

The expression and function of miRNA-106 in pediatric osteosarcoma

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Abstract. – OBJECTIVE: This study is to investigate the expression and biological function of miRNA-106b in osteosarcoma.

PATIENTS AND METHODS: Freshly resected osteosarcoma tissues and the corresponding para-carcinoma tissues were collected from 54 patients. Then miR-106b level in the carcinoma tissues was detected by real-time PCR. To test the function of miR-106b, osteosarcoma cell line U2-OS was transfected with miR-106b inhibitor in vitro. Then, the proliferation, cell cycle, invasion and metastasis of U2-OS cells were detected by CCK-8 assay, flow cytometry, and transwell respectively. The PI3K/AKT signaling pathway activity after treatment was detected by Western blot.

RESULTS: miR-106b level was significantly higher in osteosarcoma, and its expression was positively correlated with the lung metastasis and the clinical stages. *In vitro* experiments showed that the proliferation, invasion, and migration of U2-OS osteosarcoma cells were inhibited after miR-106b inhibition. The transition from G1 to S phase of U2-OS osteosarcoma cells was inhibited after miR-106b inhibition. The Western blot analysis demonstrated that both the AKT phosphorylation in PI3K/AKT pathway and the PI3Kp100 expression were significantly reduced when the expression of miR-106b was down-regulated.

CONCLUSIONS: miR-106b level was significantly up-regulated in osteosarcoma, which was positively correlated with the lung metastasis and clinical stages. The down-regulation of miR-106b inhibited the proliferation, invasion, and migration of osteosarcoma cells; thus, it may function as an oncogene to promote osteosarcoma proliferation and invasion through the PI3K/AKT signaling pathway.

Key Words:

miRNA-106, Pediatric osteosarcoma, PI3K/AKT signaling pathway.

Introduction

Osteosarcoma originates in the leaf tissues and accounts for 5% of pediatric bone cancers; it is the most primary malignant bone tumor in children and adolescents under the age of 20¹⁻³. Studies have demonstrated that about 80 % osteosarcoma occurs in long bone^{4,5}. Metaphysis of long bones surrounding the knee is at high risk for tumor formation, followed by the axial skeleton and the pelvis^{6,7}. Though the incidence of osteosarcoma is not high, its high malignancy, strong invasion ability and the earlier onset of lung metastasis may cause poor prognosis and high mortality. The 5-year survival rate after amputation is only 5-15%^{8,9}. Thus, to investigate the molecular mechanisms underlying the development of osteosarcoma is of important clinical significance.

MicroRNA (miRNA) is a kind of highly conserved small non-coding RNA molecule, with the length of 18-22 nt. miRNAs are widely presented in plants and animals, participating in the post-transcriptional regulation of genes expression^{10,11}. miRNAs are involved in almost all physiological and pathological processes^{12,13}. Each miRNA has its own specific target genes and one miRNA could usually regulate multiple genes, several miRNAs also could regulate the same genes¹⁴. In tumors, miRNA could play a role as either oncogene or anti-oncogene, and its expression is closely related to the development of tumors¹⁵.

Studies have shown the expression of miRNAs in osteosarcoma is abnormal, and it is closely related with the recurrence, metastasis and drug resistance of osteosarcoma¹⁶. The stability of miRNAs in the peripheral blood has an important role in the early diagnosis of osteosarcoma and prognostic prediction¹⁷. For example, the reduced expression

of miR-223 in human peripheral blood is closely related with early diagnosis and prognosis of patients with osteosarcoma¹⁸. By targeting the regulation of a pyrimidinic endonuclease, miR-513a-5p could promote the sensitivity of osteosarcoma cells to radiation therapy¹⁹. Jacques et al²⁰ found that miR-193a-5p could regulate the sensitivity of osteosarcoma cells to cisplatin by inhibiting p73 expression, suggesting that the combination of miRNA regulation and chemotherapy could improve the clinical therapy for osteosarcoma. These researches prompt that miRNAs are widely involved in the development of osteosarcoma. Identifying the novel miRNA molecules for diagnosis and treatment of osteosarcoma is of clinical significance. miR-106b is a tumor-associated miRNA molecule, which is identified in recent years^{21,22}. Studies^{23,24} have shown the abnormal expression of miR-106b played a role as an oncogene in liver cancer, breast cancer, chronic myelogenous leukemia and other tumors. However, the expression and biological function of miR-106b in osteosarcoma are still not clear.

