

Original Article

miR-216a expression in osteosarcoma tissues and its effect on the proliferation, migration, and autophagy of the osteosarcoma U2OS cell line

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Abstract: Objective: To study the expression of miR-216a in osteosarcoma tissues and how it works on the proliferation, migration, and autophagy of the osteosarcoma U2OS cell line. Methods: Patients diagnosed with osteosarcoma who underwent surgery in our hospital from March 2018 to March 2020 were recruited as the study cohort, and their tumor tissues and corresponding paracancerous tissues were collected to determine their miR-216a expression levels. A miR-216a inhibitor and a miR-216a negative control were transfected into U2OS cells using liposomes, and the cells were divided into a no-treatment control group (non-transfected), a negative control group (transfection of miR-NC), and a miR-216a inhibitor group (transfected with a miR-216a inhibitor). The following tests were conducted for each group of cells: cell proliferation (using the MTT method), miR-216a expression (using the qRT-PCR method), cell invasion ability (using a Transwell assay), cell migration ability (using a scratch test), and Beclin-1 protein expression (Western blot). Results: The relative expressions of miR-216a in the osteosarcoma tissues significantly exceeded their expressions in the paracancerous normal tissues ($P < 0.05$). The relative expression of miR-216a in the inhibitor group was notably lower than it was in the no-treatment control group and the negative control group ($P < 0.05$). The difference in the relative expression levels of miR-216a in the blank control group and the negative control group showed no statistical significance ($P > 0.05$). The absorbance value of the cells in the miR-216a inhibitor group at 48 h, 72 h, and 96 h at OD490 was significantly lower than it was in the no-treatment control group and negative control group ($P < 0.05$), and there was no significant difference in the absorbance value at 490 nm in the no-treatment control group or the negative control group at each time point ($P > 0.05$). The number of cells penetrating the basement membrane of the miR-216a inhibitor group was apparently less than it was in the no-treatment control group and the negative control group ($P < 0.05$), and the difference between the no-treatment control group and the negative control group was not statistically significant ($P > 0.05$). After 12 hours of scratch treatment on each group of cells, the migration force of the osteosarcoma cells in the miR-216a inhibitor group was significantly weaker than it was in the negative control group and the no-treatment control group, but the migration force in the negative control group and the no-treatment control group was not significantly changed. The relative expression degree of the Beclin-1 protein in the osteosarcoma cells in the miR-216a inhibitor group was much higher than it was in the no-treatment control group and the negative control group ($P < 0.05$), but the difference showed no statistical significance between the relative expression degrees of the Beclin-1 protein in the no-treatment control group and the negative control group ($P > 0.05$). Conclusion: The expression of miR-216a in the osteosarcoma tissue increased abnormally. Inhibiting its expression helps to restrain the proliferation in osteosarcoma cells, reduce the invasion and migration abilities of the osteosarcoma cells, and accelerate the autophagy of the osteosarcoma cells, which provides a new, prospective biological direction for curing osteosarcoma.

Keywords: miR-216a, osteosarcoma, osteosarcoma cells, proliferation, migration, autophagy

Introduction

Osteosarcoma is the most common type of primary malignant bone cancer, and it is derived from mesenchymal stem cells. Osteosarcoma

often occurs in the proximal tibia, humerus, and metaphysis of distal femur, and its typical symptoms include limited joint movement, local swelling, pain, and trabecular bone damage [1]. Osteosarcoma is extremely malignant and eas-

ily relapses and metastasizes, which leads to a poor clinical prognosis. Currently, the main clinical treatment for osteosarcoma is surgical resection combined with chemoradiotherapy, and while the therapeutic effect is not unsatisfactory, the patients' clinical prognosis is poor [2]. Therefore, it is the focus of current clinical work to seek a new diagnostic marker for osteosarcoma. microRNA (miRNA) is a type of endogenous, non-coding RNA at around 22 nucleotides in length [3]. Many studies [4, 5] have shown that miRNA is involved in many biological processes such as cell proliferation, differentiation, and apoptosis, and the ectopic expressions of various miRNAs can be detected in tumor tissues. In recent years, miR-216a has been found to be related to tumor proliferation and metastasis. According to the published research, miR-216a can inhibit pancreatic cancer by regulating the expression of JAK2 and by promoting drug tolerance and the relapse of liver cancer by inducing interstitial cell transformation [6]. However, no study has yet addressed the function of miR-216a in osteosarcoma and how it works on osteosarcoma cell proliferation and autophagy. This study investigated and analyzed the expression of miR-216a in osteosarcoma tissues and how it works on the proliferation, migration, and autophagy of osteosarcoma cells (the U2OS cell line), and our report is as follows.

