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Article

Expression Changes, Prognostic Analysis and Risk Factors of miR-625-3p and miR-449a in Osteosarcoma Patients after Surgery

Hui Zhang, Zhan Wang, Lin Liu, Fuqiang Zhang, Yuxin Song and Yaowen Qian*

Department of Orthopaedics, Gansu Provincial Hospital, Lanzhou, 730000, China
*Corresponding Author: Yaowen Qian. Email: qyw2218@163.com

Abstract: The expression changes of miR-625-3p and miR-449a in osteosarcoma patients after surgery is the critical study in this paper. Analysis of their prognosis and risk factors can be helpful in understanding the prognostic value for clinical purposes. Fifty-eight patients with osteosarcoma diagnosed in our hospital were considered as the research group (RG), and 52 health subjects at the same time were collected as the control group (CG). Fluorescence quantitative PCR (RT-PCR) was employed to test the expression levels of miR-625-3p and miR-449a in the serum of subjects in both groups before surgery and patients in the RG after surgery. The survival conditions of both groups with high and low miR-625-3p and miR-449a expression levels were compared after 5-year follow-up of patients in the RG. The prognostic factors of osteosarcoma patients were assessed through Cox regression analysis. The miR-625-3p expression level in the RG was dramatically higher than that in the CG ($p < 0.05$), and the miR-449a expression level was dramatically lower than that in the CG ($p < 0.05$). The miR-625-3p expression level in the RG after surgery was markedly lower than that before surgery ($p < 0.05$), and the miR-449a expression level was markedly higher than that before surgery ($p < 0.05$). After they were followed up for 5 years, the overall survival rate of those in the RG was 60.34%, the rate of miR-625-3p low expression group (LEG) was dramatically higher than that of high expression group (HEG) ($p < 0.05$), and the rate of miR-449a HEG was markedly higher than that of miR-449a LEG ($p < 0.05$). The relative expression of miR-625-3p and miR-449a was remarkably different from tumor size, lymph node metastasis, and Enneking stage ($p < 0.05$). Univariate Cox regression analysis identified that tumor size, lymph node metastasis, Enneking stage, miR-625-3p and miR-449a were risk factors affecting the prognosis of patients; multivariate Cox regression analysis found that the five were independent prognostic factors of them. miR-625-3p and miR-449a are differentially expressed in the postoperative serum of osteosarcoma patients, and their differential expression is an independent risk factor affecting their prognosis, which has prognostic value and can provide a reference value for clinical practice.

Keywords: Osteosarcoma; miR-625-3p; miR-449a; prognostic analysis; risk factors

1 Introduction

Osteosarcoma is a highly malignant interstitial tumor, which mainly metastasizes to the lung [1]. It is the most common malignant bone tumor in children and adolescents, with high morbidity, recurrence rate, and metastasis [2]. In Europe, 2–5 people are diagnosed with osteosarcoma every year for every 1 million people [3]. Radiotherapy or exposure to nuclear radiation will increase the risk of osteosarcoma. Patients usually receive neoadjuvant chemotherapy, followed by complete tumor resection and postoperative chemotherapy [4]. The overall survival rate of patients with local osteosarcoma has been dramatically improved as multiple drug chemotherapy schemes enter the treatment mode. However, despite the best treatment method, the all-cause mortality of osteosarcoma survivors is still higher than that of the general

population [5], and the 5-year survival rate at all stages is about 70% [6]. The underlying mechanism of osteosarcoma formation is still indistinct, and there is no reliable method for its early evaluation and prognosis analysis [7].

It is well known that microRNA is a small non-coding RNA that negatively regulates gene expression at the post-transcriptional level. Abnormal microRNA expression, through the wrong expression of microRNA target genes, can produce profound cellular effects, leading to various diseases, including cancer [8,9]. In clinical application, microRNA has potential as a biomarker for prognosis, diagnosis, and treatment [10]. Studies have shown that it can regulate the differentiation of human bone marrow mesenchymal stem cells [11], so its role in the development and progression of osteosarcoma has attracted more and more attention. Therefore, it is necessary to study the changes of microRNA regulation in osteosarcoma patients. Some studies show that miR-625-3p is useful in the development of other malignancies, up-regulates [12] in clear cell renal cell carcinoma, and is a promising predictive marker [13] in colorectal cancer. Re-expressing miR-449a can effectively inhibit tumor growth [14]; some studies show that it can inhibit breast cancer cell migration and invasion [15], but there is no research on miR-625-3p, miR-449a, and osteosarcoma.

