JBUON 2020; 25(3): 1562-1568
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Effects of miR-432 and miR-548c-3p on the proliferation and invasion of osteosarcoma cells

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Summary

Purpose: *To investigate the effects of miR-432 and miR-548c-3p on the proliferation and invasion of osteosarcoma cells.*

Methods: *A total of 67 cases of patients with osteosarcoma who came to the third Affiliated Hospital of Southern Medical University from April 2015 to May 2018 formed the experimental group, and 63 healthy individuals who came to this hospital for physical examination during the same period formed the control group. The expressions of miR-432 and miR-548c-3p in sera of each group were detected by RT-PCR to observe the changes of the expression levels of these miRs in the sera of the experimental and the control group, and the relationship between the expression levels of these miRs in the sera of patients with osteosarcoma and the grade of tumor differentiation and different pathological classification. GM-63 cells were selected as the target for in vitro experiments which were cultured and transfected. Before transfection, cells were divided into blank group (without transfection), negative control group (transfected with miR NC) and experimental group (transfected with miR-432 mimics/miR-548c-3p mimics). Cell proliferation was detected by CCK-8 method, cell apoptosis was detected by flow cytometry, and cell invasion by transwell invasion experiment.*

Results: *miR-423 and miR-548c-3p showed low expression in osteosarcoma, and the overexpression of miR-432 and miR-548c-3p in osteosarcoma cells could inhibit the proliferation of tumor cells and promote their apoptosis. miR-432 and miR-548c-3p might be tumor suppressors of osteosarcoma, and their expression levels could be used as important reference indexes to evaluate the benign and malignant levels of osteosarcoma.*

Conclusion: *These results suggested that the abnormal expressions of miR-432 and miR-548c-3p may be key factors of the occurrence and development of osteosarcoma.*

Key words: *miR-423, miR-548c-3p, osteosarcoma, cell proliferation, cell invasion*

Introduction

In adolescents, there was a common primary malignant tumor with high incidence and prone to distant metastasis, with low survival rate - osteosarcoma [1,2]. According to the statistics, the 5-year survival rate of patients with osteosarcoma who underwent single amputation was only 50-60%. Clinical treatment of osteosarcoma is usually a combination of radical surgery and chemotherapy. However, even early osteosarcoma is prone to invasion and metastasis, which makes the effect of radiotherapy and chemotherapy inconspicuous [3].

Studies have shown that microRNAs (miRs) are highly conserved non-coding single-stranded small RNAs with a length of about 22-24 bases, which play an important role in the proliferation, migration and invasion of tumor cells [4]. They can bind to mRNA sequences, leading to degradation of target mRNA and inhibition of proteins translation, thus achieving the role of cancer promoting and inhibiting factors. Their molecular mechanism is still unclear [5]. For example, miR-137, miR-146a and miR-181 have been confirmed to be involved in the

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Received: 01/04/2020; Accepted: 28/04/2020

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proliferation and invasion of osteosarcoma. Hence, whether miR-432 and miR548c-3p were involved in the occurrence or progress of osteosarcoma was exactly the purpose of this study.

As a miR [6] involved in regulating lung cancer cells [7], did miR-423 also act on other tumor cells? Unfortunately there are few reports on such a relationship with the occurrence and development of osteosarcoma [8]. However, it was found in a previous study [9] that miR-432 showed low expression in osteosarcoma cells compared with normal cells, which might be related to the occurrence of osteosarcoma, but the specific mechanism of action was not yet clear. In addition, the involvement of miR-548c-3p was also found in lung cancer tissues, while miR-542c-3p was found that it could directly target the 3' untranslated region of ITGAV, and its overexpression inhibited the mRNA and protein levels of ITGAV, which was confirmed by luciferase reporter gene detection [10]. Therefore, in this study, the expressions of miR-432 and miR-548c-3p in osteosarcoma and their influences on cell proliferation and other biological functions were investigated, so as to provide new molecular directions for the diagnosis and treatment of osteosarcoma.

