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ORIGINAL RESEARCH

Identifying the Potential Differentially Expressed miRNAs and mRNAs in Osteonecrosis of the Femoral Head Based on Integrated Analysis

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Yangquan Hao
Chao Lu
Baogang Zhang
Zhaochen Xu
Hao Guo
Gaokui Zhang

Department of Osteonecrosis and Joint Reconstruction, Honghui Hospital Xian Jiao Tong University Health Science Center, Xian, Shaanxi 710068, People's Republic of China

Purpose: Osteonecrosis of the femoral head is a common disease of the hip that leads to severe pain or joint disability. We aimed to identify potential differentially expressed miRNAs and mRNAs in osteonecrosis of the femoral head.

Methods: The data of miRNA and mRNA were firstly downloaded from the database. Secondly, the regulatory network of miRNAs–mRNAs was constructed, followed by function annotation of mRNAs. Thirdly, an in vitro experiment was applied to validate the expression of miRNAs and targeted mRNAs. Finally, GSE123568 dataset was used for electronic validation and diagnostic analysis of targeted mRNAs.

Results: Several regulatory interaction pairs between miRNA and mRNAs were identified, such as hsa-miR-378c-WNT3A/DACT1/CSF1, hsa-let-7a-5p-RCAN2/IL9R, hsa-miR-28-5p-RELA, hsa-miR-3200-5p-RELN, and hsa-miR-532-5p-CLDN18/CLDN10. Interestingly, CLDN10, CLDN18, CSF1, DACT1, IL9R, RCAN2, RELN, and WNT3A had the diagnostic value for osteonecrosis of the femoral head. Wnt signaling pathway (involved WNT3A), chemokine signaling pathway (involved RELA), focal adhesion and ECM-receptor interaction (involved RELN), cell adhesion molecules (CAMs) (involved CLDN18 and CLDN10), cytokine–cytokine receptor interaction, and hematopoietic cell lineage (involved CSF1 and IL9R) were identified.

Conclusion: The identified differentially expressed miRNAs and mRNAs may be involved in the pathology of osteonecrosis of the femoral head.

Keywords: osteonecrosis of the femoral head, miRNAs, mRNAs, signaling pathway

Introduction

Osteonecrosis of the femoral head is a disabling and progressive chronic disease, which leads to femoral head collapse and further total hip arthroplasty.[1,2] It is estimated that the age range of about 75% of patients is from 30–60 years old.[3] Pain is one of the common clinical symptoms of osteonecrosis of the femoral head.[4] However, most patients with a lesion less than 30% of the femoral head are initially asymptomatic.[5] Malizos[6,7] and Zalavras and Lieberman found that osteocytes death and bone marrow cells was the main characteristic of the early stages of osteonecrosis of the femoral head. In the next moment, the repair reaction of necrotic bone is initiated. During this process, the imbalance between bone resorption and bone reformation leads to structural damage of the femoral head, among which there is a significant degeneration and cracking of the hip articular cartilage, which accelerates the development of osteonecrosis of the femoral head.[8,9]

Correspondence: Yangquan Hao
Department of Osteonecrosis and Joint Reconstruction, Honghui Hospital Xian Jiao Tong University Health Science Center, No. 555 Youyi East Road, Xian, Shaanxi 710068, People's Republic of China
Tel +86 13991038599
Fax +86 29-87894724
Email yangquan_haodoctor@163.com

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The pathogenic mechanism of osteonecrosis of the femoral head is complex. Kerachian et al[10] found that local microvascular thrombosis resulted in decreasing blood flow in the femoral head. It was pointed out that fibroblast growth factor 2 (FGF2), insulin-like growth factor 1 (IGF1), SRY-box transcription factor 9 (SoX9), and collagen type ii α1 affected the pathogenesis of osteonecrosis of the femoral head.[11] In addition, several risks, such as the trauma, steroids, smoking, alcoholism, irradiation/chemotherapy, clotting disturbances, hyperlipidemia, hyperviscosity, autoimmune diseases, Legg-Calve-Perthes diseases, and genetic factors are possible causes of osteonecrosis of the femoral head.[12–22]

The incidence of osteonecrosis of the femoral head is on the rise, in spite of various research efforts and trials. Therefore, understanding the pathophysiology of the disease and its progression is urgently needed. Interestingly, miRNAs play roles in targeting mRNAs, and regulate diverse biological processes in bones, such as osteoblasts, osteoclasts differentiation, and bone programming.[23–26] In this study, we performed the differentially expression analysis of miRNA and mRNA in osteonecrosis of the femoral head, which may provide a novel field in understanding the pathological mechanism of osteonecrosis of the femoral head.

Materials and Methods

Datasets

The miRNA and mRNA expression profile was downloaded from the Gene Expression Omnibus database (GEO) dataset. The keywords of ((“femur head” [MeSH Terms] OR femoral head [All Fields]) AND (“necrosis” [MeSH Terms] OR necrosis [All Fields])) AND “gse” [Filter] was used to retrieve related datasets. According to above screening criteria, one miRNA dataset (GSE89587, involving ten cases and seven normal controls) and one mRNA dataset (GSE74089, involving four cases and four normal controls) were finally selected.

Identification of Differentially Expressed miRNAs and mRNAs

Firstly, the raw data of miRNAs and mRNAs was preprocessed as follows: the probes corresponding to multiple miRNAs/mRNAs were removed; and the miRNAs/mRNAs corresponding to multiple probes were left with only the one with the highest average expression. In this study, the identification method of differentially expressed miRNAs and mRNAs was referred to previous studies.[27,28] The screening criteria of differentially expressed miRNAs and mRNAs was, respectively, FDR<0.05, |log2FC|>3, and FDR<0.05, |log2FC|>1.

Correlation Analysis Between miRNAs and mRNAs

In this study, miRWalk (http://mirwalk.umm.uni-heidelberg.de/) was used to predict targeted mRNAs of miRNAs. The establishment of a miRNA-target regulatory network was visualized using Cytoscape software.

Functional Enrichment of mRNAs

In order to understand the biological function of the targeted differentially expressed mRNAs of differentially expressed miRNAs, we performed functional analysis via GeneCodis3 software. FDR<0.05 was set as the criterion for selecting significantly enriched functional terms.

In vitro Validation of miRNAs and Targeted mRNAs

In total, six patients with osteonecrosis of the femoral head and seven normal controls were enrolled. All patients had not taken corticosteroids or medications for nearly a month. In addition, patients older than 80 years or without incomplete clinical information were excluded. Normal controls were matched by gender and age of the case group. Healthy individuals with a history of osteonecrosis of the femoral head and suffering from bone metabolic disorders (such as osteoporosis) were excluded. Ethical approval was obtained from the ethics committee of Honghui Hospital Xian Jiao Tong University Health Science Center (No.201904006). Those included provided informed written consent. This study was carried out in accordance with the Declaration of Helsinki.

Total RNA of the blood samples was extracted and synthesized cDNA by FastQuant Reverse Transcriptase (TIANGEN). Then real-time PCR was performed in an ABI 7300 Real-time PCR system with SYBR® Green PCR Master Mix (Applied Biosystems). Has-U6 was used for the internal reference of miRNA. ACTB and GAPDH were used for the internal reference of mRNA.

Electronic Validation and Diagnostic Analysis of Targeted mRNAs in GSE123568 Dataset

The GSE123568 dataset (peripheral blood sample) involved 30 patients with osteonecrosis of the femoral head and 10

normal controls, and was used for electronic validation and ROC analysis of targeted mRNAs. The expression result of these mRNAs was shown by box plots.

Results

Expression Pattern of miRNA and mRNA

There were 24 differentially expressed miRNAs (supplementary Table 1), and 901 differentially expressed mRNAs (supplementary Table 2) were identified. All differentially expressed miRNAs and the top 20 differentially expressed mRNAs are shown in Tables 1 and 2, respectively. The volcano plot and heat map of all miRNAs and top 100 mRNAs are shown in Figures 1 and 2, respectively.