In this study, we mainly study the changes of miR-625-3p and miR-449a microRNA expression before and after osteosarcoma surgery to analyze its prognosis and risk factors for clinical reference.

2 Information and Methods

2.1 Clinical Data

Fifty-eight patients diagnosed as osteosarcoma in our hospital were taken as the research group (RG). According to Enneking stage, 34 patients were in Stage I, 13 patients in Stages II and 11 patients in stage III. Fifty-two health subjects at the same time were taken as the control group (CG), among which the laboratory test indexes of healthy people were normal and there was no congenital immune deficiency.

Inclusion criteria: The subjects were accompanied by their families when they were admitted to the hospital, with complete clinical and pathological data, and they underwent routine examinations such as liver and kidney function and electrocardiogram; the postoperative pathological report of patients in the RG was confirmed as osteosarcoma. Exclusion criteria: Patients had previous history of mental illness and family history of mental illness, with expected survival time <3 months; those had history of severe organ diseases, or drug dependence; those were unable to cooperate with examination due to aphasia, dysphoria, unconsciousness and communication disorder.

This study was approved by the Ethics Committee of our hospital and it was understood by the subjects and their families in advance. They agreed and signed a complete informed consent form.

2.2 Blood Sample Collection and Testing Methods

Altogether 5 mL of fasting venous blood was collected on the morning of the second day of admission and the fifth day after surgery. Meanwhile, the same blood samples were taken from the CG, and they were put into anticoagulants, coagulated 60 min (20–25ºC), and centrifuged 15 min at 1369.55xp (4ºC) with a centrifuge (Sichuan Shuke Instruments Co., Ltd., TG 112, Chengdu, Sichuan, China). The upper serum was separated and stored in a refrigerator at –70ºC for centralized detection.

Total RNA in serum was extracted in view of the instructions of TRIzol kit (Shanghai Mingjing Biology, 5003050, Shanghai, China). In order to eliminate the contamination of genomic DNA, template RNA was digested and treated with DNase1 (RNA free) (Shanghai Hengfei Biotechnology Co., Ltd., K003399P, Shanghai, China). The purity and concentration were determined by ultraviolet spectrophotometer (Peking University Medical Industrial Park Science and Technology Co., Ltd., UV-1100, Beijing, China), and RNA integrity was tested by 1.5% agarose gel electrophoresis. The RNA concentration was adjusted to 500 ng/μL, and the RNA sample was reverse transcribed into cDNA through cDNA reverse transcription kit (Shanghai Lianqiao Biotechnology Co., Ltd., 4368814, Shanghai, China) in strict conformity with the instructions. The RT-PCR system was 20 μL: 2x Ultra SYBR one step Qrt-PCR Buffer 10 μL, RNA template 2 μL, Nuclease-free water 5.5 μL, upstream and downstream primers 1 μL each, Super enzyme mix 0.5 μL. RT-PCR reaction conditions were as belows: pre-denaturation at 95ºC for 10 min, denaturation at 60ºC for 15 s, annealing, extension at 60ºC for 1 min, a total of 40 cycles. Primers in

this experiment were devised by Primer Premier 5.0 (Premier, Palo Alto, CA, USA) and produced by Tianjin Saierbio Biotechnology Co., Ltd. (Tianjing, China) miR-625-3p and miR-449a both employed U6 as internal reference. The specific primer sequences were shown in Tab. 1. We found that the cycle number Ct value of the fluorescence signal was the cycle number corresponding to the inflection point when the background enters the exponential growth stage during the amplification process; the miR-625-3p and miR-449a relative expression levels were calculated by $2^{-\Delta Ct}$.