Methods

General data

A total of 67 patients with osteosarcoma (35 male and 32 female, mean age 47.8±3.5 years) who came to the third Affiliated Hospital of Southern Medical University from April 2015 to May 2018 formed the experimental group. There were 37 patients with grade I-II tumor differentiation, 30 with grade III-IV, 29 patients

Table 1. Clinicopathological characteristics

Characteristics	*Experimental group n (%)*	*Control group n (%)*	*t/x2*	*p value*
Gender			0.016	0.900
Male	35 (52.23)	30 (47.62)		
Female	32 (47.76)	33 (52.38)		
Age, years			0.034	0.854
≥47	39 (58.21)	38 (60.32)		
<47	28 (41.79)	25 (39.68)		
BMI			0.854	0.334
≥20	37 (55.22)	33 (52.38)		
<20	30 (44.77)	30 (47.62)		
Grade of differentiation				
Grade I-II	37 (55.22)	-	-	-
Grade III-IV	30 (44.77)	-	-	-
Case classifications				
Osteosarcoma	29 (43.28)	-	-	-
Chondrosarcomas	23 (34.32)	-	-	-
Giant cell tumor of bone	9 (13.43)	-	-	-
Bone marrow neoplasms	6 (8.96)			
Coagulation functions (mean±SD)				
APTT s	28.89±2.71	29.02±2.78	0.278	0.781
PT s	11.97±1.02	12.05±0.99	0.467	0.462
FIB g/l	3.14±0.23	3.17±0.22	0.781	0.436
TT s	14.78±1.62	14.55±1.59	0.840	0.402
Hepatic function index				
Total serum protein (g/L)	71.67±2.54	71.91±2.67	0.542	0.589
Glutamic-pyruvic transaminase (μmol/L)	26.13±4.27	26.19±4.08	0.084	0.933
Total bilirubin (μmol/L)	11.25±2.08	11.13±2.11	0.336	0.737
Renal function index (μmol/L)				
Creatinine	69.45±4.23	70.12±4.19	0.934	0.352
Serum urea	5.78±0.79	5.71±0.87	0.497	0.620
Uric acid	297.56±13.73	295.09±14.01	1.046	1.297

with osteoblastic osteosarcoma, 23 patients with chondrogenic osteosarcoma, 9 patients with giant cell tumor of bone, and 6 patients with bone marrow tumor. A total of 63 healthy subjects who came to the third Affiliated Hospital of Southern Medical University for physical examination during the same period formed the control group. There was no significant difference between the two groups in terms of gender, age, body mass index (BMI) and other factors ($p>0.05$).

Inclusion and exclusion criteria

Inclusion criteria: Patients pathologically diagnosed with osteosarcoma.

Exclusion criteria: Patients with other serious organ dysfunction or cancers. Patients with communication and mental disorders. Patients who did not cooperate with the medical staff. All subjects and their families agreed to participate in the study and signed the informed consent forms. This study was approved by the ethics committee of the third Affiliated Hospital of Southern Medical University (Table 1).

Materials and reagents

Unknown sources of bone sarcoma cells GM-63 were bought from Shanghai Zeye Biological Technology Co. Ltd. Real-time quantitative PCR was bought from BioRad Company, United States. Flow cytometry CytoFLEX LX was purchased from Beckman Company, United States. DMEM culture medium was purchased from Gibco Company, United States. Fetal bovine serum (FBS) and trypsin were purchased from Hyclone, United States. Trizol reagent was purchased from Invitrogen Company, USA, qPCR kit and minScript reverse transcription kit were purchased from TaKaRa, Dalian, China. miR-432 mimics, miR-548c-3p mimics, miR NC and internal reference U6 primers were designed and synthesized by Shanghai Jima Company. The CCK-8 reagent was purchased from Promega, USA and the Annexin V-FITC/PI cell apoptosis kit was purchased from Jiangsu Kaiji Biological Co. Ltd, Jiangsu, China.