Network of miRNAs–mRNAs

Depending on the targeted analysis, 2,137 miRNA–mRNA pairs (involving 24 miRNA and 457 mRNA) were identified (supplementary Table 3). The established regulatory network of miRNA–targeted mRNA is illustrated in Figure 3. In the network, there were 481 nodes and 1,205 edges. The top 10 differentially expressed miRNAs that targeted the most differentially expressed mRNAs were hsa-miR-378c, hsa-miR-3191-5p, hsa-let-7a-5p, hsa-miR-28-5p, hsa-miR-3200-5p, hsa-miR-532-5p, hsa-miR-106b-3p, hsa-miR-339-3p, hsa-miR-5195-5p, and hsa-miR-3200-3p. In addition, the sub-network of miRNA-target mRNAs between hsa-miR-378c, hsa-let-7a-5p, hsa-miR-28-5p, hsa-miR-3200-5p, hsa-miR-532-5p, and their targeted mRNAs are shown in Figure 4. In addition, we used the TargetScan (http://www.targetscan.org/vert_72/) software to further validate the targeted relationship between miRNA and mRNA, such as hsa-miR-378c-DACT1 and hsa-miR-28-5p-RELA (supplementary Figure 1).

Table 1 All Differentially Expressed miRNAs in Osteonecrosis of the Femoral Head

Symbol	LogFC	*P*-value	FDR	Up/Down
hsa-miR-3191-5p	−3.15065	3.62E-07	0.000358	Down
hsa-miR-4511	−4.24635	5.12E-06	0.000514	Down
hsa-miR-5195-5p	−3.1202	5.88E-06	0.000514	Down
hsa-miR-128-3p	3.473807	7.27E-06	0.000514	Up
hsa-miR-374c-5p	3.70425	1.30E-05	0.000722	Up
hsa-miR-532-5p	3.811221	2.07E-05	0.000764	Up
hsa-miR-140-5p	3.711132	2.09E-05	0.000764	Up
hsa-miR-3200-3p	3.396543	2.39E-05	0.000764	Up
hsa-miR-181a-5p	3.934233	2.62E-05	0.000786	Up
hsa-miR-28-5p	3.249402	4.52E-05	0.001064	Up
hsa-miR-3200-5p	3.499118	6.03E-05	0.001152	Up
hsa-miR-106b-3p	3.17598	7.23E-05	0.001167	Up
hsa-miR-130a-3p	3.227388	7.73E-05	0.001184	Up
hsa-miR-126-5p	3.329919	7.94E-05	0.00119	Up
hsa-miR-4762-5p	−3.29506	0.000158	0.001839	Down
hsa-miR-378c	3.525841	0.000171	0.00185	Up
hsa-miR-374a-5p	3.48526	0.000189	0.001889	Up
hsa-miR-29c-5p	3.161635	0.000221	0.002036	Up
hsa-miR-126-3p	3.196734	0.000246	0.002164	Up
hsa-let-7a-5p	3.626154	0.000248	0.002164	Up
hsa-miR-339-3p	3.235942	0.000261	0.002187	Up
hsa-miR-301a-3p	3.358697	0.000278	0.002292	Up
hsa-miR-4711-3p	−3.28102	0.001108	0.005396	Down
hsa-miR-141-3p	3.03568	0.016527	0.03819	Up

Abbreviations: FC, fold change; FDR, false discovery rate.

Table 2 Top 20 Differentially Expressed mRNAs in Osteonecrosis of the Femoral Head

Symbol	LogFC	*P*-value	FDR	Up/Down
C10orf105	3.03737	9.90E-12	2.15E-07	Up
ARL4C	2.533696	2.73E-11	2.55E-07	Up
EGR2	2.551482	3.52E-11	2.55E-07	Up
LRRC15	2.360616	5.31E-11	2.68E-07	Up
AMTN	3.203584	6.15E-11	2.68E-07	Up
IL11	2.257176	7.78E-11	2.82E-07	Up
FAP	2.516977	2.84E-10	8.71E-07	Up
VEGFC	1.98741	3.44E-10	8.71E-07	Up
FZD10	2.128076	3.60E-10	8.71E-07	Up
MMP13	2.670397	5.32E-10	1.16E-06	Up
MSMP	−3.01757	1.28E-09	1.74E-06	Down
VIT	−1.35337	3.11E-09	3.06E-06	Down
HLA-DRB4	−1.49327	3.17E-09	3.06E-06	Down
RNASE1	−1.55781	3.23E-09	3.06E-06	Down
RCAN2	−1.70034	4.41E-09	3.75E-06	Down
DACT1	−1.65655	5.18E-09	3.75E-06	Down
APOD	−1.67708	5.18E-09	3.75E-06	Down
TYROBP	−1.27919	8.36E-09	4.92E-06	Down
CTSH	−1.55528	1.03E-08	5.16E-06	Down
HLA-DRA	−1.92425	1.35E-08	5.44E-06	Down

Abbreviations: FC, fold change; FDR, false discovery rate.

Functional Analysis of Targeted mRNAs

GO and KEGG analysis of targeted mRNAs is shown in supplementary Table 4. The top five significant enrichment GO terms and all KEGG terms are presented in Figures 5 and 6, respectively. Total KEGG terms involving targeted differentially expressed mRNAs are shown in Table 3. In the KEGG terms, we found seven valuable signaling pathways including the Wnt signaling pathway (involved WNT3A), chemokine signaling pathway (involved RELA), focal adhesion and ECM-receptor interaction