Table 1: Primer sequence

		Upstream primer	Downstream primer
	miR-625-3p	5'-GTCGTATCCAGTGCAGGGTCCG-3'	5'-CGCACTGGATACGACAAAT-3'
	miR-449a	5'-GCCGATGGCAGTGTATTGTTAG-3'	5'-CAGTGCAGGGTCCGAGGT-3'
Internal reference	U6	5'-GGGTGCTCGCTTCGGCAGC-3'	5'-CAGTGCAGGGTCCGAGGT-3'

2.3 Follow-Up of Patients

At the 6, 12, 24, 36, 48 and 60th months after treatment, to record their survival, patients were followed up for 5 years by telephones and out-patient review.

2.4 Outcome Measures

The miR-625-3p and miR-449a expression levels of subjects in the RG before and after surgery in the two groups were observed. The survival situation of patients in the RG was followed up for 5 years and the relationship with the two expression levels was analyzed. The comparative expression of miR-625-3p and miR-449a in the serum of patients in the RG was compared with the clinical data: Their clinical data were collected for univariate Cox regression analysis, and then the indicators with statistically different univariate results were analyzed for multivariate Cox regression analysis so as to assess the independent prognostic factors of them.

2.5 Statistical Analysis

The collected data were statistically analyzed via SPSS20.0, and the required pictures were drawn via GraphPad 7. The counting data were expressed as [n(%)], and chi-square test was employed for comparison between groups. The measurement data were expressed as (mean ± standard deviation), and the comparison between groups adopted *t* test. The predicted value was analyzed through ROC curve. The survival rate was counted via Kaplan-Meier method, and the survival rate was assessed through Log-rank test. The relevant factors were assessed by Logistic regression analysis. The difference was statistically remarkable when $p < 0.050$.

3 Results

3.1 Comparison of General Data

The average age, body mass index (BMI), average height, smoking history and other general clinical data of subjects in the two groups were collected. There was no marked difference in general clinical data between both groups ($p > 0.05$) (Tab. 2).

Table 2: Comparison of general data of subjects in both groups ($\bar{x} \pm s$) /[n(%))

	RG (n = 58)	CG (n = 52)	t/X^2	*p*
Average age (years)	20.53 ± 2.24	21.07 ± 2.18	1.28	0.20
BMI (kg/m^2)	22.13 ± 2.12	21.68 ± 2.37	1.05	0.30

Average height (cm)	171.34 ± 9.41	172.15 ± 10.58	0.43	0.67
Smoking history			0.03	0.86
Yes	21 (36.21)	18 (34.62)		
No	37 (63.79)	34 (65.38)		
History of alcoholism			0.29	0.59
Yes	3 (5.17)	4 (7.69)		
No	55 (94.83)	48 (92.31)		
Place of residence			0.00	0.98
Cities and towns	28 (48.28)	25 (48.08)		
Countryside	30 (51.72)	27 (51.92)		
Tumor size			-	-
≤2 cm	34 (58.62)	0 (0.00)		
>2 cm	24 (41.38)	0 (0.00)		
Lymph node metastasis			-	-
Metastasis	25 (43.10)	0 (0.00)		
Non-metastasis	33 (56.90)	0 (0.00)		
Enneking stage			-	-
Stage I	34 (58.62)	0 (0.00)		
Stages II + III	24 (41.38)	0 (0.00)		

3.2 Comparison of miR-625-3p and miR-449a Expression Levels of Subjects in Both Groups before Surgery

By comparing the miR-625-3p and miR-449a expression levels in the serum of patients in the RG and the CG before surgery, we discovered that the miR-625-3p expression level in the RG was dramatically higher (3.24 ± 1.05) than that in the CG (1.02 ± 0.21) ($p < 0.05$), and the miR-449a expression level in the RG was dramatically lower (0.34 ± 0.28) than that in the CG (1.11 ± 0.37) ($p < 0.05$) (Fig. 1).