Materials and methods

Clinical data

49 patients with osteosarcoma who underwent surgery in our hospital from March 2018 to March 2020 were recruited as the study cohort, and their tumor tissues and corresponding paracancerous tissues were collected. The results of the pathological examinations confirmed the osteosarcoma, and there were no tumor cells found in periosteal tissues, which were ≥ 3 cm from the edge of the solid tumor. None of the patients received radiotherapy or chemotherapy prior to their surgery. The fresh specimens were numbered and registered after they were removed, and then they were stored in a -80°C freezer. All the specimens were used with the informed consent of the patients and their families. This study was approved by the ethics committee of our hospital.

The cell lines and main reagents

Osteosarcoma cell line-U2OS was sourced from the cell repository of The Chinese Academy of Sciences. We also obtained a cell-culture solution for DMEM (Gibco Co., USA), FBS (Gibco Co., USA), Lipofectamine Reagent Lipofectamine™ 2000 (Invitrogen Corporation, USA), Trizol Reagent (Invitrogen Corporation, USA), Real-time PCR instrument (AB company, USA), miR216a inhibitor, and the short RNA (miR-NC) of negative control (GenePharma Co.), primary antibody Beclin-1 rabbit anti human (Abcam Company, UK), Goat anti-Rabbit IgG (Abcam Company, UK), and a Transwell chamber (Corning Corporation, USA).

Cell culture and transfection

The osteosarcoma cell line-U2OS was cultured in 10% fetal bovine serum and included the DMEM medium at 37°C and 5% CO_2 . The solution was changed every two days, and subcultured to maintain the logarithmic growth period when the fusion degree of the cells reached about 90%. Then the cells were inoculated into a 6-hole culture plate. Lipofectamine 2000 was adopted to transfect the liposomes in line with the kit's instructions, and the cells were divided into the no-treatment control group (non-transfected), the negative control group (transfection of miR-NC), and the miR-216a inhibitor group (transfection of the miR-216a inhibitor). We continued to cultivate them for 24 h after the transfection.

Cell proliferation

The cells in each group were inoculated into a 96-hole plate with a density of 1×10^4 cells per hole. A complete medium containing penicillin-streptomycin was added and cultured for 24 h, 48 h, 72 h, and 96 h before removal. Each hole was filled with 20 μL MTT (5 mg/ml), and then we placed the cells in the incubator for 4 hours. We then carefully poured off the liquid and added 150 μL dimethyl sulfoxide (DMSO) to each well. The wells were then incubated at room temperature for 10 min using a shaker, and then we measured and recorded the absorbancy values (the OD values) at 490 nm in each group of cells, and drew the corresponding cell growth curves. The change of the cell proliferation ability was observed in each mol-

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ecule group, and 5 replicate wells were set in each group.

qRT-PCR quantification

The total RNA from the osteosarcoma tissues, the paracancerous normal tissues, and the osteosarcoma cells in each group was extracted using the Trizol method. The purity and integrity of the RNA were determined using a uv-visible spectrophotometer and agarose electrophoresis respectively, and then the wells were stored in the freezer at -80°C for testing. According to primer sequences described in the literature [7], the upstream primer of miR-216a was 5'-GTCGTATCCAGTGC GTGTCGGACG TAG-3', the downstream primer was 5'-GGCAATTGCACTGGATACGACTCACAGTT-3'; we used U6 as an internal reference gene, the upstream primer was 5'-GCTTCGGCAGCACATATACTAAAAT-3', and the downstream primer was 5'-CGCTTACGAATTTTC GGRGTCAT-3'. The PCR reaction conditions were 95°C for 10 s, 60°C for 20 s, 72°C for 10 s. A total of 40 cycles were completed, and each experiment was repeated three times. The relative expression degrees of miR-216a were counted according to $2^{-\Delta\Delta\text{Ct}}$.