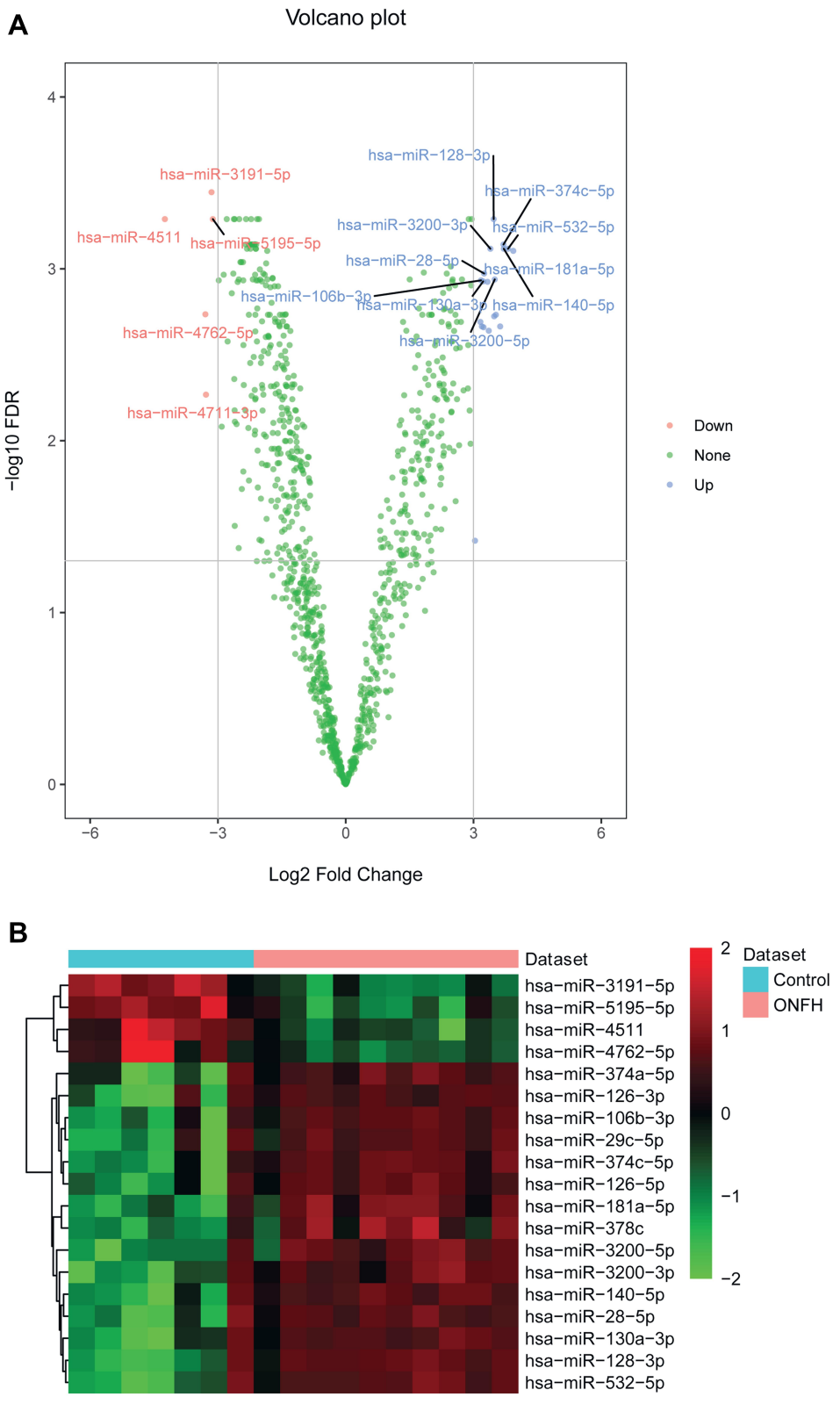


Figure 1 The volcano plot and heat map of all differentially expressed miRNAs in osteonecrosis of the femoral head. (**A**) The volcano plot of all differentially expressed miRNAs. The X and Y axis represents Log2 Fold Change and –log10 FDR, respectively. Blue and red represents up-regulated and down-regulated miRNAs, respectively. (**B**) The heat map of all differentially expressed miRNAs.

Abbreviation: ONFH, osteonecrosis of the femoral head.

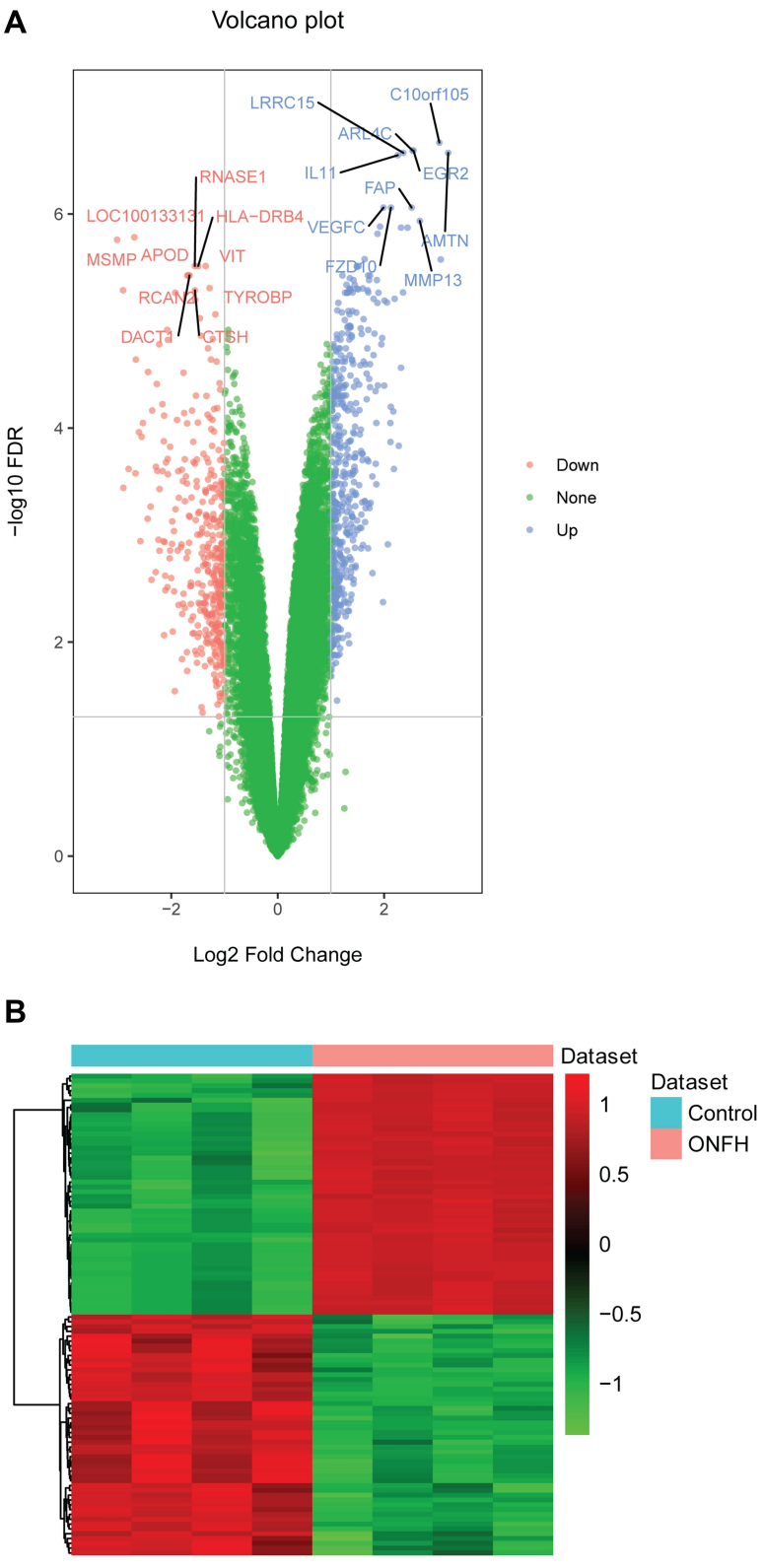


Figure 2 The volcano plot and heat map of the top 100 differentially expressed mRNAs in osteonecrosis of the femoral head. (**A**) The volcano plot of the top 100 differentially expressed mRNAs. The X- and Y-axes represent Log2 Fold Change and –log10 FDR, respectively. Blue and red represent up-regulated and down-regulated mRNAs, respectively. (**B**) The heat map of the top 100 differentially expressed mRNAs.
Abbreviation: ONFH, osteonecrosis of the femoral head.