Expressions of miR-432 and miR-548c-3p in the serum of patients detected by RT-PCR [11]

In the morning 3 ml fasting venous blood was obtained from all subjects and centrifuged at 3000 r/min for 5 min. After centrifugation, the serum was placed in a refrigerator at -80°C for later measurement. The total RNA was extracted by adding Trizol reagent into the serum reagent tube and the purity and concentration of RNA were determined by ultraviolet spectrophotometer. One µg total RNA was taken and cDNA was synthesized according to the instructions of reverse transcription kit. The ratio of optical density (OD) 260/OD280 was 1.8, and the ratio of OD260/OD230 was 2.2. Two µg synthesized DNA were taken for PCR in reaction conditions of predenaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 60°C for 45 s. The expression levels of miR-432 and miR-548c-3p were detected with U6 as the internal reference. The primer sequences of miR-432 and miR-548c-3p are shown in Table 2, and the experiments were repeated in triplicate.

Cell culture, transmission and transfection

Bone sarcoma GM-63 cell line was placed in RMI1640 medium containing 10% FBS, and cultured at 37°C and 5% CO_2 in incubator. When the cell confluence reached 80%, they were digested with 25% trypsin. After digestion, the cells were transfected at 37°C and 5% CO_2. The expressions of transfected miR-432 and miR-548c-3p and miR-NC were detected by qRT-PCR after 48-h transfection, and the cells were collected for further experiments.

The effects of cell proliferation detected by CCK-8 [12]

Transfected cells were collected and placed in well plates at a density of 3×10^4/well. The cells were inoculated in 96-well plates with 100 µl cells/well with 37°C and 5% CO_2. Ten µl CCK8 solution was added to each well on days 1, 2, 3, 4, 5, and the culture was continued in the incubator after the addition of reagents. OD value was measured at 450 nm using a microplate reader after 2-h culture to detect cell proliferation and to draw growth curves. The experiments were performed in triplicate.

The effects of cell invasion detected by transwell assay

The cells were collected 24 h after transfection, with 3×10^5 cells/well. Then the cells were inoculated on 6-well plates, washed twice with PBS, and inoculated in the upper chamber. 200 µl DMEM culture medium was added to the upper chamber, and 500ml DMEM with 20% FBS was added to the lower chamber. The cells were cultured at 37°C for 48h. The substrate and cells that did not pass through the surface of the upper chamber were wiped out and the remaining cells were rinsed with PBS 3 times, then fixed with paraformaldehyde for 10min, rinsed with double distilled water 3 times, dried and stained using 0.5% crystal violet. The cell invasion was observed with a microscope.

Cell apoptosis rate assessed by flow cytometry

Annexin V-FITC/PI double staining combined with flow cytometry [13] was used to detect apoptosis. Gm-63

Table 2. Correlated primer sequences list

Primer	*Sense primer*	*Reverse primer*
miR-432	5'-CGACGCGTACTCAAACACTTCGGACATGG-3	5'-CCCAAGCTTCAAAGAGCAACAGAGAGTAGCA-3'
miR-548c-3p	5'-TGTGACAGATTGATAACT-3'	5'-CGTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGCACTGGATACGACCTGCGGTTTCAGT-3'
U6	5'-ATTGGAACGATACAGAGAAGATT-3'	5'-GGAACGCTTCACGAATTTG-3'

cells transfected with miR432, miR-548c-3p and miR NC were inoculated at the density of 3×10^5/well in 6-well plates. After 24-h incubation, they were washed twice with PBS and 5 µl Annexin V-FITC were added. After incubation at room temperature for 10 min, 10 µl PI were added, and then incubated in the dark for 20 min at room temperature. Finally, flow cytometry was used to detect apoptosis. The experiments were repeated in triplicate.

Statistics

SPSS 18.0 software (BinZinsight, Beijing Information Technology Co., Ltd.) was used in this study to carry out the statistical analyses of data and GraphPad Prism 6 software was used to draw all images of the experiment. Enumeration data were compared by chi-square test and measurement data were expressed as mean±standard deviation. T-test was used for comparison between two groups, and one-way analysis of variance (ANOVA) was used to make comparisons among groups. P<0.05 signified statistically significant differences.

