Int J Clin Exp Med 2017;10(5):7767-7773
www.ijcem.com /ISSN:1940-5901/IJCEM0042398

Original Article

The effect of miR27b on osteosarcoma cell proliferation and invasion

Chengliang Yang[1], Dong Xuan[1], Tianyu Chen[3], Jian Chen[4], Lizhu Tang[2], Zhihua Yang[2], Zhidan Li[1], Eryi Deng[1]

Departments of [1]Oncology, [2]Tumour, Central People's Hospital of Zhanjiang, Zhanjiang, China; [3]Department of Orthopaedic, The Third Affiliated Hospital of Southern Medical University, Guangzhou, China; [4]Three Gorges Central Hospital of Chongqing, Chongqing 404000, China

Received July 19, 2016; Accepted January 3, 2017; Epub May 15, 2017; Published May 30, 2017

Abstract: Osteosarcoma is one of malignant bone tumors mainly occurred in young children. miR-27b was found closely associated with the development of a variety of malignant tumors. In this study, the effect of miR-27b on the proliferation, invasion, and migration of osteosarcoma MG-63 cells and the relationship between miR-27b and JNK/p38 signaling pathway were investigated. Osteosarcoma MG-63 cells were conventional cultured. miRNA-27b mimics, miRNA-27b inhibitor, mimic control, inhibitor control were transfected into MG-63 cells. PCR was used to detect the expression of miRNA-27b and MAP2K4 in transfected MG-63 cells. MTT assay was used to measure MG-63 cell proliferation activity. Luciferase reporter assay was used to analyze target genes. Transwell assay was used to measure invasion and migration (Matrigel) capability changes after transfection. Western blotting was used to measure the expression levels of MAP2K4, JNK1, and p38 in MG-63 cells after transfection. In miRNA-27b mimic group, the expression level of miRNA-27b significantly increased and MAP2K4 mRNA expression level decreased significantly. In miRNA-27b inhibitor group, expression level of miRNA-27b decreased while MAP2K4 mRNA expression level increased significantly ($P < 0.05$). Transfection of miRNA-27b inhibitor significantly decreased cell proliferation ($P < 0.05$). Capability of invasion and migration of MG-63 cells was significantly enhanced by transfection of miRNA-27b mimic ($P < 0.05$). miRNA-27b inhibitor significantly reduced capability of invasion and migration of MG-63 cells ($P < 0.05$). Transfection of miRNA-27b inhibitor significantly enhanced the expression of MAP2K4, JNK1, and p38 in MG-63 cells, and transfection of miRNA-27b mimic significantly inhibited the expression of MAP2K4, JNK1, and p38 in MG-63 cells ($P < 0.05$). miRNA-27b can inhibit MAP2K4 expression and activate JNK/p38 signaling pathway to suppress the proliferation, invasion, and migration of MG-63 cells.

Keywords: miR-27b, osteosarcoma, proliferation, Invasion, JNK/p38

Introduction

Osteosarcoma is a common malignant bone tumor which mainly occurs in children and adolescents. It is highly malignant with poor prognosis and lower survival rate [1]. Many treatments including surgery, chemotherapy, and neoadjuvant chemotherapy have been carried out in recent years. Although the survival rate is greatly improved, many patients are unable to adhere to chemotherapy because of the side effects of chemotherapy drugs, leading to tumor recurrence, metastasis, or forced amputation [2]. Micro RNAs (microRNAs, miRNAs) are short (70-80 nt), non-coding single-stranded RNA which have a strong regulatory role [3]. miRNAs bind to 3' untranslated region (3'-UTR) of their target mRNAs (*Bcl-Xl, Bcl-2, RAS, etc*), leading to inhibition gene expression which play an important role in the regulation of cell growth, development, and tumorigenesis [4]. Previous studies indicated that miR-27b level was decreased in prostate cancer and overexpression of miR-27b could inhibit androgen to suppress the metastasis of prostate cancer [5]. Expression of tumor suppressor gene, E-cadherin was overexpressed by miR-27b to inhibit prostate cancer [6, 7]. Furthermore, miR-27b has also been demonstrated to be involved in the pathogenesis of other types of cancers, including Non-small cell lung cancer [8], breast cancer [9], etc. Given the closely association of miR-27b with several cancers, whether it is involved in the development of osteosarco-