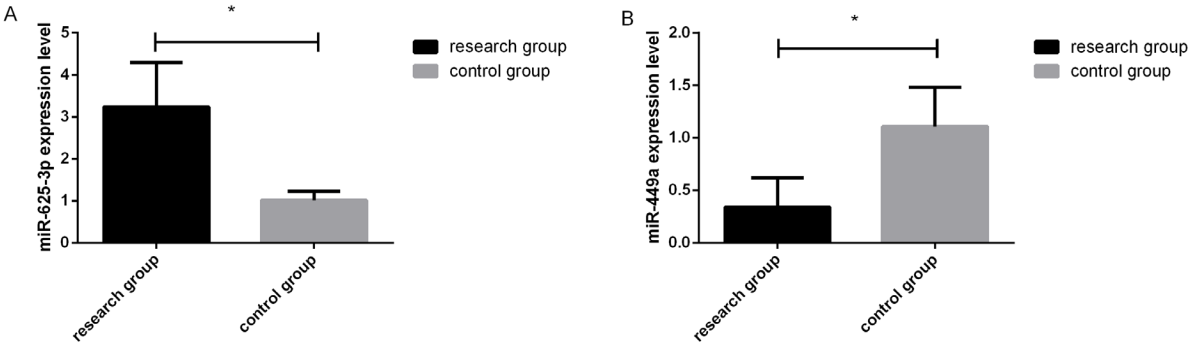

Figure 1: Comparison of miR-625-3p and miR-449a expression levels of subjects in both groups before surgery. A. The miR-625-3p expression level in the RG before surgery was markedly higher than that in the CG ($p < 0.05$); B. The miR-449a expression level in the RG was dramatically lower than that in the CG ($p < 0.05$). Note: * indicates the comparison between the two groups ($p < 0.05$)

3.3 Comparison of miR-625-3p and miR-449a Expression Levels before and after Surgery in RG

By comparing the miR-625-3p and miR-449a expression levels in patients' serum before and after surgery, we discovered that the miR-625-3p expression level after surgery (1.56 ± 0.44) was dramatically lower than that before surgery (3.24 ± 1.05) ($p < 0.05$), and the miR-449a expression level after surgery (0.82 ± 0.38) was remarkable higher than that before surgery (0.34 ± 0.28) ($p < 0.05$) (Fig. 2).

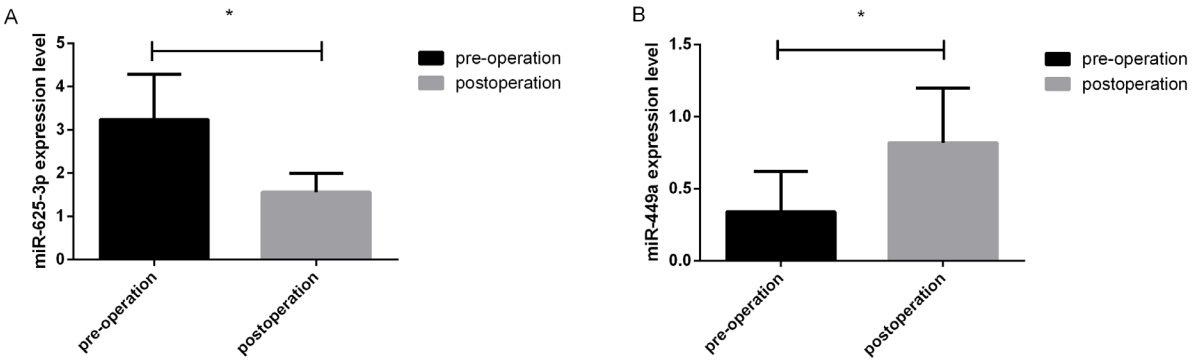

Figure 2: Comparison of miR-625-3p and miR-449a expression levels before and after surgery in RG. A. The miR-625-3p expression level in the RG after surgery was dramatically lower than that before surgery ($p < 0.05$); B. The miR-449a expression level in the RG after surgery was dramatically higher than that before surgery ($p < 0.05$). Note: * indicates the comparison between the two groups ($p < 0.05$)

3.4 Relationship between Survival of Patients in RG and miR-625-3p, miR-449a

Statistics on the overall survival rate of patients in the RG showed that 58 patients were followed up without defects, and the overall survival rate was 60.34%. According to the median expression of miR-625-3p and miR-449a before surgery, they were divided into high and low expression groups respectively. We observed the overall survival rate of patients in both groups, and the rate of the miR-625-3p low expression group (LEG) was markedly higher than that of the high expression group (HEG). There was marked difference in survival between both groups ($p < 0.05$), while the overall survival rate of miR-449a HEG was remarkably higher than that of miR-449a LEG, and there was marked difference in survival between both groups ($p < 0.05$) (Fig. 3).