Results

The expressions of miR-432 and miR-548c-3p in the sera of the two groups

The relative expression of miR-432 in the serum of patients in the experimental group was 0.42±0.09, and the relative expression of miR-548c-3p in the serum of patients was 0.59±0.11, which was significantly lower than those of the control group. The differences were statistically significant (p>0.05). More details are shown in Table 3 and Figure 1.

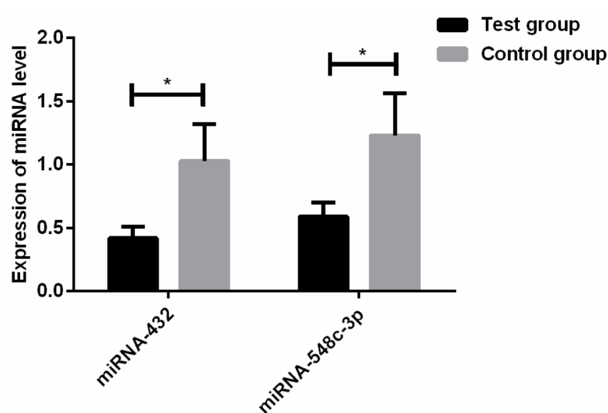

Figure 1. Expression of two groups of miR-432 and miR-548c-3p in tumor cells and normal cells. *P<0.05.

The expressions of miR-432 and miR-548c-3p in the three groups of cells after transfection

After transfection, PCR was used to detect the expressions of miR-432 and miR-548c-3p in the cells. The mean expressions of miR-432 and miR-548c-3p were 12.32±3.96 and 11.45±3.02, respectively, which were significantly higher than those of the blank group and control group. The differences were statistically significant, and more details are shown in Figure 2.

The effects of miR-432 and miR-548c-3p on cell proliferation

Cell proliferation was detected at different time points after transfection, and no significant difference in the cell proliferation ability of the three groups on day 1 and 2 was detected (p>0.05). How-

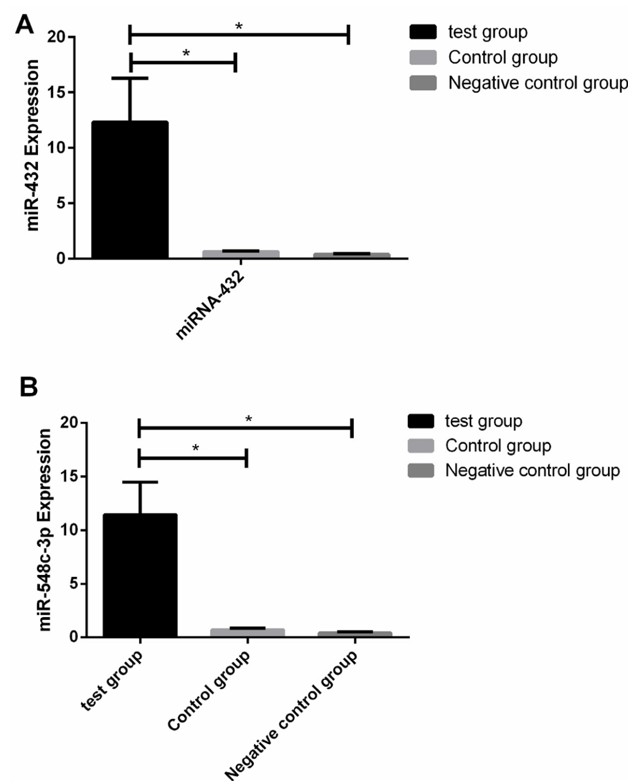

Figure 2. Expressions of miR-432 and miR-845c-3p in cells after transfection. **A:** Expression of miR-432 after transfection for 48h in gm-63 cells; **B:** Expression of miR-548c-3p after transfection for 48h in gm-63 cells. *P<0.05.