In this paper, we studied the expression and functions of miR-106b in osteosarcoma. Our findings may provide the experimental and theoretical basis for understanding the molecular mechanisms of miR-106b in osteosarcoma.

Patients and Methods

Collection of Clinical Samples and Data

Osteosarcoma samples from 54 patients were studied. There were 35 males and 18 females with the mean age of 12.3 years, including 32 cases with lung metastasis. All patients did not receive radiation therapy, chemotherapy or other anti-cancer therapy. All cases were confirmed as osteosarcoma by clinical, radiological and histological diagnosis. Fresh osteosarcoma samples were resected from the 54 patients and then stored in the hospital from October 2013 to December 2015. The para-carcinoma tissues that were 5 cm far away from the cancer tissues were also collected. All the clinical information of the patients was collected. Prior written and informed consent were obtained from every patient, and the study was approved by the Ethics Review Board of Yishui Central Hospital of Linyi City.

Culture of Osteosarcoma Cell Line

U2-OS cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured

in DMEM complete medium (Gibco, Grand Island, NY, USA) at 37°C, 5% CO₂. Cells were passaged when the growth density reached 80-90%; the medium was changed once every two days.

RNA Extraction and RT-PCR Analysis

Osteosarcoma and para-carcinoma tissues were frozen in liquid nitrogen and then pulverized. Total RNAs were extracted by TRIzol® isolation reagent (Thermo Fisher Scientific, Waltham USA). Then the RNA concentration and quality were tested by gel electrophoresis and UV spectrophotometer 260/280 analysis. RNA was reverse transcribed into cDNA and stored at -20°C until use. Real-time PCR was performed on Step one Plus Real-time PCR System (Thermo Fisher Scientific, Waltham USA). The GAPDH was used as internal control.

Transfection

U2-OS osteosarcoma cells were cultured with DMEM complete medium in 24-well plate. Totally 20 uM miR-106b inhibitor was mixed with 2 ul lipo2000 (Thermo Fisher Scientific, Waltham USA) in 50 ul Opti-MEM medium (Thermo Fisher Scientific, Waltham USA) and incubated for 6 hours. The mixture was added into cell culture when cell density was 70%-90%. The medium was replaced after 6 hours of incubation. Then the cells were cultured for 48 h.

Test Proliferation of Osteosarcoma Cell by CCK-8 Assay

U2-OS cells were cultured in 96-well plate at 3000 cells/well. After 24 h of mir-106b transfection, the cells were cultured for 24 h, 48 h, and 72 h in fresh medium respectively. Then CCK-8 (Beyotime Biotechnology, Beijing, China) was added and incubated for 30 min. The absorbance of each well was measured at 490 nm wavelength by spectrophotometer. The cell proliferation curve was plotted.

Osteosarcoma Cell Invasion and Migration by Transwell Assay

The 24-well transwell chamber with 8 µm aperture (Corning Inc., Corning, NY, USA) was used. The 2 × 10⁵ U2-OS cells were added into the upper chamber in serum-free 1640 medium. The lower chamber was added with 600 ul 1640 medium containing 10% FBS. After 24 h, cells in the upper chamber were wiped off. Cells in the lower chamber were fixed with 4% formal-

dehydrate for 10 min and stained with Giemsa. The number of cells through the membrane was calculated, and then the cell migration was assessed.

Extracellular matrix environment was simulated by Matrigel (Thermo Fisher Scientific, Waltham, MA, USA) in cell invasion assay. Totally 100 μ l Matrigel in 1640 medium was smeared on the upper chamber and incubated at 37°C for 60 min. The procedures for cell invasion were the same as described above. The results were observed 72 h later.

Cell Cycle by Flow Cytometry

After 24 h transfection of miR-106b inhibitor, 1×10^6 cells were collected and washed with pre-cooled PBS for two times. The cell cycle was analyzed by Cell Cycle Assay Kit (BD, Franklin Lakes, NJ, USA) according to the manufacture's instruction. Then the cell cycle was tested by flow cytometry, and the results were analyzed using Modfit software (BD Biosciences, San Jose, CA, USA).

