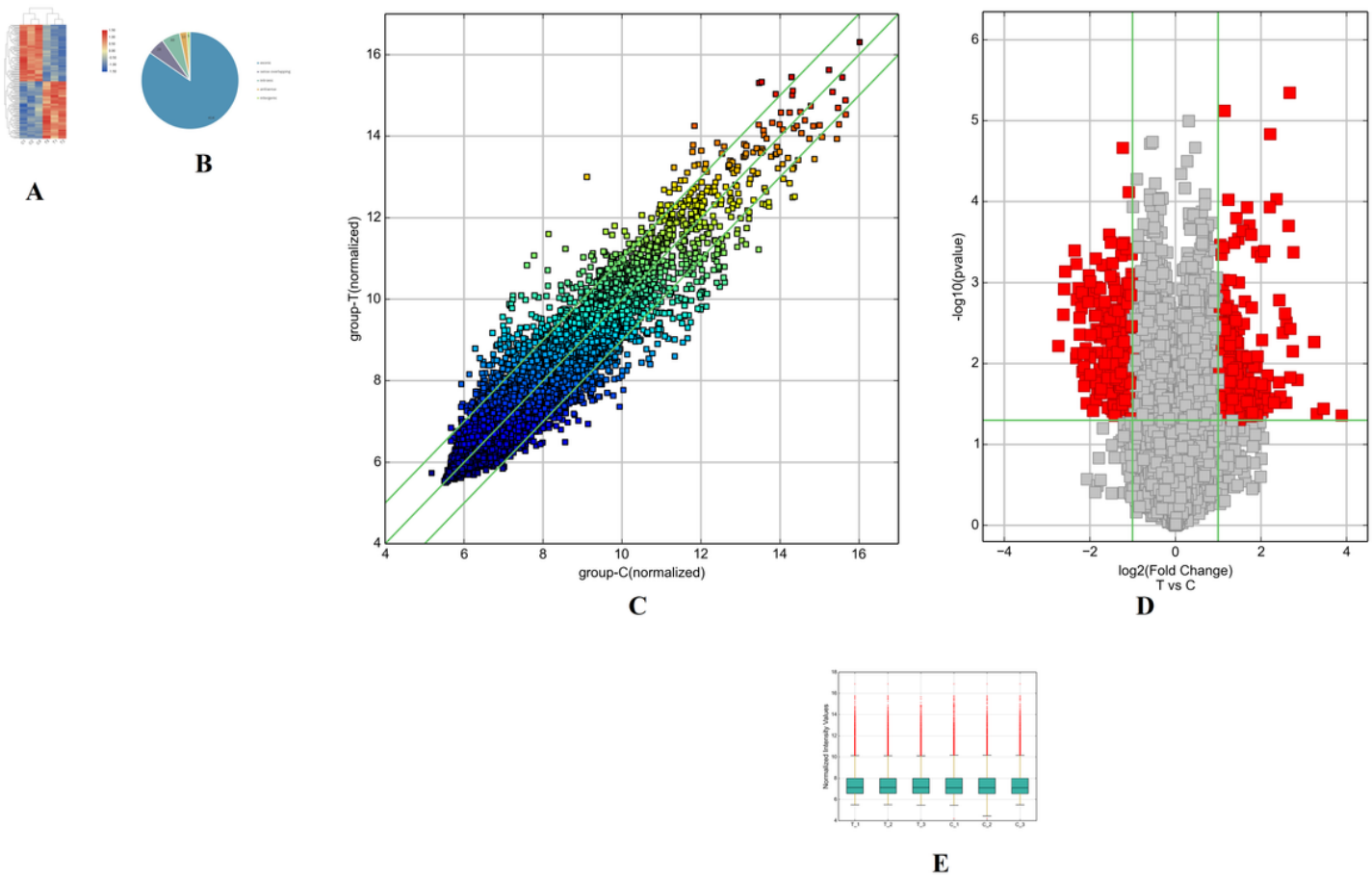


Figure 2

A. Heatmap showing expression profiles of different CircRNAs. (T-C) B. The CircRNAs are classified into 5 types: "exonic", "intronic", "antisense", "sense overlapping" and "intergenic" C. The values of X and Y axes in the Scatter-Plot are the normalized signal values of the samples (log2 scaled) or the averaged normalized signal values of groups of samples (log2 scaled). The green lines are Fold Change Lines. The CircRNAs above the top green line and below the bottom green line indicated more than 2.0 fold change of circRNAs between the two compared samples. D. The vertical lines correspond to 2.0-fold up and down, respectively, and the horizontal line represents a p-value of 0.05. So the red point in the plot represents the differentially expressed circRNAs with statistical significance. E. The green box represents the data subject, the middle horizontal line in the green box is the median, and the upper and lower horizontal lines are the quartiles. The size and range of the green box are close, indicating that the overall chip data distribution is close.