Table 3. The expressions of miR-432 and miR-548c-3p

Factors	*Experimental group n=67*	*Control group n=63*	*t*	*p value*
miR-432	0.42±0.09	1.03±0.29	16.40	<0.001
miR-548c-3p	0.59±0.11	1.23±0.33	15.02	<0.001

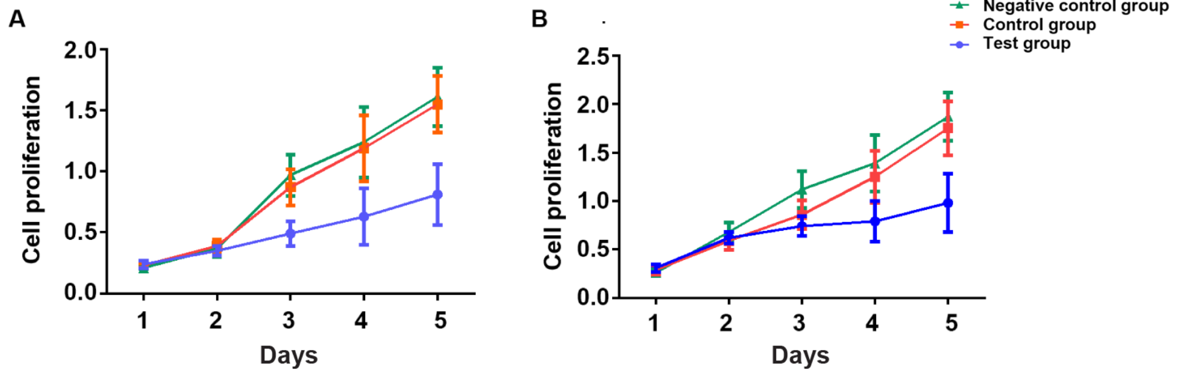

Figure 3. The effects of miR-432 and miR-548c-3p on cell proliferation. **A:** Expression of miR-432 on cell proliferation in different time points. **B:** Expression of miR-548c-3p on cell proliferation in different time points. P<0.05.

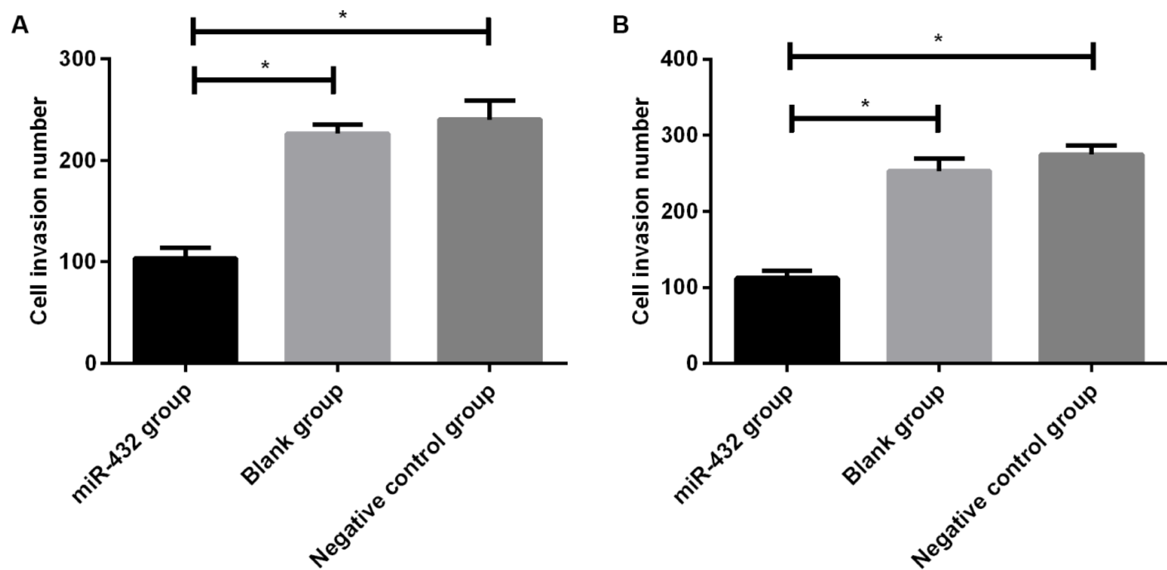

Figure 4. The effects of miR-432 and miR-548c-3p on cell invasion. **A:** Expression of miR-432 on cell invasion in different groups. **B:** Expression of miR-548c-3p on cell invasion in different groups. *P<0.05.