Western Blot

After 48 h of transfection, the cells were washed with pre-cooled PBS for two times. Then RIPA lysis buffer with 1% PMSF was added and incubated for 15 min. Total proteins were collected by centrifugation. The proteins were separated by SDS-PAGE and transferred to PVDF membrane. Then the membrane was blocked by 50 g/L milk at room temperature for 1 h. After blocking, primary antibodies [anti-AKT 1:1000, anti-p-AKT 1:1000, anti-PI3K-p110 α 1:1000, anti-PI3Kp85 1:1000 (CST, Boston, USA), anti-GAPDH 1:4000 (Beyotime Bi-

otechnology, Beijing, China)] were added and incubated at 4°C overnight. After washing with TBST, HRP-conjugated secondary antibodies [goat-anti-mouse-HRP, 1:5000; goat-anti-rabbit-HRP, 1:2000 (ABCAM, Cambridge, MA, USA)] were added and incubated at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The developed film was scanned using the AlphaImager gel imaging systems (AlphaImager, Santa Clara, CA, USA). And the Western blot images were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as an internal control.

Statistical Analysis

Statistical analysis was performed by using SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA). All measurement data were expressed as mean $\pm$ SD. The Student's *t*-test was used. $p < 0.05$ was considered statistically significant.

Results

miR-106b Expression and its Clinical Significance in Osteosarcoma Tissue

To determine the expression of miR-106b in osteosarcoma tissue, Real-time PCR was performed. The results showed that the expression of miR-106 in osteosarcoma tissues was significantly higher than that in the para-carcinoma tissues ($p < 0.05$) (Figure 1A). Besides, the expression of miR-106 in patients with lung metastasis (L1) was significantly higher than that in patients without lung metastasis (L0) ($p < 0.05$) (Figure 1B). And the miR-106b expression of patients in phase III

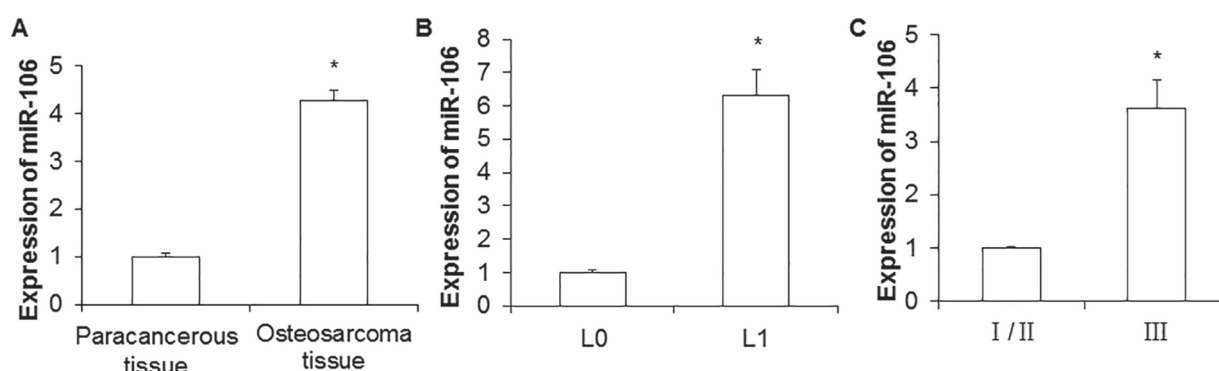


Figure 1. Analysis of miR-106b expression. The miR-106b expression was detected with RT-PCR. **A**, The miR-106b expression in osteosarcoma tissue and paracancerous tissue. **B**, The expression of miR-106 in patients with lung metastasis (L1) and patients without lung metastasis (L0). **C**, The miR-106b expression in osteosarcoma patients in phase III and phase I/II. * $p < 0.05$.

was significantly higher than that in phase I/II of clinical stages ($p < 0.05$) (Figure 1C). These results suggest that miR-106 expression might have a close relationship with the invasion and metastasis of osteosarcoma.

