

Regulation of osteosarcoma proliferation and apoptosis by *miR-489* through targeting *SOX4* gene.

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Abstract

Objective: To explore the expression of microRNA-489 (*miR-489*) in osteosarcoma, so as to figure out the role and mechanism involved in proliferation and invasion control of osteosarcoma cells for *miR-489*. **Methods:** By comparison *miR-489* expression levels in osteosarcoma and normal colonic tissues, or in osteosarcoma cell lines with different metastasis potency by qRT-PCR. In SW480 cell stably overexpressing *miR-489* by transfection of *miR-489* mimics, proliferation was assessed by MTT(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide), and the cell cycle and apoptosis of SW480 was assayed by FACS(fluorescence activated cell sorting). We predicted *SOX4* as the specific target gene of *miR-489* with the approach of bioinformatics. Then, the protein level of *SOX4* in osteosarcoma cells was detected after transfection *miR-489* mimics by Western blotting.

Results: The expression level of *miR-489* in osteosarcoma tissues was decreased when compared with the matched adjacent non-tumorous colonic tissues. The expression level of *miR-489* in HR8348 and HCT116 cells with low metastasis potency were higher than those in HT29 and SW480 cells with high metastasis potency. After transfection with *miR-489* mimics for 48 h, the growth speed was significantly inhibited in *miR-489* mimic group than those of control group and blank group (P<0.05). Compared with control group, the distribution of cell cycle was not changed but apoptotic cells were significantly increased in *miR-489*-overexpression group (P<0.05). Western blot analytic results confirmed that *SOX4* was inhibited in *miR-489*-transfected cells.

Conclusion: *miR-489*, acting as an anti-oncogene microRNA, inhibited proliferation and invasion in osteosarcoma. Overexpression of *miR-489* could suppress the growth of osteosarcoma cells through induction of cell apoptosis.

Keywords: Osteosarcoma, MicroRNA-489, *SOX4*, Apoptosis.

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Introduction

Osteosarcoma is one of the most common malignant tumor of digestive tract, the morbidity of which has gradually increased [1]. Despite the continuous advances in the treatment of surgery and chemotherapy, 5 years survival rate of patients with osteosarcoma is still low [2]. At present, the proliferation of osteosarcoma tumor cells is known to have close relationship with occurrence, development and prognosis of osteosarcoma [3]. In recent years, many studies have confirmed that MicroRNAs plays a very important role in the occurrence and development of tumor. MicroRNAs are a small molecule RNA of endogenous non encoding, and participate in the pathological process of many kinds of tumors' occurrence [4]. Recent studies showed that *miR-489* could regulate and become the tumor suppressor gene of a wide variety of tumors [5,6]. In addition, the downregulation of *miR-489* in breast

cancer cells can eventually affect the prognosis of patients with breast cancer through influencing proliferation of tumor cell [7]. This study made a quantitative analysis of the expression of *miR-489* in osteosarcoma tissues and the surrounding normal bone cancer tissues of 30 cases. At the same time, it used the *miR-489* overexpressing SW480 clone transfected and constructed by *miR-489* to further explore the effects of overexpressed *miR-489* on the biological behaviors of osteosarcoma cells, providing a theoretical basis for the treatment of osteosarcoma.

Material and Method

Specimens and cell lines

Osteosarcoma specimens come from the inpatients in the department of general surgery in People's Hospital of Hubei Province from January 2011 to December 2015. After

acquiring the fresh specimens of patients who underwent surgical resection, each cancer tissue and adjacent (5 cm away from the cancer margin) of the normal tissue were immediately kept in liquid nitrogen for spare. All cases were proved by histopathology, and 30 cases of osteosarcoma were classified as 14 cases of high differentiation, 10 cases of middle differentiation, and 6 cases of low differentiation as well as 20 cases of lymph node metastasis, 10 cases without lymph node metastasis. HR8348, HCT116, HT29 and SW480 cells were from the Wuhan University archives center for cells.

