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BMP-2 and miR-29c in osteosarcoma tissues on proliferation and invasion of osteosarcoma cells

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Abstract. Expression of bone morphogenetic protein (BMP)-2 and microRNA (miR)-29c in osteosarcoma tissues and effects on proliferation and invasion of osteosarcoma cells were investigated. A retrospective analysis of 75 patients with osteosarcoma who underwent surgery in Tianjin Baodi Hospital from May 2013 to June 2017 was conducted. A total of 75 osteosarcoma tissues and 51 normal paraneoplastic tissues were collected. RT-PCR was used to compare the expression difference of BMP-2 and miR-29c. miR-29c mimics (experimental group A) and BMP-2 siRNA plasmid (experimental group B) were transfected into human osteosarcoma cells MG-63, respectively. The transfected cells were divided into miRNA negative control (miR-NC) group (cells transfected with miR-negative control), siRNA negative control group (cells transfected with non-silent siRNA) and blank group (cells without any transfection). MTT assay was used to detect cell proliferation in each group at different time periods. Transwell insert was used to detect invasion of cells *in vitro*. The relative expression of BMP-2 in osteosarcoma tissue was significantly higher than that in paraneoplastic tissue ($P<0.05$). The relative expression of miR-29c in osteosarcoma tissue was significantly lower than that in paraneoplastic tissue ($P<0.05$). The cell survival rates in experimental groups A and B were significantly lower than those in the blank, miR-NC negative control and siRNA negative control groups on day 5 ($P<0.05$). The number of cell transmembranes in experimental groups A and B was significantly lower than those in the blank, miR-NC negative control and siRNA negative control groups ($P<0.05$). BMP-2 is over-expressed in osteosarcoma tissues, and miR-29c is under-expressed in osteosarcoma tissues. Interfering with the expression of BMP-2 and overexpression of miR-29c can inhibit the proliferation and invasion of osteosarcoma cells, indicating that BMP-2 and miR-29c may be involved in the regulation of proliferation and metastasis of osteosarcoma cells and could be used as new molecular target markers for the diagnosis and treatment of osteosarcoma.

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Introduction

Osteosarcoma is a primary malignant tumor of bone that is prone to occur in adolescent children. Its incidence rate is approximately 10% in primary malignant tumor of bone, and the main site is at tubular bone of the distal end of the bone and proximal humerus (1,2). Osteosarcoma has the characteristics of high malignancy and early distal metastasis that is also the main cause of death (3,4), which is the cause of the poor prognosis of osteosarcoma patients and the less than 20% 5-year survival rate (5). With the development of new chemotherapy techniques, the survival rate of osteosarcoma patients has increased, and the 5-year survival rate is up to 80%, but the 5-year survival rate of osteosarcoma patients with metastasis has not improved significantly (6).

In recent years, the development of molecular biology has found increasing number of molecules playing an important role in the development of osteosarcoma (7). As an organism polypeptide growth factor, bone morphogenetic protein (BMP) has been found to be highly expressed in osteosarcoma, and it is speculated that BMP can promote the growth of osteosarcoma cells (8). BMP-2, a member of BMPs family, has been found to be highly expressed in osteosarcoma (9), but its mechanism in osteosarcoma cells is not described in detail. MicroRNAs (miRNAs/miRs) have been intensely researched in tumor molecular biology in recent years, and large number of studies considered that their abnormal expression and regulation is the main cause of tumor cell production and metastasis (10). As a member of the miR-29 family, a study (11) found that miR-29c can inhibit the proliferation and metastasis of tumor cells in a variety of tumors. Therefore, it was speculated that miR-29c is a factor associated with metastasis of tumor cells (12). However, there are few reports on the expression of miR-29c in osteosarcoma cells and its effect on the biological function of osteosarcoma cells.

The expression of BMP-2 and miR-29c in osteosarcoma cells and their effects were studied in order to provide a new theoretical basis for the diagnosis and treatment of osteosarcoma in molecular biology.