Table 1. Primer sequences

Gene		Sequence (5'-3')
miRNA-27b	Forward	5'-AGCCGTCAAGAGCAATAACGAA-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
MAP2K4	Forward	5'-TGGTGGGCAGTATGTTGT-3'
	Reverse	5'-GCTATTGGCATTGGTGAA-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

ma remains unclear. In this study, selected osteosarcoma cell line MG-63 was transfected with miR-27b to investigate the role of miR-27b on the proliferation, invasion of osteosarcoma cells.

Material and methods

Experimental cells

Osteosarcoma MG-63 cell was purchased from Shanghai Baili biotech.

Reagents and instruments

miRNA-27b mimics and miRNA-27b inhibitor were purchased from U.S. GenePharma (**Table 1**). DMEM culture medium, penicillin/streptomycin, Trizol reagent, and fetal calf serum were purchased from Gibco (USA). LipofectamineTM 2000 was purchased from Invitrogen. miRNA-27b, MAP2K4, JNK1, and p38 kit were purchased from Takara Shuzo (Japan). RPMI-1640 medium and MTT were purchased from sigma (USA). Carbon dioxide incubator and inverted microscope were purchased from SANYO (Japan).

Methods

Conventional cell culture

Osteosarcoma MG63 cells were conventional cultured with RPMI1640 medium in 37°C incubator supplemented with 5% of CO_2.

Cell transfection

Osteosarcoma MG-63 cells were cultured with DMEM containing 10% fetal bovine serum, 100 u/mL penicillin, and 100 u/mL streptomycin. Cells were seeded in a cell culture plate to get a confluence rate of about 60%. miRNA-27b mimics, miRNA-27b inhibitor, mimic control, and inhibitor control were transfected into MG-63 cells with Lipofectamine 2000 as transfection reagent. Cells were changed with fresh medium 4-6 h after transfection and cultured for 48 h. Non-transfected MG-63 cells were used as blank control.

Real time-PCR detection of miRNA-27b, MAP2K4 expression in transfected MG-63 cells

Total RNA was extracted 72 h after transfection according to the manufacturer's instruction. The concentration of total RNA was measured and 200 ng of total RNA was used to synthesize cDNA for subsequent PCR reaction at: 95°C 5 min for pre-denaturation; 30 cycles of 95°C 30 s, 58°C 30 s, 72°C 30 s; and a final extension at 72°C for 10 min. The primers used in this study were summarized in **Table 1**. U6 snRNA was used as internal control.

Luciferase reporter assay

3'-untranslated region (3'-UTR) of MAP2K4 containing miRNA-27b binding site was amplified by PCR and inserted into vector. Vectors carrying mutant MAP2K4 3'-UTR was used as a control. Gene expression was measured 24 h after transfection of miRNA-27b according to manufacturer's instruction.

MTT assay

Cells with different transfection were seeded to cell culture plate at a concentration of 8×10^4/well. Cell viability was measured at 24, 48, and 72 h after inoculation. 20 μL of MTT solution (5 mg/mL) was added to each well, incubated for 4 h, stopped by adding 150 μL of dimethylsulfoxide to each well and shaking for 10 min. The absorbance at 570 nm was measured with a microplate reader.

Transwell assay of invasion and migration

Invasion assay: Matrigel was added to each chamber and incubated at 4°C overnight. Transwell chambers were hydrated in serum-free medium at 37°C for 1 h. Transfected cells were seeded in the upper chamber, RPMI1640 medium was added to the lower part. Cells were stained with Giemsa staining for observation under microscopy.