Effect of miR-106b on the Proliferation of Osteosarcoma Cell

To investigate the effect of miR-106b on the proliferation of osteosarcoma cells, the CCK-8 cell proliferation assay was performed. After the transfection of miR-106b inhibitor, the proliferation rate of osteosarcoma cells was significantly reduced, implying that the high level miR-106b should enhance the proliferation of human U2-OS osteosarcoma cells *in vitro*, and its function could be inhibited by miR-106b inhibitors (Figure 2).

Effect of miR-106b on Invasion and Migration of Osteosarcoma Cell

The invasion and migration of osteosarcoma cell after transfection of miR-106b were detected by transwell assay. Results showed that the U2-OS cell migration was significantly inhibited. The number of cells that went through the cell membrane was much less compared to the NC group (28.5 ± 5.6 vs. 73.8 ± 4.37 cells), after the transfection of miR-106b inhibitor (Figure 3A and 3B). Besides, the invasion test showed that the number of U2-OS cells (19.8 ± 2.1 vs. 39.5 ± 3.50) that passed through the chamber membrane decreased significantly (Figure 3A and 3B).

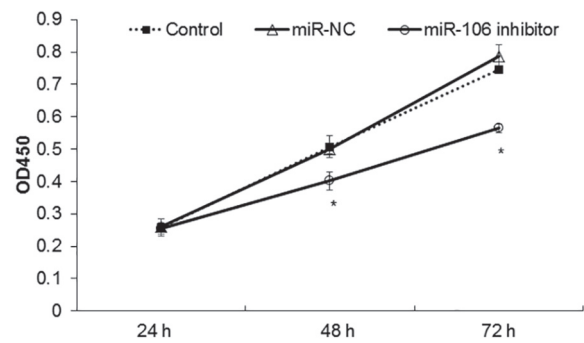


Figure 2. The function of miR-106b on U2-OS osteosarcoma cells proliferation. U2-OS cells were cultured in 96-well plate at 3000 cells/well. U2-OS cells were transfected with miR-106b inhibitor, miR-NC or un-transfected (control) and then incubated for 24 h, 48 h and 72 h. Then CCK-8 was added and incubated for 30 min. The absorbance of each well was measured at 490 nm wavelength by spectrophotometer. Compared with NC and control, * $p < 0.05$.

Promotion of G1/S Cycle Transition in Osteosarcoma U2-OS Cells by miR-106b

The influence of miR-106b on cell cycle of U2-OS cells was tested by flow cytometry. miR-106b inhibitor treated group showed G1/S phase arrest compared to the NC group (Figure 4), suggesting that miR-106b could regulate the growth of U2-OS osteosarcoma cells by regulating the G1/S phase transition.

Detection of PI3K Signaling Pathway by Western Blot

Western blot analysis showed that after transfection of miR-106b inhibitor, the phosphoryla-