Patients and methods

General information. A retrospective analysis of 75 patients with osteosarcoma who underwent surgery in Tianjin Baodi Hospital (Tianjin, China) from May 2013 to June 2017 was conducted. The average age was 21.3±9.4 years. A total of 49 patients were at stage IIB/III, and 26 patients were at stage I/IIA. A total of 75 osteosarcoma tissues and 51 normal paraneoplastic tissues were excised with the patient's consent during the surgery. All patients were diagnosed with osteosarcoma by pathology and signed an informed consent. Patients with other serious organ diseases and tumors; with communication and mental disorders; and patients not cooperating with the study were excluded. All the specimens were stored in liquid nitrogen tanks immediately after removal.

The study was approved by the Ethics Committee of Tianjin Baodi Hospital. Patients who participated in this research had complete clinical data. The signed informed consents were obtained from the patients or the guardians.

Experimental reagents and materials. Human osteosarcoma cell line MG-63 was purchased from the cell bank of Shanghai Institutes of Biological Sciences (CAS; Shanghai, China); real-time quantitative PCR instrument was purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA; fetal bovine serum (FBS) and 0.25% trypsin were purchased from HyClone; GE Healthcare Life Sciences (Logan, UT, USA); TRIzol reagent was purchased from Applied Biosystems; Thermo Fisher Scientific, Inc., (Waltham, MA, USA); DMEM medium was purchased from Gibco; Thermo Fisher Scientific, Inc.; MTT solution was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany); Transwell insert was purchased from Corning, Inc. (Corning, NY, USA); Matrigel matrix was purchased from Bejing Biodee Biotechnology Co., Ltd., (Beijing, China); CDNA reverse transcription kit, SYBR Green PCR kit and Lipofectamine 2000 transfection reagent were both purchased from Invitrogen; Thermo Fisher Scientific, Inc. All primers and transfection plasmids were synthesized and designed by Sangon Biotech Co., Ltd. (Shanghai, China).

Expression of BMP-2 mRNA and miR-29c in osteosarcoma and paraneoplastic tissues. Total RNA of BMP-2 mRNA and miR-29c were extracted by TRIzol reagent from the osteosarcoma and paraneoplastic tissues. The purity and concentration of RNA were detected by ultraviolet spectrophotometer (G-9; Runqee (Shanghai) Instruments Technology Co., Ltd.). Then, 5 μg total RNA was taken from each to reverse transcribe cDNA according to the kit instructions. Reaction parameters: 37˚C for 15 min, 42˚C for 42 min and 70˚C for 5 min. Transcribed cDNA was used for PCR amplification, β-actin was used as the internal reference for BMP-2 mRNA, and U6 was used as an internal reference for miR-29c. The primer sequences are shown in Table I. PCR reaction conditions of BMP-2 mRNA: 40 cycles of predenaturation at 94˚C for 4 min, 94˚C for 60 sec, 59˚C for 60 sec, then elongation at 72˚C for 90 sec; PCR reaction conditions of miR-29c: 40 cycles of predenaturation at 95˚C for 2 min, 95˚C for 10 sec, 60˚C for 40 sec, then elongation at 72˚C for 90 sec. The relative expression of the gene was expressed by $2^{-\Delta\Delta Cq}$ (13). Real-time fluorescence quantitative PCR detection was conducted, and the experiment was repeated 3 times.

Cell culture, passage and transfection. Human osteosarcoma cells MG-63 were cultured in a medium containing 10% PBS DMEM at 37˚C and 5% CO_2. When the adherent cell confluence reached 85%, 25% trypsin was added for digestion, then the cells were cultured in medium to complete the passage. BMP-2 siRNA and miR-29c were transfected into logarithmic phase cells after passage. Untransfected cells were the blank group, cells transfected with miR-29c and BMP-2 siRNA were the experimental groups A and B, respectively. Cells transfected with miRNA negative control (miR-NC) were the miR-NC negative control group, cells transfected with non-silent siRNA were the siRNA negative control group. Lipofectamine 2000 and miR-29c mimics, BMP-2 siRNA, miR-NC and siRNA were mixed according to the instructions of Lipofectamine 2000 kit, and incubated at room temperature for 5 min. Finally, the mixture was mixed with cells and then transfected at 37˚C and 5% CO_2. The transfection efficiency of miR-29c mimics and BMP-2 siRNA in MG-63 cells after 48 h transfection was measured.

