Int J Clin Exp Pathol 2015;8(3):2461-2472
www.ijcep.com /ISSN:1936-2625/IJCEP0005460

Original Article

MicroRNA-196a overexpression promotes cell proliferation and inhibits cell apoptosis through PTEN/Akt/FOXO1 pathway

Yong Shang[1], Li-Qing Wang[2], Quan-Yi Guo[3], Tu-Long Shi[1]

[1]Department of Orthopaedics, Air Force General Hospital of PLA, Beijing 100142, People's Republic of China; [2]Department of Chronic Diseases, Haidian Center For Disease Control and Prevention, Beijing 100142, People's Republic of China; [3]Institute of Orthopaedics, Chinese PLA General Hospital, Beijing 100142, People's Republic of China

Received January 3, 2015; Accepted February 25, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: MicroRNAs (miRNAs) are endogenous, non-coding, small RNAs, which play a critical role in regulating varieties of the biological and pathologic processes. MiR-196a has been reported to take part in tumorigenic progression of osteosarcoma (OS). However, the effects of miR-196a on OS are still unclear. The objective of this study is to investigate the molecular mechanism of miR-196a in osteosarcoma cells. In the present study, the expression of miR-196a in OS cell lines was detected by real-time PCR. We found that the expression level of miR-196a was markedly up-regulated in osteosarcoma cell lines compared with normal osteoblastic cells. Then, the miR-196a mimic was transiently transfected into MG63 and U2OS cells using Lipofectamine™ 2000 reagent. Subsequently, the MTT and Brdu-ELISA results showed that up-regulation of miR-196a promoted the cell viability and proliferation. Our results also showed that miR-196a mimic accelerated cell cycle progression of MG63 and U2OS cells by down regulation of p21 and p27, and upregulation of cyclin D1. In addition, overexpression of miR-196a suppressed apoptosis of MG63 and U2OS cells due to increasing BCL2L2 and MCL-1 expressions, and then inactivating caspase-3. Eventually, the effect of miR-196a mimic on the PTEN/phosphoinositide 3-kinase (PI3K)/Akt signaling pathway was explored by Western blot. From our results, transfection of miR-196a decreased the expression of PTEN and increased the phosphorylation of PI3K and Akt. Taken together, miR-196a should be an oncogene in osteosarcoma. The possible mechanism was that overexpression of miR-196a promoted proliferation of MG63 and U2OS cells by modulating the PTEN/PI3K/Akt signaling pathway.

Keywords: MicroRNA-196a, osteosarcoma, proliferation, cell cycle, apoptosis, PTEN/PI3K/Akt signaling pathway

Introduction

Osteosarcoma (OS) is the most common and primary bone tumor in children and adolescent [1]. It happens mainly around areas with active bone growth and repairation. Emerging evidence indicates OS was induced by genetic and epigenetic alterations which disturb mesenchymal stem cells to differentiate into osteoblast [2]. Over the past decade, Advances in OS therapy have improved patient outcomes [3], resulting in dramatically improving the five-year survival rate of OS patients to approximately 60~70%. However, outcome is still poor and most of them are dead eventually due to local relapse or pulmonary metastases after surgical resection and intensive-chemotherapy [4, 5]. Hence, identification of the effective molecules or signaling pathways contributed to regulating tumor growth and metastasis is greatly required for improving the therapy of OS.

MicroRNAs (miRNAs) are a wide class of endogenous, small (approximately 22 nucleotides long), non-coding RNAs molecules [6]. MiRNAs play a critical role in inhibiting gene expression posttranscriptionally by translational repression or degradation. Up to now, miRNAs have been identified as both tumor suppressors and oncogenes, which is dependent on the role of their target genes. MiRNAs are frequently aberrantly altered in diverse cancers, including breast cancer [7], colon cancer [8], glioblastoma [9], lung cancer [10], and OS [11]. Moreover,

it has been reported that miR-196a was up-regulated in many kinds of cancers including pancreatic cancer, which are closely related to poor survival rate [12]. Zhang et al. showed that miR-196a overexpression could promote the proliferation and colony formation of cervical cancer cells [13]. MiR-196a has also been showed to promote proliferation and invasion of non-small cell lung cancer cell [14]. However, the expression and molecule mechanism of miR-196a in OS are still unknown.