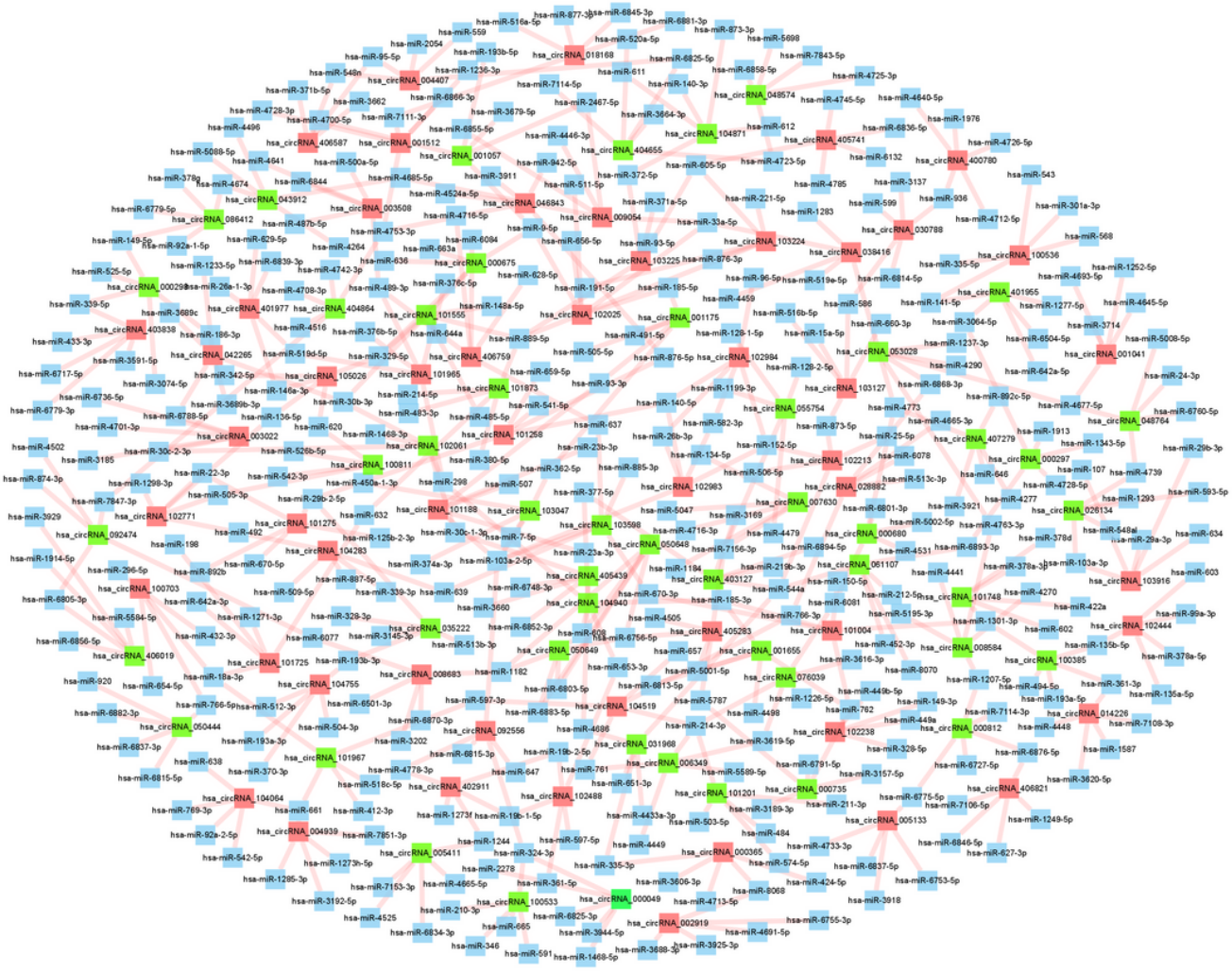


Figure 3

The view of circRNA-miRNA double network. The network includes 2445 miRNAs, 489 circRNAs, 3507 edges. The blue nodes represented miRNA, the red nodes represented up-regulated circRNAs, and the green nodes represented down-regulated circRNAs.

probeID	P-value	FDR	Regulation	group-T(raw)	group-T(normalized)	miRNA
ASCRP3000139	0.017678705	0.12505503	down	2062.833333	11.346281	hsa-miR-3909
ASCRP3010583	0.000255278	0.06413201	up	455.166667	8.888246	has-miR-6844

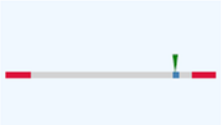
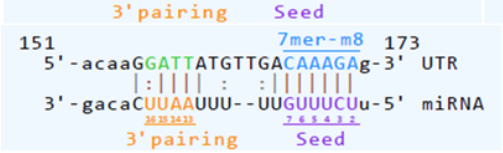
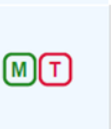
2D Structure	Local AU	Position	Conservation	Predicted By
				
				

Figure 4

A snippet of the detailed annotation for circRNA/miRNA interaction.

