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ORIGINAL ARTICLE

MiR-505-5p inhibits proliferation and promotes apoptosis of osteosarcoma cells via regulating RASSF8 expression

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Summary

Purpose: *The purpose of this study was to observe the effects of micro ribonucleic acid (miR)-505-5p on the proliferation and apoptosis of osteosarcoma cells, and to further investigate its potential mechanism.*

Methods: *Human osteosarcoma U2-OS cell lines were divided into Control group, miR-505-5p nonsense sequence (NS) group and miR-505-5p inhibitor group. Subsequently, cell proliferation and apoptosis in each group were observed. Finally, the effect of miR-505-5p on the in vivo growth of osteosarcoma was explored by means of subcutaneous tumor formation assay.*

Results: *The expression of miR-505-5p in the cancer tissues was remarkably higher than in normal paracancer tissues of osteosarcoma patients. U2-OS cell lines cultured in vitro in miR-505-5p inhibitor group manifested notably weakened proliferative ability after transfection with miR-505-5p inhibitor. Colony formation assay showed that the number of colonies formed in miR-505-5p inhibitor group was obviously smaller than that in Control group. The results of Western blotting assay indicated that the inhibition of miR-505-5p markedly increased the expression of Bax and decreased Bcl-2 in cancer cells ($p<0.05$). Furthermore, it was revealed that the inhibited miR-505-5p could distinctly up-regulate the protein expression level of RASSF8 in cancer cells. Furthermore, the miR-505-5p inhibition was able to prominently repress the subcutaneous tumor formation ability of osteosarcoma cells.*

Conclusions: *The expression level of miR-505-5p is raised significantly in the cancer tissues of osteosarcoma patients, and the inhibition of miR-505-5p can up-regulate RASSF8 to suppress the proliferation and promote the apoptosis of osteosarcoma cells.*

Key words: *miR-505-5p, osteosarcoma, proliferation, apoptosis, RASSF8*

Introduction

Osteosarcoma is the most common primary malignant bone tumor that originates from primitive mesenchymal cells. There are few cases of classic osteosarcoma, with about 3 cases among 1,000,000 people, accounting for 0.2% of all the malignant tumors [1]. Osteosarcoma mainly occurs in the metaphysis of long bones of the four limbs and rarely appears in soft tissues. The prevalence age of osteosarcoma displays a bimodal distribution, with the childhood and young adults as the first peak and the old age as the second peak. A few decades ago, the patients with osteosarcoma were usually treated with amputation, but only less than 20% of the patients could be cured completely [2]. Nowadays, preoperative chemotherapy, local resection and postoperative adjuvant chemotherapy are considered as the standard therapeutic methods for osteosarcoma patients. Nevertheless, the prognosis

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of the patients is still poor, and the survival is less than 30% [3]. Therefore, it is of great significance for the prevention and treatment of osteosarcoma in the future to further elaborate the pathological characteristics and molecular mechanism of this disease.

Micro ribonucleic acids (miRs) are a group of single-stranded non-coding RNAs with a length of 20-24 nt, which exist in eukaryotes and possess regulatory functions [4]. MiRs can regulate the expression of many genes by binding to specific genes in a targeted manner, so they play vital roles in cell proliferation, differentiation, invasion, apoptosis and other activities [5]. Large numbers of clinical and basic studies have revealed that abnormal changes occur in the expression profiles of miRs during tumorigenesis, which promote or inhibit the occurrence and development of tumors by affecting the proliferation, apoptosis, invasion and epithelial-mesenchymal transition (EMT) of tumor cells [6]. For example, miR-410 can reduce the proliferative and invasive abilities of osteosarcoma cells through repressing the expression of VEGF [7]. MiR-187 suppresses the proliferation migration and invasion of human osteosarcoma cells by targeting MAPK7 [8]. Moreover, the overexpression of miR-126 can prominently enhance the sensitivity of osteosarcoma cells to epigallocatechin-3-gallate-induced apoptosis [9]. However, the expression and function of miR-505-5p in osteosarcoma patients have not been researched and reported yet.