MTT assay for cell proliferation. Cells in each group after 48 h of transfection were inoculated in a 96-well cell culture plate, and approximately 100 μl of cell fluid was inoculated into each well, with a cell density of $2x10^3$ cell/ml, approximately 200 cells. Next, 20 μl MTT solution was added into each well on the 1st, 2nd, 3rd and 5th day, and then cultured in the incubator for 4 h. Then, 150 μl dimethyl sulfoxide was added, after 10 min of shaking, the absorbance was measured at the wavelength of 490 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated three times.

Transwell inserts for cell invasion in vitro. Matrigel was first diluted at the ratio of 1:8, then the upper surface of the bottom membrane of the Transwell chamber was coated with the dilution. It was placed at 37˚C for 30 min to polymerize Matrigel into a gel, and the basement membrane was hydrated before use. The transfected MG-63 cells were treated with starvation for 24 h, then resuspended with FBS free DMEM medium, and the cell density was adjusted to $1x10^5$/ml. Cells were seeded in Transwell inserts with the 24-well plate, and each well was filled with 100 μl of cell suspension. Then, 600 μl DMEM medium containing 10% FBS was added to the lower chamber of the 24-well plate, and then cultured in the incubator at 37˚C for 6 h. After culture, the supernatant was removed with cotton swabs, then the chambers were washed with PBS. Cells in the lower chamber were immobilized with 95% ethanol solution for 15 min at 37˚C, then washed with PBS and stained with 0.1% crystal violet. After staining, the number of cell migration in random 6 wells was calculated by microscope (XSP-L130; Shanghai Puqian Optical Instrument Co., Ltd., Shanghai, China) to get the average value, and the experiment was repeated three times.

Statistical analysis. SPSS 20.0 software package (IBM Corp., Armonk, NY, USA) was used for statistical analysis of the experimental data. The enumeration data were measured by

Table I. Primer sequences.

Genes	Upstream primers	Downstream primers
BMP-2	5'-TTGCGGCTGCTCAGCATGTT-3'	5'-TTCCGAGAACAGATGCAAGATG-3'
miR-29c	5'-ACACTCCAGCTGGGTAGCACCATTTGAAAT-3'	5'-TGGTGTCGTGGAGTCG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

BMP, bone morphogenetic protein.

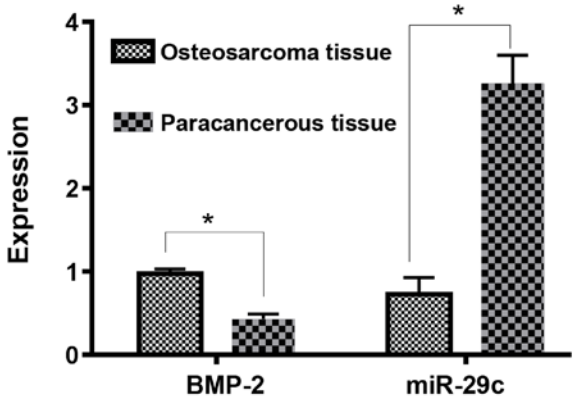

Figure 1. Relative expression of BMP-2 mRNA in osteosarcoma and paraneoplastic tissues. The relative expression of BMP-2 mRNA in osteosarcoma tissue was significantly higher than that in paraneoplastic tissue, and the difference was statistically significant (P<0.05). The relative expression of miR-29c in osteosarcoma tissue was significantly lower than that in paraneoplastic tissue, and the difference was statistically significant (*P<0.05). BMP, bone morphogenetic protein.

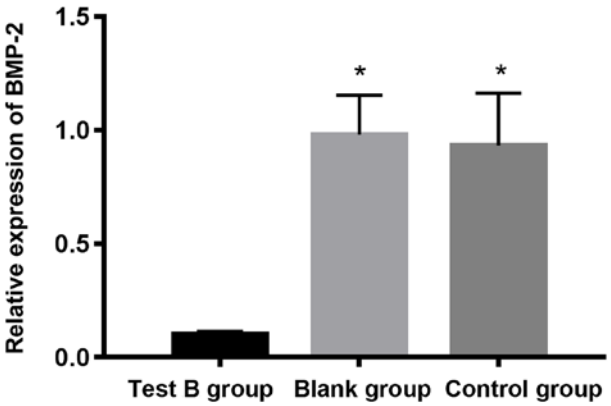

Figure 2. Comparison of relative expression of BMP-2 in cells of each group after transfection. RT-qPCR detection showed that the expression of BMP-2 in experimental group B was significantly lower than that in the siRNA negative control and blank groups (P<0.05), and there was no significant difference between the siRNA negative control and blank groups (P>0.05). Compared with experimental group B, *P<0.05. BMP, bone morphogenetic protein.