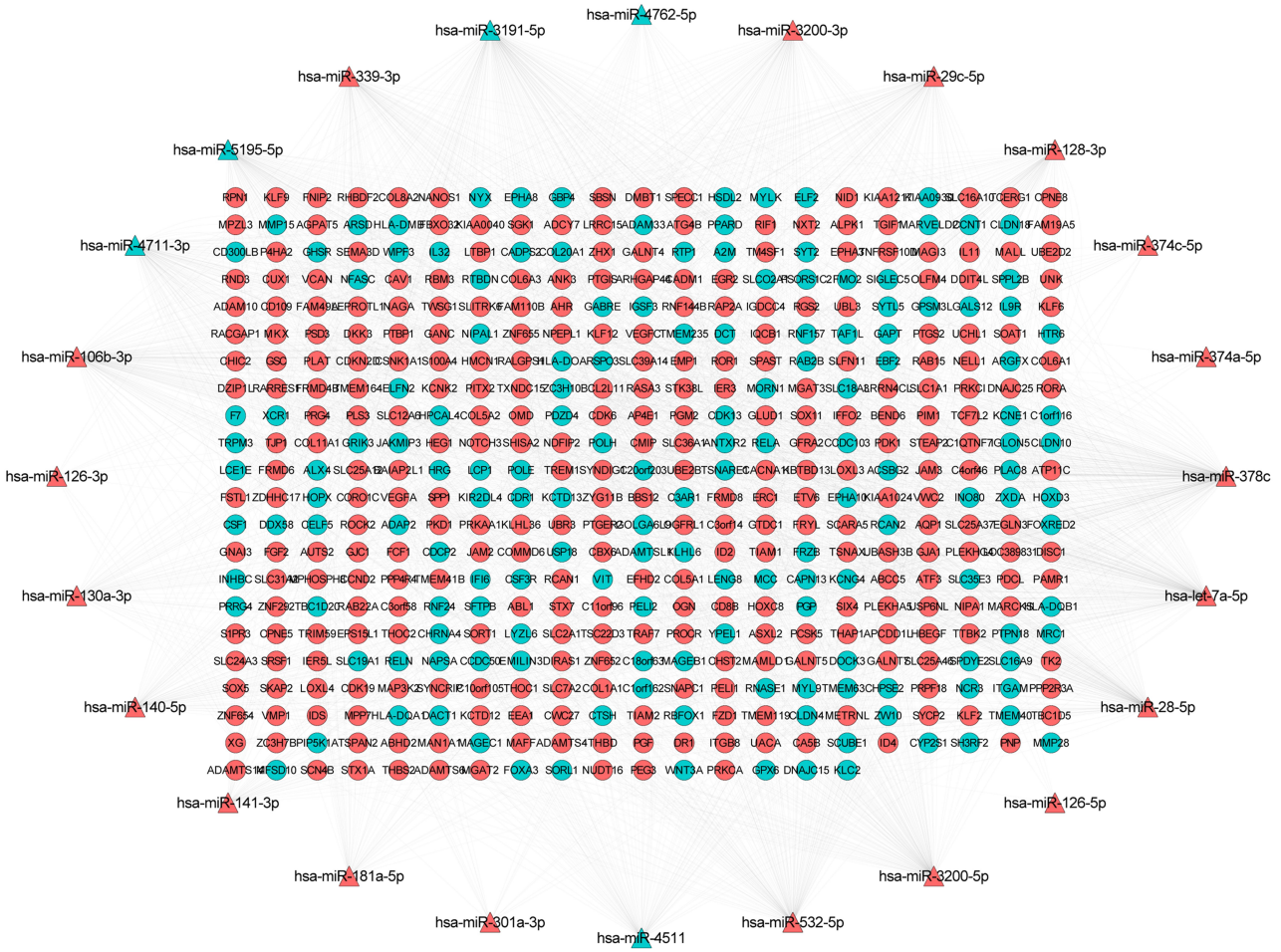


Figure 3 The network of miRNA-target mRNAs between 24 miRNAs and 457 mRNAs in osteonecrosis of the femoral head. The triangle and circule represent the differentially expressed miRNAs and targeted differentially expressed mRNAs, respectively. The red and green color represent up-regulation and down-regulation, respectively.

(involved RELN), cell adhesion molecules (CAMs) (involved CLDN18 and CLDN10), cytokine–cytokine receptor interaction, and hematopoietic cell lineage (involving CSF1 and IL9R).

In vitro Validation

Six patients with osteonecrosis of the femoral head and seven normal controls were incorporated in our study. Clinical information of these individuals is shown in Table 4. As mentioned above, hsa-miR-28-5p was one of the top 10 differentially expressed miRNAs that targeted the most differentially expressed mRNAs. WNT3A, RELA, and RELN were involved in KEGG pathways. RCAN2 was in the top 20 down-regulated mRNAs. We selected hsa-miR-28-5p, WNT3A, RCAN2, RELA, and RELN for validation (Figure 7). The relative expression of hsa-miR-28-5p was significantly up-regulated, the relative expression of WNT3A, RCAN2, RELA, and RELN was down-regulated in patients with osteonecrosis of the femoral head. The validated result was in line with the bioinformatics analysis.

Expression Validation and Diagnostic Analysis of Targeted mRNAs

The GSE123568 dataset was firstly utilized to validate the expression of CLDN10, CLDN18, CSF1, DACT1, IL9R, RCAN2, RELN, and WNT3A (Figure 8). The expression of these mRNAs was all significantly down-regulated, which is in line with the bioinformatics analysis. In addition, the ROC curve analysis was performed to assess the diagnosis ability of these mRNAs in the GSE123568 dataset (Figure 9). The AUC of these mRNAs was more than 0.7, which suggested that they had a diagnostic value for osteonecrosis of the femoral head.

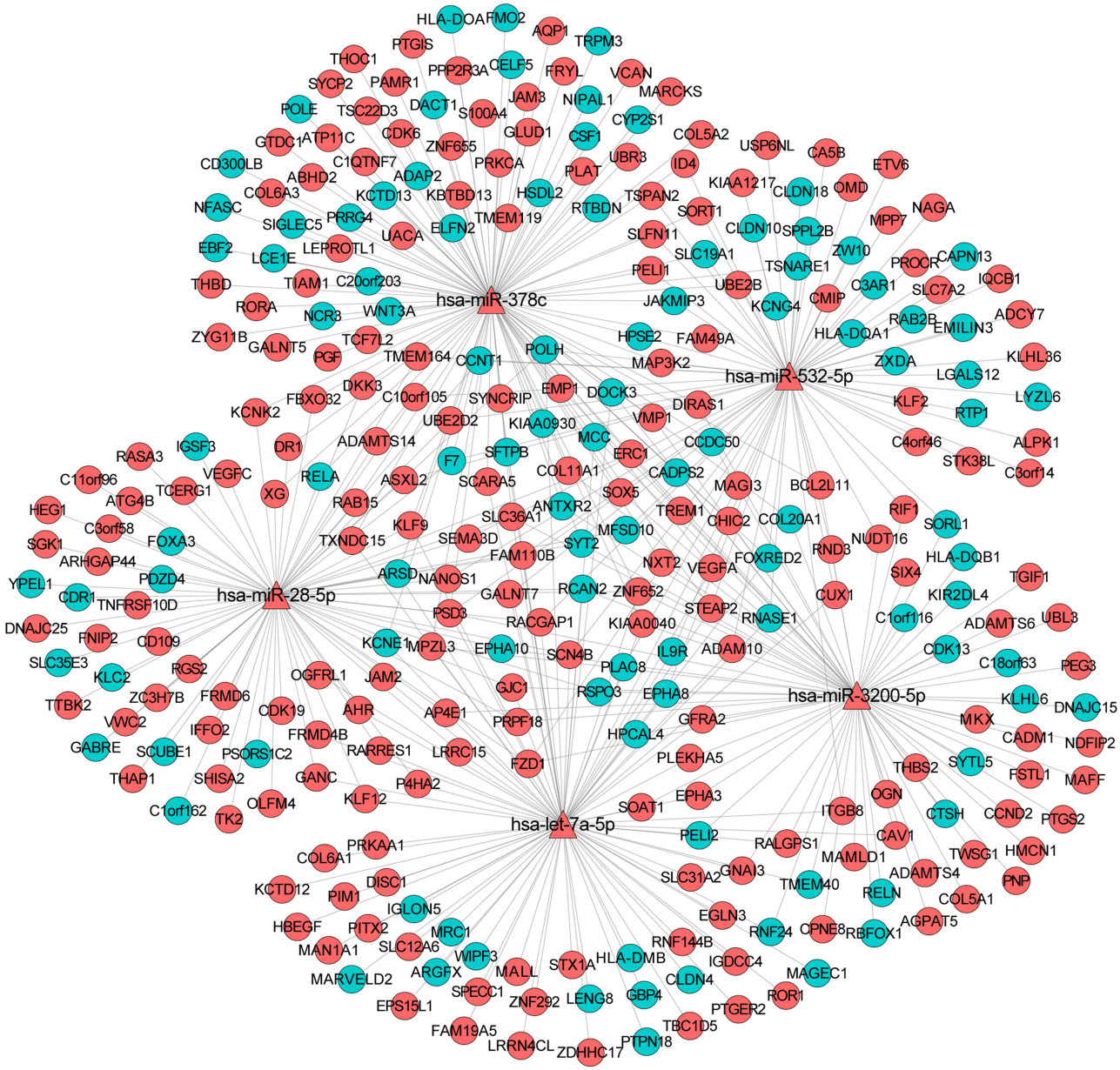
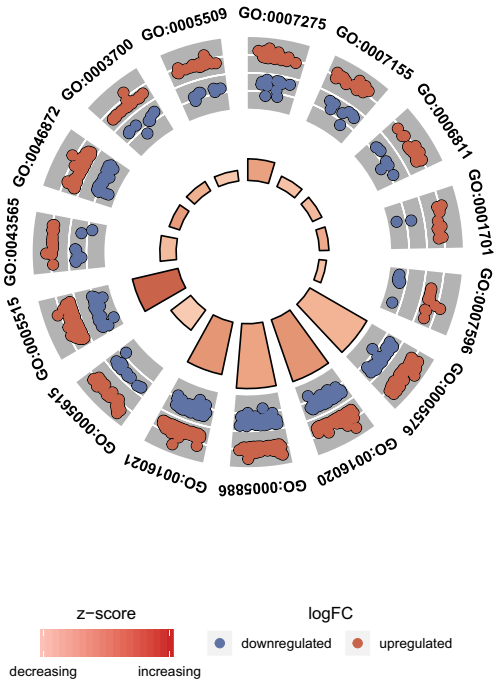