Western blot experiments

After the routine lysis of the cells, the protein determination was conducted using Coomassie brilliant blue staining. After the electrophoresis, electrotransformation, and sealing, Beclin-1 antibody (1:1000) was added separately, and incubated in a refrigerator a 4°C overnight. After washing the membrane, HRP AffiniPure Goat Anti-Rabbit IgG (H+L) (1:20000) was added for the incubation, rinsed with PBS, and then we added DBA for the color development. The analysis software for gel optical density Gel pro4.0 was used to analyze the grayscale of each group of cells. The cumulative absorbance value of the target band and the absorbance value of the internal reference GAPDH were measured under the same conditions, and the ratio of the two was used as the relative expression.

Transwell invasion assay

The cells of each group were digested at 24 hours post-transfection and then resuspended without serum BSA. The cells was added to the

chamber, 2×10^4 per hole, and 10% fetal bovine serum and the included medium were added to the lower chamber. We then cultured the cells at 37°C for 24 hours without CO_2 . The cells were then fixed for 20 min at room temperature using 95% ethanol and stained with hematoxylin for 10 min, and then they were cleaned three times using distilled water. We placed the solution under an inverted microscope for observation, randomly selected five visual fields, and recorded the number of cells in each group that had passed through the basement membrane.

Scratch test

We inoculated the transfected cells into a 6-well culture plate, and we discarded the culture solution when the cell density covered around 85% of the bottom area of the well. We drew a straight line with a $10 \mu\text{l}$ pipette tip, making sure the line passed across the largest area of cell density as much as possible. Then we washed both sides with PBS, and added 3-5 ml serum-free medium. We placed it in the incubator to continue the cultivation for 48 h, and we observed the cell migration under the microscope.

Statistical analysis

We used SPSS 22.0 to process the data. The measurement data were expressed as $(\bar{x} \pm s)$. Variance was employed to analyze the three sets of measurement data, LSD-t tests were adopted for the further comparisons, and t tests were used to compare the two sets of measurement data. $P < 0.05$ was considered a statistically significant difference.

Results

Comparison of the miR-216a expressions in the osteosarcoma and paracancerous tissues

The relative expression degrees of miR-216a in the osteosarcoma tissues was notably higher than it was in the paracancerous tissues ($P < 0.05$). See **Table 1** and **Figure 1**.

Comparison of the miR-216a expressions in the osteosarcoma cells in the various groups

The relative miR-216a expression level in the miR-216a inhibitor group was much less than it

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Table 1. Comparison of the miR-216a expressions in the osteosarcoma tissues and the paracancerous normal tissues ($\bar{x} \pm s$)

Group	Number of cases	The relative expression of miR-216a	t	P
Osteosarcoma tissues	49	1.974±0.482	11.973	0.000
Paracancerous normal tissues	49	1.072±0.214		

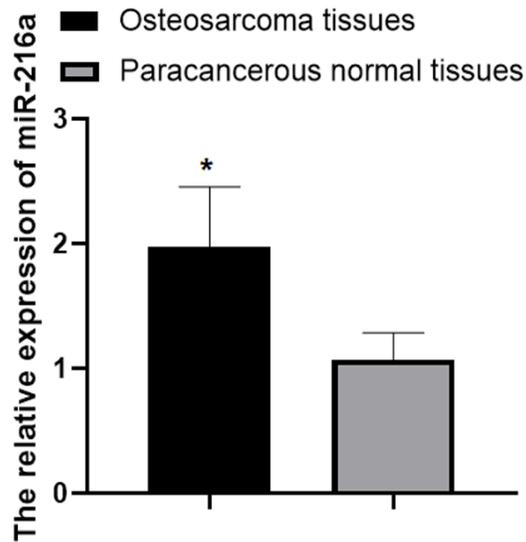


Figure 1. Comparison of the miR-216a expressions in the osteosarcoma tissues and the paracancerous normal tissues. Compared with the paracancerous normal tissues, * $P < 0.05$.

