Pharmacogenomics and Personalized Medicine

Dovepress

open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

The Clinical Significance of miR-21 in Guiding Chemotherapy for Patients with Osteosarcoma

Li-Zhi Li
Zhuang-Zhuang Wu
Zhi Lv

Department of Orthopaedics, The Second Hospital of Shanxi Medical University, Taiyuan, 030001, People's Republic of China

Objective: The present study aims to explore the correlation between osteosarcoma (OS) chemosensitivity and the expression levels of serum and tumor tissue micro-ribonucleic acid-21 (miR-21).

Methods: The relevant miR-21 expression levels in 30 patients with OS were detected, and the gender, age, tumor location, pathological type, Enneking stage, and miR-21 expression changes before and after chemotherapy were retrospectively analyzed.

Results: Serum and tumor tissue miR21 expression levels were significantly higher in patients with OS than in control subjects; the serum miR-21 expressions before and after chemotherapy were not related to patient age and gender. The effective chemotherapy group showed significant differences in miR-21 expression levels before and after chemotherapy.

Conclusion: Serum and tumor tissue miR-21 expression levels in patients with OS are closely related to the effects of chemotherapy, making miR-21 a potential biomarker and therapeutic target for the diagnosis and evaluation of chemotherapy effects on patients with OS.

Keywords: osteosarcoma, tumor tissue, serum, miR-21, chemotherapy

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor. It usually occurs in teenagers and is characterized by early lung metastasis and high degrees of malignancy and invasion.[1] At present, neoadjuvant chemotherapy or surgery combined with preoperative and postoperative chemotherapy is used in clinical treatment. Epidemiological investigation has revealed a 5-year survival rate of approximately 55–68%; however, in patients with early recurrence and metastasis after treatment, the rate falls to approximately 20%.[2] The tumor recurrence and metastasis rates are closely related to the effects of tumor chemotherapy; therefore, selecting sensitive chemotherapeutic drugs in the early tumor stages for chemotherapeutic effect improvement is considered the key to OS treatment; it is also the hot spot of current OS research.

Micro-ribonucleic acid (miRNA) is a highly conserved endogenous, non-coding small ribonucleic acid (RNA) nucleotide sequence. It is widely distributed in animals, plants, viruses, and other organisms, and the gene contains approximately 19–25 nucleotide sequences. Previous studies have revealed that miRNA plays an important role in organism growth and differentiation regulation; it is also involved in many physiological and pathological processes, such as cell proliferation, differentiation, apoptosis, tumorigenesis, invasion, and metastasis. Thus, a gene expression imbalance can promote the occurrence and development of various tumors.[3–7]

Correspondence: Li-Zhi Li
Department of Orthopaedics, The Second Hospital of Shanxi Medical University, NO. 382 Wuyi Road, Taiyuan, 030001, People's Republic of China
Tel +86 351-3365105
Email lzlsun_09dr@aliyun.com

Received: 24 May 2021
Accepted: 16 August 2021
Published: 29 September 2021

miR-21 is widely expressed in human cells and tissues; it is also highly expressed in many human tumor cells, such as gastric cancer, non-small cell lung cancer, colon cancer, and liver cancer.[8] Furthermore, miRNA-21 (miR-21) is involved in the regulation of multiple tumor suppressor genes and apoptosis-related proteins, including tropomyosin-1, programmed death protein 4, metalloproteinase inhibitor 3, Fas ligand, heterogeneous RNA protein K, and phosphatase and tensin homolog (PTEN) 5. It can negatively regulate the expressions of these genes and participate in tumor growth, invasion, and metastasis.[9] It has been suggested that miR-21 targets the transforming growth factor β1 (TGF-β1) signaling pathway to promote cell proliferation in OS; miR-21 knockdown inhibits OS proliferation and promotes PTEN and TGF-β1 protein expressions in human osteoblast-like cells and human U2OS cell lines.[10] Moreover, TGF-β1 inhibitor treatment has been shown to counter the inhibitory effects of miR-21 knockdown on OS cell proliferation.[11]

However, many patients with OS experience a low response to therapeutic drugs, which is responsible for subsequent aggressive progression and unfavorable outcomes. In recent years, a close connection has been found between miRNAs and the respective mechanisms of pathogenesis and drug resistance in different cancer types. Furthermore, a competitive endogenous RNA regulatory network has been established; however, this network requires further investigation.[12–15] Inspiringly, miRNAs seem to play an emerging role in OS drug resistance.[16,17]

Recent studies have discovered that the expression level of this gene has a high application value in predicting the sensitivity of tumors to chemotherapeutic drugs,[18–20] and that miRNAs exist in the blood circulation, which allows them to serve as tumor biomarkers.[21] Serum miRNA has many advantages as a tumor-screening biomarker: (1) a specimen is easy to obtain with little loss and can be acquired repeatedly, and (2) serum miRNA is very stable and resistant to ribonuclease (RNase) degradation. It can even maintain stability in an acid-based environment as well as throughout boiling, repeated freeze–thaw cycles, long-term storage, and other treatment methods.[22,23] Ziyan et al verified that miR-21 is closely related to OS occurrence, development, and prognosis. They further found that miR-21 expression was significantly higher in OS than in healthy tissues and that the inhibition of miR-21 expression can reduce the invasion and metastasis of OS cells in vitro.[24] The aim of the present study is to explore the effect of chemotherapy on miR-21, find a molecular biological indicator to evaluate the effect of chemotherapy, and improve the accuracy of chemotherapy to better guide clinical practice.

Materials and Methods

The present study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Second Hospital of Shanxi Medical University. All participants provided a signed informed consent form before the study was conducted.

Main Experimental Instruments

The main experimental instruments were as follows: (1) a biosafety cabinet (provided by Heartorce, China); (2) a cryogenic, high-speed centrifuge (provided by Eppendorf, Germany); (3) a magnetic multifunctional agitator (provided by Jiangsu Guosheng instrument factory, China); (4) a cell incubator (provided by Thermo Fisher Scientific, USA); (5) a fluorescent quantitative polymerase chain reaction (PCR) machine (IQ-5.0) (provided by Bio-Rad, USA); (5) an inverted optical microscope (provided by Olympus, Japan); (6) a cell counting board (provided by Shanghai Qiujing Instrument, China); (7) a NanoDrop One ultra-micro spectrophotometer (provided by Thermo Fisher Scientific, USA); (8) 10 μL, 200 μL and 1000 μL pipette tips (provided by Axygen, USA); and (9) 200 μL, 500 μL, and 1500 μL Eppendorf (EP) tubes (provided by Axygen, USA).

Main Experimental Reagents

The main experimental reagents were as follows: (1) RPMI1640 (provided by Gibco, USA); (2) DEME (provided by Gibco, USA); (3) fetal calf serum (provided by Gibco, USA); (4) a miRNA extraction kit (provided by Qiagen, USA); (5) a reverse transcription and amplification kit (provided by Qiagen, USA); and (6) 0.25% trypsin (provided by Gibco, USA).

Cell miRNA Extraction

It was necessary to ensure that the target cells were in the logarithmic growth phase before the experiment began.

First, the medium in the culture flask (T25) was poured out, rinsed twice with sterile phosphate-buffered saline, and immediately digested with 0.25% trypsin-ethylenediamine tetra-acetic acid (EDTA). The medium was centrifuged at 1000 rpm for 5 min and the supernatant sucked out thoroughly. Next, 700 μL of QIAzol cracking

liquid was added and mixed in well, and the sample was left standing for pyrolysis at room temperature for 5 min. A volume of 140 μL of chloroform was added, the sample shaken well for 15 s, left to stand at room temperature for 3 min, and centrifuged at 4°C and 12,000 rcf for 15 min. Finally, the upper water phase liquid was sucked out and put into a new collecting tube. The volume of anhydrous ethanol (usually 525 μL) × 1.5 was added, fully pipetted, and mixed in well. Then, 700 μL was sucked out, the sample added to a 2-mL micro column, and the tube cover closed gently. The substance was centrifuged at 8000 rcf for 15 s at room temperature and the circulating fluid discarded. The previous step was repeated until all samples were centrifuged. Next, 700 μL of RWT buffer was added to the micro column, the cover of the tube closed gently, and the sample centrifuged at room temperature and 8000 rcf for 15 s. The circulating fluid was then discarded. A volume of 500 μL of RWT buffer was added to the micro column again and the tube cover closed lightly. The sample was centrifuged at room temperature and 8000 rcf for 15 s and the circulating fluid discarded. After the previous step was repeated, the micro column was placed in a new epoxy resin EP tube and centrifuged at full speed for 1 min. The micro column was placed in a new 1.5-mL EP tube, 50 μL of RNase-free water was added, and the miRNA was eluted by centrifugation at room temperature and 8000 rcf for 1 min. The RNA concentration was detected using a NanoDrop spectrophotometer.