In this research, the expression of miR-505-5p in cancer and paracancer tissues of osteosarcoma patients as well as in osteosarcoma cells was detected, and the influences of miR-505-5p on the proliferation and apoptosis of human osteosarcoma U2-OS cell lines were observed, so as to provide reference bases for further optimizing the treatment protocols for osteosarcoma patients in the future.

Methods

Tissue specimens

The cancer and paracancer tissue specimens were collected from 45 osteosarcoma patients treated with operation in our hospital. After the bloodiness was removed using normal saline, all the specimens were cut into pieces, put into Eppendorf (EP) tubes and stored in a refrigerator at -80°C. Otherwise, the specimens were not cut off but fixed with 10% neutral formalin. This study was approved by the Ethics Committee of Yanan People's Hospital. Signed written informed consents were obtained from all participants before the study entry.

Human osteosarcoma U2-OS cell lines

The human osteosarcoma U2-OS cell lines were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences, and were cultured with Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) in a constant-temperature (37°C) incubator, followed by subculture once every 2-3 d. Then the osteosarcoma cells were seeded into 6-well plates supplemented with medium and cultured in the constant-temperature incubator for 36 h. When the cell density in the culture dish reached 70-80%, the cells were transfected with miR-505-5p nonsense sequence (NS) and miR-505-5p inhibitor added into the medium according to the instructions of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Finally, the cells were cultured again in the constant-temperature (37°C) incubator.

Detection of mRNA expression of miR-505-5p in cancer and paracancer tissues via reverse transcription-polymerase chain reaction (RT-PCR) assay

First, total RNAs were extracted from the cancer and paracancer tissues as well as the human osteosarcoma U2-OS cell lines using TRIzol method (Invitrogen, Carlsbad, CA, USA). Next, the concentration and purity of the RNAs extracted were measured by an ultraviolet spectrophotometer, with A_{260}/A_{280}=1.8-2.0. Later, the RNAs were synthesized into complementary DNAs (cDNAs) which were stored in the refrigerator at -80°C. Subsequently, the RT-PCR assay was performed in a system containing 2.5 μL of 10× Buffer, 1 μL of cDNAs, 0.5 μL of forward primer (20 μmol/L), 0.5 μL of reverse primer (20 μmol/L), 10 μL of LightCycler® 480 SYBR Green I Master (2×) and 5.5 μL of ddH2O. The RT-PCR amplification system was the same. Primer sequences used in the study are shown in Table 1.

Determination of proliferation status of each group of cells through cell counting kit-8 (CCK-8) assay

The cells in the logarithmic growth phase in each group were inoculated into 96-well plates and cultured in an incubator with 5% CO2 at 37°C for 0, 12, 24, 48 and 72 h, followed by discarding of the medium and preparation of developing reagent in the dark at a proportion of medium: CCK-8 solution =10:1 (Dojindo, Kumamoto, Japan). After that, 110 μL of developing reagent was added into each well of the 96-well plate for incubation in the constant-temperature (37°C) incubator for 2 h, and the optical density (OD) value at 540 nm in each group was determined using ultraviolet spectrophotometer.

Table 1. Primer sequences of indexes for RT-PCR

Target gene	*Primer sequence*
GAPDH	
Forward	5'-GACATGCCGCCTGGAGAAAC-3'
Reverse	5'-AGCCCAGGATGCCCTTTAGT-3'
MiR-505-5p	
Forward	5'-GTCCACGAACCCGTAAGGT-3'
Reverse	5'-CATCTTACACGATCGCGTCCA-3'

Colony formation assay

The cells in each group were cultured into the logarithmic phase and then digested into single cell suspension with 0.25% trypsin, ensuring a proportion of single cells >95%. Next, the cell suspension was inoculated into the 6-well plates, with about 500 cells in each well. Subsequently, each well was added with 2 mL of RPMI-1640 medium containing 10% FBS, which was replaced every 2 days. Ten days later, the cells were fixed in formaldehyde and stained with crystal violet, and the number of colonies in each well plate was recorded.