PTEN is initially recognized as a multifunctional tumor suppressor continually down-regulated in many types of human tumors [15]. PTEN negatively regulates the PI3K/Akt signaling pathway through dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3), eventually taking part in regulating the proliferation, cell cycle, apoptosis, migration, invasion and metastasis during the development and progression of cancers [16]. Recently, it has been reported that PTEN may be regulated by miRNAs in many types of cancers, including breast [7], colon [8], hepatocellular carcinoma [17], cervical [18], bladder cancer [19] and osteosarcoma [20-22]. Especially, Zhou et al. found that miR-20b promotes cell growth of breast cancer cells partly by targeting PTEN [7]. Valeri et al. demonstrated that miR-135b could promote tumor transformation and progression in colon cancer by down-regulating PTEN expression [8]. Jiang et al. reported that overexpression of miR-492 decreased PTEN expression in Hepatocellular Carcinoma cells [17]. Sun and his team found that miR-222 promoted the proliferation and migration through modulation of PTEN in cervical cancer cells [18]. In addition, Feng et al. demonstrated that miR-19a acts as an oncogenic gene in bladder cancer cells, and its oncogenic role was dependent on targeting PTEN [19]. Some newly studies also showed that miR-128, -17, and -181a were involved in decrease of PTEN expression in osteosarcoma [20-22].

In this paper, we demonstrated that the expression of miR-196a was significantly up-regulated in osteosarcoma cells compared with normal osteoblastic cell, and miR-196a acts an important regulatory role in osteosarcoma cell proliferation by regulation of the PTEN/PI3K/Akt signaling pathway. Our findings demonstrated that miR-196a might be a novel target for further studies of the therapy of osteosarcoma.

Materials and methods

Chemicals and reagents

Dimethylsulfoxide (DMSO), propidium iodide (PI) and 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (USA). Roswell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) were purchased from Gibco Co. (New York, USA). BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Penicillin/streptomycin was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China). Annexin V-FITC Apoptosis Detection Kit was obtained from Nanjing KeyGen Biotech Co. (Nanjing, China).

Cell culture

Osteosarcoma cell lines MG63, HOS, SaOS-2, and U2OS were cultured in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2. hFOB1. 19 human osteoblasts were cultured in DMEM/Ham's F-12 containing 10% FBS and Geneticin (400 μg/mL) at 37°C in 5% CO_2 incubator.

miRNA transfection

To enhance miR-196a expression in MG63 and U2OS cells, MG63 and U2OS cells were transfected with miR-196a mimics, which served as the miR-196a group. MG63 and U2OS cells transfected with miR-negative control (miR-NC) were used as miR-NC group. One day before transfection, cells at about 40 to 60% confluency were changed to the antibiotic-free media. After 24 h, the cells were transfected with 50 nM miR-196a mimics using Lipofectamine™ 2000 reagent according to the manufacturer's instruction.

Reverse transcription polymerase chain reaction

Total RNA from MG63 and U2OS cells was extracted using Trizol reagent (Invitrogen). Two microgram RNA was used for gene-specific reverse transcription polymerase chain reaction (RT-PCR) using one-step RT-PCR kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. PCR was implemented in 50 μL reaction volume including 1 μL of dNTPs (10 mM), 5 μL complementary DNA

Table 1. Sequence of primers for RT-PCR

Gene	Primer Sequence
miR-196a	F: 5'-GCTCTGGCTCCGTGTCTTCACTCCC-3'
	R: 5'-TGCCCCAGCACAGCCCCCGTCCCTC-3'
p21	F: 5'-TGTCCGTCAGAACCCATGC-3'
	R: 5'-AAAGTCGAAGTTCCATCGCTC-3'
p27	F: 5'-TAATTGGGGCTCCGGCTAACT-3'
	F: 5'-TGCAGGTCGCTTCCTTATTCC-3'
cyclin D1	F: 5'-TGAGAGAAAAAGGTCCTACG-3'
	R: 5'-GTAGCAGCTACTGTAGACAG-3'
PTEN	F: 5'-CCAGGACCAGAGGAAACCT-3'
	R: 5'-GCTAGCCTCTGGATTTGA-3'
GAPDH	F: 5'-GAGTCAACGGATTTGGTCGTATTG-3'
	R: 5'-CCTGGAAGATGGTGATGGGATT-3'

solution, 1 μL of each primer (50 PM), 1 μL Taq DNA polymerase (Invitrogen), water (33 μL), 3 μL of $MgCl_2$ (15 mM), and 5 μL of PCR buffer (10×) on a thermal cycler (Thermo Scientific, Waltham, MA). Denaturation was performed at 94°C for 1 minute, annealing at 59°C for 1 minute, and elongation at 72°C for 1 minute for 32 cycles, followed by 72°C for 10 minutes. Primers used were listed in **Table 1**. The amplified products were electrophoresed on 2% agarose gels using Gel-Pro Analyzer 6.0 to analyze. The levels for each gene were counted by standardizing the quantified mRNA amount to the GAPDH mRNA. Each sample was assessed in triplicate.