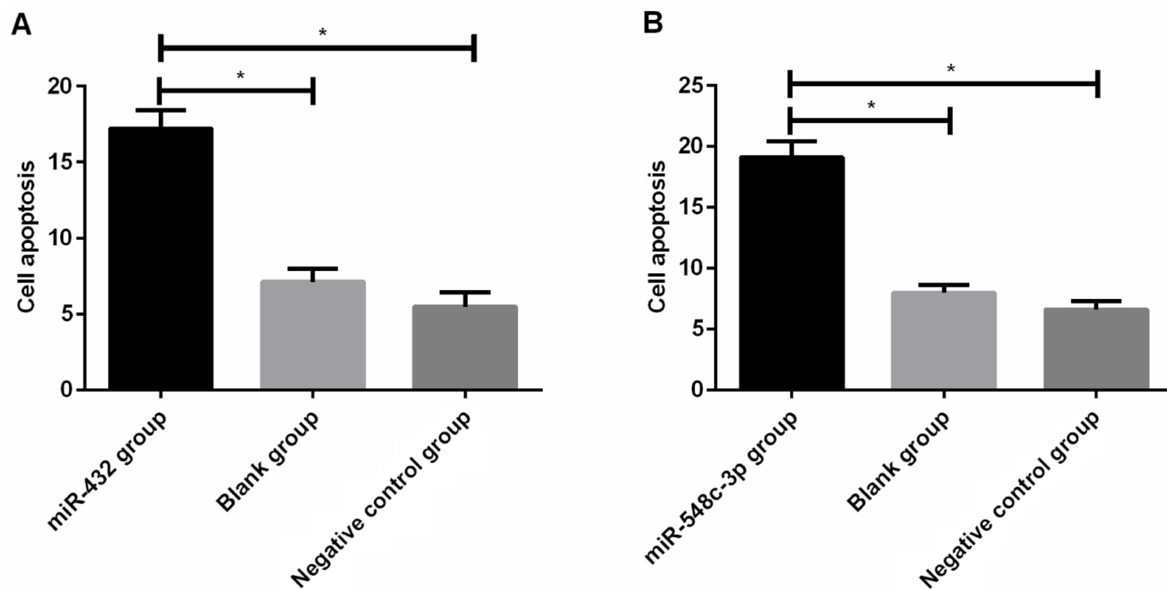

Figure 5. The effects of miR-432 and miR-548c-3p on cell apoptosis. **A:** Expression of miR-432 on cell apoptosis in different groups. **B:** Expression of miR-548c-3p on cell apoptosis in different groups. *P<0.05.

ever, the cell proliferation ability of the experimental group was significantly lower than that of the blank group and the negative control group from day 3. These differences were statistically significant ($p<0.05$). More details are shown in Figure 3.

The effects of miR-432 and miR-548c-3p on cell invasion

After 3 days, the mean number of transmembrane cells in the miR-432 group was 103.4±10.5, which was significantly lower than in the control group (226.7±8.5) and the negative control group (240.3±18.5) ($p<0.05$). The mean number of transmembrane cells in the miR-548c-3p group was 112.45±9.4, which was also significantly lower than in the control group (253.14±16.7) and the negative control group (274.76±11.8) ($p<0.05$). More details are shown in Figure 4.

The effects of miR-432 and miR-548c-3p on cell apoptosis

The mean apoptosis rate was 5.49 ± 0.94 in the negative control group, 7.12±0.86 in the blank group, and 17.21 ± 1.23 in the miR-432 group. The mean apoptosis rate of miR-548c-3p group was 19.11±1.32, that of the blank group was 7.98±0.64, and that of the negative control group was 6.62±0.71. The apoptosis rate of the experimental group was significantly higher than that of the negative control group and the blank group ($p<0.05$). There was no significant difference in the apoptosis rate between the negative control group and the blank group ($p>0.05$). More details are shown in Figure 5.