RNA Concentration and Purity Determination

The RNA concentration was determined using an ultraviolet spectrophotometer. The sampling arm was unfolded, 2 μL of diethyl pyrocarbonate water transferred to the detection platform using a pipette, and the sampling arm lowered. The test platform was dried using absorbent paper, and the process was repeated three times.

The sampling arm was unfolded again, 2 μL of RNA droplet transferred onto the detection platform using a pipette, the sampling arm lowered, and the measure button pressed to obtain the sample absorbance and concentration.

After testing one sample, the test solution was wiped off with absorbent paper. The above-described steps were repeated to test the remaining samples. An RNA purity (A260/A280) of 1.8–2.1 was considered qualified, A260/A280 < 1.8 was considered polluted by organic matter, and A260/A280 > 2.1 was considered degraded.

Complementary DNA Reverse Transcription

An RNA reverse transcription (RT) was performed in accordance with the instructions included in the Quick-start Protocol miScript® II RT kit. The reaction system consisted of the following: (1) 4 μL 5x miScript HiSpec Buffer; (2) 2 μL 10x miScript Nucleics Mix; (3) 2 μL RNase-free H_2O Variable and miScript Reverse Transcriptase Mix; and (4) 1 μL template miRNA. The reaction conditions were 37°C for 60 min, 95°C for 5 min, and 4°C until the end of the process.

After the above-mentioned system was mixed, it was immediately centrifuged. The RT procedure was as follows: 37°C for 60 min and 95°C for 5 min. At the end of the reaction, the complementary DNA (cDNA) products were immediately taken out and quickly placed on ice for cooling or stored at a temperature of less than −20°C. Internal control primer (U6) usage was consistent with the miRNA detection method.

Quantitative MiRNA PCR

Amplification was performed using a Quick-start Protocol miScript SYBR® Green PCR kit, with the obtained cDNA as the template. The SYBR Green Kit comprising the Taq enzyme, deoxynucleotide mix, PCR buffer, and SYBR Green I. U6 was used as the internal control of the relative miR-134 content. The relative value calculated by the $2^{-\Delta\Delta Ct}$ method was regarded as the final result. miR-21: 5′-CTGGACGGTGCCAGGT-3′ 5′-CGGGTCTTTCGTTAG CCTGC-3′ U6: 5′-CTCGCTTCGG CAGCACA-3′ 5-AAC GCTTCACGAATTTGCGT-3′. A volume of 2.5 μL 2x Quanti Tect SYBR Green PCR Master Mix 1, 2.5 μL 10x miScript Universal Primer, 2.5 μL 10x miScript Universal Primer, 6.5 μL RNase-free H_2O, and 1 μL template miRNA. The procedure for carrying out quantitative miRNA PCR is shown in Table 1.

OS Tissue miR-21 Expression

Samples were collected from 30 patients with OS to study their tissue miR-21 expressions. Two samples were taken from each patient, the first from the OS tissue at the time of the biopsy before chemotherapy and the second from the resected tumor after chemotherapy. In addition, five cases of healthy bone tissue were obtained as controls. Next, quantitative RT-PCR (QRT-PCR) was performed to detect miR-21 expression in the OS tissue obtained before and after chemotherapy as well as the healthy bone tissues.

Table 1 QPCR Reaction Procedure of miRNA

Loop	Step	Temperature	Time	Content
1X	Pre-denaturation	95°C	15min	Enzyme activation
40X	Amplification reaction	94°C 55°C 70°C	15sec 30sec 30sec	PCR Template denaturation Annealing Extension (signal collection)
1X	Analysis of melting curve	85°C		Analysis of melting curve

Abbreviation: QPCR, quantitative polymerase chain reaction.

Tissue miR-21 Extraction

Approximately 30–50 mg tumor tissue was weighed out, and 500 μL of QIAzol lysate was added. The tissue was then cut into small pieces on ice and prepared into homogenate using a tissue homogenizer. Again, 700 μL of QIAzol lysate was added and mixed well; the tissue was then left to stand at room temperature for 5 min for pyrolysis. Next, 140 μL of chloroform was added to the mixture, and the tissue was shaken well for 15 s and left to stand at room temperature for 3 min. Centrifugation was carried out at 4°C and 12,000 rcf for 15 min. Finally, the upper water phase liquid was sucked out and put into a new collecting tube. The volume of anhydrous ethanol (usually 525 μL) × 1.5 was added; it was subsequently fully pipetted and mixed. Next, 700 μL of the sample was taken and added to a 2-mL micro column. The tube cover was closed carefully, and the sample was centrifuged at room temperature and 8000 rcf for 15 s; the circulating fluid was then discarded. The previous step was repeated until all samples were fully centrifuged. A volume of 700 μL of RWT buffer was added to the micro column, the cover of the tube closed gently, and the sample centrifuged at room temperature and 8000 rcf for 15 s; the circulating fluid was then discarded. The same procedure was carried out using 500 μL of RWT buffer. In each case, the micro column was placed in a new 1.5-mL EP tube, and 50 μL of RNase-free water was added. The miRNA was eluted by centrifugation at room temperature and 8000 rcf for 1 min, and the concentration of RNA was detected using the NanoDrop spectrophotometer.

Serum miR-21 Expression in Patients with OS

Serum Collection from Patients with OS

In the present study, two serum samples were also collected from each of the 30 patients with OS. The serum obtained before chemotherapy after diagnosis was used as the first sample, and the serum obtained after chemotherapy was used as the second sample. Serum samples were also collected from five healthy subjects to act as controls. Serum preservation involved the following: All subjects fasted for eight h and had 5 mL of blood taken from the elbow vein; EDTA was then added for anticoagulation. The blood was centrifuged at 4°C and 3000 rpm for 15 min. Upper plasma was extracted, placed in a 1.5-mL enzyme-free EP tube, and stored in a −80°C refrigerator to ensure plasma quality and result accuracy.

Serum miR-21 Extraction from Patients with OS

First, 200 μL of plasma was mixed with 1000 μL of QIAzol lysate and left to stand at room temperature for 5 min. Then, 200 μL of chloroform was added to the plasma; the mixture was shaken well for 15 s, left to stand at room temperature for 3 min, and centrifuged at 4°C and 15,000 rcf for 15 min. The upper water phase liquid was sucked out and placed into a new collecting pipe, and the volume of absolute alcohol (usually 525 μL) ×1.5 was added to the water phase liquid, pipetted fully, and mixed via shaking. A 700 μL sample was sucked out and added to a 2-mL micro column; the cover was closed carefully, and the mixture was centrifuged at room temperature and 8000 rcf for 15 s. The circulating fluid was then discarded. The previous step was repeated until all samples were centrifuged. Next, 700 μL of RWT buffer was added to the micro column, and the cover was closed gently. The substance was centrifuged at room temperature and 10,000 rcf for 15 s and the circulating fluid discarded. Next, 500 μL of retinal pigment epithelial cell buffer was added to the micro column again, the cover closed carefully, and the substance centrifuged at room temperature and 10,000 rcf for 2 min. The circulating fluid was then discarded. The previous step was repeated once, and the micro column was placed in a new EP tube and centrifuged at full speed for 1 min. The micro column was then placed in a new 1.5-mL EP tube, to which 16 μL of

RNase-free water was added. It was centrifuged at room temperature and 8000 rcf for 1 min to elute the miRNA. The concentration of miRNA was detected by the NanoDrop spectrophotometer, and the extracted miRNA was stored at −80°C to prevent degradation.

QRT-PCR was performed to detect the miR-21 expressions in the serum obtained before chemotherapy and after chemotherapy and the serum of the healthy control subjects.

Bioinformatics Analysis

A bioinformatics analysis was conducted in the present study to identify the proteins and signaling pathways interacting with miR-21; several proteins with obvious differences were found. Future in-depth research is planned.