Measurement of expressions of related proteins via Western blotting assay

First, the solution in the medium was discarded, and the medium was rinsed by phosphate-buffered saline (PBS) for 3 times. Second, 1,000 μL of cell lysate was added into each dish and shaken sufficiently for 10 min. Then the cells at the bottom of the dish were fully scrapped using a brush and put into prepared Eppendorf (EP) tubes. Third, the cells collected were lysed in an ultrasonication instrument for no more than 15 s (1-2 s/time). After standing for 15 min, the cells were centrifuged at 12,000 r/min for 0.5 h. Fourth, the supernatant was aspirated and subpackaged into EP tubes. Later, the protein concentration was determined through bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometric assay, and all the sample proteins were maintained at a constant volume and equal concentration. Next, the proteins were subpackaged and preserved in a refrigerator at -80°C. Fifth, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted, after which the proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland) and incubated with primary antibodies at 4°C overnight. Following the incubation with goat anti-rabbit secondary antibodies in the dark for 1 h, the protein bands were scanned and quantified using an Odyssey membrane scanner (Seattle, WA, USA), with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as the internal reference of proteins.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining for cells

The cells were seeded into 24-well plates containing coverslips, and the cells on the coverslips were fixed in paraformaldehyde for 30 min and then washed with PBS for 3 times. After permeation with 0.2% Triton X-100, TUNEL reagent (Solarbio, Beijing, China) was added dropwise onto the coverslips carefully for 1-h incubation. Subsequently, the nuclei were labeled by propidium iodide (PI) dye. Finally, the cells were observed, photographed and counted under a fluorescence microscope after the staining.

Subcutaneous tumor formation assay in nude mice

A total of 30 nude mice were randomly divided into 3 groups, identical to the grouping of cells. Matrigel and PBS were prepared into mixture at 1:1, and the 3 groups of osteosarcoma cells were resuspended into single-cell suspension. After the nude mice in the 3 groups were anesthetized by diethyl ether, the 3 groups of cells (10^7) were inoculated into the loose skin on the lateral side of right forelimb. Six weeks later, the mice were euthanized by cervical dislocation, and the tumor masses were isolated and measured.

Statistics

All the data were analyzed using SPSS 22.0 software (IBM, Armonk, NY, USA). The measurement data were presented as mean ± standard deviation, and t-test was performed for data comparison between two groups. $P<0.05$ suggested that the difference was statistically significant.