Reagents and instruments

RNA reverse transcription kit (Promega Co.USA); SYBR reagent (TAKARA Co. Japan); DMEM high glucose medium and serum of fetal bovine(Hyclone Co. USA); liposome lipfectamine2000 (Invitrogen Co. USA); real time fluorescence quantitative PCR (qRT-PCR) instrument (ABI Co. USA); *MiR-489* mimics and mimics NC (Ruibo Co. Guangzhou); *miR-489* primer and U6 primer (Ruibo Co. Guangzhou); Protein Extraction Kit (Bi Yuntian); *SOX4* antibody and glyceraldehyde -3- phosphate dehydrogenase (GAPDH) , and antibody was purchased from Santa Cruz Co.USA; MTT reagent (Sigma Co. USA).

qRT-PCR

According to the instruction of Trizol kit for RNA extraction, osteosarcoma tissues and intestinal mucosa tissues specimens adjacent to carcinoma or osteosarcoma tumor clone were collected, with 1 ml Trizol added for 10 min cell lysis and total RNA extraction. Total RNA was transcribed into cDNA according to the instruction of used reverse transcription kit. Reaction conditions: 37°C, 15 min; 98°C, 5 min. qPT-PCR reaction system: 1 µl reversely transcribed cDNA (final solubility of 5 ng), 2 × SYBR Premix Ex Taq™ II Mix 10 µl, upstream primer 0.5 µmol/L and downstream primer 0.5µmol/L, DEPC H₂O 8 µl with the total volume of 20 µl. Reaction conditions: 95°C, 30 s; 95°C, 5 s; 60°C, 15 s; 72°C 20 s; amplified 35 cycles. With U6 as reference, 2^{-CT} method was used to analyse the results.

Cell culture and transfection

With complete culture medium DMEM containing 10% fetal bovine serum, SW480 cells were cultured under the condition of 37°C and 5% CO₂. The growth state of the cells was observed under inverted microscope, and cells were tested while cells are presenting the fusion of 70%-80%. The experiment was divided into 3 groups equally, non-transfection group (Blank control), negative control group (mimics NC) and transfection group (*miR-489* mimics). 2 µL negative control storage solution or *miR-489* mimics storage solution (20 µmol/L) was added into 100 µL Opti-MEM in each group with incubated at room temperature for 5 min. And 100 µL Opti-MEM was used for the dilution of 1 µL lipo2000 in each group with mild mixed and incubated at room temperature for 5 min. Then the above two liquid were gently mixed and incubated at room temperature for 20 min. The mixed liquid

was poured into 96 well plates and cultured for 6 h before the replacement of fresh complete culture medium DMEM containing 10% fetal bovine serum.

MTT used for detecting cell proliferation

The SW480 cells in logarithmic growth phase were collected and inoculated in 96 pore plates with 3 × 10⁴ per pore plate. According to the above grouping and methods, transfection was conducted as 5 parallel holes set for each group, transfecting after 24 h, 48 h, 72 h. MTT 20 µl (5 mg/ml) was added to each hole and continuously cultured in the incubator under 37°C, 5% CO₂ for 4 h, and culture solution was carefully sucked from holes after centrifugation. Then DMSO 150 µl was poured into each hole with oscillation under protecting form light for 15~20 min. At last, microplate reader was adopted to detected the absorbance value (A490) for each hole at the site of 490 nm.

Flow cytometry analysis for cell cycle distribution

Collect the cells after 48 h transfection from each group, wash them with PBS for twice, and make centrifugation in 1000 g for 5 min. Discard liquid supernatant, slowly add 75% ethanol after precooling at -20°C, and save it under 4°C. Take the cell suspension with washed by PBS, and discard the supernatant after centrifugation in 2000 g for 5 min. Dye for 30 min with PI dye 1.0 ml , use 488 nm excitation wavelength for sample determination, and 620 nm bandpass filter for PI fluorescence detection. Collect more than 10000 fluorescence signals from each sample, and obtain the percentage of cells in each phase from the total cells number. Repeat 3 times in each group.