Chi-square test. The measurement data are presented as the mean ± standard deviation. Independent t-test was used for the comparison between the two groups, one-way ANOVA was used for multigroup comparison, and Bonferroni was used for post hoc comparison. GraphPad Prism 6 software was used to draw figures in this experiment. P<0.05 was considered to indicate a statistically significant difference.

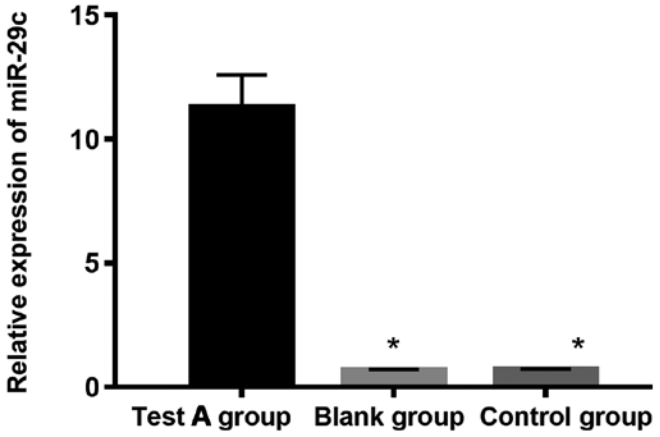

Figure 3. Comparison of relative expression of miR-29c in cells of each group after transfection. The expression of miR-29c in experimental group A was significantly higher than that in negative control and blank groups (P<0.05), and there was no significant difference between negative control and blank groups (P>0.05). Compared with experimental group A, *P<0.05.

Results

Expression of BMP-2 mRNA and miR-29c in osteosarcoma and paraneoplastic tissues. The relative expression of BMP-2 mRNA in osteosarcoma tissue (0.979±0.053) was significantly higher than that in paraneoplastic tissue (0.431±0.062), and the difference was statistically significant (P<0.05). The relative expression of miR-29c in osteosarcoma tissue (0.733±0.195) was significantly lower than that in paraneoplastic tissue (3.261±0.341), and the difference was statistically significant (P<0.05; Fig. 1).

Relative expression of BMP-2 mRNA and miR-29c in cells of each group after transfection. The expressions of miR-29c in experimental group A, miR-NC negative control and blank groups were 11.319±1.276, 0.688±0.027 and 0.714±0.021, respectively. The expression of miR-29c in experimental group A was significantly higher than that in miR-NC negative control and blank groups (P<0.05), and there was no significant difference between negative control and blank groups (P>0.05). The expression of BMP-2 mRNA in experimental group B, siRNA negative control and blank groups was 0.102±0.013, 0.981±0.173 and 0.932±0.231, respectively. The expression of BMP-2 mRNA in experimental group B was significantly lower than that in siRNA negative control and blank groups (P<0.05), and there was no significant difference between siRNA negative control and blank groups (P>0.05; Figs. 2 and 3).

Table II. Comparison of cell proliferative ability in each group.

Time	Experimental group A	Experimental group B	Blank group	miR-NC negative control group	siRNA negative control group	F	P-value
1st day	0.62±0.14	0.67±0.08	0.66±0.15	0.64±0.13	0.65±0.12	0.070	0.978
3rd day	0.83±0.22[a]	0.82±0.21[a]	1.39±0.23	1.31±0.21	1.23±0.24	3.881	0.117
5th day	1.13±0.21[a]	1.09±0.24[a]	2.06±0.27	2.12±0.25	2.09±0.27	13.97	<0.050

[a]Compared with blank and negative control groups, P<0.05.

Table III. Comparison of the number of cell transmembranes in each group.

Indicator	Experimental group A	Experimental group B	Blank group	miR-NC negative control group	siRNA negative control group	F	P-value
Cell transmembrane number	115.19±18.91[a]	113.62±19.13[a]	291.35±33.51	288.21±31.33	289.75±30.96	36.55	<0.050

[a]Compared with blank group, miR-NC negative control and siRNA negative control groups, P<0.05.