Database of miRNA Target Genes

The miRDB is a miRNA database containing software-predicted miRNA target genes. Taking miRDB as the sole reference would result in a relatively high false positive result rate; therefore, the best approach is combining the predicted results of multiple databases. The present study compares target genes from three microRNA databases; the intersection (42 intersection genes in total) is taken to make up for the shortcomings of using a single software algorithm.

FunRich3.1.3

FunRich3.1.3 is an independent software tool mainly used for the functional enrichment and interaction network analysis of genes and proteins. At present, the FunRich3.1.3 tool is used to process various gene and protein data sets. The software was used for enrichment analyses of gene ontology (GO) function and biological pathway of differentially expressed genes. The core biological processes, molecular functions, and cellular components of GO were visualized.

7.1.3 GO, Biological signaling pathway, and Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes.

GO is a common method for defining and describing gene and protein functions as well as identifying high-throughput genome or transcriptome data biological characteristics. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a large-scale molecular data set generated from molecular level information (especially genome sequencing and other high-throughput experiments) and database resources for understanding the advanced functions and utility of biological systems, such as cells, organisms, and ecosystems. The KEGG database is a collection of hand-drawn KEGG pathway maps; each pathway map includes a network of molecular interactions and reactions, connecting genes in the genome with gene products (mainly proteins) in the pathway. KEGG pathway analysis is the process of mapping the target gene into the KEGG pathway map. There are certain similarities between the biological signaling pathway and KEGG.

Statistical Methods

The Statistical Analysis Software (SAS) 9.2 (SAS INSTITUTE INC, USA) was used for data analysis. For the counting data, the rates, the Minimum (Min), Q_{25} (Upper quartile), Median (M), Q_{75} (Lower quartile), Maximum (Max) and Standard Deviation (SD) were used for statistical descriptive, and the Chi-square test, Kruskal–Wallis method, and *t*-test method were used for statistical analysis. The box plot delineated the interquartile range (P_{25} –P_{75}), within which the horizontal line depicts the median (P_{50}) and the whiskers indicate the minimum and maximum values. *Indicated a statistically significant difference between groups in the Kruskal–Wallis method. A *P* value of <0.05 was considered statistically significant.

Results

Correlation Between Serum miR-21 Expression with Different Demographic and Pathological Characteristics in Patients with OS (Table 2)

The clinical data of all the patients were collected to understand the correlation between serum miR-21 expression and the clinicopathological characteristics of the 30 patients with OS. The miR-21 expression was significantly correlated with the Enneking clinical stages and lung metastases; however, miR-21 expression was not correlated with gender, age, tumor location, histological classification, and tumor size ($P < 0.05$).

Relationship Between the Tumor Tissue miR-21 Expression Level and Chemotherapy Effectiveness

In the effective chemotherapy group, there was a significant difference in the tumor tissue miR-21 expression levels in patients with OS before and after chemotherapy (median: 1.7 vs 1.0, $P < 0.05$); however, the ineffective chemotherapy group showed no significant difference (median: 0.9 vs 1.3, $P > 0.05$) (Table 3).

Table 2 Correlation Between Serum microRNA-21 (miR-21) Level and Clinicopathological Characteristics of Osteosarcoma Patients (n = 30)

Clinicopathological Characteristics	n	miR-21 Expression Level		*P* value
		High	Low	
Age (Year)				
< 18	19	12(63.2%)	7(36.8%)	*P*= 0.712
≥ 18	11	6(54.5%)	5(45.5%)	
Gender				
Male	17	10(58.8%)	7(41.2%)	*P*=1.000
Female	13	8(61.5%)	5(38.5%)	
Tumor location				
Femur or tibia	21	13(61.9%)	8(38.1%)	*P*=1.000
Other	9	5(55.6%)	4(44.4%)	
Histological classification				
Conventional osteosarcoma	13	7(53.8%)	6(46.2%)	*P*= 0.711
Other types of osteosarcoma	17	11(64.7%)	17(35.3%)	
Enneking stage				
I	9	3(33.3%)	6(66.7%)	*P*=0.042
II	21	16(76.2%)	5(23.8%)	
Tumor size				
<5cm	12	3 (25.0%)	9(75.0%)	*P*=0.860
≥5cm	18	4 (22.2%)	14(77.8%)	
Lung metastasis				
Yes	18	14 (11.1%)	2 (88.9%)	P= 0.015
No	12	4(41.7%)	8 (58.3%)	

Correlation Between the Serum miR-21 Expression and Chemotherapy Effectiveness

In the effective chemotherapy group, there was a significant difference in serum miR-21 expression levels in patients with OS before and after chemotherapy (4.04 ± 1.91 vs 2.95 ± 1.43, P < 0.05); however, the ineffective chemotherapy group showed no significant difference in miR-21 expression before and after chemotherapy (2.12 ± 1.43 vs 1.79 ± 0.88, P > 0.05) (Table 4).

Table 4 Correlation Between Serum miR-21 Expression and the Effect of Chemotherapy

Chemotherapy Necrosis Rate	n	Expression of miR-21 in Tumor Tissue	
		Before Chemotherapy	After Chemotherapy
≥90%	22	4.04±1.91	2.95±1.43
<10%	8	2.12±1.43	1.79±0.88
p value		<0.001	

Note: The difference of serum miR-21 level between groups was compared by *t*-test, and the value was expressed as Mean±SD, p<0.05.

Chemotherapy Regimens for Patients with OS in Different Periods

Neoadjuvant chemotherapy for patients with OS is divided into four periods: (1) Preoperative neo-assistance (two cycles); (2) postoperative assistance (six cycles); (3) recurrence and metastasis during postoperative assistance; and (4) recurrence and metastasis after chemotherapy completion (Table 5).

OS Tissue miR-21 Expression

The miR-21 expression in the OS tissues obtained before and after chemotherapy and in the healthy bone tissues are shown in Figure 1 (*P < 0.05).

Serum miR-21 Expression in Patients with OS

The respective expression levels of serum miR-21 in the healthy control subjects, before chemotherapy, and after chemotherapy indirectly prove that chemotherapy is effective (*P < 0.05) (Figure 2).

Database of miRNA

TargetScan[25] can predict biological miRNA targets by searching for the presence of conserved 8mer and 7mer sites

Table 3 Correlation Between the Expression of miR-21 in Tumor Tissue and the Effect of Chemotherapy Necrosis Rate

Chemotherapy Necrosis Rate	n	Expression of miR-21 in Tumor Tissue										*P*
		Before Chemotherapy					After Chemotherapy					
		Min	Q_{25}	M	Q_{75}	Max	Min	Q_{25}	M	Q_{75}	Max	
≥90%	22	1.1	1.5	1.7	2.3	3.2	0.5	0.7	1.0	1.2	1.6	<0.001
<10%	8	0.6	0.7	0.9	1.6	2.0	0.8	0.9	1.3	2.4	2.6	0.07

Table 5 Osteosarcoma Chemotherapy Regimen

Preoperative	Postoperative Assistance	Recurrence and Metastasis	Recurrence and Metastasis After
Neo-Assistance (2 Cycles)	**(6 Cycles)**	**During Postoperative Assistance**	**The Completion of Chemotherapy**
EH 100mg/m^2 1–3d	EH 100mg/m^2 1–3d	EH 100mg/m^2 3d	IFO 1.5–2g/m^2 5d
NDP 100mg/m^2 1–3d	NDP 100mg/m^2 1–3d	NDP 100mg/m^2 3d IFO 1.5–2g/m^2 5d	ET 100mg/m^2 3d AHC 12mg/d 3W

Abbreviations: EH, EpirubicinHydrochloridefor; NDP, nedaplatin; ET, Etoposide; IFO, IfosfamideforInjection; AHC, Anlotinib Hydrochloride Capsules.

matching the seed region of each miRNA. The miRDB is a database for miRNA target prediction and functional annotations. It employs an improved algorithm for miRNA target prediction, including 3.5 million predicted targets regulated by 7000 miRNAs in five species. There are several differences between RNA22[26] and other algorithms: RNA22 (1) is trained using known miRNAs, not with experimentally validated heteroduplexes; (2) permits combinations of G:U wobbles and bulges in the seed region of a heteroduplex; and (3) neither relies on nor requires a target's conservation across genomes. The target genes used in the present study were found in three miRNA databases, and the intersection (42 intersection genes) was obtained (Figure 3).

