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# RNA Sequencing of Osteosarcoma Gene Expression Profile Revealed that miR-214-3p Facilitates Osteosarcoma Cell Proliferation via Targeting Ubiquinol-Cytochrome c Reductase Core Protein 1 (UQCRC1)

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Statistical Analysis C
Data Interpretation D
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**Background:** Osteosarcoma (OS) is a common primary malignant bone tumor for which the molecular mechanisms remain unclear. Studies on coding and non-coding RNAs are needed to determine the molecular mechanism.

**Material/Methods:** To explore the potential roles of miRNAs and mRNA in OS, we determined the miRNA and mRNA expression profile of 3 pairs of OS and paracancerous tissues from patients with OS by sequencing and bioinformatics analysis. The expression levels of critical miRNAs and mRNAs were verified in 10 pairs of OS and paracancerous tissues. An miRNA inhibitor and mimics were used to investigate the interactions between miRNAs and target genes. The cell counting kit-8 assay was performed to evaluate OS cell proliferation after miRNA interference.

**Results:** A total of 184 miRNAs and 2501 mRNAs were identified (fold-change >2.0 or <2.0, P<0.05), with up-regulation of 82 miRNAs and 1320 mRNAs and down-regulation of 102 miRNAs and 1181 mRNAs in OS tissue. The protein protein interaction network revealed that UQCRC1 (ubiquinol-cytochrome c reductase core protein 1) is a critical gene and a potential target gene of miR-214-3p. Both UQCRC1 and miR-214-3p were significantly differentially expressed in OS tissue and cell lines (down and up-regulated, respectively). Down-regulated miR-214-3p expression increased UQCRC1 expression and suppressed OS cell proliferation. In contrast, overexpression of miR-214-3p decreased UQCRC1 expression and promoted OS cell proliferation.

**Conclusions:** High miR-214-3p expression may promote OS cell proliferation by targeting UQCRC1, providing insight into a potential therapeutic target for preventing and treating OS.

**MeSH Keywords:** High-Throughput Nucleotide Sequencing • MicroRNAs • Osteosarcoma • Transcriptome

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## Background

Osteosarcoma (OS) is a type of bone tumor arising from primitive transformed cells of mesenchymal origin in teenagers and young adults [1,2]. Currently, most cases of OS are treated clinically by surgery combined with chemotherapy [3]. However, it is estimated that the incidence of OS is 4–5/1,000,000 and the 5-year survival rate for metastatic OS is less than 20% [4,5]. The origin and etiology of OS involve complicated genome rearrangement, highly variable patterns of gene expression, and high metastatic capacity [6,7]. Therefore, studies are needed to investigate the underlying molecular mechanisms and new strategies for diagnosis and treatment of OS.

MicroRNA (miRNAs) are a large class of small non-coding RNAs consisting of approximately 20–25 nucleotides and have been demonstrated to regulate approximately 30% of genes via 3' untranslated regions [8,9]. Abnormal expression of miRNAs has been widely reported in cancer and plays key roles in many biological processes, such as cell proliferation, differentiation, and apoptosis [10,11]. Studies of OS in recent years has revealed important roles for miRNAs, such as miR-184, miR-664, and miR-885-5p, among others [12,13]. However, few studies have been performed using high-throughput sequencing to evaluate OS tissues compared to sequencing of OS cell lines. Additional studies of critical miRNAs and mRNAs as well as their biological roles in OS tissues are needed.

In the present study, we performed RNA-seq to investigate the miRNA and mRNA expression profiles of OS tissue. Bioinformatics analysis and further experiments were conducted to identify the key genes involved in OS dysregulation. Our study provides insight into the molecular mechanisms of OS.

## Material and Methods

The research design was as follows.

### Patients and controls

A total of 10 patients with primary OS (age range 10–63 years, 4 males/6 females) treated at the Department of Orthopedics of the Second Hospital of Jilin University between May 2017 and December 2018 were enrolled in this study. The diagnosis of OS was confirmed by pathological analysis. The patients were not treated with radiotherapy or chemotherapy before the surgery. Patients with concurrent congenital diseases or tumor-related diseases were excluded. OS tissues and their matched adjacent normal tissues were obtained from patients who underwent complete resection surgery. All tissue samples were immediately frozen in liquid nitrogen. This research was approved by the Ethics Committee of Jilin University (2016.169), The Second Hospital, and all patients involved in this research signed written informed consents. The clinical and demographical characteristics of the patients are summarized in Table 1.