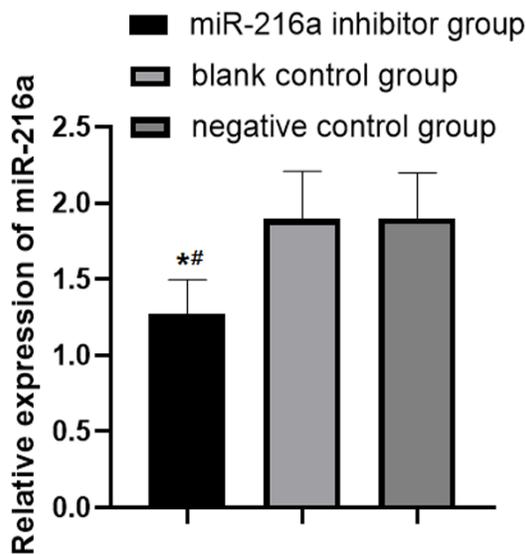


Figure 2. The relative expression degrees of miR-216a in the U2OS cells in the various groups. Compared with the blank control group, * $P < 0.05$. Compared with the negative control group, # $P < 0.05$.

was in the no-treatment control group and negative control group ($P < 0.05$), but the difference was not statistically significant for the relative expression of miR-216a between the

no-treatment control group and the negative control group ($P > 0.05$), as illustrated in **Figure 2**.

MTT assays determined the proliferation of the osteosarcoma cells in each group

The absorbance values of the miR-216a inhibitor at 48 h, 72 h, and 96 h at OD490 were notably less than the corresponding values in the no-treatment control group and negative control group ($P < 0.05$). The differences were not statistically significant for the absorbance values at 490 nm at each time point in the no-treatment control group and the negative control group ($P > 0.05$). See **Table 2** and **Figure 3**.

Transwell invasion assays to determine the invasions of the osteosarcoma cells in the various groups

The quantity of cells penetrating the basement membrane of the miR-216a inhibitor group was definitely less than of the quantity in the no-treatment control group and the negative control group ($P < 0.05$), and no significant difference was found in the quantity of cells that penetrated the basement membrane between the no-treatment control group and the negative control group ($P > 0.05$), as shown in **Table 3** and **Figure 4**.

Each group's osteosarcoma cell migration ability was measured using a scratch test

After 48 hours treatment with the scratch tests in the three groups of cells, the migration abilities of the osteosarcoma cells in the miR-216a inhibitor group were notably weaker than they were in the negative control group and no-treatment control group, but there was little apparent difference in the cell migration between the negative control group and the no-treatment control group, as shown in **Figure 5**.

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Table 2. MTT assays with the absorbance at 490 nm for each group of cells ($\bar{x} \pm s$)

Group	24 h	48 h	72 h	96 h
Blank control group	0.119±0.028	0.182±0.023	0.261±0.030	0.389±0.045
Negative control group	0.126±0.031	0.179±0.019	0.256±0.032	0.378±0.041
miR-216a inhibitor group	0.112±0.024	0.157±0.020* [#]	0.205±0.027* [#]	0.289±0.035* [#]

Note: Compared with the blank control group, * $P < 0.05$. Compared with the negative control group, [#] $P < 0.05$.

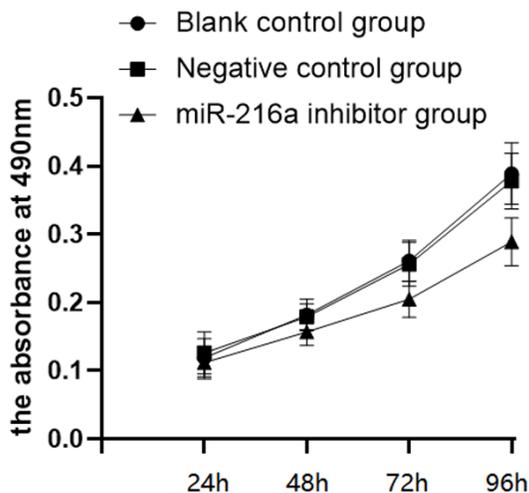


Figure 3. MTT assays with the absorbance at 490 nm for the cells in each group.

The protein expression status of Beclin-1 in the osteosarcoma cells as determined using Western blot

The relative expression degrees of the Beclin-1 proteins in the osteosarcoma cells of the miR-216a inhibitor group increased significantly compared to the no-treatment control group and the negative control group ($P < 0.05$), but there was no significant difference in the relative expression of the Beclin-1 protein in the no-treatment control group compared to the negative control group ($P > 0.05$), see **Table 4** and **Figure 6**.