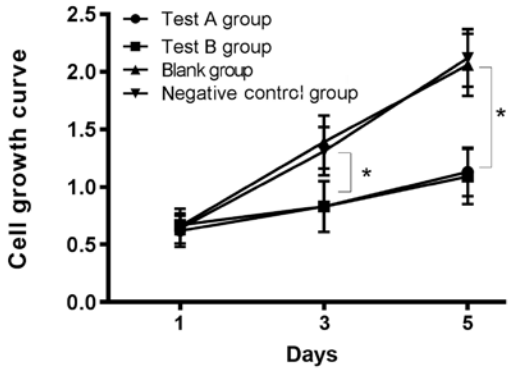

Figure 4. Comparison of cell proliferation in each group. There was no significant difference in proliferation ability between the five groups on the 1st and 3rd day after transfection (P>0.05). While the proliferation ability in the experimental groups A and B was significantly lower than that in the blank, the miR-NC negative control and the siRNA negative control groups on the 5th day, the difference was statistically significant (P<0.05). *P<0.05.

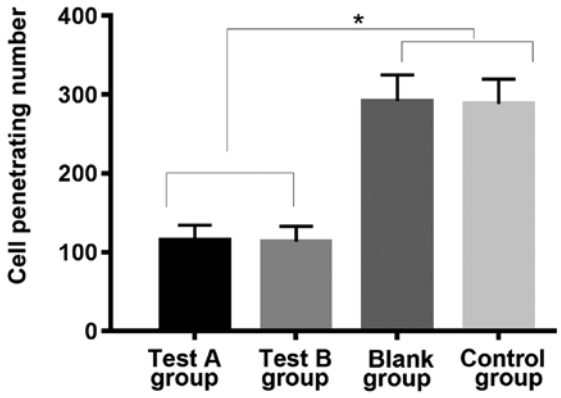

Figure 5. Comparison of the number of cell transmembranes in each group. The number of cell transmembranes in the experimental groups A and B was significantly lower than that in the blank, the miR-NC negative control and the siRNA negative control groups, and the difference was statistically significant (P<0.05). *P<0.05.

Comparison of cell proliferation in each group. There was no significant difference in proliferation ability among the five groups on the 1st and 3rd days after transfection (P>0.05), but the proliferation ability in experimental groups A and B was significantly lower than that in the blank, miR-NC negative control and siRNA negative control groups on the 5th day, and the difference was statistically significant (P<0.05). There was no significant difference in proliferation ability between experimental groups A and B on the 1st, 3rd and 5th days (P>0.05), and there was also no significant difference between the blank, the negative control and the siRNA negative control groups on the 1st, 3rd and 5th days (P>0.05; Table II and Fig. 4).

Comparison of cell invasion in each group. The number of cell transmembranes in the experimental group A (115.19±18.91) and group B (113.62±19.13) were significantly lower than that in the blank group (291.35±33.51), the miR-NC negative control group (288.21±31.33) and the siRNA negative control group (289.75±30.96), and the difference was statistically significant (P<0.05). However, there was no significant difference in the number of cell transmembranes between experimental group A and B, and between the blank, miR-NC negative control and siRNA negative control groups (P>0.05; Table III and Fig. 5).

Discussion

Osteosarcoma, a primary malignant bone tumor, originates from mesenchymal stem cells (14). Most of its biological behavior is highly malignant with the characteristics of early metastasis, high mortality and high disability rate (15). A study (16) has shown that metastasis of osteosarcoma is