Migration experiments: All steps are the same with invasion assay except that no artificial basement membrane was used.

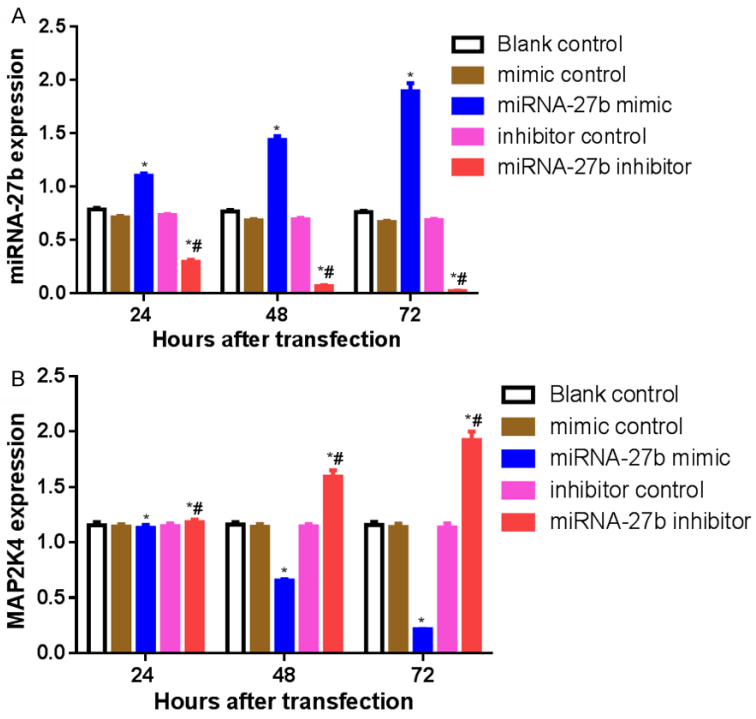

Figure 1. Expressions of miRNA-27b (A) and MAP2K4 (B) after transfection. *Compared to Blank control, $P < 0.05$. #Compared to mimic, $P < 0.05$.

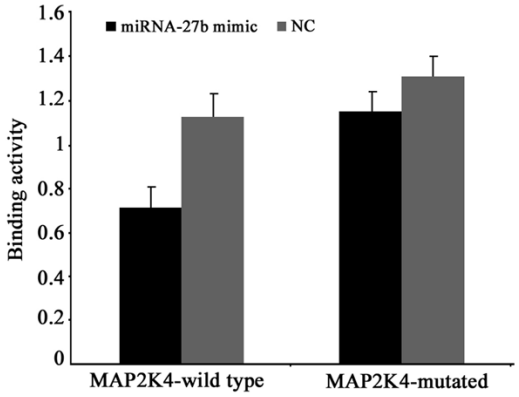

Figure 2. Analysis of the binding of miRNA-27b and MAP2K4 3'-UTR. *, Compared to control group (NC), $P < 0.05$. 3'-untranslated region (3'-UTR) of MAP2K4 containing miRNA-27b binding site was amplified by PCR and inserted into vector. Vectors carrying mutant MAP2K4 3'-UTR was used as a control. Gene expression was measured 24 h after transfection of miRNA-27b according to manufacturer's instruction.

Western blot analysis of MAP2K4, JNK1, and p38

40 μg of cell lysates was loaded to 8% SDS-PAGE to resolve and then transferred to PVDF membrane. Membrane was then blocked at room temperature for 1 h and incubated with first antibody (anti-MAP2K4 antibody, anti-JNK1 antibody, and anti-p38 antibody were diluted to 1:200, antiβ-actin antibody was diluted to 1:500) at 4°C overnight. Membrane was washed with TBST and secondary antibodies (1:2000) were added and incubated for 1 h. Membrane was then washed and developed. Quantity One software was used for optical density analysis.