Discussion

Osteosarcoma is usually a malignant bone tumor in teenagers with a high degree of malignancy and a low 5-year survival rate. Moreover, most patients show distant metastasis [8]. Therefore, it has become the focus of clinical scholars to seek new and effective therapeutic strategies to enhance the survival rate of patients as well as prevent or delay the metastasis of osteosarcoma. According to relevant

research reports [9], miRNAs have a wide range of gene regulation functions and are closely involved in various pathophysiological processes such as growth and development of the body, cell proliferation, and the growth and inhibition of tumors. By complementing and binding to the 3'UTR region of target gene mRNA, miRNAs promote the degradation of mRNA or inhibit its translation [10]. miR-216a is a miRNA associated with tumor proliferation and metastasis discovered in recent years [11]. As described in published studies [12], miR-216a can inhibit pancreatic carcinoma by regulating the expression of JAK2, and it can promote the drug tolerance and the relapse of liver cancer by inducing interstitial cell transformation. However, correlated research has yet to be found for the function of miR-216a in osteosarcoma.

Autophagy is widespread in eukaryotes and highly conservative in its progress. It degrades damaged, degenerated, or aging substances and organelles in cells through lysosomes [13]. According to published studies [14, 15], the emergence and progression of tumors are tightly correlated with autophagy. When a cell lacks autophagy, DNA damage is activated, and this interferes with apoptosis and ultimately promotes tumor formation. In addition, it plays an important role in regulating the tumor microenvironment. Autophagy of tumor stroma can be involved in the process of tumor recurrence, metastasis, drug resistance, and other processes through the regulation of microcystin [16]. Beclin-1 is a homologue of yeast ATG6. As an essential gene for autophagy initiation involved in the formation of autophagosome, Beclin-1 can combine with Vps34 and Vps15 to form the Beclin-1-pi3k complex, which can recruit lipid substances in the cytoplasm and thus form autophagy membranes [17, 18]. In addition, Beclin-1 can interact with the protein of apoptosis inhibitor Bcl-2 to double regulate apoptosis and autophagy [19]. Current studies

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Table 3. The number of cells that penetrated the basement membrane in each group was tested using Transwell invasion assays ($\bar{x} \pm s$)

Group	Number of cells that penetrate the basement membrane	F	P
Blank control group	44.05±5.82	25.359	0.000
Negative control group	43.28±5.92		
miR-216a inhibitor group	24.27±2.31* [#]		

Note: Compared with the blank control group, * $P < 0.05$. Compared with the negative control group, [#] $P < 0.05$.