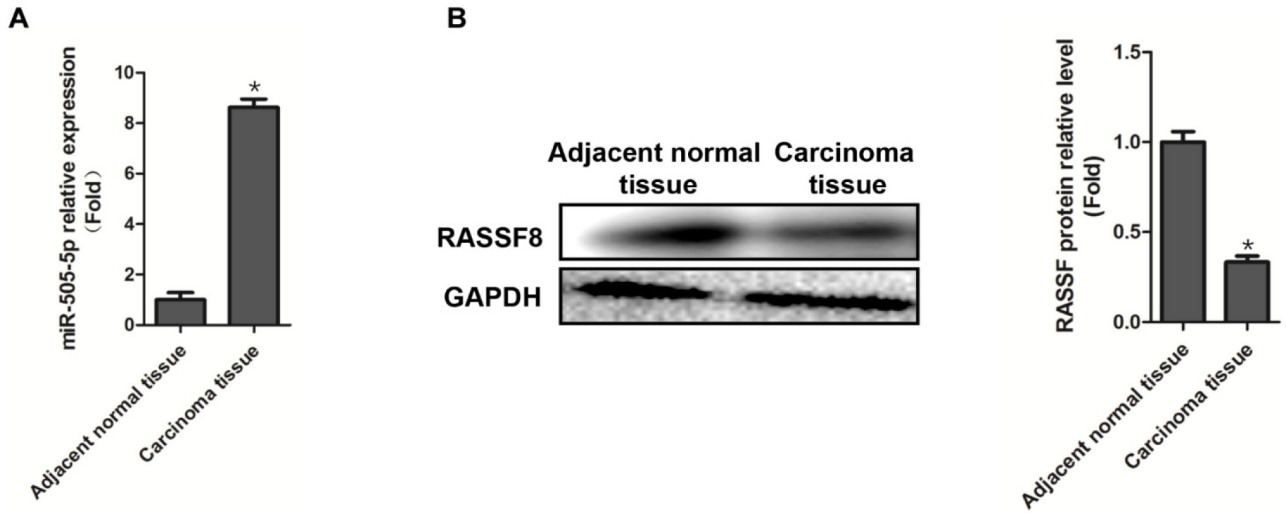

Figure 1. Expression level of miR-505-5p in cancer tissues and normal paracancer tissues of osteosarcoma patients. Adjacent normal tissues: normal paracancer tissues, Carcinoma tissues: cancer tissues. *p<0.05.

Results

Expression of miR-505-5p in cancer tissues and normal paracancer tissues of osteosarcoma patients

The expression level of miR-505-5p in the cancer tissues and normal paracancer tissues of 45 osteosarcoma patients was measured through RT-PCR assay (Figure 1) which indicated that the expression level of miR-505-5p in the cancer tissues of osteosarcoma patients was remarkably higher than in the normal paracancer tissues, about 8.65-fold higher that in the paracancer tissues ($p<0.05$). Previous studies have demonstrated that Ras association domain family 8 (RASSF8) is a direct target of miR-505-5p. Therefore, the protein expression level of RASSF8 in the normal paracancer and cancer tissues was tested by means of Western blotting assay. The results manifested that the cancer tissues had a notably lower protein expression level of RASSF8 than the normal paracancer tissues ($p<0.05$).

Construction of human osteosarcoma cell lines with miR-505-5p knockdown

In order to investigate the effects of miR-505-5p on the proliferation and apoptosis of human osteosarcoma cells, the human osteosarcoma U2-OS cell lines with targeted knockdown of miR-505-5p were constructed. It was shown in the results of RT-PCR assay that the miR-505-5p inhibitor obviously reduced the expression of miR-505-5p in human osteosarcoma U2-OS cell lines ($p<0.05$), suggesting that the human osteosarcoma cells with specific miR-505-5p knockdown were constructed successfully (Figure 2).

Effect of miR-505-5p knockdown on the proliferation of human osteosarcoma cells

According to the results of CCK-8 assay for cell proliferation (Figure 3), the proliferative ability of the human osteosarcoma cells was markedly weakened by inhibition of miR-505-5p at 24, 48 and 72 h ($p<0.05$), while no apparent impact on the proliferative ability of the tumor cells was observed at 12 h ($p>0.05$).

Effect of miR-505-5p knockdown on colony formation ability of human osteosarcoma cells

The results of colony formation assay showed that the number of colonies of osteosarcoma cells

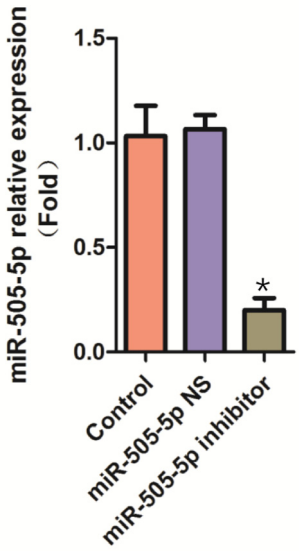

Figure 2. Construction of human osteosarcoma cell lines with miR-505-5p knockdown. Control: Control group, miR-505-5p NS: miR-505-5p nonsense sequence group, miR-505-5p inhibitor: miR-505-5p inhibitor group. *$p<0.05$.