Statistical analysis

SPSS17.0 was used for statistical analysis. All data are expressed as mean ± standard deviation. χ^2 test was used to analyze counting data. Analysis of variance was used to do the comparison between groups. $P < 0.05$ was considered statistically significant.

Results

Upregulation of miRNA-27b and downregulation of MAP2K4 in MG-63 after transfection

Results showed that in miRNA-27b mimic group, the expression level of miRNA-27b was significantly up-regulated in a time-dependent manner. In contrast, the level of MAP2K4 mRNA was significantly down-regulated in a time-dependent manner ($P < 0.05$). In miRNA-27b inhibitor group, the expression level of miRNA-27b was significantly down-regulated in a time-dependent manner and the level of MAP2K4 mRNA was significantly up-regulated in a time-dependent manner ($P < 0.05$) (**Figure 1**).

MAP2K4 mutation affects miRNA-27b binding

Luciferase density of MG-63 cells co-transfected with both MAP2K4 3'-UTR and miRNA-27b mimic was significantly decreased ($P < 0.05$). Mutation of MAP2K4 3'-UTR inhibited the binding of MAP2K4 3'-UTR and miRNA-27b, leading to increased luciferase activity (**Figure 2**).

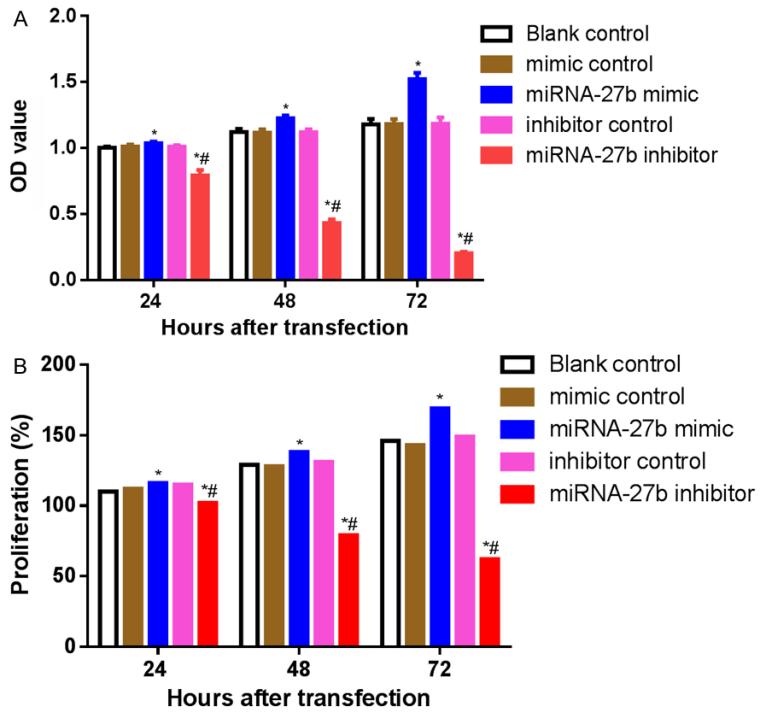

Figure 3. Analysis of MG-63 cell proliferation after transfection. A. OD value, B. Proliferation (%). *Compared to Blank control, $P < 0.05$. #Compared to mimic, $P < 0.05$.

Increased cell proliferation after transfection

MTT assay was used to measure MG-63 cell proliferation after transfection. Results showed that OD value and ratio of proliferation of miRNA-27b mimic group were significantly increased in a time-dependent manner ($P < 0.05$). In contrast, OD value and ratio of proliferation of miRNA-27b inhibitor group were significantly decreased in a time-dependent manner ($P < 0.05$) (**Figure 3**).

Transwell assay of invasion and migration of MG-63 cells after transfection

Results showed that transfection of miRNA-27b mimic significantly increased the invasion and migration of MG-63 cells, whereas transfection of miRNA-27b inhibitor significantly decreased the invasion and migration of MG-63 cells ($P < 0.05$) (**Figure 4**).