### miRNA and mRNA sequencing

The miRNA and mRNA sequencing of 3 pairs of OS and paracancerous tissues from patients with OS were performed at Beijing Novogene Co., Ltd. (Beijing, China). A total of 3 μg total RNA per sample was used as input material to prepare the miRNA and mRNA libraries. Sequencing libraries were generated using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs, Ipswich, MA, USA) and rRNA-depleted RNA by NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs). Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's

**Table 1.** Clinical characteristics of the subjects.

| No. | Sex | Age | Anatomic location | Stage |
|---|---|---|---|---|
| 1 | F | 63 | Femur | IIB |
| 2 | M | 59 | Femur | IIB |
| 3 | F | 54 | Tibia | IIA |
| 4 | M | 42 | Scapula | IIB |
| 5 | F | 37 | Femur | IIB |
| 6 | F | 35 | Femur | IIB |
| 7 | M | 15 | Femur | IIB |
| 8 | M | 14 | Femur | IIA |
| 9 | F | 12 | Femur | IIA |
| 10 | F | 10 | Femur | IIA |





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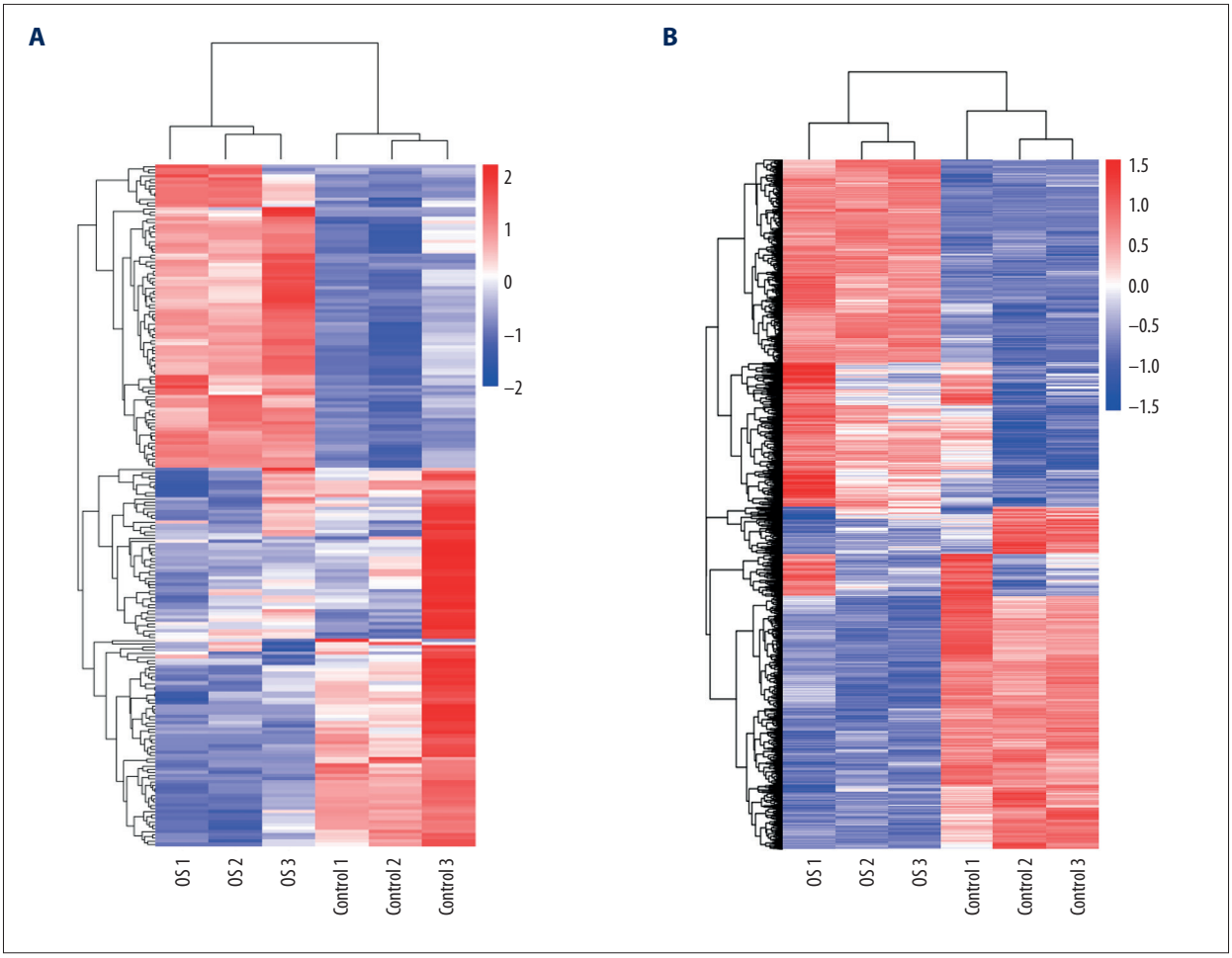


**Figure 1.** Hierarchical clustering of aberrantly expressed miRNAs (**A**) and mRNAs (**B**) in OS. Each row represents a miRNA or mRNA and each column represents an OS sample. Red or blue colors represents high or low relative expression level, respectively.

instructions. The library preparations of miRNA were sequenced on an Illumina Hiseq 2500/2000 platform and 50-base pair (bp) single-end reads were generated. For mRNA, the libraries were sequenced on an Illumina Hiseq 4000 platform and 150-bp paired-end reads were generated.

### Cell culture and RNA extraction

Human OS cell lines, including MG63, U2OS, and HOS, and human osteoblast cell line hFOB1.19 were acquired from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics (penicillin and streptomycin). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

### Real-time RT-PCR analysis

Total RNA of miRNA and mRNA were reverse-transcribed to cDNA by using an All-in-one™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA) and Oligo (dT) priming method (Prime Script TMRT Reagent Kit; TaKaRa, Shiga, Japan), respectively. RT-qPCR (Applied Biosystems 7500, Foster City, CA, USA) was performed using power SYBR® Green PCR Master Mix (Applied Biosystems). The expression levels of miRNA and mRNAs were normalized to the levels of U6 and GAPDH, respectively.