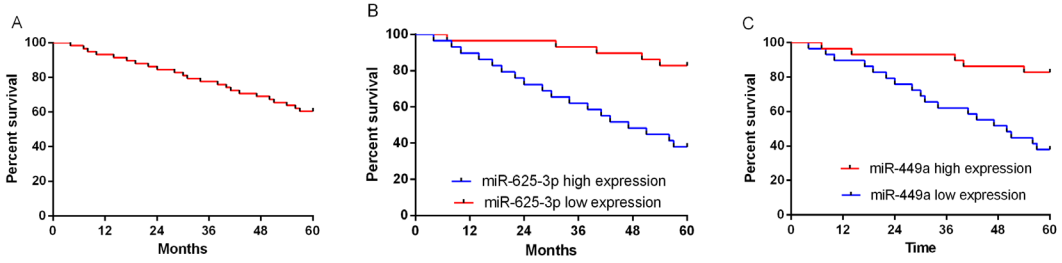

Figure 3: 5-year survival rate of patients. A. The overall survival rate of patients was 60.34%; B. The overall survival rate of miR-625-3p HEG was markedly lower than that of LEG, and there was marked difference between both groups ($p < 0.05$); C. The overall survival rate of miR-449a LEG was dramatically lower than that of miR-449a HEG, and there was obvious difference between both groups ($p < 0.05$)

3.5 Cox Regression Analysis

According to the comparison between the relative expression of miR-625-3p and miR-449a in the serum of patients in the RG and clinical data, there was no statistical difference between the relative expression of miR-625-3p and miR-449a and age, BMI, smoking history, drinking history and place of residence ($p > 0.05$), but there was marked difference between the two and tumor size, lymph node metastasis and Enneking stage ($p < 0.05$). (Tabs. 3 and 4). The clinical data of patients were collected for univariate Cox regression analysis, and it manifested that tumor size, lymph node metastasis, Enneking stage, miR-625-3p and miR-449a were risk factors affecting prognosis. Subsequently, multivariate Cox regression analysis was conducted for indicators with statistically different univariate results, and it showed that tumor size, lymph node metastasis, Enneking stage, miR-625-3p and miR-449a were independent prognostic factors (Tab. 5 and Tab. 6).

Table 3: Relationship between relative expression of miR-625-3p in patients' serum and clinical data

Factor	miR-625-3p relative expression		t/c^2 value	*p* value
	High expression (n = 29)	Low expression (n = 29)		
Age (years)			0.28	0.59
≤20 (n = 24)	13 (44.83)	11 (37.93)		
>20 (n = 34)	16 (55.17)	18 (62.07)		
BMI (kg/m^2)	22.11 ± 2.18	22.09 ± 2.20	0.03	0.97
Smoking history			0.67	0.41
Yes (n = 21)	9 (31.03)	12 (41.38)		
No (n = 37)	20 (68.97)	17 (58.62)		
History of alcoholism			0.35	0.55
Yes (n = 3)	2 (6.90)	1 (3.45)		
No (n = 55)	27 (93.10)	28 (96.55)		
Place of residence			0.28	0.60
Cities and towns (n = 28)	13 (44.83)	15 (51.72)		
Countryside (n = 30)	16 (55.17)	14 (48.28)		
Tumor size			13.93	0.00
≤2 cm (n = 34)	10 (34.48)	24 (82.76)		
>2 cm (n = 24)	19 (65.52)	5 (17.24)		
Lymph node			5.70	0.02

Metastasis (n = 25)	17 (58.62)	8 (27.59)		
Non-metastasis (n = 33)	12 (41.38)	21 (72.41)		
Enneking stage			10.24	0.00
Stage I (n = 34)	11 (37.93)	23 (79.31)		
Stages II + III (n = 24)	18 (62.07)	6 (20.69)		