Expression of MAP2K4, JNK1, and p38 in MG63 cells after transfection

Western blot was used to measure the expression changes of MAP2K4, JNK1, and p38 in MG-63 cells after transfection. Results showed that compared with mimic control or blank control group, transfection of miRNA-27b inhibitor significantly increased the expression levels of MAP2-K4, JNK1, and p38, whereas transfection of miRNA-27b mimic significantly decreased the expression levels of MAP2K4, JNK1, and p38 ($P < 0.05$, **Figure 5**).

Discussion

Osteosarcoma is commonly occurred in children and adolescents and is ranked No. 4. Deaths from osteosarcoma account for 8.9% of deaths from all malignant tumors. The disability-adjusted life-years of osteosarcoma are 10 years more than that of the gastrointestinal cancer, lung cancer, and breast cancer [7, 8]. More and more studies have found that multiple genes affect the occurrence, development and prognosis of osteosarcoma [9]. MicroRNAs (miRNAs) are endogenous non-coding single-stranded RNA which can bind to 3'-UTR of target genes' mRNAs to degrade target mRNAs, inhibit translation, and suppress gene expression [11]. Researchers have found that many miRNAs involved in the development and progression of osteosarcoma, including miR-29a, miR-29c, miR-195, miR-181a, miR-181b, miR-26a, and miR-218 [12-14]. Previous studies also found that miRNA-27b can inhibit the development of prostate cancer. In this study, the effects of miRNA-27b on the proliferation and invasion of osteosarcoma MG-63 cells were investigated.

In the present study, miRNA-27b mimics, miRNA-27b inhibitor, mimic control, and inhibitor control were transfected into human osteosarcoma MG-63 cells. Results showed that transfection of miRNA-27b mimics increased the level of miRNA-27b and decreased the level of MAP2K4 mRNA in a time-dependent manner. In contrast, transfection of miRNA-27b inhibitor decreased the level of miRNA-27b and increased the level of MAP2K4 mRNA in a time-

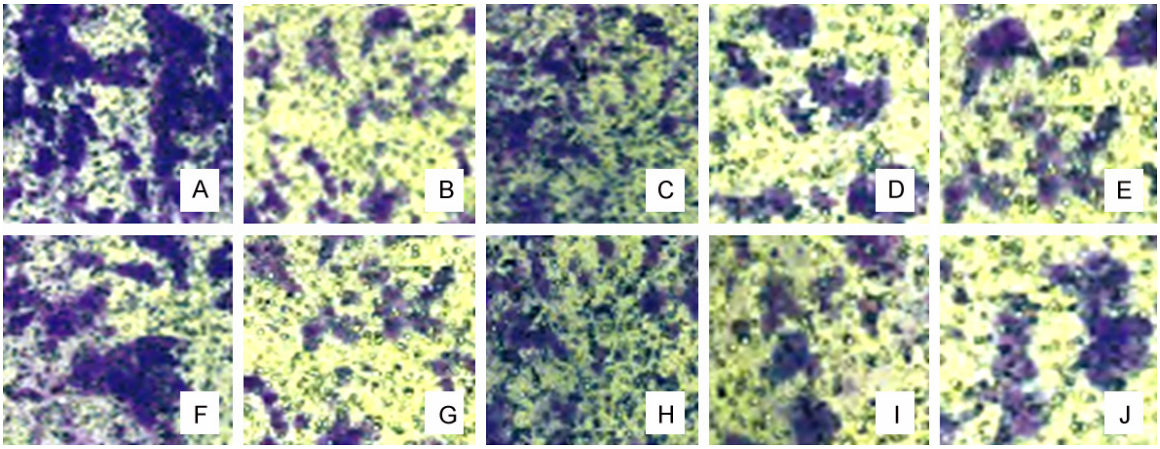

Figure 4. Invasion and migration of MG-63 after transduction. A. Invasion of MG-63-miRNA-27b mimic; B. Invasion of MG-63-miRNA-27 inhibitor; C. Invasion of MG-63-mimic control; D. Invasion of MG-63-inhibitor control; E. Invasion of MG-63-miRNA-control; F. Migration of MG-63-miRNA-27b mimic; G. Migration of MG-63-miRNA-27 inhibitor; H. Migration of MG-63-mimic control; I. Migration of MG-63-inhibitor control; J. Migration of MG-63-miRNA-control.