Quantification and the fold-change of miRNA and mRNA expression were calculated with the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate.

### Functional enrichment analysis

The functions and pathway enrichment of differentially expressed genes were analyzed using multiple online databases.





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**Table 2.** The top 10 up- and down-regulated miRNAs.

| miRNAs | Regulation | log2 (fold change) | pval | Sequence |
|---|---|---|---|---|
| hsa-miR-181b-3p | Up | 5.4514 | 4.81E-12 | CUCACUGAACAAUGAAUGCAA |
| hsa-miR-449a | Up | 4.8894 | 5.68E-08 | UGGCAGUGUAUUGUUAGCUGGU |
| hsa-miR-181a-3p | Up | 4.7456 | 1.39E-16 | ACCAUCGACCGUUGAUUGUACC |
| hsa-miR-34c-5p | Up | 4.601 | 1.88E-10 | AGGCAGUGUAGUUAGCUGAUUGC |
| hsa-miR-301b-3p | Up | 4.457 | 1.10E-05 | CAGUGCAAUGAUAUUGUCAAAGC |
| hsa-miR-140-5p | Up | 3.9584 | 1.23E-11 | CAGUGGUUUUACCCUAUGGUAG |
| hsa-miR-181b-5p | Up | 3.7059 | 3.16E-10 | AACAUUCAUUGCUGUCGGUGGGU |
| hsa-miR-3910 | Up | 3.6595 | 1.17E-05 | AAAGGCAUAAAACCAAGACA |
| hsa-miR-199a-5p | Up | 3.6392 | 5.26E-10 | CCCAGUGUUCAGACUACCUGUUC |
| hsa-miR-34b-3p | Up | 3.5794 | 2.51E-05 | CAAUCACUAACUCCACUGCCAU |
| hsa-miR-378i | Down | 6.7007 | 1.23E-16 | ACUGGACUAGGAGUCAGAAGG |
| hsa-miR-378d | Down | 6.3599 | 5.62E-55 | ACUGGACUUGGAGUCAGAAA |
| hsa-miR-378g | Down | 6.0021 | 7.04E-24 | ACUGGGCUUGGAGUCAGAAG |
| hsa-miR-133a-5p | Down | 5.9774 | 2.01E-10 | AGCUGGUAAAAUGGAACCAAAU |
| hsa-miR-378c | Down | 5.9466 | 2.36E-40 | ACUGGACUUGGAGUCAGAAGAGUGG |
| hsa-miR-133a-3p | Down | 5.7551 | 5.23E-09 | UUUGGUCCCCUUCAACCAGCUG |
| hsa-miR-885-5p | Down | 5.7188 | 4.92E-16 | UCCAUUACACUACCCUGCCUCU |
| hsa-miR-122-3p | Down | 5.6206 | 2.83E-12 | AACGCCAUUAUCACACUAAAUA |
| hsa-miR-378a-3p | Down | 5.5687 | 6.89E-25 | ACUGGACUUGGAGUCAGAAGGC |
| hsa-miR-378f | Down | 5.5423 | 1.86E-19 | ACUGGACUUGGAGCCAGAAG |

Gene Ontology (GO, *http://www.geneontology.org/*) describes genes and gene products involved in molecular function (MF), cellular component (CC), and biological process (BP) [14,15]. GO is a widely used method for identifying characteristic biological attributes of high-throughput genome or transcriptome data. Kyoto Encyclopedia of Genes and Genomes (*http://www.genome.jp/kegg/*), which stores information on how molecules and genes are networked, was used for pathway mapping [16]. The Database for Annotation, Visualization and Integrated Discovery (*https://david.ncifcrf.gov/*) [17] is a website that lists gene annotation, visualization, and integrated discovery function, and thus, can systematically extract biological meaning from large gene or protein lists.

### Integration of protein–protein interaction (PPI) network and module analysis

The Search Tool for the Retrieval of Interacting Genes (STRING) (*http://string-db.org*) [18] is an online tool designed to evaluate the differentially expressed mRNA-encoded proteins and PPI information. The OmicsBean (*http://www.omicsbean.com:88/*) database was used to construct a protein interaction relationship network and analyze the interaction relationships of differentially expressed candidate genes based on the STRING analysis results.

### MicroRNA mimics, inhibitor, and transfection

The miRNA miR-214-3p mimics and inhibitor were synthesized by GenePharma (Shanghai GenePharma Co., Ltd., Shanghai, China). The sequences of the miR-214-3p mimics were as follows: Sense 5'-ACAGCAGGCACAGACAGGCAGU-3', Antisense 5'-UGCCUGUCUGUGCCUGCUGUUU-3'; miR-214-3p mimics control: Sense 5'-UUCUCCGAACGUGUCACGUTT-3', Antisense 5'-ACGUGACACGUUCGGAGAATT-3'; miR-214 inhibitor: 5'-ACUGCCUGUCUGUGCCUGCUGU-3'; miR-214 inhibitor control: 5'-CAGUACUUUUGUGUAGUACAA-3'. A total of $2\times10^5$ OS cells was seeded into each well of a 6-well plate and transiently transfected with 50 nM miR-214-3p mimic/inhibitor and NC/inhibitor NC to knock down or overexpress miR-214-3p using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. miR-214-3p levels were quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) after transfection for 48 h.