Functional Enrichment of 42 Intersection Genes

A total of 42 intersection genes were input into the FunRich3.1.3 software. The GO analysis results revealed that biological processes were primarily enriched in signal transduction, whereas the molecular function was primarily enriched in the receptor signaling complex scaffold activity and cell components were mainly enriched in focal adhesion. The results of the Biological Pathway analysis revealed that the processes were mainly enriched in the downstream signaling pathways of CDC42 activity regulation, the CDCA2 signaling pathway, the ALK1 signaling pathway, and the activated FGFR (Figure 4).

Protein–Protein Interaction Network Core Gene Screening

Based on information obtained from the public STRING protein interaction database, a protein–protein interaction network of 20 core genes was created. The 10 genes most highly related to the connectivity of surrounding genes in the Cytoscape database were then screened; the results

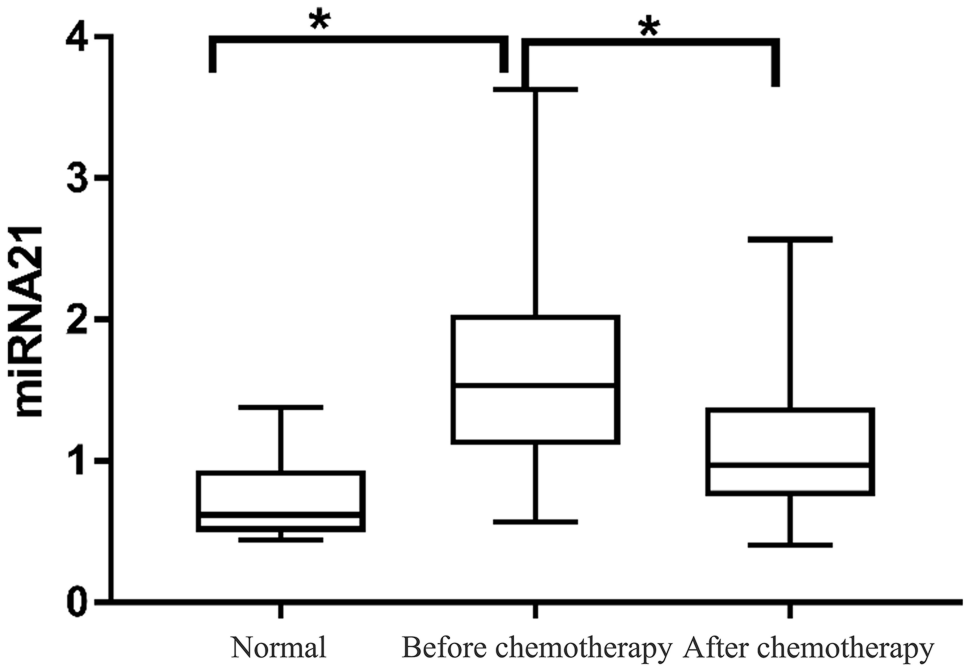


Figure 1 Expression of miR-21 in healthy bone tissue, OS tissue before chemotherapy, and OS tissue after chemotherapy. The miR-21 expression is higher in OS tissues than in healthy bone tissues and is reduced after chemotherapy (*P < 0.05).

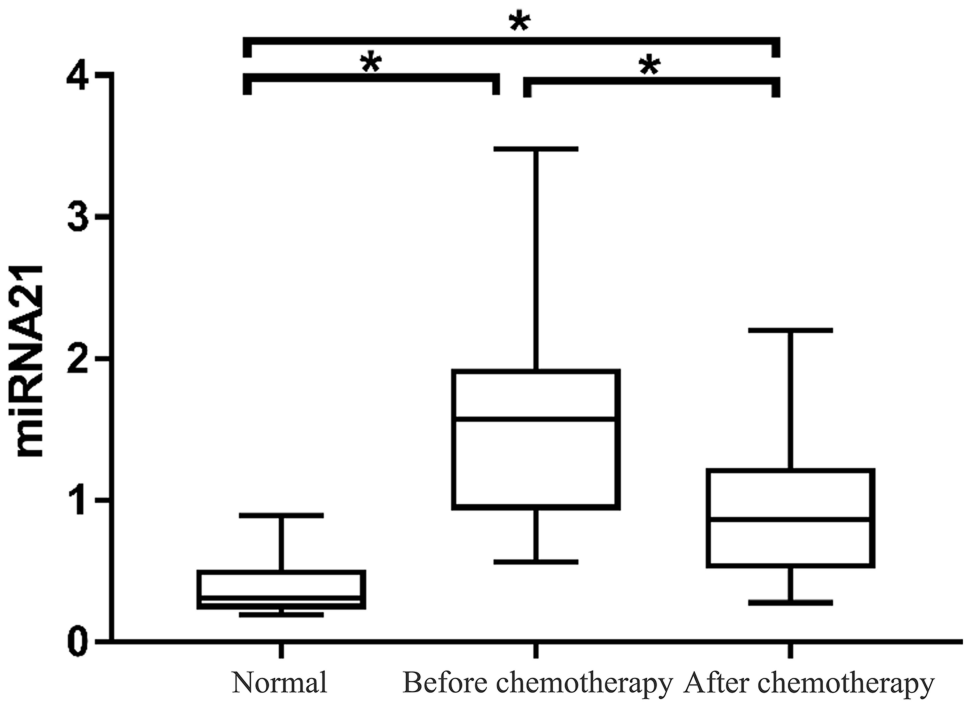


Figure 2 Expression of miR-21 in healthy serum, OS serum before chemotherapy, and OS serum after chemotherapy. The results showed that the serum miR-21 expression was higher in patients with OS than in the control subjects. After chemotherapy, the miR-21 expression decreased (*P < 0.05).

showed that PIK3R1 had the strongest relation (Figures 5 and 6).

Tumor Core Gene Expression

In the present study, the expressions of the above-mentioned 10 core genes in tumor tissues and healthy tissues were also detected in the Gene Expression Profiling Interactive Analysis (GEPIA) database. The results clearly showed that the expressions of FRS2, PIKFYVE, PIK3R1, and SOCS6 were up-regulated, while the expressions of other genes were down-regulated. The results also indicated that, at present, the data concerning OS (a major type of malignant sarcoma originating from mesenchymal tissue) are limited. Hence, the investigators believe that the results of the comparison between sarcomas and healthy tissues in GEPIA also have a certain reference significance (Figure 7).

Discussion

OS is a common primary malignant bone tumor occurring in adolescents. It has been argued that the 5-year survival rate of patients with OS has significantly improved with the gradual development of medical technology.[27] However, others claim that the 5-year survival rate has shown little improvement after 20 years of development[28,29] due to patients with OS developing multidrug resistance after chemotherapy. When cancer cells develop this resistance, the possibility of recurrence increases greatly. Therefore, the cancer cell sensitivity to chemotherapeutic drugs determines the effect of anti-cancer treatment. Finding relatively good tumor markers is urgently required in order to provide early diagnosis for patients and monitor the signs related to individualized chemotherapy in real time.

Drug resistance is the main reason for OS treatment refractoriness. Recent studies have revealed the emerging roles of miRNAs in OS chemoresistance under the mechanisms of DDR, apoptosis avoidance, autophagy induction, CSC activation, and alteration in signal pathways. According to the above-listed preclinical studies, these drug-resistance-related miRNAs are expected to supplement or replace existing diagnosis or prognosis biomarkers and serve as promising candidates for therapeutic targets to overcome drug resistance in the future.