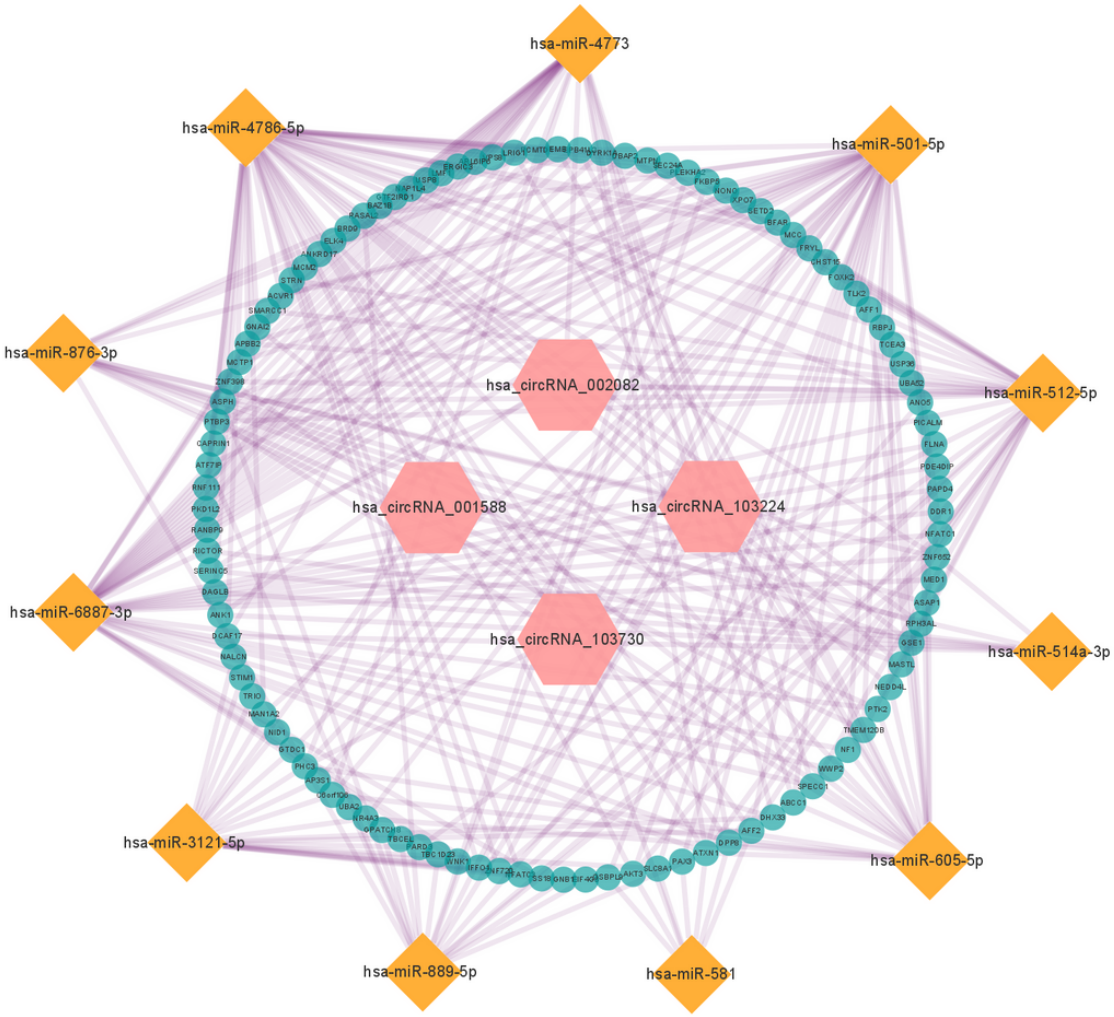


Figure 5

The view of circRNA-miRNA-mRNA triple network. The network includes 11 miRNAs, 4 circRNAs, 106 target genes, 272 edges. The orange nodes represented miRNA, the red nodes represented circRNAs, and the green nodes represented target genes.