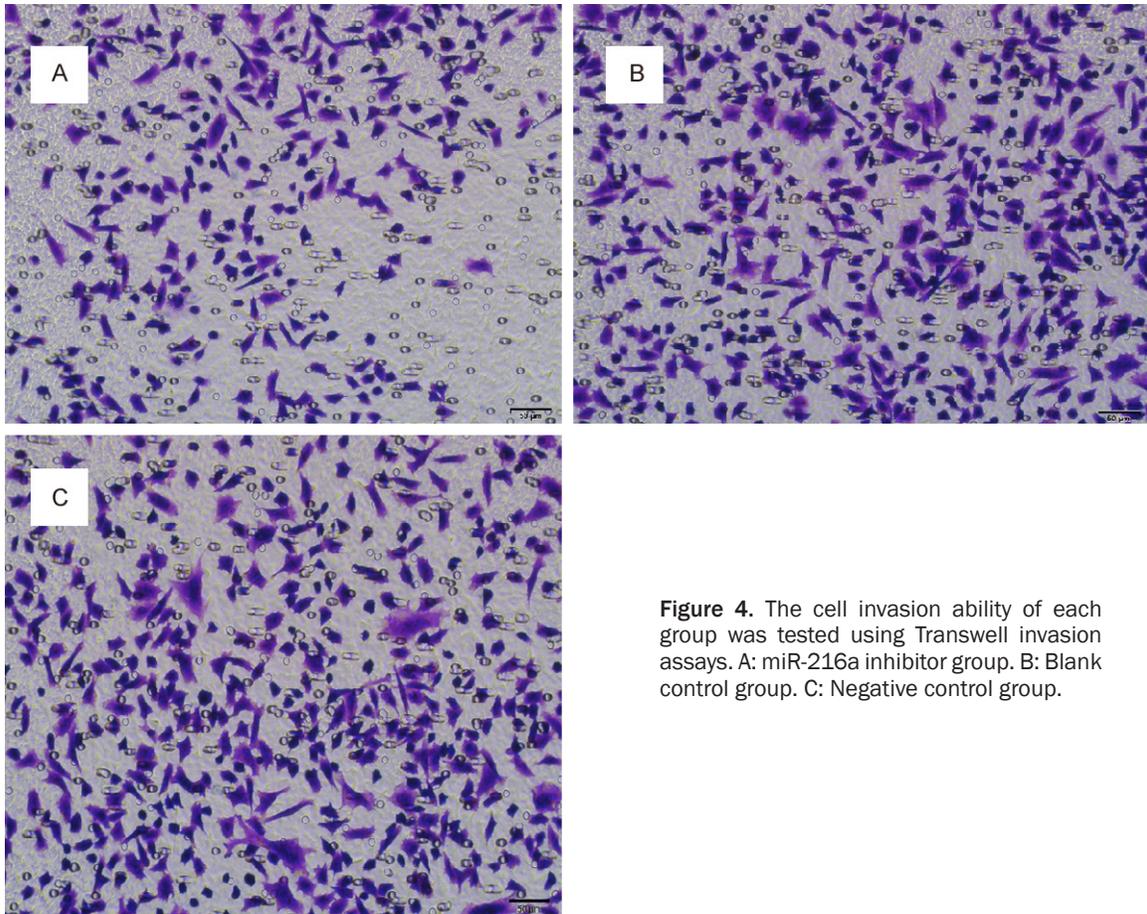


Figure 4. The cell invasion ability of each group was tested using Transwell invasion assays. A: miR-216a inhibitor group. B: Blank control group. C: Negative control group.

have found that Beclin-1 can be affected in the emergence and progression of tumors by regulating autophagy [20].

This study explored and analyzed miR-216a expression in osteosarcoma tissues and how it works on the proliferation, migration, and autophagy of osteosarcoma cells (U2OS). The results showed that the relative expression degree of miR-216a in osteosarcoma is significantly higher than it is in the paracancerous normal tissues. As shown in the results reported by other scholars [21], there is an upregula-

tion of miR-216a expression in osteosarcoma tissues, which may play the role of a tumor-promoting gene in osteosarcoma. In addition, the results of the transfection of the miR-216a inhibitors through liposomes showed that the miR-216a expression level in the miR-216a inhibitor group was greatly reduced, suggesting that it can be effective in the transfection of miR-216a inhibitors to inhibit the expression degree of miR-216a in osteosarcoma cells. Further analysis of the effect of miR-216a interference on osteosarcoma cells showed that the cell proliferation of the miR-216a inhibitor

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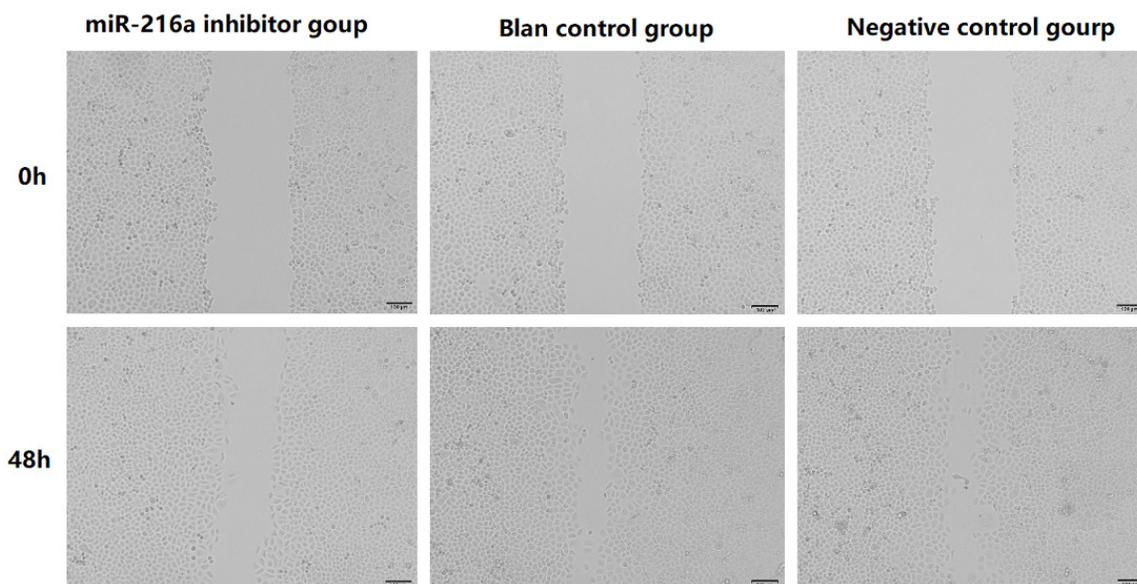