### Cell Counting Kit-8 assay

The OS cell line (MG63) was seeded into 96-well plates at a density of 3000 cells per well in a volume of 100 µL, and proliferation ability was detected using Cell Counting Kit-8 (Dojindo





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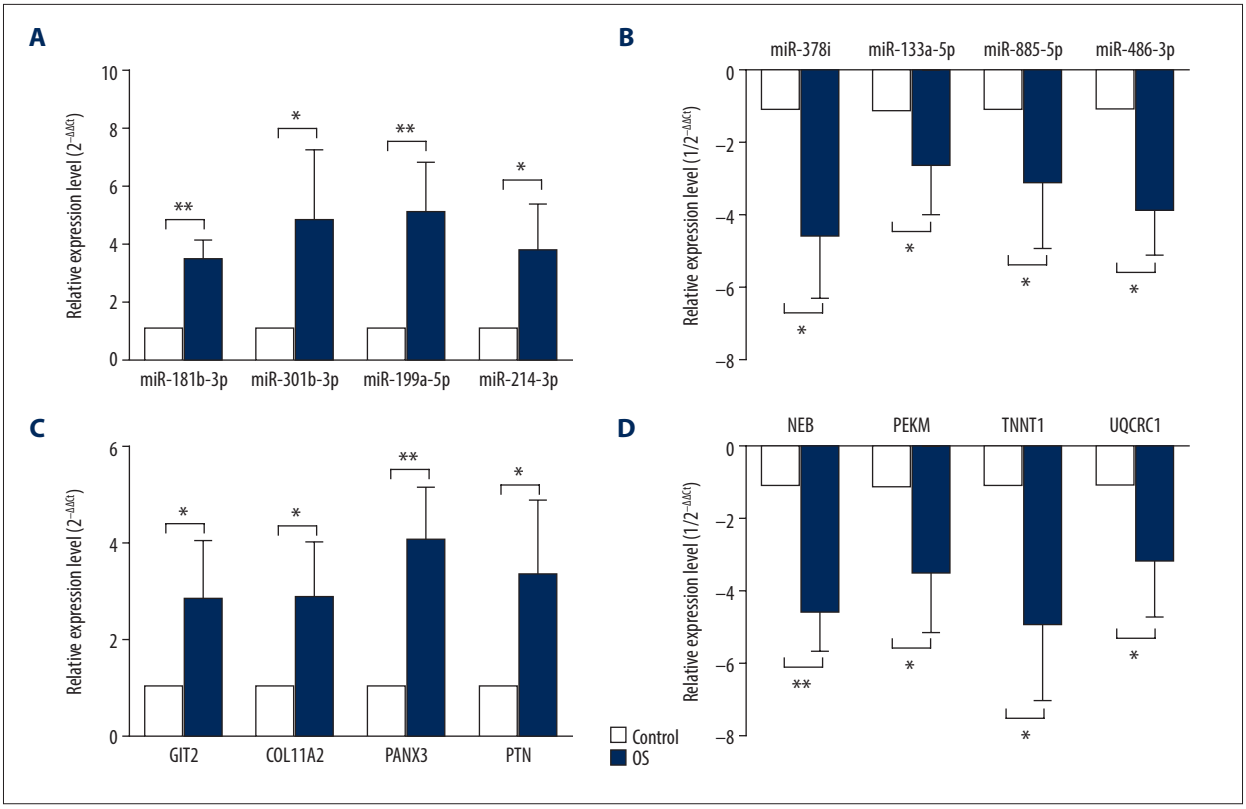


**Figure 2.** Verification of the miRNA and mRNA sequencing data by RT-qPCR. miRNA (**A, B**) and mRNA (**C, D**) sequencing data were validated by RT-qPCR between 10 OS tissues (OS group) and 10 paired adjacent non-tumor tissues (Control group). * P<0.05 and ** P<0.01.

Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The optical density (OD) values were measured at 450 nm.

### Data analysis

SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. A |fold-change| ≥2 and adjusted P-value <0.05 indicated that the miRNA and mRNA were significantly differentially expressed. Unpaired Student's *t*-test was used for comparisons between groups. The adjusted P-value was evaluated with the Benjamini-Hochberg method for multiple tests and <0.05 was regarded as the criterion for pathway enrichment. Fisher's exact test was used to evaluate the significance of GO terms. RT-qPCR and CCK-8 data were expressed as the mean ±SD.

## Result

### Differential expression of miRNAs and mRNAs in patients with OS

In total, 184 miRNAs and 2501 mRNAs were identified, among which 82 miRNAs and 1320 mRNAs were up-regulated, while 102 miRNAs and 1181 mRNAs were down-regulated (fold-change >2.0, P value <0.05). The expression profiles of miRNA and mRNA were distinguishable in hierarchical clustering analysis (Figure 1). The detailed information of the top 10 miRNAs (up- and down-regulated) is shown in Table 2.