Table 4: Cox regression analysis

Factor	Univariate Cox			Multivariate Cox		
	Exp(B)	95CI%	Sig.	Exp(B)	95CI%	Sig.
Age	0.894	0.429–1.834	0.761			
BMI	0.633	0.772–1.168	0.628			
Smoking history	2.010	0.931–4.288	0.069			
History of alcoholism	0.443	0.171–1.149	0.090			
Place of residence	0.779	0.382–1.698	0.598			
Tumor size	0.141	0.058–0.349	0.000	0.229	0.091–0.580	0.003
Lymph node metastasis	0.251	0.130–0.513	0.000	0.321	0.151–0.679	0.002
Enneking stage	5.310	2.743–10.252	0.000	3.110	1.643–6.242	0.002
miR-625-3p	3.191	1.861–5.512	0.000	2.681	1.450–4.929	0.001

Table 5: Relationship between relative expression of miR-449a in patients' serum and clinical data

Factor	miR-449a relative expression		t/c^2 value	*p* value
	High expression (n = 29)	Low expression (n = 29)		
Age (years)			0.18	0.67
≤20 (n = 24)	11 (37.93)	13 (44.83)		
>20 (n = 34)	18 (62.07)	16 (55.17)		
BMI (kg/m^2)	22.10 ± 2.21	22.08 ± 2.17	0.03	0.97
Smoking history			0.07	0.78
Yes (n = 21)	11 (37.93)	10 (34.48)		
No (n = 37)	18 (62.07)	19 (65.52)		
History of alcoholism			0.35	0.55
Yes (n = 3)	2 (6.90)	1 (3.45)		
No (n = 55)	27 (93.10)	28 (96.55)		
Place of residence			0.28	0.60
Cities and towns (n = 28)	15 (51.72)	13 (44.83)		
Countryside (n = 30)	14 (48.28)	16 (55.17)		
Tumor size			10.24	0.00
≤2 cm (n = 34)	23 (79.31)	11 (37.93)		
>2 cm (n = 24)	6 (20.69)	18 (62.07)		
Lymph node			115.82	0.00
Metastasis (n = 25)	5 (17.24)	20 (68.97)		
Non-metastasis (n = 33)	24 (82.76)	9 (31.03)		
Enneking stage			7.11	0.01
Stage I (n = 34)	22 (75.86)	12 (41.38)		
Stages II + III (n = 24)	7 (24.14)	17 (58.62)		

Table 6: Cox regression analysis

Factor	Univariate Cox			Multivariate Cox		
	Exp(B)	95CI%	Sig.	Exp(B)	95CI%	Sig.
Age	1.074	0.595–1.927	0.816			
BMI	1.151	0.634–2.068	0.660			
Smoking history	1.243	0.738–2.196	0.602			
History of alcoholism	1.431	0.741–2.802	0.284			
Place of residence	1.064	0.677–2.128	0.827			
Tumor size	0.258	0.129–0.510	0.000	0.328	0.158–0.687	0.002
Lymph node metastasis	3.189	1.853–5.498	0.000	2.676	1.454–4.936	0.003
Enneking stage	0.151	0.062–0.349	0.000	0.238	0.095–0.580	0.001
miR-449a	5.310	2.743–10.250	0.000	3.194	1.639–6.234	0.001

4 Discussion

Osteosarcoma is a primary bone tumor with an extremely high malignant degree [16]. Its morbidity and survival rate are quite different in the world. In Argentina, the morbidity of osteosarcoma is similar to that of high-income countries, but the survival rate in all regions is relatively low [17]. The treatment method of osteosarcoma has hardly changed in the past 30 years [18]. The high incidence period is puberty, which indicates that bone growth and puberty hormones play a vital role in the disease [19]. The somatic genome of osteosarcoma is highly aneuploid, showing extensive intratumoral heterogeneity with a mutation rate higher than that of most other pediatric cancers [20]. According to the research results recently, microRNA is an essential component of biological and metabolic pathways that regulate development stage, signal transduction, cell maintenance and differentiation [21]. Hence, their imbalance may expose individuals to malignancies. It has been proved that miRNA expression imbalance in different types of tumors, such as osteosarcoma, may cause tumor development [22,23]. In this research, to study the prognostic value and risk factors for clinical reference, the miR-625-3p and miR-449a expression levels in serum of osteosarcoma patients were detected.