The metastasis and invasion of tumor cells is managed by miR-21, an endogenous non-coding small RNA with a length of 20–25 nt, by regulating the expression of many tumor metastasis suppressor genes

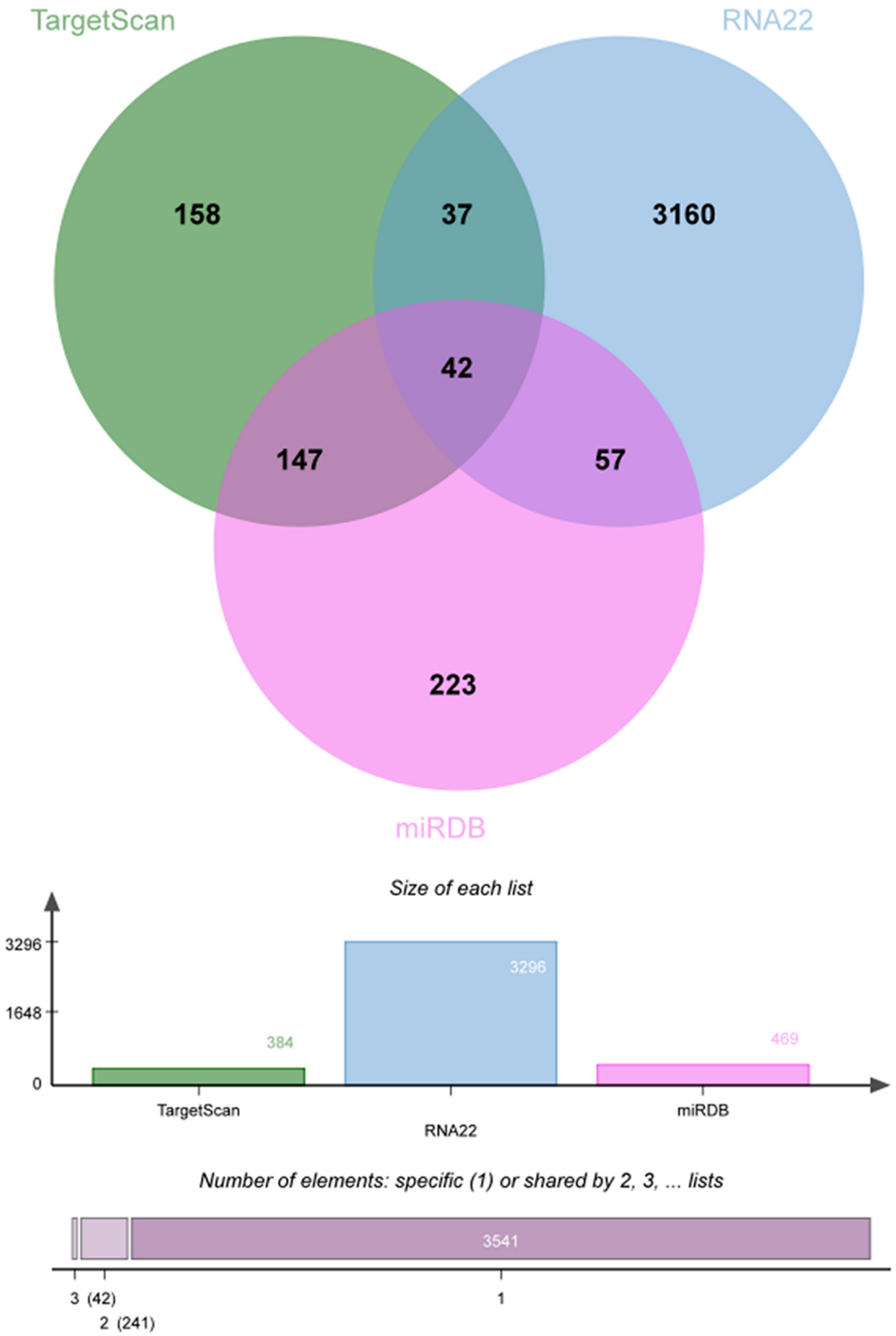


Figure 3 Venn diagram of mRNAs included in the TargetScan, RNA22, miRDBnetwork; the green area shows the number of mRNAs targeted by TargetScan; the blue area shows the number of mRNAs targeted by RNA22; and the pink area shows the number of mRNAs targeted by miRDB. The area in the middle indicates the number of mRNAs included in the TargetScan, RNA22, and miRDBnetwork.

in cells.[30] Recent studies[31,32] report a certain correlation between miR-21 expression and drug tolerance. It has also been found that a change in miR-21 expression affects breast cancer cell sensitivity to Adriamycin.[28] Furthermore, Zadeh et al[33] reported that increasing miR-21 expression in malignant glioma cells led to drug resistance to a variety of chemotherapeutic drugs.

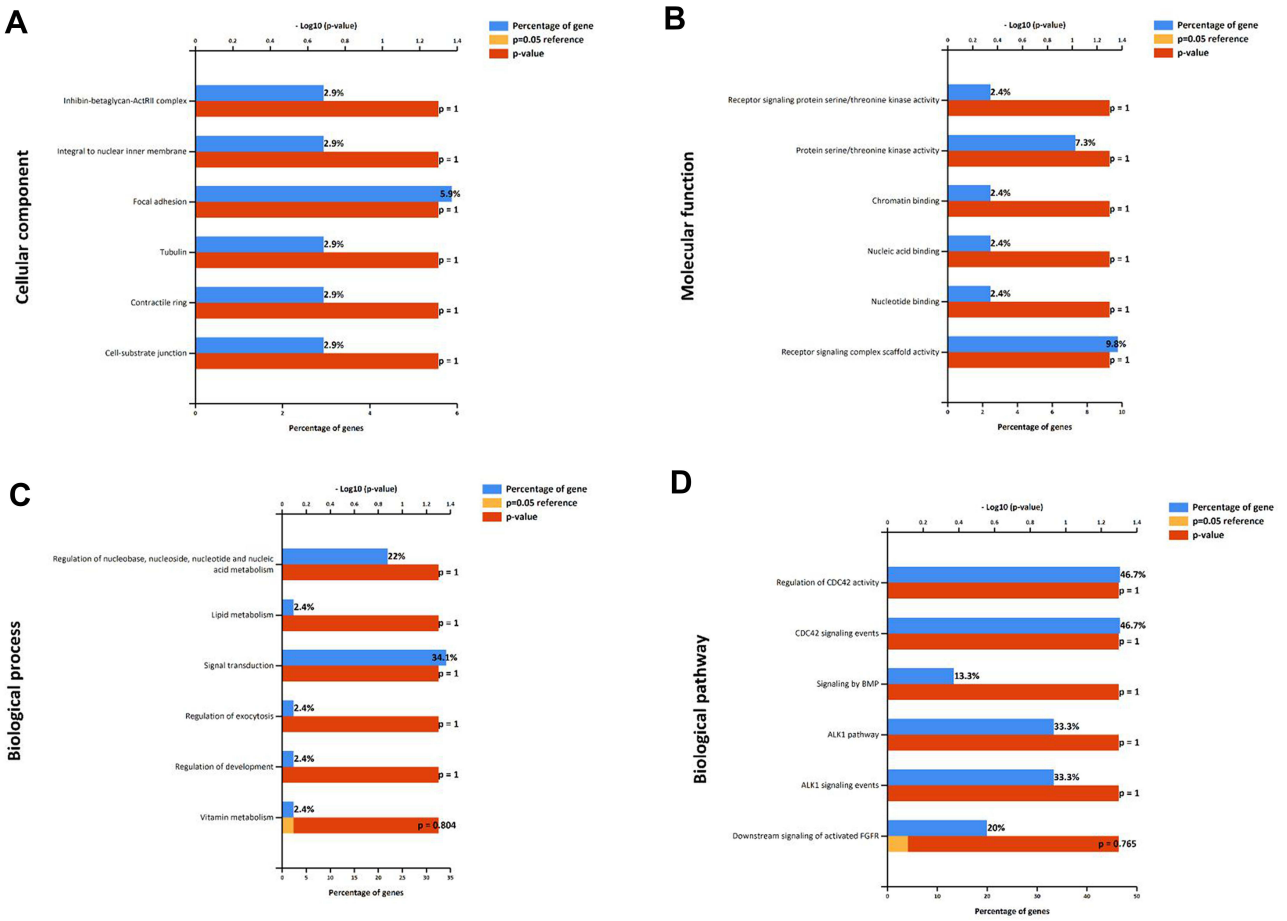
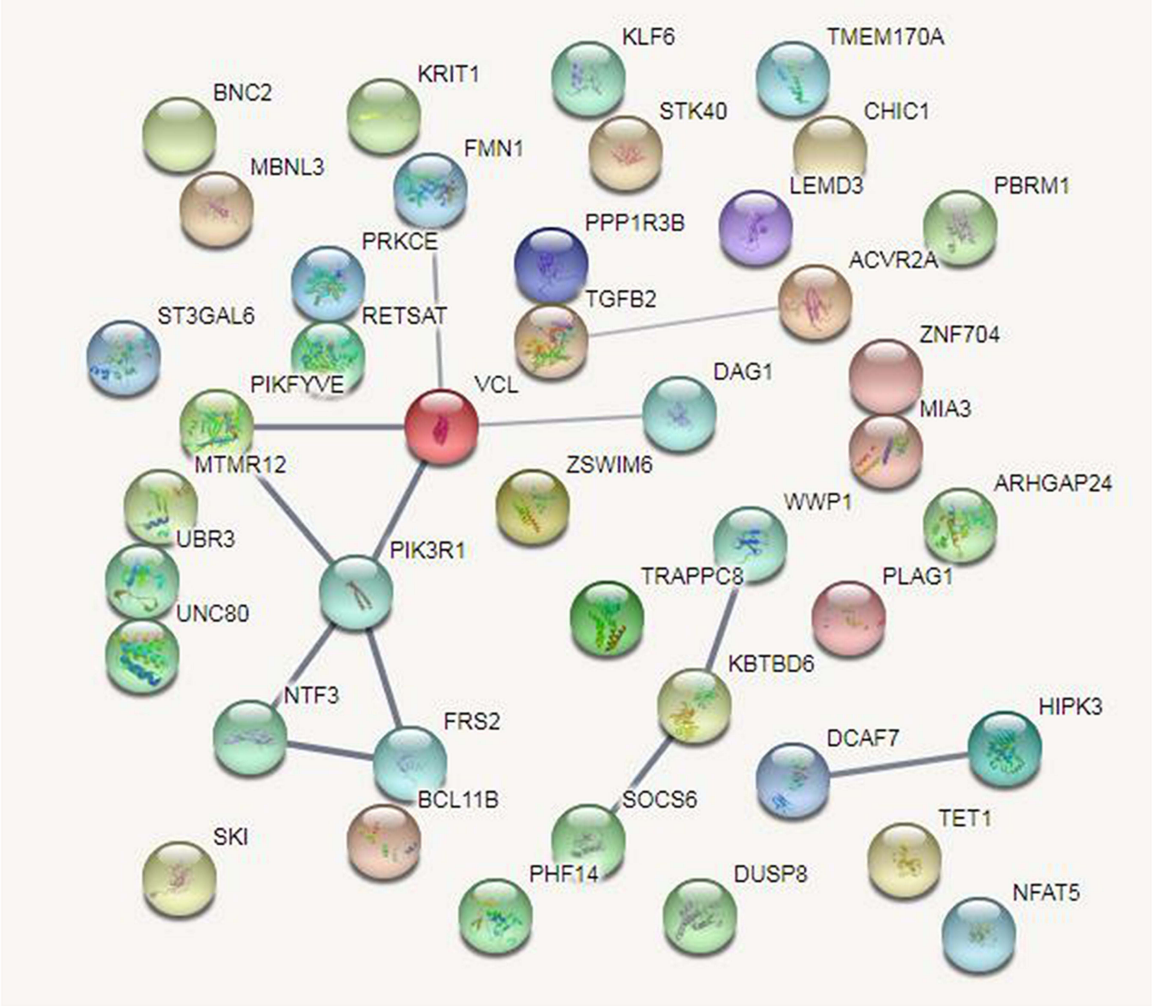