### RT-qPCR to verify the accuracy of miRNA and mRNA sequencing data

To validate the reliability of the sequencing data, we selected 8 miRNAs and 8 mRNAs (4 up-regulated and 4 down-regulated, respectively) and measured their expression levels in 10 patients (OS tissues versus pericarcinomatous tissues) by RT-qPCR. In agreement with the sequencing data, the 4 up-regulated miRNAs were miR-181b-3p, miR-301b-3p, miR-199a-5p, and miR-214-3p and the mRNAs were GIT2, COL11A2, PANX3, and PTN (Figure 2A, 2B). The 4 down-regulated miRNAs were miR-378i, miR-133a-5p, miR-885-5p, and miR-486-3p and mRNAs were NEB, PFKM, TNNT1, and UQCRC1 (Figure 2C, 2D).

### GO and pathway enrichment analysis

GO analysis was conducted to identify the key roles performed by the differentially expressed genes. Cell growth and/or





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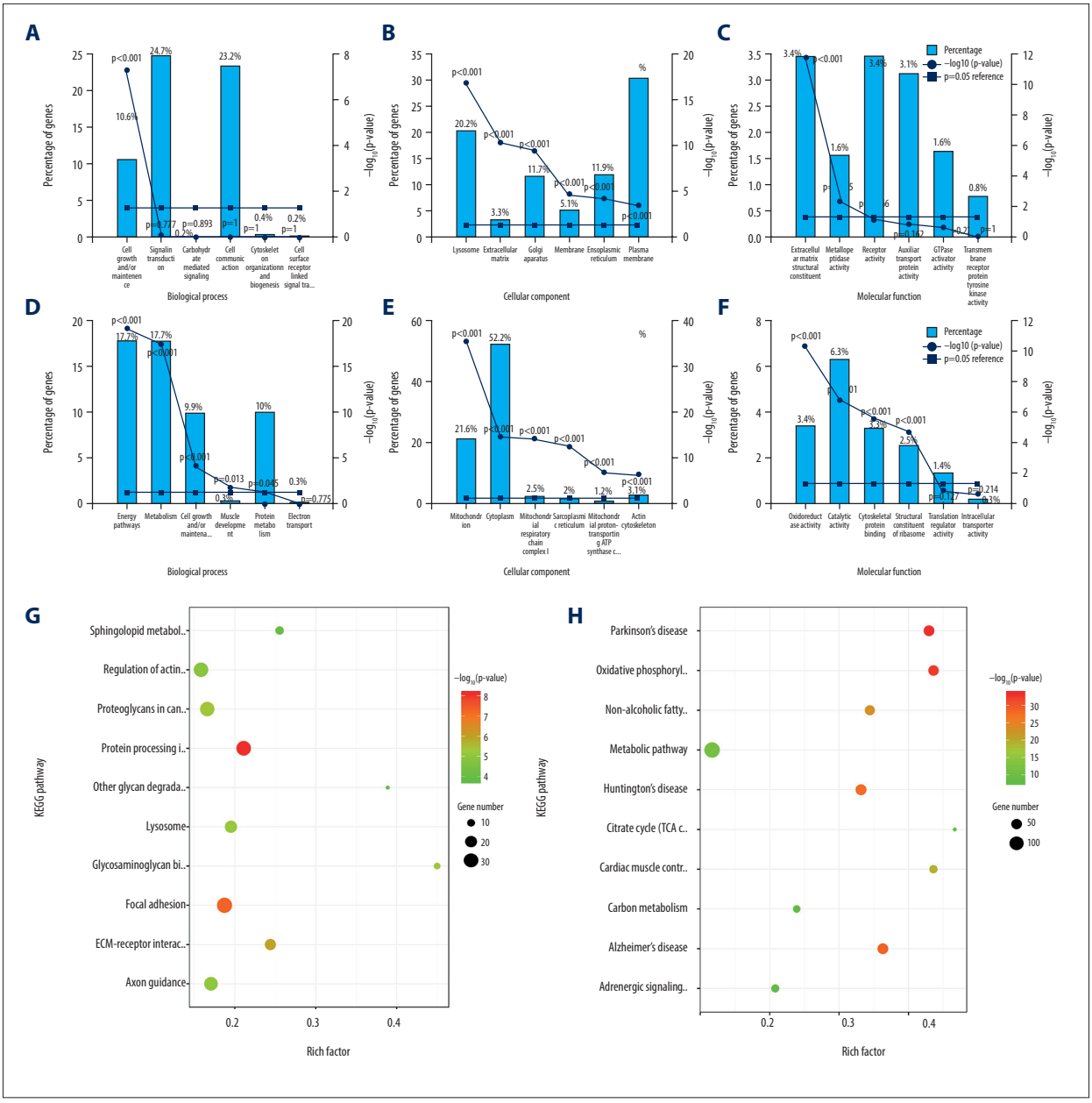


**Figure 3.** Gene ontology and pathway enrichment analysis of differential expression genes. Enriched up-regulated (**A–C**) and down-regulated (**D–F**) GO terms. Bubble charts for up- (**G**) and down-regulated (H) pathway enrichment analysis.

maintenance, lysosome and extracellular matrix structural constituent were the most significantly up-regulated terms in BP, CC, and MF (Figure 3A–3C). Energy pathways, mitochondrion, and oxidoreductase activity were the most significantly down-regulated terms enriched in each of the 3 categories (Figure 3D–3F). The pathway enrichment results showed that 54 pathways exhibited significantly differentially expressed genes, including 20 pathways with up-regulated genes and 34 pathways with down-regulated genes (Figure 3G, 3H).