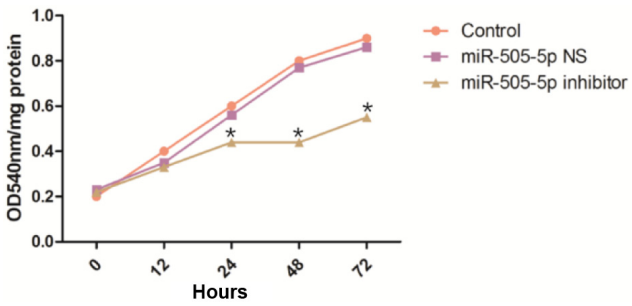

Figure 3. Effect of miR-505-5p knockdown on proliferation of human osteosarcoma cells. Control: Control group, miR-505-5p NS: miR-505-5p nonsense sequence group, miR-505-5p inhibitor: miR-505-5p inhibitor group. *$p<0.05$.

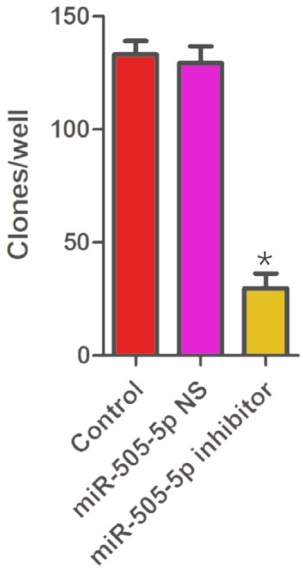

Figure 4. Effect of miR-505-5p knockdown on colony formation ability of human osteosarcoma cells. Control: Control group, miR-505-5p NS: miR-505-5p nonsense sequence group, miR-505-5p inhibitor: miR-505-5p inhibitor group. *$p<0.05$.

formed was evidently decreased on 10 days after inhibiting miR-505-5p [(137±9) *vs.* (32±7)] ($p<0.05$), implying that the miR-505-5p inhibitor has the potential to repress the colony formation of osteosarcoma cells (Figure 4).

Effect of miR-505-5p knockdown on apoptosis of human osteosarcoma cells

Western blotting assay and TUNEL staining were utilized to detect the expressions of apoptosis-related proteins and genomic DNA fragmentation, respectively, in the 3 groups of cells, so as to further explore the effect of miR-505-5p on the apoptosis of human osteosarcoma cells. It was shown that the expression level of anti-apoptotic protein Bcl-2 declined clearly, while that of pro-apoptotic protein Bax rose distinctly in miR-505-5p inhibitor group ($p<0.05$) (Figure 5A). Meanwhile, the percentage of TUNEL-positive cells in miR-505-5p inhibitor group was evidently higher than that in Control group ($p<0.05$) (Figure 5B). Both assays demonstrated that the miR-505-5p inhibitor significantly enhances the apoptosis of human osteosarcoma cells.

Effect of miR-505-5p knockdown on protein expression of RASSF8 in human osteosarcoma cells

As shown in the results of Western blotting assay (Figure 6), the protein expression level of RASSF8 was elevated prominently in miR-505-5p inhibitor group compared with the Control group ($p<0.05$).

Effect of miR-505-5p knockdown on subcutaneous tumor formation ability in nude mice

Based on the results of subcutaneous tumor formation assay (Figure 7), the knockdown of miR-505-5p could remarkably suppress the *in vitro* tumor formation ability of human osteosarcoma cells ($p<0.05$), further corroborating that the miR-505-5p inhibitor is able to resist the growth of osteosarcoma cells to some extent.