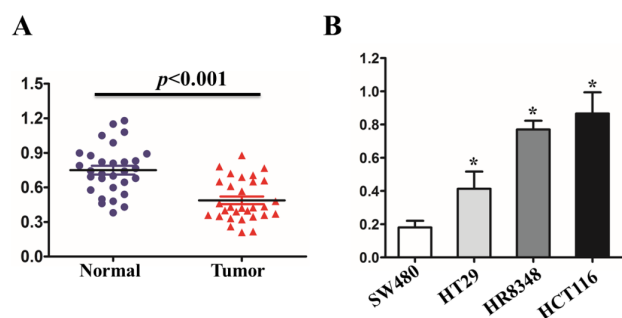


Figure 1. *miR-489* expression in CRC tissues and in CRC cell lines with different metastasis potency. A, *miR-489* expression was tested using qRT-PCR in 30 pairs of human CRC tissues (Tumor) and adjacent non-tumorous tissues (Normal), and its expression was normalized to the level of U6 small nuclear RNA (U6) expression in each sample; ** $P < 0.001$. B, *miR-489* expression detected by qRT-PCR in four groups of CRC cells; * $P < 0.05$.

Detection of Western blot for SOX4 protein expression

Put total protein 40 µg into 10% Polyacrylamide gel for electrophoresis. Transfer the protein to nitrocellulose membrane by semi dry electroblotting, and separately add the first corresponding antibody (1:1000 dilution) for overnight under 4°C after 5% skim milk closing for 40 min. Conduct

room temperature balance on the second day for 40 min, respectively add the second antibody labelled by horseradish peroxidase enzyme after washing the membrane, and incubate for 1 h under 37°C. After washing the membrane, add Enhanced Chemiluminescence reagent (ECL), and put the membrane into the X-ray cassette for pressing, developing and fixing. Compare the gray value between the target band and internal reference band with the expression of GAPDH as a reference.

Statistical methods

The data were analysed by statistical software SPSS 12.0. The experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and means of the two groups were compared by t test; many groups were compared by variance analysis of single factor with P<0.05 as statistically significant difference.

Result

Expression of miR-489 in osteosarcoma tissues and osteosarcoma cells

The results of qRT-PCR showed that compared with adjacent normal bone tumor tissues, the expression amount of miR-489 in 30 cases of human osteosarcoma tissues was significantly decreased (P<0.001), which could be seen as Figure 1A. In order to verify the expression pattern of miR-489 in osteosarcoma cells, qRT-PCR was used to detect the expression of miR-489 in four strains of osteosarcoma cells with different potential of metastatic and migration. The results showed that the expression level of miR-489 in osteosarcoma clone with high metastatic potential like SW480, HT29 was significantly reduced compared to those with low metastatic potential like HR8348, HCT116 (P<0.05), which could be seen as Figure 1B.

Table 1. Effect of miR-489 on cell proliferation evaluated by MTT assay.

| Groups | 24 h | 48 h | 72 h |
|----------------|----------------|----------------|----------------|
| Blank control | 0.356 ± 0.048 | 0.626 ± 0.024 | 0.956 ± 0.026 |
| mimics NC | 0.402 ± 0.032 | 0.641 ± 0.035 | 0.920 ± 0.038 |
| miR-489 mimics | 0.218 ± 0.024* | 0.306 ± 0.028* | 0.385 ± 0.046* |

Note: *P<0.05, miR-489 mimics vs. Blank control group

The detection by MTT on the effect of miR-489 on the proliferation of human osteosarcoma SW480

Respectively use negative control agent or treat SW480 cells by MiR-489 mimics for 24, 48, 72 h, and measure absorbance value at the site of 490 nm by microplate reader to express the proliferation of SW480 cells. The results showed that compared with non transfection group and negative control group, absorbance value at the site of 490 nm in miR-489 mimics group decreased significantly (P<0.05), which could be seen in Table 1. The experimental results suggest that low

expression of miR-489 in osteosarcoma tissue may promote tumor cell proliferation.

The detection by FCM on the effect of miR-489 on cell cycle distribution and apoptosis

The detection by flow cytometry method (FCM) on the effect of miR-489 on cell cycle distribution and apoptosis of osteosarcoma cell line SW480 was detected by FCM results showed that after the transfection of miR-489 mimics for 48 h, there were no statistically significant differences in the number of cells in each phase of cell cycle between the groups (P>0.05). However, it was observed that after overexpression of miR-489, the apoptosis of SW480 cells was promoted, and compared with control group, cell apoptosis rate of miR-489 mimics treatment group was significantly higher with statistically significant differences (P<0.05). The experimental results indicated that overexpression of mir-489 could induce the apoptosis of osteosarcoma cells, which could be seen in Table 2.