### PPI network of differentially expressed genes

Based on the STRING and Omics Bean databases, a PPI network of differentially expressed genes was established. A total of 410 interactions (edges), 40 proteins (nodes), and 10 pathways were involved in the PPI network according to their internal correlation (Figure 4). Importantly, ubiquinol-cytochrome c reductase core protein 1 (UQCRC1) played a critical role in the network. In all 21 nodes, 7 pathways were found to interact with UQCRC1.





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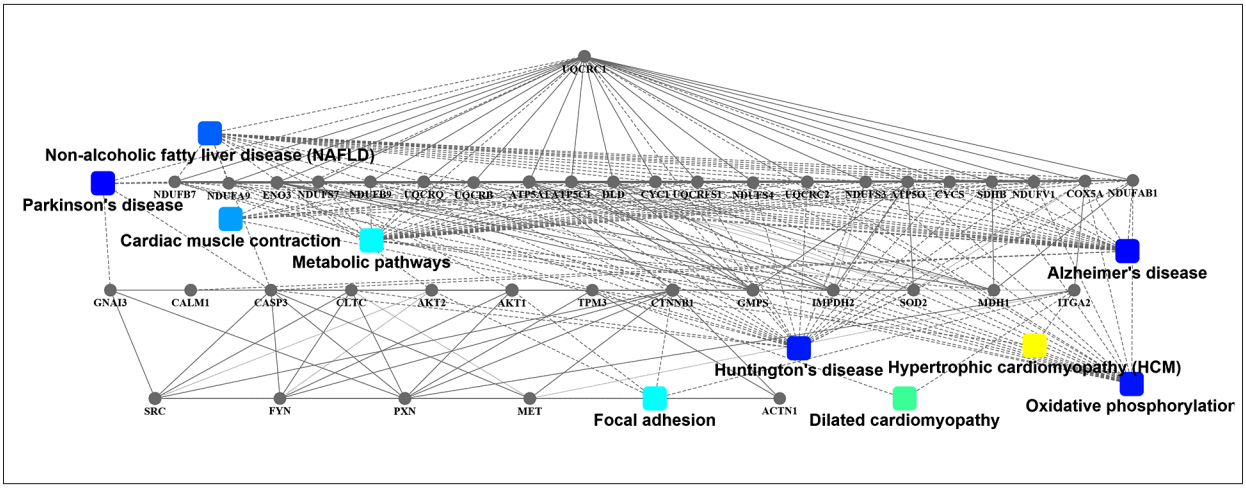


**Figure 4.** The result of PPI network analysis. The circle nodes represent proteins and the rectangles represent KEGG pathway or biological process. Pathways were colored with gradient color from yellow to blue (yellow for smaller P-value and blue for bigger).

### UQCRC1 is a direct target of miR-214-3p

To explore the mechanism of UQCRC1, potential target miRNAs of UQCRC1 were predicted using Target Scan Human 7.2 [19]. Sixty miRNAs were successfully predicted, with 5 miRNAs (miR-214-3p, miR-512-3p, miR-3688-5p, miR-6866-5p, and miR-520a-3p) detected by miRNA expression profiling of OS (Figure 5A). As UQCRC1 was confirmed to be down-regulated in the mRNA expression profile, only the up-regulated miRNA (miR-214-3p) was considered as a potential target gene, as the other 4 miRNAs were down-regulated. The binding site between UQCRC1 and miR-214-3p is shown in Figure 5B. The expression of miR-214-3p and UQCRC1 was further verified in 3 OS cell lines (MG63, U2OS, HOS). Consistent with the sequencing data, miR-214-3p and UQCRC1 were up-regulated and down-regulated, respectively (Figure 5C, 5D).

The regulatory function of miR-214-3p in OS was verified using an miRNA inhibitor or mimics to knock down or overexpress miR-214-3p. The expression of UQCRC1 in MG63 cells was examined by RT-qPCR. The results showed that UQCRC1 expression was up-regulated when miR-21-3p was knocked down (Figure 5E). When miR-21-3p was over-expressed by miR-21-3p mimics, UQCRC1 expression was substantially down-regulated (Figure 5F).

### Effect of miR-214-3p on OS cell proliferation

The function of miR-214-3p in the proliferation of OS cells was measured by CCK8-assay. The proliferation of MG63 cells was recorded every 24 h after transfection with the microRNA inhibitor or mimics. The proliferation of MG63 cells transfected with miR-214-3p mimics was significantly higher than control subject (non-transfected cells) at days 3, 4, 5, and 6. However, OS cells transfected with miR-214-3p inhibitor exhibited significantly decreased cell proliferation (Figure 6).