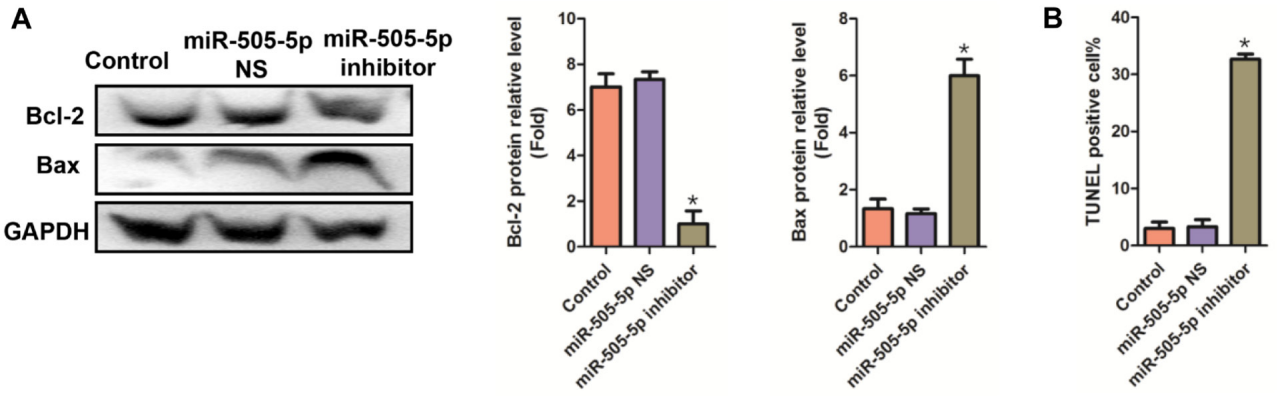

Figure 5. Effect of miR-505-5p knockdown on apoptosis of human osteosarcoma cells. Control: Control group, miR-505-5p NS: miR-505-5p nonsense sequence group, miR-505-5p inhibitor: miR-505-5p inhibitor group. *p<0.05.

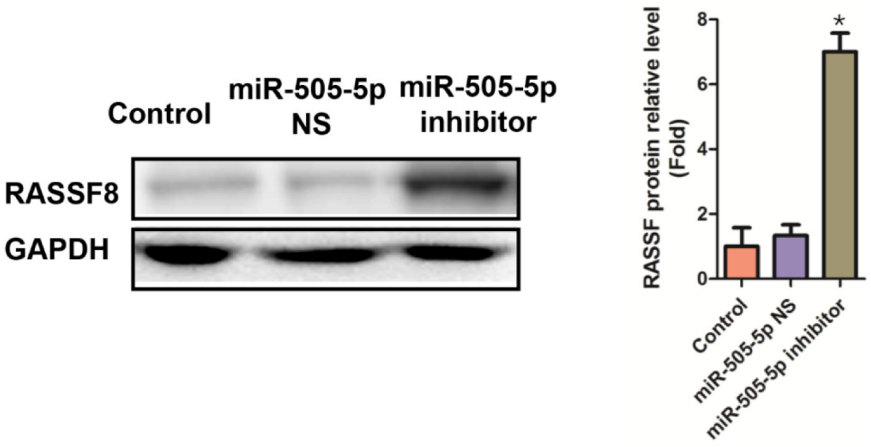

Figure 6. Effect of miR-505-5p knockdown on protein expression of RASSF8 in human osteosarcoma cells. Control: Control group, miR-505-5p NS: miR-505-5p nonsense sequence group, miR-505-5p inhibitor: miR-505-5p inhibitor group. *p<0.05.

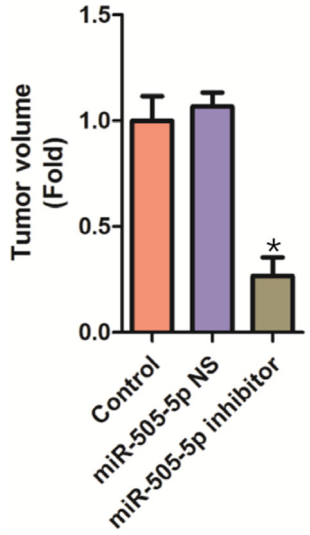

Figure 7. Effect of miR-505-5p knockdown on subcutaneous tumor formation ability in nude mice. Control: Control group, miR-505-5p NS: miR-505-5p nonsense sequence group, miR-505-5p inhibitor: miR-505-5p inhibitor group. *p<0.05.