Figure 4 The sub-network of miRNA-target mRNAs between hsa-miR-378c, hsa-let-7a-5p, hsa-miR-28-5p, hsa-miR-3200-5p, hsa-miR-532-5p, and their targeted mRNAs in osteonecrosis of the femoral head. The triangle and circule represent the differentially expressed miRNAs and targeted differentially expressed mRNAs, respectively. The red and green color represent up-regulation and down-regulation, respectively.

Discussion

Hsa-miR-378c, a hypoxia-regulated miRNA, is reported to be down-regulated in rheumatoid arthritis and osteosarcomas.[29,30] The level of hsa-miR-378c was increased in osteonecrosis of the femoral head in this study. Moreover, three down-regulated mRNAs including Wnt family member 3A (WNT3A), dishevelled binding antagonist of beta catenin 1 (DACT1), and colony stimulating factor 1 (CSF1) were all regulated by hsa-miR-378c. Significantly, WNT3A, DACT1, and CSF1 had a diagnostic value for osteonecrosis of the femoral head. WNT3A can induce chondrocytes proliferation and alter the extracellular matrix synthesis function of the chondrocytes.[31] In addition, WNT3A and hypoxia could act together to promote angiogenesis by regulating cell death.[32] It is noted that liposome-reconstituted recombinant human WNT3A protein has been used to treat osteonecrosis defect.[33] The expression of DACT1 is found in primary chondrocytes and vascular endothelial cells.[34,35] Käkönen and



ID	Description
GO:0007275	multicellular organismal development (BP)
GO:0007155	cell adhesion (BP)
GO:0006811	ion transport (BP)
GO:0001701	in utero embryonic development (BP)
GO:0007596	blood coagulation (BP)
GO:0005576	extracellular region (CC)
GO:0016020	membrane (CC)
GO:0005886	plasma membrane (CC)
GO:0016021	integral to membrane (CC)
GO:0005615	extracellular space (CC)
GO:0005515	protein binding (MF)
GO:0043565	sequence−specific DNA binding (MF)
GO:0046872	metal ion binding (MF)
GO:0003700	sequence−specific DNA binding transcription factor activity (MF)
GO:0005509	calcium ion binding (MF)

Figure 5 Top five significantly enriched GO terms of targeted differentially expressed mRNAs in osteonecrosis of the femoral head. The z-score clustering in the GO terms of targeted differentially expressed mRNA is shown below. The red and blue color represent up-regulated and down-regulated mRNA, respectively.
Abbreviations: BP, biological process; CC, cellular component; MF, molecular function; FC, fold change.

Mundy[36] found that CSF-1 could interact with osteoblast to regulate the RANK–RANKL pathway to stimulate osteoclast precursors, ultimately leading to osteolysis. Additionally, CSF1, combined with VEGF-A, induces angiogenesis and recruitment of pericyte to neovessels.[37] Our result suggested that WNT3A, DACT1, and CSF1 may play roles in bone remodeling and angiogenesis under the regulation of hsa-miR-378c in the process of osteonecrosis of the femoral head.

Hsa-let-7a-5p is down-regulated in osteogenic differentiation, while up-regulated during osteoclastogenesis, which indicates the role of hsa-let-7a-5p in the bone imbalance.[38–40] Under mechanical tension, hsa-let-7a-5p is remarkably increased in cartilage endplate chondrocytes.[41] In this study, we found that hsa-let-7a-5p was up-regulated in osteonecrosis of the femoral head. Furthermore, a regulator of calcineurin 2 (RCAN2) and interleukin 9 receptor (IL9R) were down-regulated and targeted by hsa-let-7a-5p. It is worth mentioning that RCAN2 and IL9R could be considered as diagnostic biomarkers for osteonecrosis of the femoral head. RCAN2, an angiogenesis related gene, is transcribed activation by vascular endothelial growth factor (VEGF).[42–45] The expression of RCAN2 is negatively correlated with cartilage proliferation and differentiation.[46] It is reported that disruption of RCAN2 could lead to reducing bone mass, which is related to increased osteoclast function and reduced osteoblast function.[47] IL9R is associated with hematopoietic cell lineage.[48] Our result indicated that hsa-let-7a-5p and its target (RCAN2 and IL9R) could be involved in osteonecrosis of the femoral head.

Deregulation of hsa-miR-28-5p is related to rheumatic diseases, such as axial spondyloarthritis and rheumatoid arthritis.[49,50] Herein, we first found that hsa-miR-28-5p was up-regulated in osteonecrosis of the femoral head. In addition, the down-regulated RELA proto-oncogene, NF-kB subunit (RELA, also called p65) was one of the targets of hsa-miR-28-5p. It is reported that RELA is the most potent transcriptional factor of hypoxic induction factor 2 (HIF2) that regulates chondrocyte differentiation and cartilage degradation.[51] Lacking or deletion of RELA inhibits the expression of cartilage catabolic factors such as matrix metalloproteinases 9 (MMP9), SRY-box transcription factor 9 (SOX9), nitric oxide synthase 2 (NOS2), and cyclooxygenase 2 (COX2) in chondrocytes, which results in reduced bone loss and accelerated cartilage degeneration.[52–57] In addition, RELA is regarded as an angiogenesis modulating agent.[58] It is suggested that RELA may be involved in cartilage degeneration of osteonecrosis of the femoral head under that regulation of hsa-miR-28-5p.