Table 2. Cell cycle distribution and apoptosis of osteosarcoma cells SW480 after miR-489 mimics treatment.

| Groups | G0/G1 | Cell cycle distribution S | G2/M | Cells apoptosis | of |
|----------------|--------------|---------------------------|--------------|-----------------|----|
| Blank control | 57.35 ± 0.53 | ± 30.36 ± 0.56 | 12.29 ± 0.88 | 5.06 ± 0.74 | |
| mimics NC | 58.45 ± 1.42 | ± 26.63 ± 1.25 | 14.92 ± 1.64 | 5.02 ± 0.36 | |
| miR-489 mimics | 57.36 ± 2.09 | ± 30.58 ± 1.54 | 14.06 ± 1.54 | 26.28 ± 0.26* | |

Note: *P<0.05, miR-489 mimics vs. Blank control group

Osteosarcoma cells, SOX4 gene is a downstream target genes of miR-489

The miRNA target gene prediction software (Miranda, Pictar and Targetscan) was used to analyze downstream target genes of miR-489, and it was found that 15 candidate target genes might be combined with miR-489 (Figure 2A). It is shown in Figure 2B the possible combination region of miR-489 and SOX4 gene 3'UTR. Western blot results showed that after the transfection of miR-489 mimics for 48 h, comparing with the control group, the grey level of SOX4 protein band in the miR-489 mimics treatment group decreased significantly, as shown in Figures 2C and 2D. The results suggested that the low expression of miR-489 in osteosarcoma could promote tumor cells' growth through up-regulating the expression of SOX4 gene.

Discussion

The abnormal expression of microRNA and up-regulated expression microRNA in tumors from a variety of human tumor phases including osteosarcoma, generally have effects of oncogenic genes, and promote the occurrence and development of tumor through negative regulation of tumor suppressor gene

or regulation of differentiation and apoptosis channel of tumor cells. However, microRNA with the down-regulated expression in tumor tissues usually acts as a tumor suppressor gene, because of its down-regulated expression, the inhibition function of cancer genes is removed, promoting the development of tumors [8]. This topic study showed that the expression amount of *miR-489* in osteosarcoma was significantly reduced compared with that of adjacent normal bone tumor tissues. It indicates that *MiR-489* acts as a tumor suppressor gene in the occurrence and development of osteosarcoma and it is helpful for the early diagnosis of osteosarcoma to detect its expression amount.

In order to further verify whether the down-regulated expression of *miR-489* could promote the proliferation, invasion and metastasis of osteosarcoma, we conducted a series of cell function experiments. Using fluorescence quantitative PCR to detect expression of *miR-489* in osteosarcoma clone with different metastatic potential, it was

shown that compared with the expression level of *miR-489* of low metastatic potential HR8348, HCT116, the level of *miR-489* in SW480 cells with high metastatic potential was significantly reduced. Statistically significant differences indicated that the down-regulated expression of *miR-489* promoted the increasing metastasis and invasion ability of osteosarcoma cell. The MTT experiment showed that overexpression of *miR-489* could inhibit the growth of osteosarcoma cells; further study found that after *miR-489* mimics transfected by osteosarcoma cells SW480 for 48 h, the distributions in each tumor cell cycle phase had no significant differences, but the percentage of apoptotic cells increased significantly in the *miR-489* mimics treatment group. These results suggested that in SW480 cells, the mechanism of up-regulated expression of *miR-489* leading to cell growth inhibition might be the promotion of cell apoptosis rather than cell cycle arrest.