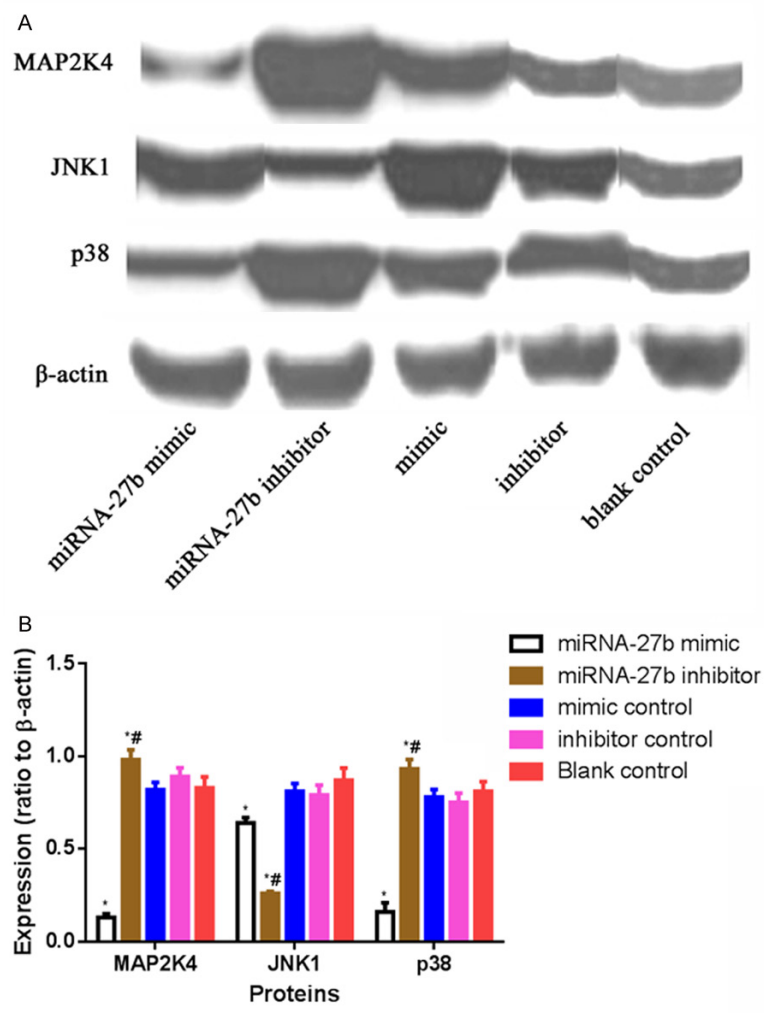

Figure 5. Expression of MAP2K4, JNK1, and p38 in MG63 cells after transfection. A. Representative western blot graph. B. Quantitative analysis of expression of MAP2K4, JNK1, and p38.

dependent manner. These results suggest that miRNA-27b has certain inhibitory effect on the expression of MAP2K4. Luciferase reporter assay showed that miRNA-27b can bind to 3'-UTR of MAP2K4 to negatively regulate the expression of MAP2K4. Inhibition of miRNA-27b resulted in the increase of MAP2K4 in MG-63 cells.