one of the main causes of failure in treatment and death of patients. The metastasis mechanism of malignant tumors is that the tumor cells that are detached from lesion enter the blood or lymphatic system and the body circulation, and then proliferate and form metastasis focus in the metastatic organs, which is basically consistent with the metastasis mechanism of osteosarcoma (17). In recent years, studies (18,19) have found that the occurrence, development and metastasis of osteosarcoma are related to the abnormal expression of genes, and BMP-2 and miR-29c are two of these factors. In the development of the skeletal system, BMPs, an important growth factor, have been a hot topic in the occurrence and development of osteosarcoma (20). A previous study on the detection of BMPs in osteosarcoma tissues of osteosarcoma patients found that the BMP-2, BMP-6 and other BMPs were highly expressed (21). At present, there is no detailed description of the cell proliferation signaling pathway and migration mechanism of BMP-2 in osteosarcoma. However, a study of the effect of BMP-2 on osteoblasts proliferation (22) has found that BMP-Smad signaling pathway plays an important role in the proliferation of osteoblasts. miR-29c is a member of the miRNA-29 family. A study found that miR-29c can inhibit the migration of nasopharyngeal carcinoma tumor cells in nasopharyngeal carcinoma, and also explained its mechanism is to inhibit the migration of nasopharyngeal carcinoma cells by targeting activator TIAM1 in Rho (23). A large number of studies have found that miR-29c can inhibit tumor metastasis in leukemia (24) and other malignancies. It has also been suggested that in granulocytic leukemia, miR-29c can inhibit the combination of CDK26 and downstream factors by targeting CDK26, thereby inhibiting the proliferation of tumor cells (25). Although the expression of miR-29c in osteosarcoma was studied and found that the expression of miR-29c is low in osteosarcoma, its mechanism on osteosarcoma cells has not been described in detail (26). Besides, there are few studies on the effects of BMP-2 on osteosarcoma cells. Therefore, the expression of BMP-2 and miR-29c in osteosarcoma and its effect on osteosarcoma cells were investigated in this study, in order to provide theoretical basis and data of the relationship between miR-29c and BMP-2 for future studies, and new ideas and directions for the diagnosis and treatment of osteosarcoma.

The content of BMP-2 and miR-29c in osteosarcoma tissue and paraneoplastic tissue of patients with osteosarcoma was measured, and it was found that the relative expression of BMP-2 in osteosarcoma tissue was significantly higher than that in paraneoplastic tissue, and the difference was statistically significant ($P<0.05$). The relative expression of miR-29c in osteosarcoma tissue was significantly lower than that in paraneoplastic tissue, and the difference was statistically significant ($P<0.05$). Guo *et al* (27) found that BMP-2 could be detected in all osteosarcoma tissues, and BMP-2 was associated with cancer metastasis. Di Fiore *et al* (28) found that miR-29c expression was lower in osteosarcoma tissue than that in normal tissue. Our conclusions are consistent with the above studies. miR-29c and BMP-2 siRNA were transfected into human osteosarcoma MG-63 cells respectively, then the proliferation and invasive abilities of cells in these groups were compared after successful transfection. The results showed that there was no significant difference in proliferation ability among the five groups on the 1st and 3rd day after transfection, but the proliferation ability in the experimental groups A and B was significantly lower than that in the blank, miR-NC negative control and siRNA negative control groups on the 5th day. There was no significant difference in proliferation ability between the experimental groups A and B, the blank, the miR-NC negative control and the siRNA negative control groups. The number of cell transmembranes in the experimental groups A and B was significantly lower than that in the blank, the miR-NC negative control and the siRNA negative control groups. However, there was no significant difference in the number of cell transmembranes between experimental groups A and B, and between the blank, the miR-NC negative control and the siRNA negative control groups. These results suggest that interfering with the expression of BMP-2 and overexpression of miR-29c can inhibit the proliferation and invasion of osteosarcoma cells. In the study of the mechanism of miR-29c on colon cancer cells (29), it was found that miR-29c inhibited cell proliferation and metastasis by acting on its target genes PHLDB2 and p53, which was consistent with our conclusions. However, there are no reports on the association between miR-29c and BMP-2, and only a few reports on the effect of BMP-2 on osteosarcoma cells. We also found that there was no correlation between the two factors by searching their relationship through online software, but whether the two factors regulate osteosarcoma cells through other signaling pathways remains to be further explored.

In conclusion, BMP-2 is over-expressed in osteosarcoma tissues, and miR-29c is under-expressed in osteosarcoma tissues. Interfering with the expression of BMP-2 and overexpression of miR-29c can inhibit the proliferation and invasion of osteosarcoma cells, which indicates that BMP-2 and miR-29c may be involved in the regulation of proliferation and metastasis of osteosarcoma cells and could be used as new molecular target markers for the diagnosis and treatment of osteosarcoma. However, the detailed mechanism of osteosarcoma cells still need to be explored.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XC was in charge of the study, performed PCR and wrote the manuscript. XC and YZ were responsible for MTT assay. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tianjin Baodi Hospital (Tianjin, China). Patients who participated

in this study had complete clinical data. Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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