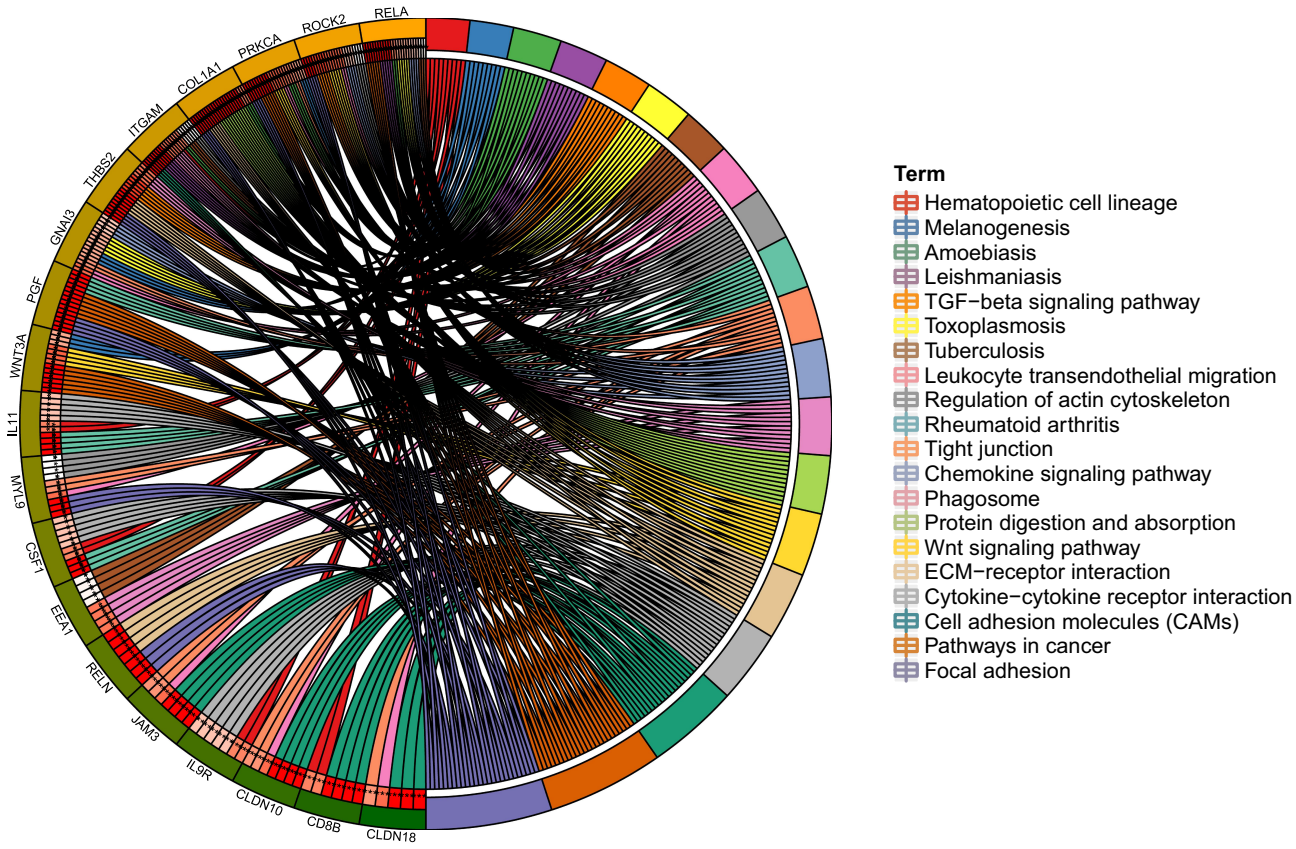


Figure 6 KEGG signaling pathways of targeted differentially expressed mRNAs in osteonecrosis of the femoral head. Different colors represent different signaling pathways; mRNA outside the circle represents the enriched one of mRNAs in the particular signaling pathway.

Previous studies on hsa-miR-3200-5p in orthopedic disease are very rare, and only a recent report showed significantly higher expression of hsa-miR-3200-5p in the osteosarcoma.[59] In our study, we found that the expression level of hsa-miR-3200-5p was increased in osteonecrosis of the femoral head. Moreover, down-regulated reelin (RELN) was targeted by hsa-miR-3200-5p. It is noted that RELN had a diagnostic value for osteonecrosis of the femoral head. RELN, expressed in osteoblast lineage cells, is considered as a stromal cell-specific and hematopoietic cell-lineage marker.[60] Clinically, RELN is a potential molecular target candidate for diagnosis and therapy of rheumatoid arthritis.[61] Our result indicated that hsa-miR-3200-5p and RELN may play a critical role in osteonecrosis of the femoral head.

Hsa-miR-532-5p plays roles in the regulation of the adaptation to hypoxia in endothelial cells.[62] Hsa-miR-532-5p is differentially expressed in chondrocytes from distinct regions of developing human cartilage.[63] In the present study, we found that hsa-miR-532-5p was up-regulated in osteonecrosis of the femoral head. Furthermore, claudin 18 (CLDN18) and claudin 10 (CLDN10) were down-regulated and regulated by hsa-miR-532-5p. In addition, CLDN18 and CLDN10 were associated with disease diagnosis. Elevated expression of CLDN18 is found in osteoblasts.[64,65] Knock-out of CLDN18 leads to reduced bone mass from hyperactive osteoclasts.[64] The expression of CLDN10 is increased in osteosarcoma osteoblast cells.[66] Our result suggested that CLDN18 and CLDN10 could be associated with bone loss under the regulation of hsa-miR-532-5p in osteonecrosis of the femoral head.

Based on KEGG analysis, we found seven valuable signaling pathways including the Wnt signaling pathway (involved WNT3A), chemokine signaling pathway (involved RELA), focal adhesion and ECM-receptor interaction (involved RELN), cell adhesion molecules (CAMs) (involved CLDN18 and CLDN10), cytokine–cytokine receptor interaction, and hematopoietic cell lineage (involved CSF1 and IL9R) in osteonecrosis of the femoral head. The Wnt/β-catenin pathway induces VEGF to

Table 3 Total KEGG Terms Involved Targeted Differentially Expressed mRNAs in Osteonecrosis of the Femoral Head

Terms	Count	*P*-value	FDR	mRNAs
Focal adhesion	19	2.09E-11	3.15E-09	RELN,PGF,MYLK,ROCK2,CCND2,SPP1,PRKCA,COL5A2,CAV1,COL1A1, THBS2,VEGFC,COL5A1,ITGB8,COL6A1,VEGFA,MYL9,COL11A1,COL6A3
Pathways in cancer	16	7.87E-06	0.00017	CDK6,PPARD,EGLN3,FGF2,PGF,ABL1,WNT3A,CSF3R,PRKCA,FZD1, VEGFC,TCF7L2,RELA,SLC2A1,VEGFA,PTGS2
Cell adhesion molecules (CAMs)	15	1.25E-10	9.43E-09	JAM3,JAM2,HLA-DOA,NFASC,ITGAM,CD8B,CADM1,HLA-DQA1, CLDN18,CLDN10,HLA-DQB1,ITGB8,HLA-DMB,VCAN,CLDN4
Tight junction	11	1.35E-06	4.07E-05	JAM3,JAM2,TJP1,GNAI3,PRKCA,MAGI3,CLDN18,CLDN10,PRKCI,MYL9, CLDN4
ECM-receptor interaction	10	1.70E-07	8.54E-06	RELN,SPP1,COL5A2,COL1A1,THBS2,COL5A1,ITGB8,COL6A1,COL11A1, COL6A3
Leukocyte transendothelial migration	10	2.71E-06	6.83E-05	JAM3,JAM2,ROCK2,ITGAM,GNAI3,PRKCA,CLDN18,CLDN10,MYL9, CLDN4
Cytokine-cytokine receptor interaction	9	0.00791727	0.031461	TNFRSF10D,IL11,INHBC,CSF3R,CSF1,XCR1,VEGFC,VEGFA,IL9R
Protein digestion and absorption	9	9.24E-07	3.49E-05	SLC36A1,SLC1A1,COL5A2,COL1A1,COL5A1,COL6A1,COL11A1, COL6A3,SLC16A10
Regulation of actin cytoskeleton	9	0.00196338	0.01098	FGF2,TIAM1,MYLK,ROCK2,ITGAM,PIP5K1A,TIAM2,ITGB8,MYL9
Phagosome	9	9.15E-05	0.001256	HLA-DOA,ITGAM,HLA-DQA1,THBS2,STX7,EEA1,HLA-DQB1,MRC1, HLA-DMB
Tuberculosis	8	0.00214245	0.011554	HLA-DOA,ITGAM,HLA-DQA1,RELA,EEA1,HLA-DQB1,MRC1,HLA-DMB
Wnt signaling pathway	8	0.000859587	0.006181	PPARD,ROCK2,CCND2,WNT3A,PRKCA,FZD1,TCF7L2,CSNK1A1
Rheumatoid arthritis	8	1.58E-05	0.000299	PGF,IL11,HLA-DOA,CSF1,HLA-DQA1,HLA-DQB1,VEGFA,HLA-DMB
Chemokine signaling pathway	7	0.0121892	0.044892	TIAM1,ROCK2,ADCY7,GNAI3,XCR1,TIAM2,RELA
TGF-beta signaling pathway	7	0.000108744	0.001368	ROCK2,INHBC,PITX2,LTBP1,ID4,THBS2,ID2
Toxoplasmosis	7	0.00128054	0.007734	HLA-DOA,GNAI3,HLA-DQA1,RELA,HLA-DQB1,PDK1,HLA-DMB
Melanogenesis	7	0.000330342	0.003118	WNT3A,ADCY7,GNAI3,PRKCA,FZD1,TCF7L2,DCT
Leishmaniasis	7	2.67E-05	0.000448	HLA-DOA,ITGAM,HLA-DQA1,RELA,HLA-DQB1,HLA-DMB,PTGS2
Amoebiasis	7	0.000421534	0.003744	ITGAM,PRKCA,COL5A2,COL1A1,COL5A1,RELA,COL11A1
Hepatitis C	6	0.00865149	0.033497	DDX58,CLDN18,CLDN10,RELA,PDK1,CLDN4
Lysosome	6	0.00552762	0.023848	NAPSA,NAGA,CTSH,SORT1,AP4E1,IDS
Axon guidance	6	0.00722661	0.029492	EPHA8,ABL1,ROCK2,GNAI3,SEMA3D,EPHA3
Hematopoietic cell lineage	6	0.000824081	0.006222	IL11,ITGAM,CSF3R,CSF1,CD8B,IL9R
Gap junction	6	0.00111969	0.007045	TJP1,ADCY7,GNAI3,PRKCA,MAP3K2,GJA1
Epithelial cell signaling in Helicobacter pylori infection	6	0.000260077	0.002805	JAM3,JAM2,TJP1,ADAM10,HBEGF,RELA

(*Continued*)

Table 3 (Continued).