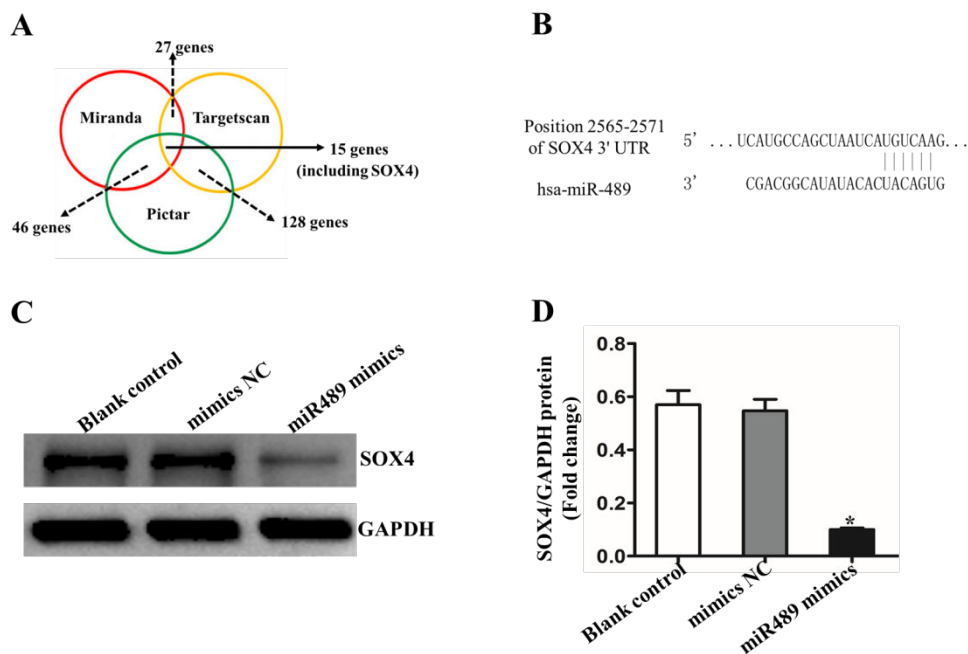


Figure 2. *MiR-489* bound directly to the *SOX4* 3'-UTRs and down-regulated its expression. *A*, A schematic diagram of *miR-489* predicted targets using three algorithms (*Miranda*, *Pictar* and *Targetscan*), and identified 15 candidate genes which may interact with *miR-489*. *B*, A putative *miR-489*-binding site exists in the 3'-UTR of the *SOX4* mRNA, and 6-nucleotide deletion were generated in the binding site. *C*: expressions of *SOX4* in each group cells detected by Western blot assay; *B*: average relative gray of *SOX4* expressions in 3 groups of SW480 cells, *GAPDH* as a loading control, * $P < 0.05$.

As known to all, the function of microRNA mainly depends on the regulation of the target gene expression. Due to imprecision of the pairing between microRNA and target mRNA in mammalian, each microRNA can regulate multiple target genes, and multiple microRNAs can have a mutual target gene, thus forming a complex regulatory network to precisely regulate the expression of functional genes [9]. Therefore, it is very important for understanding the role of microRNA in the occurrence and development of cancer to successfully predict the target genes of microRNA. *SOX4* gene is an important member of SOX (SRY (sex determining region Y) -box)

family, and participates in the regulation of embryonic cells development, decisions of cells' fate through encoding transcription factor as well as takes part in various types of biological behavior from tumors [10]. Certain studies showed that the expression of silencing *SOX4* gene could lead to decreased expression level of activation factor Bcl-10 of nuclear transcription factor κ B in tumor cell, while the stability of apoptosis inhibitor Survivin protein was enhancing, so that tumor apoptosis increased [11]. In addition, in liver cancer cells, *SOX4* protein can promote apoptosis of tumor cells through activating the approach of Caspase [12]. In this study,

the author used a variety of miRNA target gene prediction software to predict tumor cell regulating factor *SOX4* as downstream target gene of *miR-489*. This topic study found that compared with the control group, the expression amount of *SOX4* in overexpression *miR-489* of osteosarcoma cells decreased significantly. These results indicated that *miR-489* inhibited the growth of tumor cells and induced their apoptosis, which was mainly achieved through the down-regulation of *SOX4* gene expression.

It has showed a rising trend in the morbidity and mortality of osteosarcoma in recent years, which may be gradual process involved by many factors and accumulated by multiple stages. *MiR-489* can inhibit the growth of osteosarcoma by regulating the multiple target genes, however, *miR-489* still has many unknown target genes to be identified and verified. The interaction between the target genes and the role of *miR-489* in the regulation network of cancer inhibition genes remains to be elucidated. Besides, the mechanism of down-regulated expression in *miR-489* from osteosarcoma tissues needs further confirm. It is believed that with in-depth understanding of *miR-489*, using *miR-489* as marker for diagnosis and targets for biological treatment will bring new hope for the diagnosis and treatment of osteosarcoma.

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