Figure 4 A total of 42 intersection genes in the STRING network by consolidating known and predicted protein–protein association data for a large number of organisms. Colored lines between the proteins indicate the various types of interaction evidence. Calculate the value of each gene through the topological network algorithm using the Cytoscape cytoHubba app and find its key gene (PIK333R) and sub-network by sorting. Cellular component (**A**). Molecular function (**B**). Biological process (**C**). Biological pathway (**D**).

In the present study, serum and bone tissue miR-21 expressions were detected in patients with OS and control subjects. The results revealed that the expression levels were significantly higher in patients with OS in the control subjects; the difference was statistically significant (Figures 1 and 2). Hence, it is speculated that miR-21 can be used as a marker for early OS diagnosis. Moreover, miR21 expression was significantly correlated ($P < 0.05$) with the Enneking clinical stages and lung metastases. Furthermore, miR-221 expression was not correlated with gender, age, etc. (Table 2). One study demonstrated an increase in serum miR-21 in patients with OS; this is correlated with lung metastasis but not with the Enneking clinical stages.[34] The findings of the present study are partly consistent with the above-mentioned previous studies and may be related to the small sample size.

In addition, the serum miR-21 expression level in patients with OS was positively correlated with the expression in corresponding OS tissues. In the effective chemotherapy group, the tumor tissue and serum miR-21 expression levels in patients with OS before and after chemotherapy were significantly different. In contrast, there was no significant difference in miR-21 expressions before and after chemotherapy in the ineffective chemotherapy group (Tables 3 and 4). These findings suggest that the serum and tumor tissue miR-21 expression levels may be related to OS chemosensitivity. These oncogenic or tumor suppressor miRNAs play a role in chemotherapy sensitivity by the mechanisms of DDR, apoptosis avoidance, autophagy induction, activation of CSCs, and alteration in signal pathways.

Literature[35] reports that lowering miR-21 production could effectively reduce the occurrence of malignant OS



Rank	Node
1	PIK3R1
1	VCL
3	FRS2
3	NTF3
3	PIKFYVE
3	KBTBD6
7	ACVR2A
7	SOCS6
7	WWP1
7	DCAF7

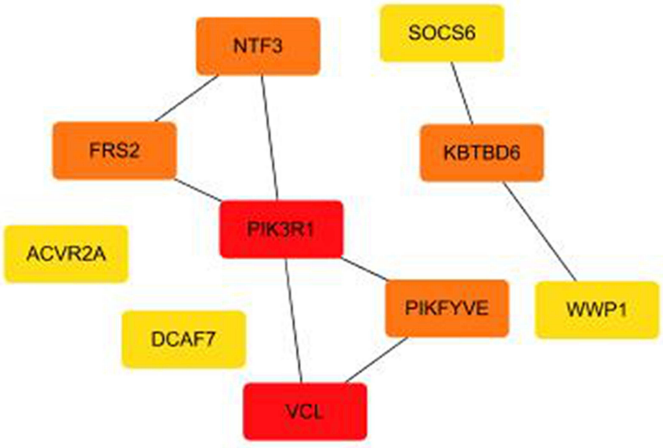


Figure 5 Intersection genes in the STRING network by consolidating known and predicted protein–protein association data for a large number of organisms. Colored lines between the proteins indicate the various types of interaction evidence. Calculate the value of each gene through the topological network algorithm and find its key gene (PIK333R) and sub-network by sorting.

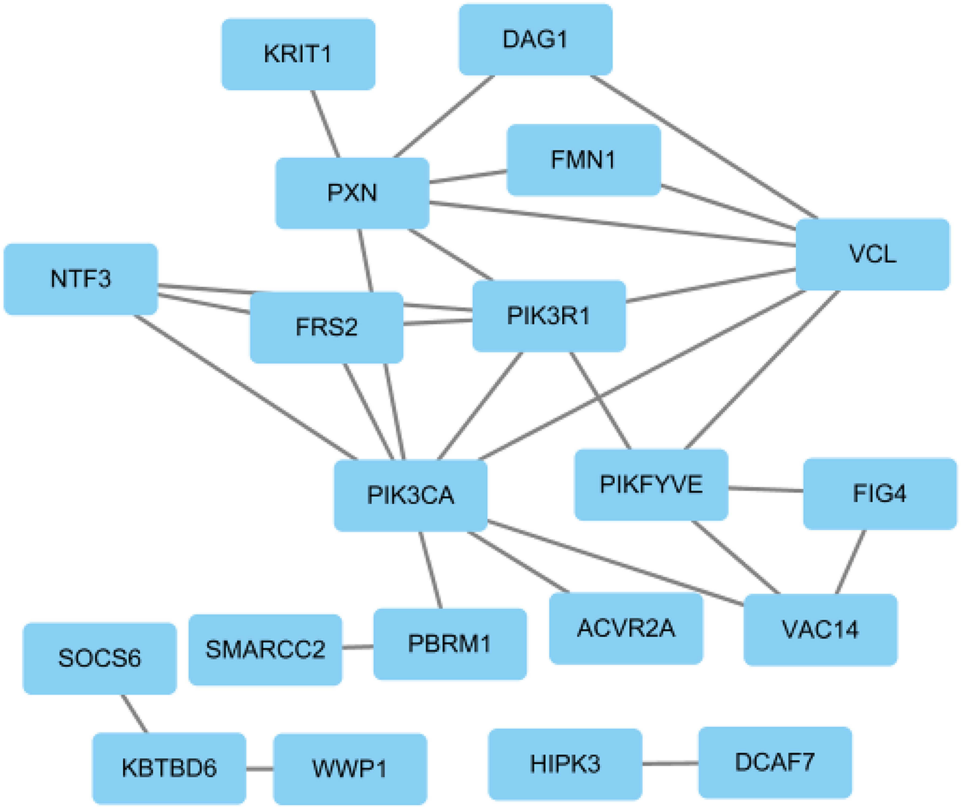


Figure 6 Calculate the interaction score between each protein through the STRING network, with 0.4 as the cut-off value, and select 20 highly correlated proteins.