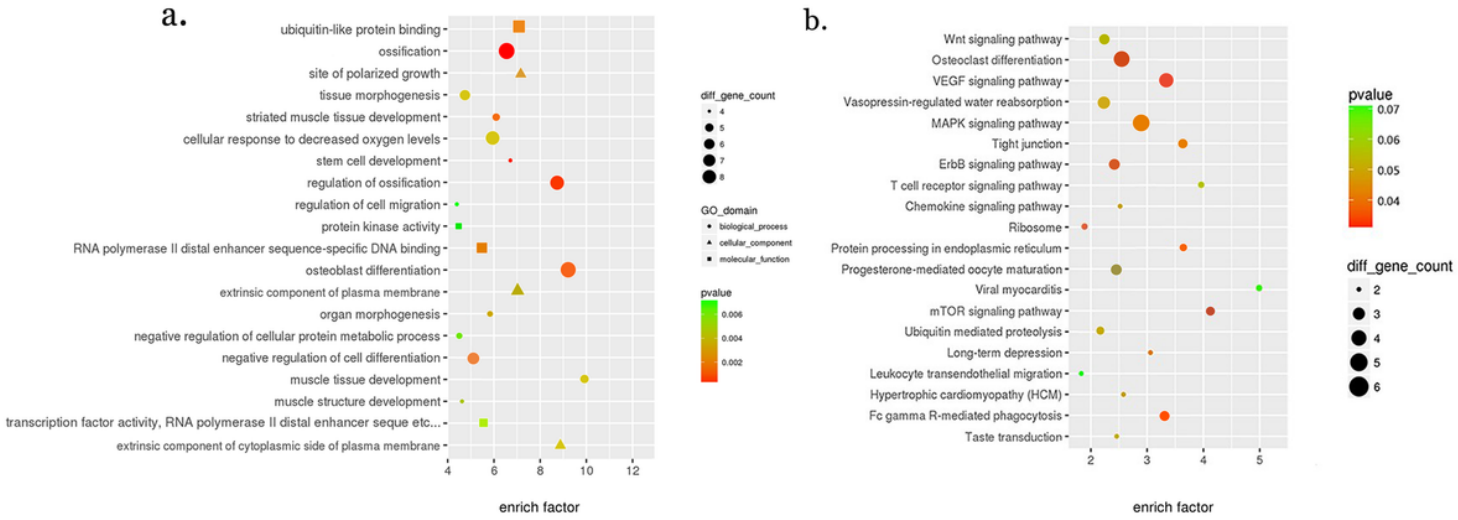


Figure 6

a. The result of GO analysis. The circle marks represented the biological process, the triangle marks represented the cellular component, and the rectangle marks represented the molecular function. b. Results of the KEGG pathway analysis.