Figure 5. The migration ability of the osteosarcoma cells in each group was determined using scratch tests.

Table 4. The relative expression of the Beclin-1 protein in the osteosarcoma cells of each group ($\bar{x} \pm s$)

Group	The relative expression of the Beclin-1 protein	F	P
Blank control group	1.257±0.165	11.143	0.000
Negative control group	1.216±0.207		
miR-216a inhibitor group	1.752±0.223*.#		

Note: Compared with the blank control group, * $P < 0.05$. Compared with the negative control group, # $P < 0.05$.

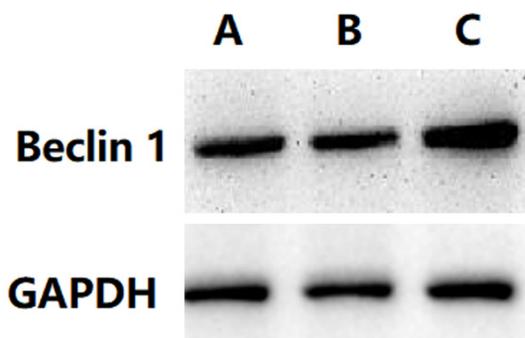


Figure 6. The Beclin-1 protein expressions of the osteosarcoma cells in each group. A: Blank control group. B: Negative control group. C: miR-216a inhibitor group.

group was remarkably decreased compared to the no-treatment control group and the negative control group. The result, which is consistent with our conjecture, suggests that the abnormally lifted expression degree of miR-

216a may work to promote osteosarcoma. However, the inhibition of miR-216a expression helps to inhibit the proliferation of osteosarcoma cells, which may be a potential biological indicator for osteosarcoma cell therapy. The number of cells that penetrate the basement membrane in the miR-216a inhibitor group was notably less than it was in the no-treatment control group and the negative control group. After 48 hours treatment using a scratch test for the three groups of cells, the migration ability of the osteosarcoma cells in miR-216a inhibitor group was notably less than it was in the negative control group and the no-treatment control group. This indicated that interference with the miR-216a expression in osteosarcoma can reduce the invasion and migration ability of osteosarcoma cells, which is the key to preventing tumor metastasis and recurrence. In addition, the study analyzed the expression of the autophagy-related protein Beclin-1 in three groups of cells. The results indicated that the relative expression degree of the Beclin-1 protein in the osteosarcoma cells in the miR-216a inhibitor group increased significantly compared to the no-treatment control group and the negative control group. This suggests that interference with the expression of miR-216a in osteosarcoma may play a corresponding tumor suppress-

sive effect by promoting tumor cell autophagy, and further proved that miR-216a plays a key role in the emergence and progression of osteosarcoma, which is similar to the results reported by other scholars [22].

To sum up, miR-216a expression is abnormally elevated in osteosarcoma tissues. The inhibition of miR-216a expression in osteosarcoma cells helps to prevent the proliferation and reduce the invasion and migration of osteosarcoma cells and supports autophagy in osteosarcoma cells, which provides a new prospective biological direction for curing osteosarcoma.

Disclosure of conflict of interest

None.

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- through regulating NF- κ B signaling pathway. *Eur Rev Med Pharmacol Sci* 2018; 22: 7633-7639.
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