cell line proliferation, thus effectively decreasing the occurrence and progression of OS. Wu et al[36] also pointed out that serum miR-21 was highly expressed in patients with OS, which is closely related to metastasis and chemosensitivity. However, the specific miRNA mechanism is still not fully understood, and further research is required for its confirmation.[37] The present study revealed that PI3KR1 was most highly related to the connectivity of surrounding genes. The phosphoinositide 3-kinase (PI3K)/RAC-α serine/threonine-protein kinase (AKT)/mammalian target of rapamycin signaling pathway is one of the important pathways dysregulated in OS.[38–40] The expression of the tumor suppressor PTEN may be regulated by miR-21; PTEN downregulation or deletion activates the PI3K/AKT (phosphoinositide 3-kinase/RAC-α serine/threonine protein kinase) signaling pathway, which leads to cancer development, invasion, and metastasis.[41,42]

It has been determined that miR-21 primarily has oncogenic roles in cancers, including OS.[43] A study revealed a positive connection between Bcl-2 expression and miR-21 that inhibited apoptosis and induced CDDP resistance; meanwhile, Bcl-2 siRNA ameliorated miR-21-induced resistance.[44] Another recent study identified Spry2 as an indirect target of miR-21 and confirmed the positive role of miR-21 in OS drug resistance.[29] Nevertheless, serum and tumor tissue miR-21 expression levels are closely related to chemotherapy efficacy; this provides an experimental basis for early OS diagnosis and chemosensitivity prediction.

In the present study, the bioinformatics analysis of miR-21, along with its related proteins and signaling pathways, was conducted to explore its action mechanism. The results revealed a correlation between miR-21 and FRS2, PIKFYVE, PIK3R1, and SOCS6; however, the specific mechanism still requires further study.

The effect of chemotherapy is evaluated by detection of the serum miR-21 expression level in patients with OS in order to change the chemotherapy regimen and improve the treatment effect. The bioinformatics analysis and previous studies show that miR-21 may regulate the progression of OS by interacting with the PI3K signaling pathway.

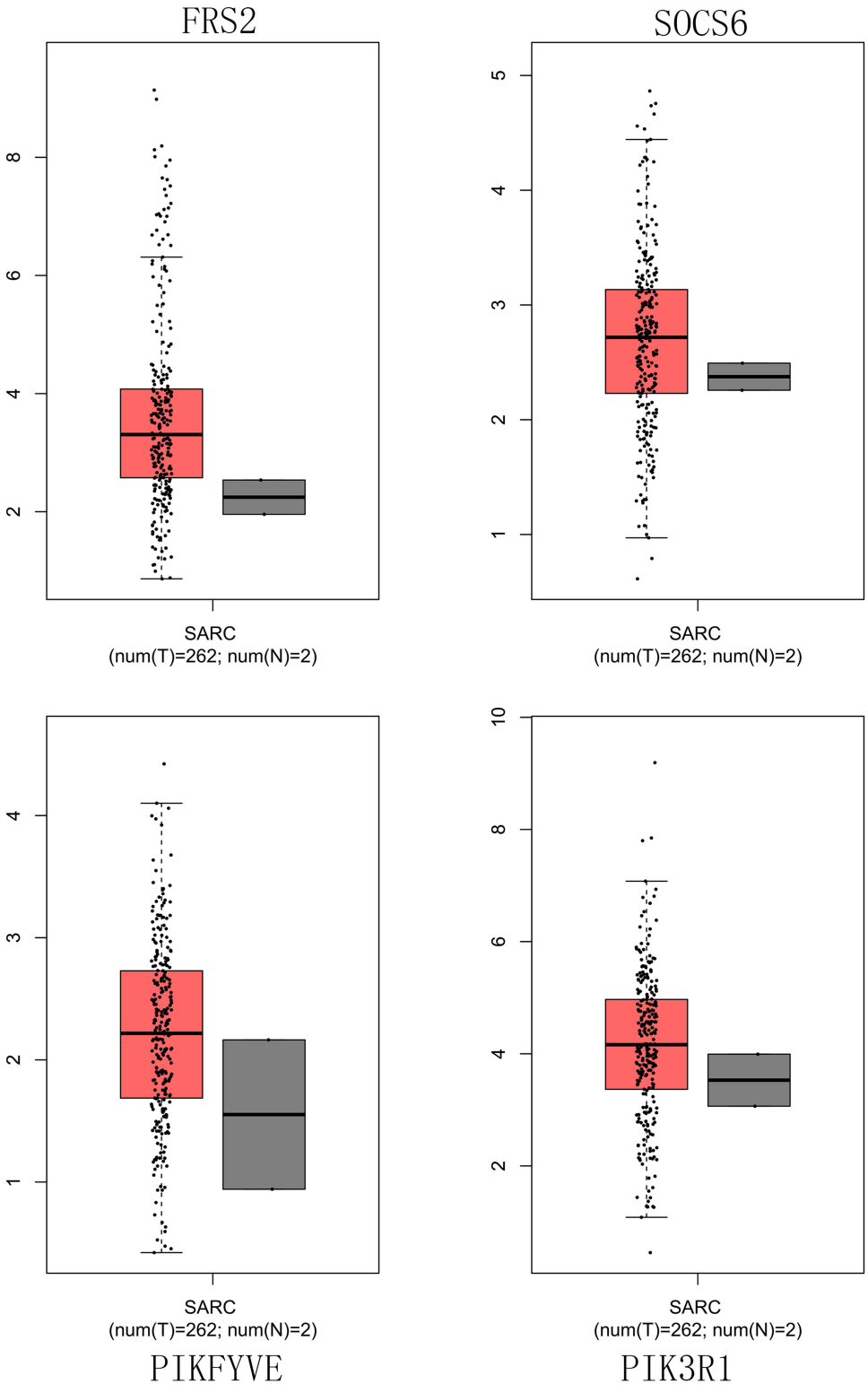


Figure 7 FRS2, PIKFYVE, PIK3R1 and SOCS6 expressions in the TCGA database in OS and healthy tissues.

Acknowledgment

This study was supported by key research and development project in Shanxi province (201703D321014-2).

Disclosure

The authors report no conflicts of interest for this work.