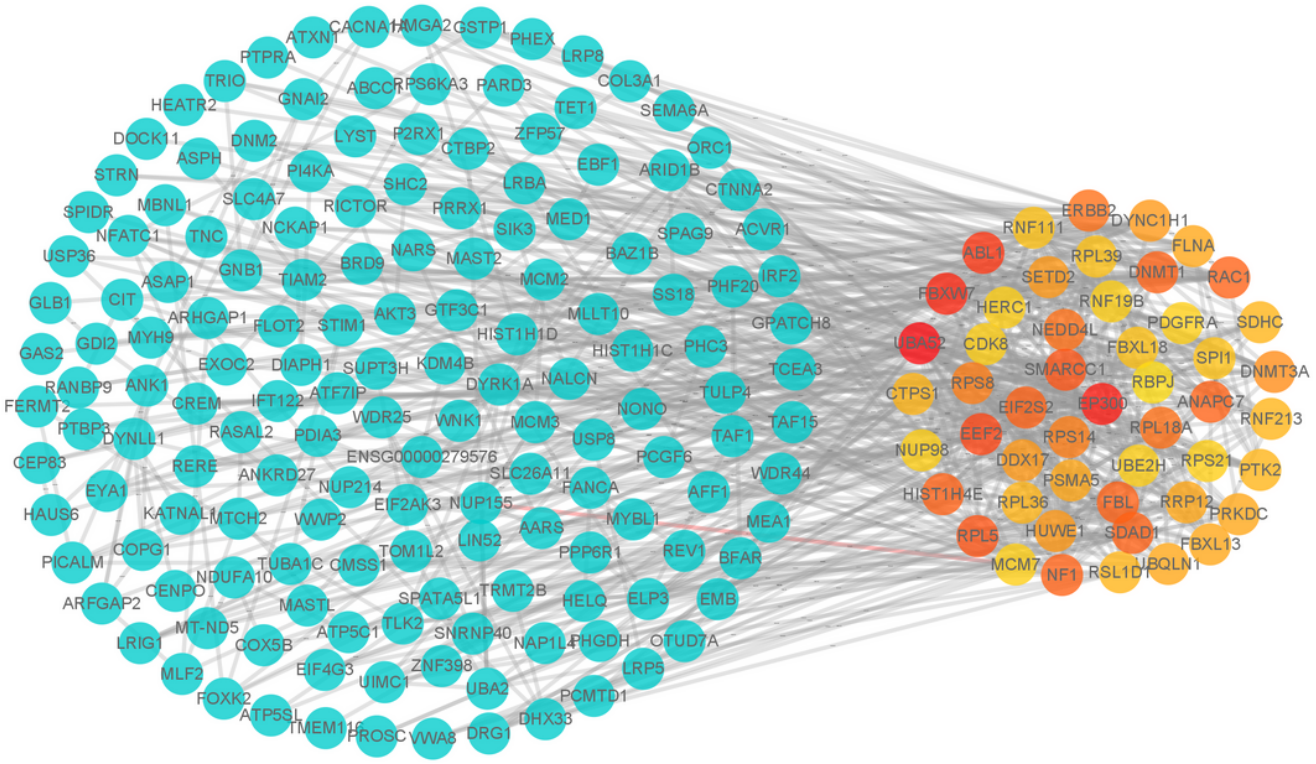


Figure 7

Network composed of 206 nodes and 707 edges. The orange circles represent the 50 highest degree genes and the circles with green represent the others genes.