Discussion

Osteosarcoma is the most common primary bone tumor, but its absolute incidence rate is relatively low among malignant tumors. It may emerge inside the bone (intramedullary cavity or cortical cavity), on the bone surface and outside the bone, so the prognosis of the patients not only depends on the histological features and grades of tumor but also has an association with the location of tumor [10,11]. Although osteosarcoma occurs most frequently in patients at the age ranging from 5 years to early adulthood, its morbidity rate can reach the peak again in the populations aged 65 years old, which is probably related to past history of Paget's disease or radiotherapy [12]. Hence, it is necessary to deeply clarify the aberrant gene regulatory networks during the occurrence and development of osteosarcoma, so as to provide certain evidence for targeted gene therapy or drug therapy for the disease in the future.

As a kind of endogenous non-coding small RNAs with 20-24 nucleotides in length, miRs are capable of regulating the expressions of various genes in organisms at the post-transcriptional level [13]. Specifically, miRs can bind to the 3'UTR of target genes to inhibit the translation of corresponding proteins and they are vital players in multiple life activities of cells [14]. Recent studies have uncovered that the abnormal expressions of nucleases Drosha and Dicer as well as 3'-5' exonuclease during the generation of miRs can increase the susceptibility of osteosarcoma patients, illustrating that miRs have important significance in the occurrence and development of osteosarcoma [15]. In the case of osteosarcoma, miRs can serve as anti-oncogenes or oncogenes to modulate the differentiation, proliferation, apoptosis, invasion and cycle of cancer cells. For instance, miR-217 inhibits the invasion and metastasis of osteosarcoma cells by binding to WASF3 in a targeted manner [16]. MiR-138 can evidently enhance the sensitivity of osteosarcoma cells to cisplatin through targeting EZH2 [17]. On the contrary, miR-374 activates the Wnt/β-catenin signaling pathway to facilitate the migration of osteosarcoma cell by repressing WIF-1 in a targeted manner [18]. Moreover, miR-19b decreases the mitofusin 1 (Mfn1)-induced apoptosis of tumor cells by means of targeted inhibition on the expression of Mfn1 in osteosarcoma cells, thus exerting pro-oncogenic effects [19]. In addition, it has been discovered in some clinical studies that miRs can predict the prognosis and survival status of osteosarcoma patients. For example, the high expression of miR-19a in osteosarcoma patients signifies poor prognosis of patients. To be more specific, osteosarcoma patients with a high expression of miR-19a have bigger tumors, higher clinical stage and positive rate of distant metastasis, and poorer chemotherapy response [20]. In contrast, the expression of miR-126 is lowered distinctly in osteosarcoma tissues, and the osteosarcoma patients with lowly expressed miR-126 have a higher clinical stage and a shorter overall survival [21]. In this research, it was found that the expression of miR-505-5p was clearly higher in osteosarcoma tissues. The results of *in vitro* experiments uncovered that inhibiting the expression of miR-505-5p in osteosarcoma tissues could markedly weaken the proliferative ability and promoted the apoptosis of tumor cells.

RASSF8 gene is located near KRAS2 gene in the chromosome 12, which contains a Ras-association domain and encodes a type of protein evolving from fish into humans, with preferable antitumor effects. Previous studies have elaborated that RASSF8 is able to exert tumor-suppressive effects via regulating the Wnt and NF-κB signaling pathways [22]. MiR-505 mediates the resistance of colorectal cancer to methotrexate by conjugating with RASSF8 in a targeted manner, demonstrating that RASSF8 can serve as a direct-action target downstream of miR-505 [23]. In this research it was shown that the protein expression of RASSF8 was notably lower in the cancer tissues than in the paracancer tissues of osteosarcoma patients. Based on the results of *in vitro* experiments, the RASSF8 expression was apparently up-regulated after inhibiting the expression of miR-505-5p, im-

plying that miR-505-5p may stimulate the growth of osteosarcoma cells by targeted repression of the anti-oncogene RASSF8.

Conclusions

In conclusion, it was discovered for the first time in this research that miR-505-5p was lowly expressed in osteosarcoma, which might promote the proliferation and inhibit the apoptosis of osteosarcoma cells by suppressing RASSF8 in a targeted manner.

Conflict of interests

The authors declare no conflict of interests.

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