Terms	Count	*P*-value	FDR	mRNAs
Staphylococcus aureus infection	6	3.08E-05	0.000466	HLA-DOA,ITGAM,HLA-DQA1,C3AR1,HLA-DQB1,HLA-DMB
Viral myocarditis	6	0.000185105	0.00215	ABL1,HLA-DOA,CAV1,HLA-DQA1,HLA-DQB1,HLA-DMB
Complement and coagulation cascades	5	0.00169723	0.009857	THBD,A2M,PLAT,C3AR1,F7
Gastric acid secretion	5	0.00266688	0.01299	MYLK,ADCY7,GNAI3,PRKCA,KCNK2
Renal cell carcinoma	5	0.00235692	0.011863	EGLN3,PGF,VEGFC,SLC2A1,VEGFA
Pancreatic cancer	5	0.00235692	0.011863	CDK6,PGF,VEGFC,RELA,VEGFA
Antigen processing and presentation	5	0.000864725	0.005935	HLA-DOA,CD8B,HLA-DQA1,HLA-DQB1,HLA-DMB
Autoimmune thyroid disease	4	0.00292128	0.013367	HLA-DOA,HLA-DQA1,HLA-DQB1,HLA-DMB
Type I diabetes mellitus	4	0.00101917	0.006691	HLA-DOA,HLA-DQA1,HLA-DQB1,HLA-DMB
Intestinal immune network for IgA production	4	0.0026893	0.01269	HLA-DOA,HLA-DQA1,HLA-DQB1,HLA-DMB
N-Glycan biosynthesis	4	0.00398592	0.017702	RPN1,MAN1A1,MGAT2,MGAT3
Shigellosis	4	0.00867634	0.032753	ABL1,ROCK2,UBE2D2,RELA
Acute myeloid leukemia	4	0.006843	0.028703	PPARD,TCF7L2,RELA,PIM1
Allograft rejection	4	0.000549845	0.004613	HLA-DOA,HLA-DQA1,HLA-DQB1,HLA-DMB
Graft-versus-host disease	4	0.000627861	0.00499	HLA-DOA,HLA-DQA1,HLA-DQB1,HLA-DMB
Asthma	4	0.000305331	0.003074	HLA-DOA,HLA-DQA1,HLA-DQB1,HLA-DMB

Abbreviation: FDR, false discovery rate.

promote neovascularization.[67,68] In addition, the signaling pathway plays a key role in regulating chondrocyte proliferation. It is found that the Wnt/β-catenin pathway is involved in the process of cartilage damage.[69] It is noted that the Wnt/β-catenin pathway is associated with the pathogenesis of early stage femoral head osteonecrosis via regulating of transcriptional regulator Myc-like (c-Myc) that affects the cell apoptosis and cell cycle.[70] Chemokines are involved in angiogenesis and wound healing. It is reported that chemokines secreted from chondrocytes alter functional abilities of subchondral bone osteoblasts.[71] The chemokine signaling pathway is significantly enriched in immature articular cartilage after osteonecrosis of the femoral head.[72] Focal adhesion, involved in cell growth, shape, and movement, attach chondrocytes to the pericellular cartilage matrix and link to intracellular organelles. It has been shown that focal adhesion is a remarkably enriched biological pathway in the immature articular cartilage after osteonecrosis of the femoral head.[72] Cell adhesion molecules, such as cadherins, selectins, and immunoglobulin superfamily proteins, are associated with angiogenesis.[73–75] In addition, cell adhesion molecules play a vital role in regulating cartilage matrix turnover.[76,77] ECM-receptor interaction is associated with angiogenesis, chondrogenesis, and cartilage degeneration.[78] It is found that ECM-receptor interaction is significantly enriched in hip cartilage with osteonecrosis of the femoral head.[79] It has been identified that cytokine–cytokine receptor interaction is one of the most dramatically important pathways in osteonecrosis of the femoral head.[72,80] Osteoclasts originated from hematopoietic stem cells are involved in maintaining bone integrity. Normal femoral head shows trabecular bones surrounded by bone marrow endowed with hematopoietic cells. Infiltration of

Table 4 Clinical Information of Enrolled Individuals in vitro Validation

Group	Gender	Age	Weight	Pain	Function	Malformation	Joint Activities	Cartilage Injury of Hip Joint	ARCO Stage
NC	Male	53	77	No	Good	No	Normal	No	No
	Male	62	68	No	Good	No	Normal	No	No
	Female	58	63	No	Good	No	Normal	No	No
	Female	58	63	No	Good	No	Normal	No	No
	Male	51	70	No	Good	No	Normal	No	No
	Female	63	55	No	Good	No	Normal	No	No
	Female	49	62	No	Good	No	Normal	No	No
Case	Male	50	80	Yes	Limp	Shortening	Adduction abduction limited and buckling 90°	Yes	IV
	Male	39	72	Yes	Limp	Shortening	Adduction abduction limited and buckling 90°	Yes	IV
	Male	46	66	Yes	Limp	Shortening	Adduction abduction limited and buckling 80°	Yes	IV
	Female	35	62	Yes	Limp	Shortening	Adduction abduction limited and buckling 90°	Yes	IV
	Female	58	67	Yes	Limp	Shortening	Adduction abduction limited and buckling 90°	Yes	IV
	Male	20	65	Yes	Limp	Shortening	Adduction abduction limited and buckling 90°	Yes	IV

Abbreviation: NC, normal controls.

hematopoietic cell to the ischemic area plays a significant role in regulating ischemia-induced angiogenesis.[81]

In conclusion, the epigenetic modifications of hsa-miR-378c-WNT3A/DACT1/CSF1, hsa-let-7a-5p-RCAN2/IL9R, hsa-miR-28-5p-RELA, hsa-miR-3200-5p-RELN, and hsa-miR-532-5p-CLDN18/CLDN10, and seven signaling pathways (Wnt signaling pathway, chemokine signaling pathway, focal adhesion, cell adhesion molecules (CAMs), ECM-receptor interaction, cytokine-cytokine receptor interaction, and hematopoietic cell lineage) may be involved in osteonecrosis of the femoral head. In addition, CLDN10, CLDN18, CSF1, DACT1, IL9R, RCAN2, RELN, and WNT3A had a diagnostic value for osteonecrosis of the femoral

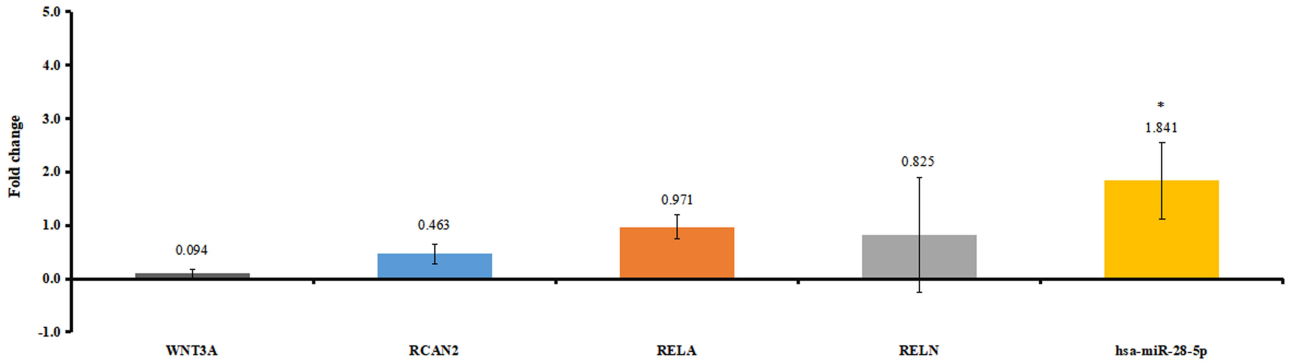


Figure 7 The in vitro validation of differentially expressed miRNAs and targeted differentially expressed mRNAs. Fold change >1 and fold change <1 represent up-regulation and down-regulation, respectively. *P<0.05.