References

1. Geller DS, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. *Clin Adv Hematol Oncol*. 2010;8(10):705–718.
2. Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. *Cancer Treat Res*. 2009;152:3–13.
3. Sun K, Wang W, Zeng JJ, et al. Micro RNA-221 inhibits CDKN1C/p57 expression in human colorectal carcinoma. *Acta Pharmacol Sin*. 2011;32(3):375–384. doi:10.1038/aps.2010.206
4. Hameed M, Doffman H. Primary malignant bone tumors-recent developments. *Semin Diagn Pathol*. 2011;28(1):86–101. doi:10.1053/j.semdp.2011.02.002
5. Sielari VA, Qin L. Targeting the osteosarcoma cancer stem cell. *J Orthop Surg Res*. 2010;5:78. doi:10.1186/1749-799X-5-78
6. Steinmann P, Walters DK, Arlt MJ, et al. Antimetastatic activity of honokiol in osteosarcoma. *J Cancer*. 2012;118(8):2117–2127. doi:10.1002/cncr.26434
7. Broadhead ML, Clark JC, Myers DE, Dass CR, Choong PF. The molecular pathogenesis of osteosarcoma: a review. *Sarcoma*. 2011;2011:959248. doi:10.1155/2011/959248
8. Zheng W, Zhao J, Tao Y, et al. MicroRNA-21: a promising biomarker for the prognosis and diagnosis of non-small cell lung cancer. *Oncol Lett*. 2018;16:2777–2782.
9. Lopezanton M, Lambie M, Lopezcabrera M, et al. miR-21 Promotes Fibrogenesis in Peritoneal Dialysis. *Am J Pathol*. 2017;187:1537. doi:10.1016/j.ajpath.2017.03.007
10. Li C, Xu B, Miu X, Deng Z. Inhibition of miRNA-21 attenuates the proliferation and metastasis of human osteosarcoma by upregulating PTEN. *Exp Ther Med*. 2018;15:1036–1040.
11. Hu X, Li L, Lu Y, Yu X, Chen H. miRNA-21 inhibition inhibits osteosarcoma cell proliferation by targeting PTEN and regulating the TGF-β1 signaling pathway. *Oncol Lett*. 2018;16:4337–4342.
12. Di Leva G, Croce CM. Roles of small RNAs in tumor formation. Trends. *Mol Med*. 2010;16(6):257-267.
13. Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet*. 2011;12(12):861-874. doi:10.1038/nrg3074
14. Garofalo M, Croce CM. MicroRNAs as therapeutic targets in chemoresistance. *Drug Resist Updates*. 2013;16(3–5):47-59. doi:10.1016/j.drup.2013.05.001
15. Lee JW, Kim N, Park JH, et al. Differential microRNA expression between gastric cancer tissue and non-cancerous gastric mucosa according to Helicobacter pylori status. *J Cancer Prev*. 2017;22(1):33-39.
16. Song B, Wang Y, Xi Y, et al. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. *Oncogene*. 2009;28(46):4065-4074. doi:10.1038/onc.2009.274
17. Gougelet A, Pissaloux D, Besse A, et al. Micro-RNA profiles in osteosarcoma as a predictive tool for ifosfamide response. *Int JCancer*. 2011;129(3):680-690.
18. Dong Y, Yu J, Ng SS. MicroRNA dysregulation as a prognostic biomarker in colorectal cancer. *Cancer Manag Res*. 2014;6(3):405–422.
19. Calin GA, Dumitru CD, Shimizum, et al. Frequent deletions and down regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2002;99(24):15524–15529. doi:10.1073/pnas.242606799
20. Qi P, Gao CF. Study progress of miRNA in hepatocytes. *China Biotechnol*. 2008;28(12):94–101.
21. Kinet V, Halkein J, Dirkx E, Windt LJ. Cardiovascular extracellular microRNAs: emerging diagnostic markers and mechanisms of cell-to-cell RNA communication. *Front Genet*. 2013;12(4):214.
22. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;105(30):10513–10518. doi:10.1073/pnas.0804549105
23. Ma R, Jiang T, Kang X. Circulating microRNAs in cancer: origin, function and application. *J Exp Clin Cancer Res*. 2012;31(1):38. doi:10.1186/1756-9966-31-38
24. Ziyan W, Shuhua Y, Xiufang W, Xiaoyun L. MicroRNA-21 is involved in osteosarcoma cell invasion and migration. *Med Oncol*. 2011;28(4):1469–1474. doi:10.1007/s12032-010-9563-7
25. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19:92–105. doi:10.1101/gr.082701.108
26. Miranda KC, Huynh T, Tay Y, et al. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. *Cell*. 2006;126(6):1203–1217. doi:10.1016/j.cell.2006.07.031
27. Ouyang L, Liu P, Yang S, Ye S, Xu W, Liu X. A three-plasma miRNA signature serves as novel biomarkers for osteosarcoma. *Med Oncol*. 2013;30(1):340. doi:10.1007/s12032-012-0340-7
28. Namløs HM, Meza-Zepeda LA, Barøy T, et al. Modulation of the osteosarcoma expression phenotype by microRNAs. *PLoS One*. 2012;7(10):e48086.
29. Vanas V, Haigl B, Stockhammer V, Sutterlüty-Fall H. MicroRNA-21 Increases Proliferation and Cisplatin Sensitivity of Osteosarcoma-Derived Cells. *PLoS One*. 2016;11(8):e0161023.
30. Qu K, Lin T, Pang Q, et al. Extracellular miRNA-21 as a novel biomarker in glioma: evidence from meta-analysis, clinical validation and experimental investigations. *Oncotarget*. 2016;7(23):33994–34010. doi:10.18632/oncotarget.9188
31. Gong B, Liu WW, Nie WJ, et al. MiR-21/RASA1 axis affects malignancy of colon cancer cells via RAS pathways. *World J Gastroenterol*. 2015;21(5):1488–1497. doi:10.3748/wjg.v21.i5.1488
32. Yamanaka S, Olaru AV, An F, et al. MicroRNA-21 inhibits Serpini1, a gene with novel tumour suppressive effects in gastric cancer. *Dig Liver Dis*. 2012;44(7):589–596. doi:10.1016/j.dld.2012.02.016
33. Zadeh MM, Ranji N, Motamed N. Deregulation of miR-21 and miR-155 and their putative targets after silibinin treatment in T47D breast cancer cells. *Iran J Basic Med Sci*. 2015;18(12):1209–1214.
34. Hua Y, Jin Z, Zhou F, et al. The expression significance of serum MiR-21 in patients with osteosarcoma and its relationship with chemosensitivity. *Eur Rev Med Pharmacol Sci*. 2017;21:2989–2994.
35. Zhang R, Xia T. Long non-coding RNA XIST regulates PDCD4 expression by interacting with miR-21-5p and inhibits osteosarcoma cell growth and metastasis. *Int J Oncol*. 2017;51(5):1460–1470. doi:10.3892/ijo.2017.4127
36. Wu ZY, Liu Y, Yang SH. The significance of serum microRNA-21 expression and its sensitivity to chemotherapeutic drugs in osteosarcoma patients. *J Pract Orthop*. 2015;21(7):607–610.
37. Jing YL, Lv Z. Research progress of microRNA in osteosarcoma. *Cancer Res Clin*. 2016;28(9):638–641.
38. Graziano AC, Cardile V, Avola R, Vicario N, Parenti C, Salvatorelli L. Wilms' tumor gene 1 silencing inhibits proliferation of human osteosarcoma MG-63 cell line by cell cycle arrest and apoptosis activation. *Oncotarget*. 2017;8:13917–13931. doi:10.18632/oncotarget.14715
39. Wang L, Tang B, Han H, Mao D, Chen J. miR-155 Affects Osteosarcoma MG-63 Cell Autophagy Induced by Adriamycin Through Regulating PTEN-PI3K/AKT/mTOR Signaling Pathway. *Cancer Biother Radiopharm*. 2018;33:32-38. doi:10.1089/cbr.2017.2306

40. Zhang J, Yu XH, Yan YG. PI3K/Akt signaling in osteosarcoma. *Clin Chim Acta*. 2015;444:182–192. doi:10.1016/j.cca.2014.12.041
41. Wu YR, Qi HJ, Deng DF, Luo YY, Yang SL. MicroRNA-21 promotes cell proliferation, migration, and resistance to apoptosis through PTEN/PI3K/AKT signaling pathway in esophageal cancer. *Tumour Biol*. 2016;37:12061-12070. doi:10.1007/s13277-016-5074-2
42. Xue R, Lei S, Xia ZY, et al. Selective inhibition of PTEN preserves ischaemic post-conditioning cardioprotection in STZ-induced Type 1 diabetic rats: role of the PI3K/Akt and JAK2/STAT3 pathways. *Clin Sci (Lond)*. 2016;130:377–392. doi:10.1042/CS20150496
43. Pfeffer SR, Yang CH, Pfeffer LM. The Role of miR-21 in Cancer. *Drug Dev Res*. 2015;76(6):270-277. doi:10.1002/ddr.21257
44. Ziyan W, Yang L. MicroRNA-21 regulates the sensitivity to cisplatin in a human osteosarcoma cell line. *Ir J Med Sci*. 2016;185(1):85-91. doi:10.1007/s11845-014-1225-x