## Discussion

As a high-grade aggressive soft tissue tumor with high mortality, early diagnosis of OS is extremely important for successful treatment. Studies are urgently needed to identify sensitive and specific biomarkers of OS. Over the past decade, numerous studies have evaluated the molecular mechanisms of OS. Multiple miRNAs and protein-coding genes have been described and identified as OS biomarkers [20–23]. However, the expression profile of miRNA and mRNA in human OS tissues remains unclear. Furthermore, the regulatory relationship between miR-214-3p and UQCRC1 in OS has not been reported. In the current study, we determined the miRNA and mRNA expression profiles of OS tissue from patients. Total of 184 miRNAs and 2501 mRNAs were found to be differentially expressed (fold-change ≥2, P value <0.05). Bioinformatics analysis revealed UQCRC1 as the key molecular target among differentially expressed genes. We further predicted and verified that UQCRC1 is a direct target of miR-214-3p. Additionally, overexpression of miR-214-3p significantly facilitated the proliferation of OS cells (P<0.05). In contrast, knockdown of miR-214-3p significantly decreased OS cell proliferation (P<0.05).

However, in pathway enrichment analysis of differentially expressed genes, our results did not reveal a large number of pathways involved in OS. Proteoglycans in cancer and PI3K/AKT signaling pathway were significantly enriched as up-regulated pathways. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the most important oncogenic pathways in human cancer and a key target pathway for treating OS [24,25].





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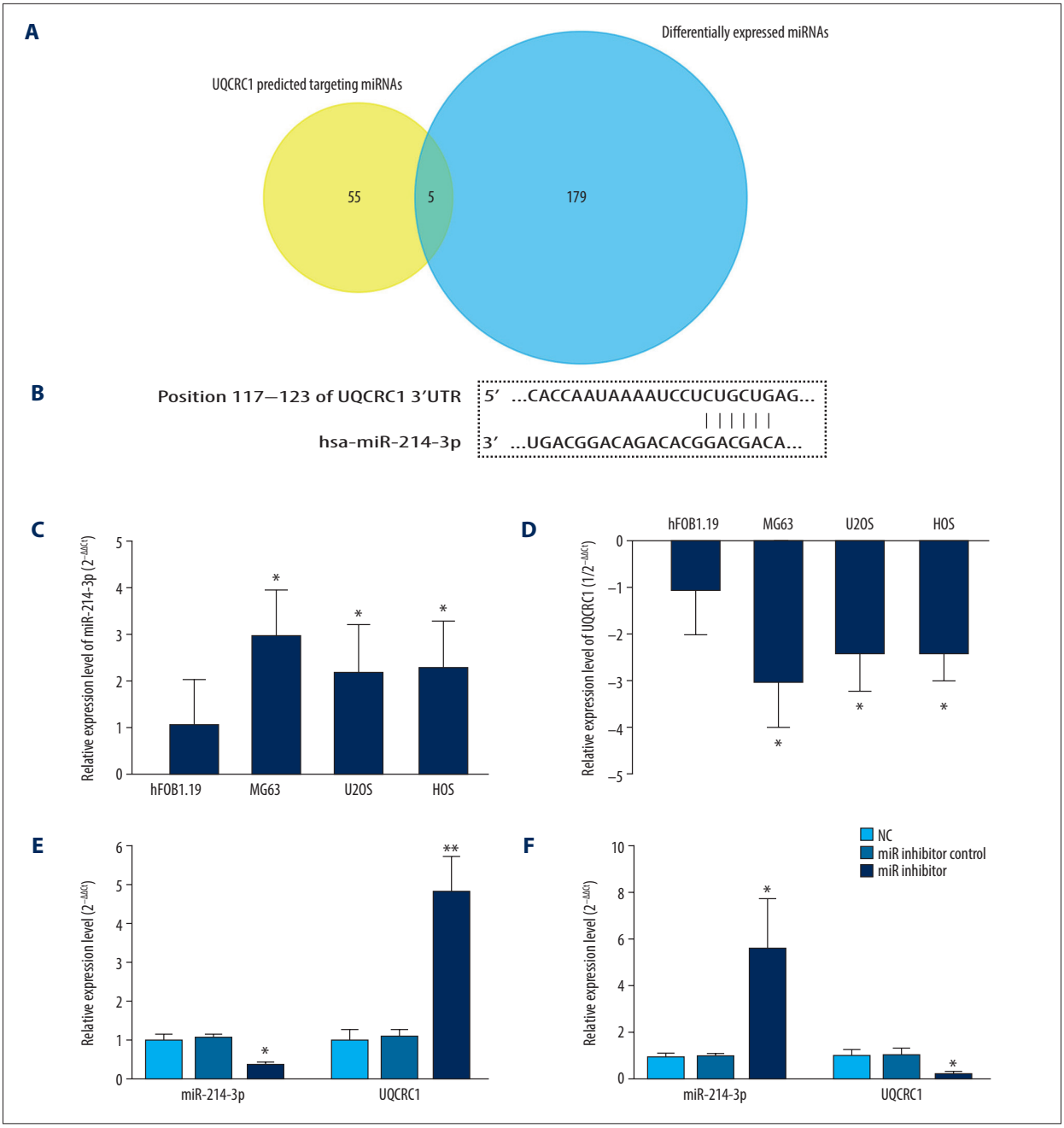


**Figure 5.** UQCRC1 is a direct target of miR-214-3p. (**A**) Venn diagram. (**B**) The binding site between UQCRC1 and miR-214-3p. (**C, D**) Expression of miR-214-3p and UQCRC1 in 3 OS cell lines. (**E**) UQCRC1 was significantly increased after knocking-down miR-214-3p by miRNA inhibitor in MG63 cells. (**F**) Overexpression miR-214-3p by miR-214-3p mimics significantly suppressed UQCRC1 expression in MG63 cells. All experiments were performed in triplicate. * P<0.05 and ** P<0.01.