Discussion

Osteosarcoma is a primary malignant tumor with high incidence among adolescents. In recent years, however, the survival rate of patients has been improving, which is related to the improvement of chemotherapy and radiotherapy techniques. However, it is still a clinical problem that tumor cells are prone to distant metastasis. miRs can be considered to fundamentally inhibit and reduce the invasion and metastasis of tumor cells [14,15]. Studies have shown that a variety of miRs have abnormal expression in osteosarcoma. For example, miR-128 has been found to have an effect on the proliferation and invasion of osteosarcoma cells, and the mechanism of action has been attributed to the regulation on the targeted gene STAT3 [16]. Due to this previous data this study investigated the expressions of miR-432 and miR-548c-3p in osteosarcoma, and their effects on tumor cell proliferation and invasion.

MiR-432 and miR-548c-3p have shown low expression in cervical cancer, lung cancer, ovarian cancer and other tumor cells, which could participate in the occurrence and development of these cells. Moreover, overexpression of miR-432 has been shown to inhibit cell proliferation [17]. Wang et al [18] also found that the overexpression of miR-548-3p could inhibit the proliferation and cloning ability of liver cancer cells. We wondered whether miR-548c-3p could have the same properties. Another study [19] showed that miR-548-3p could be used as a tumor suppressor in the invasion and metastasis of breast cancer cells, while miR-548-3p and miR-548c-3p belonged to the same family and had been involved in the occurrence and development of breast cancer tumor cells [20]. It was speculated that this factor could play the same anticancer effect in osteosarcoma, which was also the reason why this factor was selected in this study.

The results of this study revealed that miR-432 and miR-548c-3p in patients with osteosarcoma showed low expressions. After transfection of miR-432 and miR-548c-3p to increase the expression of GM-63 cells, it was found that the overexpression of miR-432 and miR-548c-3p could inhibit the proliferation, migration and invasion of GM-63 cells, suggesting that miR may play an anti-oncogene role in osteosarcoma.

In this study we compared the expressions of miR-432 and miRNA-548c-3p in the serum of patients with osteosarcoma and healthy subjects by PCR and the influence of different pathological factors on these miRs firstly, and found that the relative expression levels of serum miR-432 and miR-548c-3p of patients in the experimental group were significantly lower than those of subjects in the control group ($p<0.05$). In the experimental group, the lower the grade of tumor differentiation, the lower the relative expression level ($p<0.05$), and there was no significant difference in the relative expression levels of miR-432 and miR-548c-3p in different pathological types ($p>0.05$). In order to investigate the effects of miR-432 and miR-548c-3p on the biological functions of osteosarcoma, we cultured the osteosarcoma GM-63 cells and transfected miR-432 mimics and miR-548c-3p mimics. When the content of miR-432 and miR-548c-3p in GM-63 cells transfected with miR-432 mimics and miR-548c-3p mimics was significantly higher than that of miR-432 and miR-548c-3p in the blank group and the negative control group, the transfection was confirmed and the follow-up experiments were continued. We compared the conditions of proliferation and invasion of the three groups follow-up, finding that there was no significant difference in the three groups on the ability of proliferation on

day 1 and 2 ($p>0.05$). From day 3 on, however, the ability of proliferation in the experimental group was significantly lower than that of the blank group and the negative control group ($p<0.05$); the ability of invasion of the negative control group and the blank group was significantly lower than that of the experimental group ($p<0.05$). Studies have shown that miR-432 acted as an anti-tumor factor in lung cancer cells, thus inhibiting their invasion and accelerating the apoptosis, which is consistent with the results of this conjecture study.

In summary, miR-432 and miR-548c-3p expression is low in osteosarcoma, and their overexpression could inhibit the proliferation and invasion ability of tumor cells. However, the correlation between miR-432 and miR-548c-3p was not explored further in this study, so it is hoped that future researchers could conduct further in-depth research on this issue.

Conflict of interests

The authors declare no conflict of interests.

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