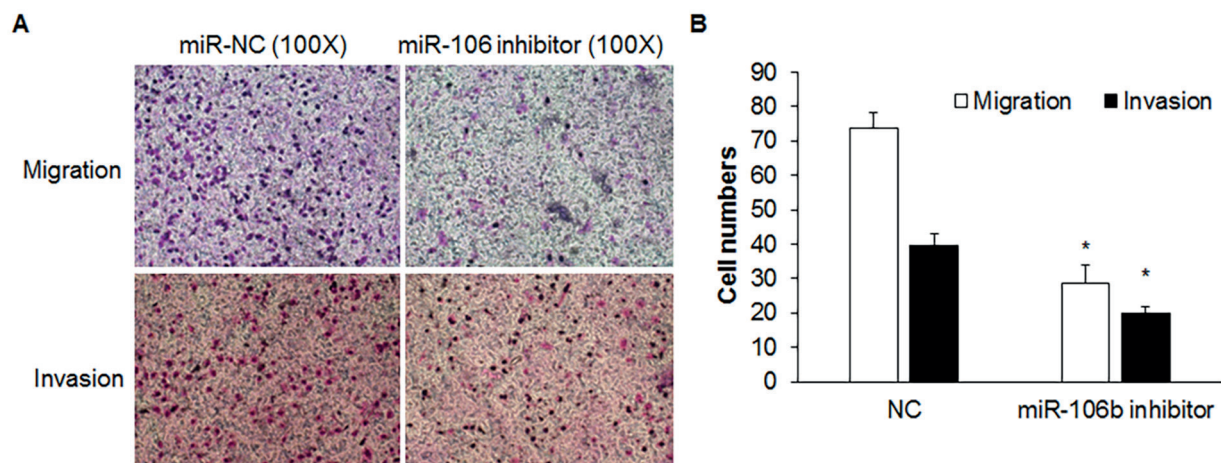


Figure 3. The function of miR-106b on the migration and invasion of U2-OS osteosarcoma cells. Transwell assay and Matrigel invasion assay was performed to evaluate the effect of miR-106 on cell migration and invasion, respectively. **A**, Representative images of cell migration and invasion (Magnification: 100X). **B**, Relative number of migrated or invaded cells. Compared with NC, * $p < 0.05$.

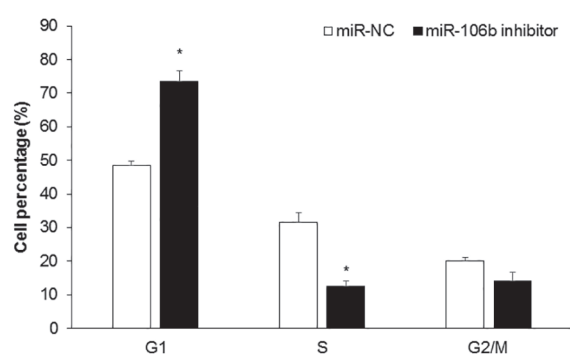


Figure 4. Percentage of cells in each cell phase. U2-OS cells transfected with miR-106b inhibitor or miR-NC. Cell cycle was analyzed with flow cytometry. Percentage of cells in each cell phase was shown. Compared with NC, * $p < 0.05$.

tion of the key protein AKT in PI3K/AKT signaling pathway was significantly reduced in U2-OS cells, as well as the expression of PI3Kp110. However, the expression of PI3Kp85 had no significant changes (Figure 5A and 5B). These results suggested that miR-106b could promote the activation of PI3K/AKT signaling pathway in osteosarcoma cells.

Discussion

In this study, we first found the increased expression of miR-106b in osteosarcoma, and it was positively correlated with lung metastasis and clinical TNM stages. After down-regulation, the miR-106b expression in osteosarcoma U2-OS cell line, the proliferation, invasion, and migration of

tumor cell were inhibited. Western blot results showed that miR-106b down-regulation could inhibit PI3K/AKT signaling pathway. Therefore, we propose that miR-106b gene may function as an oncogene in osteosarcoma by the activation of PI3K/AKT signaling pathway, thus promoting the development of osteosarcoma.

miR-106b is one of the members of the miR-17 family, located at chromosome 7q22, and is a tumor-associated miRNA molecule^{22,25}. The miR-106b expression is up-regulated in many tumors, involving in tumor invasion and metastasis²⁶. In tissues and blood of gastric cancer, miR-106b expression was significantly increased, correlated well with the invasion and metastasis of gastric cancer²⁷. The expression of miR-106b in the tumor tissue of colorectal cancer patients without lymph node metastasis was significantly higher than its adjacent tissues²⁸. In primary hepatocellular carcinoma, miR-106b also showed high expression levels, which could inhibit the apoptosis of tumor cells and promote proliferation through regulation of E2F1²⁹. Moreover, miR-106b was also involved in the regulation of p21 expression, promoting the proliferation of breast cancer cells³⁰. These results indicate that miR-106b may function as an oncogene in tumors. Our results showed that miR-106 expression in osteosarcoma tissues was significantly higher, and it was closely correlated with the lung metastasis and clinical stages.