In this study, after MTT assay was used to measure the proliferation of MG-63 cells after transfection. OD values and proliferation ratio of miRNA-27b mimic group showed significant increase in a time-dependent manner, whereas OD values and proliferation ratio of miRNA-27b inhibitor group showed significant decrease in a time-dependent manner, suggesting that miRNA-27b inhibited the proliferation of MG-63 cells. Migration and invasion assay showed that transfection of miRNA-27b mimic enhanced the capability of migration and invasion of MG-63 cells. In contrast, transfection of miRNA-27b inhibitor

suppressed the capability of migration and invasion of MG-63 cells. These results suggested that lower level of miRNA-27b inhibited the migration and invasion of MG-63 cells. Previous studies have found that miR-27b can inhibit the growth, proliferation, and metastasis of neuroblastoma via acting on the peroxisome proliferator-activated receptors [15]. Moreover, miR-27b can act angiogenic growth factor to promote the proliferation of vascular endothelial cells [16, 17]. Ye et al. found that the expression level of miR-27b in colon cancer tissue was significantly lower than that of adjacent tissue. Over-expression of miR-27b to inhibit VEGF could inhibit the proliferation of colon cancer cells [18]. Studies also found that overexpression of miR-27b resulted in the inhibition of cancer cells' proliferation, invasion, and migration, however, overexpression of c-MET resulted in the enhancement of cancer cells' proliferation, invasion, and migration, indicating that miR-27b inhibits cancer cells' proliferation through suppression of c-MET [19-23].

In this study, the protein levels of MAP2K4, JNK1, and p38 were measured by Western blot. Results showed that transfection of miRNA-27b mimic decreased the protein levels of MAP2K4, JNK1, and p38 in MG-63 cells. In contrast, transfection of miRNA-27b inhibitor increased the protein levels of MAP2K4, JNK1, and p38 in MG-63 cells, suggesting that inhibition of miRNA-27b could activate the JNK1/p38 signaling pathway.

In summary, miRNA-27b was expressed in the osteosarcoma MG-63 cells. miRNA-27b can affect MAP2K4 expression. Transfection of miRNA-27b could increase the expression level of MAP2K4 and activate JNK1/p38 signaling pathway, leading to the suppression of MG-63 cells' proliferation, invasion, and migration. miRNA-27b plays a vital role in the occurrence and progression of osteosarcoma and might be used to improve clinical treatment and prognosis of osteosarcoma.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jian Chen, Three Gorges Central Hospital of Chongqing, New City Road 165, Wanzhou District, Chongqing 404000, China. Tel: +86-0235-8103576; Fax: +86-0235-8103576; E-mail: jianchenbnm@sina.com

References

[1] Ottavian G, Jaffe N. The epidemiology of osteosarcoma. Cancer Treat Res 2009; 152: 3-13.
[2] Wafa H, Grimer RJ. Surgical options and outcomes in bone sarcoma. Expert Rev Anticancer Ther 2006; 6: 239-248.
[3] Yamamoto T, Nakai K, Matsumura A. Neutron capture therapy for glioblastoma. No Shinkei Geka 2008; 36: 293-302.
[4] Mongroo PS, Rustgi AK. The role of the miR-200 family in epithelial-mesenchymal transition. Cancer Biol Ther 2010; 10: 219-222.
[5] Rice MA, Ishteiwy RA, Magani F, Udayakumar T, Reiner T, Yates TJ, Miller P, Perez-Stable C, Rai P, Verdun R, Dykxhoorn DM, Burnstein KL. The microRNA-23b/-27b cluster suppresses prostate cancer metastasis via Huntingtin-interacting protein 1-related. Oncogene 2016; 35: 4752-61.
[6] Ishteiwy RA, Ward TM, Dykxhoorn DM, Burnstein KL. The microRNA-23b/27b cluster suppresses the metastatic phenotype of castration resistant prostate cancer cells. PLoS One 2012; 7: e52106.
[7] Lee JJ, Drakaki A, Iliopoulos D, Struhl K. MiR-27b targets PPAR gamma to inhibit growth, tumor progression and the inflammatory response in neuroblastoma cells. Oncogene 2012; 31: 3818-3825.
[8] Wan L, Zhang L, Fan K, Wang J. MiR-27b targets LIMK1 to inhibit growth and invasion of NSCLC cells. Mol Cell Biochem 2014; 390: 85-91.
[9] Takahashi RU, Miyazaki H, Takeshita F, Yamamoto Y, Minoura K, Ono M, Kodaira M, Tamura K, Mori M, Ochiya T. Loss of microRNA-27b contributes to breast cancer stem cell generation by activating ENPP1. Nat Commun 2015; 6: 7318.
[10] Ottaviani G, Jaffe N. The etiology of osteosarcoma. Cancer Treat Res 2009; 152: 15-32.
[11] Ma X, Chen R, Liu X, Xie J, Si K, Duan L. Effects of matrine on JAK-STAT signaling transduction pathways in bleomycin-induces pulmonary fibrosis. Afr J Tradit Complement Altern Med 2013; 10: 442-448.
[12] Tu B, Du L, Fan QM, Tang Z, Tang TT. STAT3 antivation by IL-6 from mesenchymal stem cells promotes the proliferation and metastasis of psteosarcoma. Cancer Lett 2012; 325: 80-88.
[13] Wang X, Goldstein D, Crowe PJ, Yang JL. Impact of STAT3 inhibition on survival of osteosarcoma cell lines. Anticancer Res 2014; 34: 6537-6545.
[14] Kurashige J, Kamohara H, Watanabe M, Hiyoshi Y, Iwatsuki M, Tanaka Y, Kinoshita K, Saito S, Baba Y, Baba H. MicroRNA-200b regulates cell proliferation, invasion, and migration