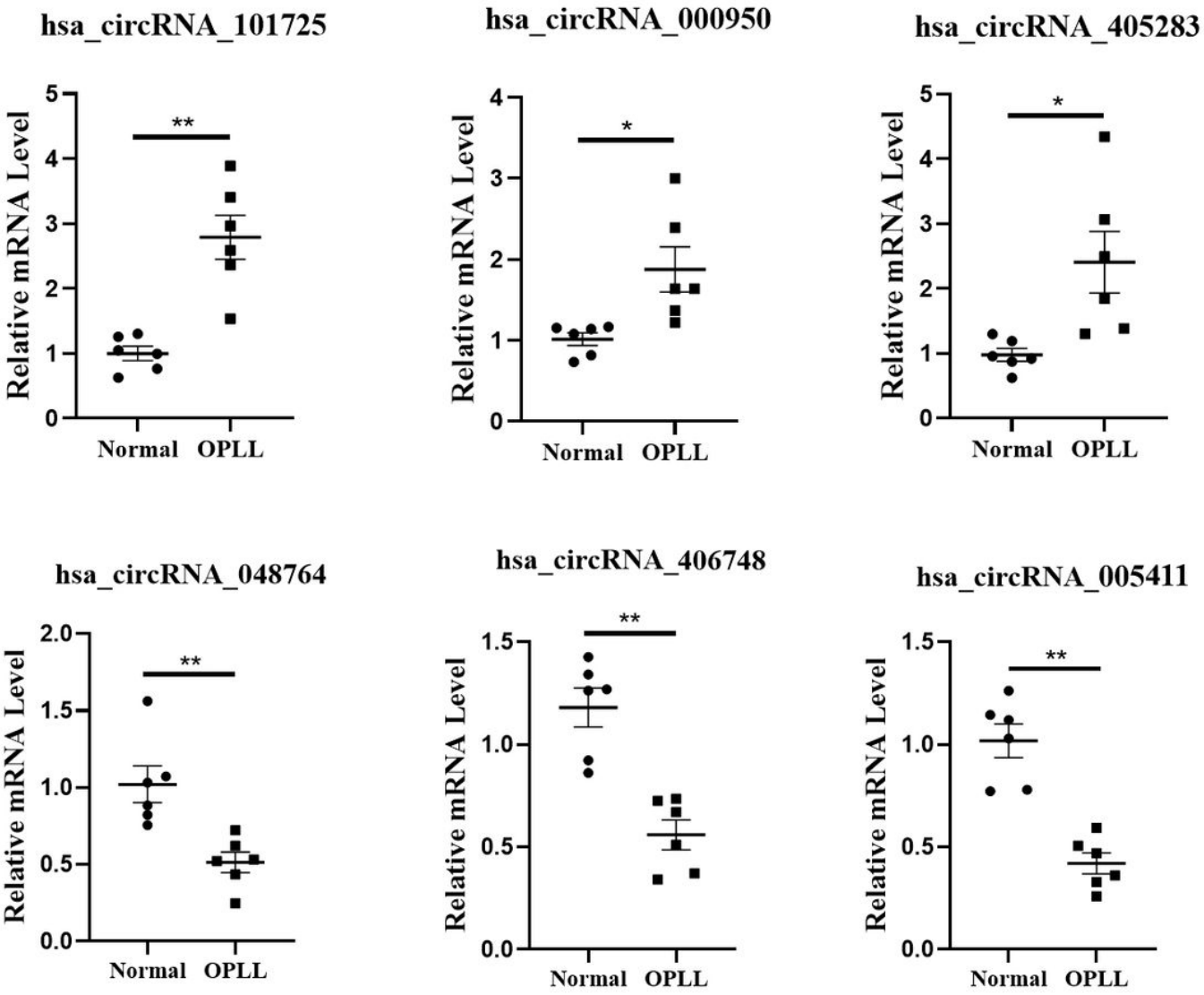


Figure 8

QRT-PCR validation of circRNAs expression. Differential expression of six representative circRNAs was validated in human ossified and normal PLL tissues by qPCR (n = 6 per group).