Several genes have been shown to be involved in dysregulation of OS through the PI3K/Akt pathway such as Wilms' tumor gene 1 [26], phosphatase and tensin homolog, mammalian target of rapamycin [25], and others. In our pathway enrichment results, 40 genes were enriched in the PI3K/Akt pathway among the differentially expressed genes. Further studies are needed to explore the potential roles of these genes in OS.

UQCRC1 is a nuclear-encoded protein localized to the inner mitochondrial membrane [27]. Numerous studies have reported that UQCRC1 is dysregulated in cancer. For instance, in breast and ovarian cancers, the expression of UQCRC1 is up-regulated [28]. However, in clear cell renal cell carcinoma [29] and gastric cancer [30], UQCRC1 is significantly down-regulated. UQCRC1 was detected as a critical gene in our PPI network.





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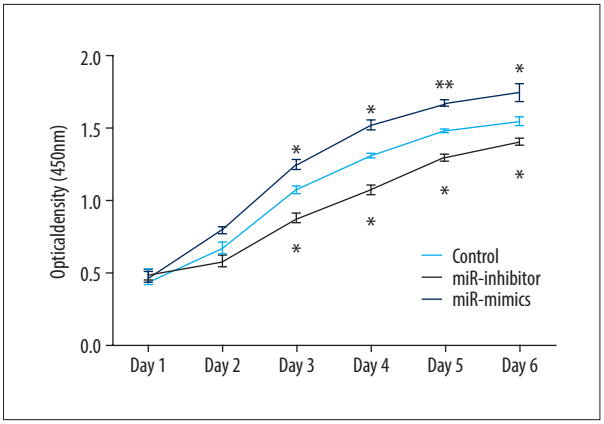


**Figure 6.** The effect of miR-214-3p in OS cells proliferation. Knocking-down miR-214-3p inhibited the cell growth of MG63 cells, while over-expressing miR-214-3p enhanced the cell growth of OS cells.

Furthermore, our results revealed that UQCRC1 was significantly down-regulated both in OS tissues and in OS cell lines (MG63, U2OS, HOS), suggesting a role in the process of OS tumorigenesis. The relationships between UQCRC1 and other key genes in the PPI network provide basis for further studies.

When mRNAs cannot accurately explain the mechanism of disease, non-coding RNAs, as the main transcripts of genes, provide guidance for in-depth studies of the disease mechanism. miR-214 is excised from the precursor hairpin by the enzyme Dicer [31], which is within the sequence of the Dmn3os transcript. miR-214 plays an important role in regulating vital processes of the cell cycle, such as apoptosis, proliferation, and angiogenesis [32]. Recent studies reported that miR-214 is highly dysregulated and variable in multiple types of cancer, such as cervical cancer [33], pancreatic cancer [34] and OS [35]. However, few studies have examined the molecular mechanisms of miR-214-3p in tumorigenesis of OS. In this study, we confirmed that miR-214-3p regulates the proliferation of OS cells by targeting UQCRC1, which may be an important regulatory target for treating OS.

miR-133a and miR-133b are members of the miR-133 family, and miR-133a-5p belongs to the miR-133a cluster [36]. miR-133a shows tumor suppressive ability, among the other miRNAs, in prostate, colorectal, esophageal, and gastric cancers [37–40]. However, the regulatory mechanism of miR-133a in OS has not been widely examined. We demonstrated that miR-133a-5p was up-regulated in the miRNA expression profile. Additionally, high expression of miR-133a-5p was validated by RT-qPCR in 10 patients (OS tissues versus pericarcinomatous tissues). The results indicate that up-regulation of miR-133a-5p weakens the anti-neoplastic effect.

Recent studies showed that miR-199a-5p was significantly associated with a variety of cancers, such as by suppressing tumorigenesis in hepatocellular carcinoma [41], which is correlated with clear cell renal cell carcinoma [42], promoting the proliferation and metastasis and epithelial-mesenchymal transition in cervical carcinoma [43], and other functions. More importantly, serum miR-199a-5p has been identified as noninvasive biomarker for detecting and monitoring OS [44]. Our study showed that miR-199a-5p expression was significantly up-regulated in OS tissue. This result is consistent with the results of previous research and expands the understanding of the important role of miR-199a-5p in OS.

There were several limitations to our study. First, few OS samples were available for sequencing, which may have affected the miRNA and mRNA expression profile data. Second, the precise targets of the miRNAs were not fully explored. Finally, the mechanisms between the differentially expressed miRNAs and mRNAs require further analysis.

## Conclusions

The miRNA and mRNA expression profiles of OS tissue from patients were identified. Differential expression of 184 miRNAs and 2501 mRNAs was detected (fold-change ≥2, P value <0.05). We also predicted and confirmed that UQCRC1 is the direct target of miR-214-3p. Additionally, miR-214-3p promotes OS cell proliferation by targeting UQCRC1. Our results provide insight for studies aimed at treating and preventing OS.

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