by directly targeting ZEB2 in gastric carcinoma. Ann Surg Oncol 2012; 19 Suppl 3: S656-664.
[15] Xia W, Li J, Chen L, Huang B, Li S, Yang G, Ding H, Wang F, Liu N, Zhao Q, Fang T, Song T, Wang T, Shao N. MicroRNA-200b regulates cyclin D1 expression and promotes S-phase entry by targeting RND3 in HeLa cells. Mol Cell Biochem 2010; 344: 261-266.
[16] Feng B, Wang R, Chen LB. Review of miR-200b and cancer chemosensitivity. Biomed Pharmacother 2012; 66: 397-402.
[17] Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep -sequencing data. Nucleic Acids Res 2011; 39: D152-157.
[18] Lee JJ, Drakaki A, Iliopoulos D, Struhl K. MiR-27b targets PPAR gamma to inhibit growth, tumor progression and the inflammatory response in neuroblastoma cells. Oncogene 2012; 31: 3818-3825.
[19] Urbich C, Kaluza D, Frmel T, Knau A, Bennewitz K, Boon RA, Bonauer A, Doebele C, Boeckel JN, Hergenreider E, Zeiher AM, Kroll J, Fleming I, Dimmeler S. MicroRNA-27a/b controls endothelial cell repulsion and angiogenesis by targeting semaphorin 6A. Blood 2012; 119: 1607-1616.
[20] Biyashev D, Veliceasa D, Topczewski J, Topczewska JM, Mizgirev I, Vinokour E, Reddi AL, Licht JD, Revskoy SY, Volpert OV. MiR-27b controls venous specification and tip cell fate. Blood 2012; 119: 2679-2687.
[21] Ye J, Wu X, Wu D, Wu P, Ni C, Zhang Z, Chen Z, Qiu F, Xu J, Huang J. miRNA-27b targets vascular endothelial growth factor C to inhibit tumor progression and angiogenesis in colorectal cancer. PLoS One 2013; 8: e60687.
[22] Su Y, Ni Z, Wang G, Cui J, Wei C, Wang J, Yang Q, Xu Y, Li F. Aberrant expression of microRNAs in gastric cancer and biological significance of miR-574-3p. Int Immunopharmacol 2012; 13: 468-475.
[23] Li DB, Xu XM. MiR-27b inhibits gastric cancer cell growth, invasion and metastasis via targeting c-MET. Modern Oncology 2015; 23: 26-31.