Some studies have shown that miR-625-3p is highly expressed in thyroid cancer tissues compared with adjacent normal tissues [24]. By comparing the miR-625-3p and miR-449a expression levels in serum of patients in the RG and the CG before surgery, it has been found that the miR-625-3p expression level in the RG was dramatically higher than that in the CG, and the miR-449a expression level was remarkably lower than that in the CG. Besides, research displayed that miR-449a was a tumor inhibitor [25], the miR-449a expression level in breast cancer tissues and cell lines reduced dramatically, and it could be used as a treatment strategy for breast cancer [26]. This indicated that miR-625-3p and miR-449a took part in the development and progression of osteosarcoma through expression. The miR-625-3p expression level in osteosarcoma patients after surgery obtained in combination with this study was markedly lower than that before surgery, and the miR-449a expression level after surgery was markedly higher than that before surgery, suggesting that the miR-625-3p and miR-449a expression levels were constantly changing with the progress of treatment.

What is more, miR-449a was associated with proliferation, cell cycle arrest, and apoptosis of osteosarcoma cells [27]. Both miR-625-3p and miR-625-5p belonged to miR-625 family, and the latter was overexpressed in gastric cancer [28] and lung cancer [29], which indicated that the two expression levels were crucial for the evaluation of therapeutic effect and prognosis recovery after treatment, and could be used as a reference index for guiding treatment methods clinically. Then, this study further carried out statistics on the overall survival rate of patients. All 58 patients were followed up without any defects, and the overall survival rate was 60.34%. According to the median expression of miR-625-3p and miR-449a

before surgery, they were divided into high and low expression groups and their overall survival rate was observed. The overall survival rate of the miR-625-3p LEG was markedly higher than that of the HEG, and the rate of the miR-449a HEG was dramatically higher than that of the miR-449a LEG. The results displayed that both miR-625-3p's high expression and miR-449a's low expression predicted poor prognosis of patients. This suggested that we could adjust and perfect the clinical treatment plan of patients by detecting the expression levels of the two indicators in the serum before treatment, and regulate their subsequent treatment plan by detecting the expression of the two indicators in the serum after treatment, thus improving their prognosis and survival rate.

The relative expression of miR-625-3p and miR-449a in patients' serum was compared with clinical data. Furthermore, we found that the relative expression of miR-625-3p and miR-449a in patients' serum had no statistical difference with age, BMI, smoking history, drinking history, and place of residence, but had a remarkable difference with tumor size, lymph node metastasis, and Enneking stage. The results showed that miR-625-3p's high expression and miR-449a's low expression might indicate that the patient's tumor diameter was >2 cm and metastasized to lymph nodes. Simultaneously, the clinical data of patients were collected for the univariate Cox regression analysis. The results manifested that tumor size, lymph node metastasis, Enneking stage, miR-625-3p, and miR-449a were risk factors affecting prognosis. Soon afterward, a multivariate Cox regression analysis was conducted for indicators with statistically different univariate results. It was found that tumor size, lymph node metastasis, Enneking stage, miR-625-3p, and miR-449a were independent prognostic factors affecting them. The results showed that the two could be used as therapeutic targets.

In this paper, we mainly determined the miR-625-3p and miR-449a expression levels in serum of osteosarcoma patients and found that the differential expression of the two could be used as potential clinical diagnostic indicators of osteosarcoma, and we also discovered that the two were independent prognostic indicators of patients through multivariate Cox regression. However, this study still has certain limitations. It is not clear how miR-625-3p and miR-449a's differential expression is caused. Their specific effects on osteosarcoma cells still need further study. Hence, we hope that the relationship between these two indicators and osteosarcoma will be further explored in future studies to verify this study further.

5 Conclusion

To summarize, miR-625-3p and miR-449a's differential expression in serum of osteosarcoma patients after surgery is expected to be a potential indicator for diagnosis, and it is an independent risk factor affecting their prognosis, which has prognostic value and can provide reference value clinically.

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