We successfully inhibit the expression of miR-106b in osteosarcoma U2-OS cells, and found that the proliferation of U2-OS cells decreased, indicating that miR-106b may pro-

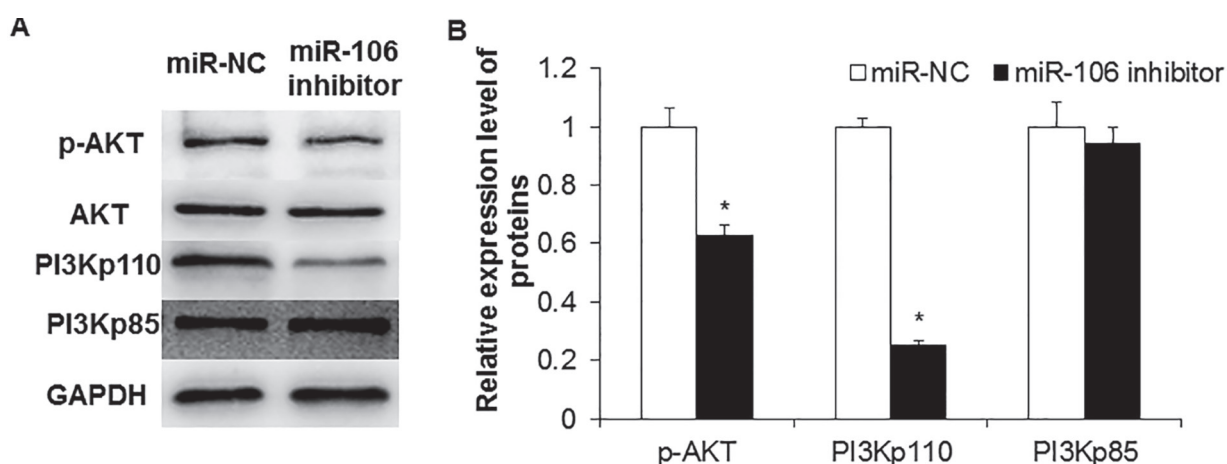


Figure 5. Analysis of relevant protein expression in PI3K/AKT signaling pathway. U2-OS cells transfected with miR-106b inhibitor or miR-NC. Protein expression levels were analyzed with Western blot. **A**, Representative Western blot results. **B**, Quantitative Western blot results. Compared with NC, * $p < 0.05$.

mote the proliferation of U2-OS. The transwell experiment results showed that miR-106b inhibition could inhibit the invasion and migration of U2-OS cells. Besides, the flow cytometry analysis showed that miR-106b inhibition could suppress U2-OS cells transition speed in G1/S phase, thereby inhibiting their proliferation. PI3K/AKT signaling pathway is an important target for cancer therapy, functions in the proliferation, tumor invasion and metastasis³¹. In breast cancer patients with metastasis, inhibition of PI3K/AKT/ mTOR signaling pathway helps to improve the patients' prognosis³². Xi et al³³ found that PI3K/AKT pathway could induce cell carcinoma stem cell phenotype in HPV16 virus-induced esophageal squamous carcinoma. Through the activation of PI3K/AKT signaling pathway, IL-11 promoted the epithelial-mesenchymal transition of anaplastic thyroid tumor cells³⁴. Studies have shown that multiple miRNA molecules are also involved in the regulation of PI3K/AKT signaling pathway. For example, miR-618 inhibited the proliferation of tumor cells in the thyroid tumor by targeting the regulation of PI3K/AKT signaling pathway³⁵. Sun et al³⁶ found that miR-7 inhibited resistant the tolerance to drugs of melanoma through targeting the regulation of MAPK and PI3K/AKT signaling pathways. miR-4638-5p was capable of inhibiting the development of castration-resistant prostate cancer by inhibiting PI3K/AKT signaling pathway³⁷. In this study, we found the down-regulation of miR-106b could inhibit the expression PI3Kp100. The p110 is the catalytic subunit of the PI3K kinase, which can enhance the level of AKT phosphorylation³⁸. Western blot results showed that p-AKT expression was significantly decreased, suggesting that PI3K/AKT pathway was inhibited. These results suggested that miR-106b over expression may activate PI3K/AKT signaling pathway, thus promoting the development of osteosarcoma cells.

Conclusions

We demonstrated that miR-106b functioned as an oncogene in the development of osteosarcoma. miR-106b may contribute to the proliferation, cycle transformation, invasion and metastasis of osteosarcoma by activating PI3K/AKT signaling pathway.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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