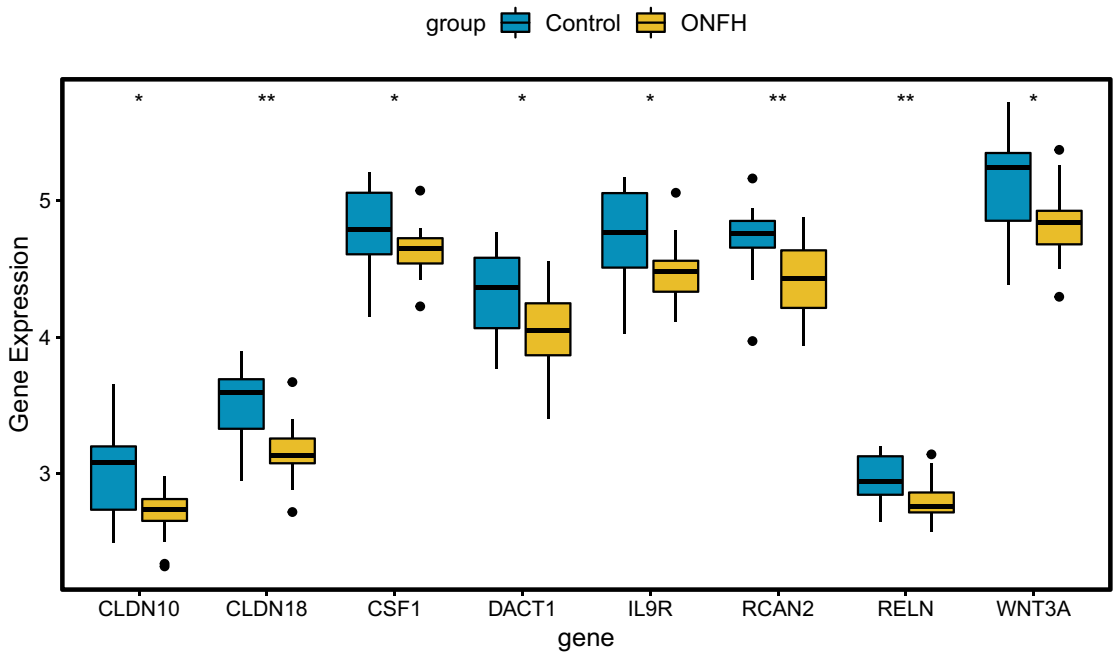


Figure 8 Expression box plots of CLDN10, CLDN18, CSF1, DACT1, IL9R, RCAN2, RELN, and WNT3A in the GSE123568 dataset. *P<0.05, **P<0.01.
Abbreviation: ONFH, osteonecrosis of the femoral head.

head. However, there are limitations of our study. Firstly, the deeper mechanism study of identified differentially expressed miRNAs, mRNAs, and relevant downstream molecules in the disease is further needed in animal models. Secondly, the regulatory relationship between identified miRNAs and targeted mRNAs is not investigated. Further in vitro experiment, such as luciferase reporter gene assay is needed in the further study.

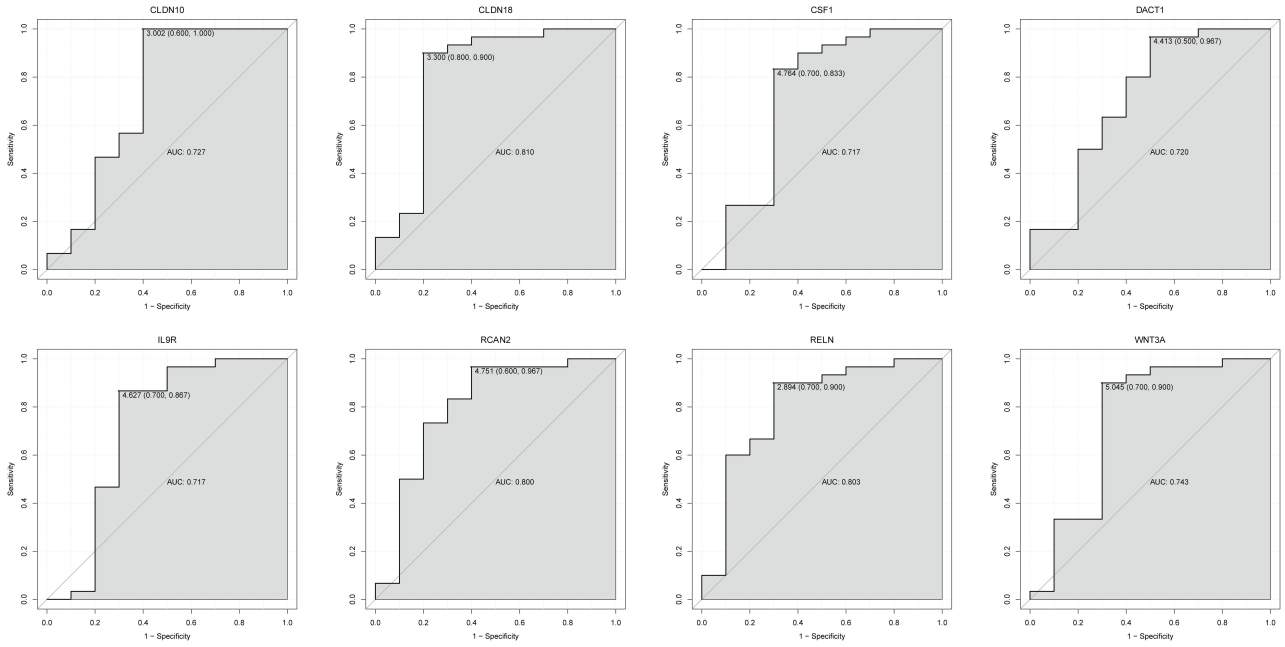


Figure 9 The ROC curves of CLDN10, CLDN18, CSF1, DACT1, IL9R, RCAN2, RELN, and WNT3A between osteonecrosis of the femoral head and normal controls. The ROC curves were used to show the diagnostic ability of these mRNAs with 1-specificity and sensitivity.

Abbreviations

CLDN10, claudin 10; CLDN18, claudin 18; CSF1, colony stimulating factor 1; COX2, cyclooxygenase 2; DACT1, dishevelled binding antagonist of beta catenin 1; RELN, reelin; CAMs, cell adhesion molecules; FGF2, fibroblast growth factor 2; GEO, Gene Expression Omnibus database; HIF2, hypoxic induction factor 2; IGF1, insulin-like growth factor 1; IL9R, interleukin 9 receptor; MMP9, matrix metalloproteinases 9; NOS2, nitric oxide synthase 2; RCAN2, regulator of calcineurin 2; SOX9, SRY-box transcription factor 9; VEGF, vascular endothelial growth factor; WNT3A, Wnt family member 3A.

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Disclosure

No competing financial interests exist and the authors report no conflicts of interest for this work.

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