Cell viability assay

MG63 and U2OS cells were seeded in 96-well dish for 24 h. After that, cells were transfected with miR-196a and miR-NC. Then, the number of viable cells was determined using MTT reagent according to the manufacturer's instructions. In brief, MTT reagent (10 μl) was added to the 100 μL medium, and incubated at 37°C for 4 h. The supernatant was removed and dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance (570 nm) of the medium was measured with Biotek Elx-800 plate reader.

Cell proliferation assay

To investigate the effect of miR-196a transfection on proliferation of MG63 and U2OS cells, 5×10^3 cells were seeded onto 96-well culture plate and allowed to grow overnight in complete DMEM. The culture medium was then removed and the cells were transfected with anti-miR-196a and anti-miR-NC for 24 h at 37°C. Cell Proliferation ELISA-BrdU (colorimetric) Kit (Roche Diagnostics, USA) was used to determine the cells proliferation according to the manufacturer's instructions.

Annexin V-FITC/PI analysis

MG63 and U2OS cells were transfected with miR-196a and miR-NC for 24 h. After treatments, cells were double-stained by using an Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Samples stained with Annexin V and PI were quantitatively analyzed at 488 nm emission and 570 nm excitation by FACSCalibur flow cytometer (Beckman Coulter, Inc., Fullerton, California, USA), and then the fluorescence was analyzed using the CellQuest software (Becton Dickinson).

Western blot analysis

Forty-eight hours after transfection, total protein was extracted from the MG63 and U2OS cells using RIPA cell lysis buffer containing proteinase and phosphatase inhibitors. The protein concentration of cell lysates was quantified by BCA Kit, and equal amounts of protein were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in 5% non-fat dry milk diluted with Tri Buffered Saline Tween-20 (TBST) (in mmol/L: Tris-HCl 20, NaCl 150, PH 7.5, 0.1% Tween 20) at room temperature for 1 h, and then probed overnight at 4°C with primary antibody. The primary antibodies and dilutions used were as follows: $p21^{WAF1}$ and $p27^{KIP1}$ were purchased from Santa Cruz Biotechnology (1:500; Santa Cruz, CA, USA); antibodies against cyclin D1, BCL2L2, MCL-1, pro-caspase-3, PTEN, phospho-PI3K (p-PI3K), total-PI3K (t-PI3K), phospho-Akt (p-Akt) and total-Akt (t-Akt) were obtained from Cell Signaling Technology Inc (1:1000; Beverly, MA, USA). The membranes were then incubated for 2 h with a goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (1:1000; Santa Cruz). Monoclonal mouse α-tubulin antibody (1:1000; Sigma) or monoclonal rabbit GAPDH (1:1000; Cell Signaling Technology) was used as an internal control. The proteins were visualized using ECL™ western blotting detection reagents (Amersham Biosciences

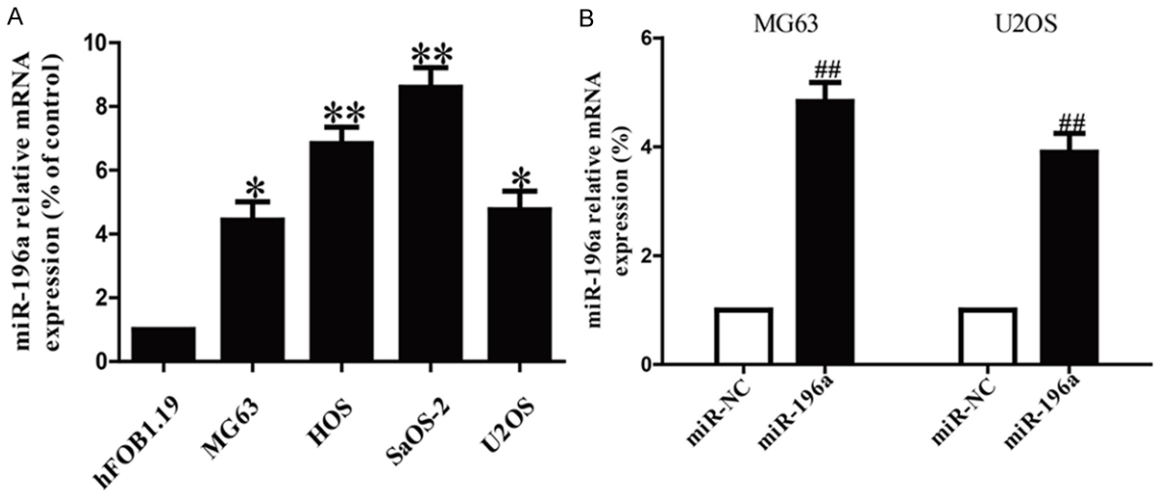

Figure 1. The mRNA level of miR-196a in OS cell lines. A. The relative expression of miR-196a in OS cell lines and hFOB1.19 human osteoblasts by real-time PCR. B. The mRNA levels of miR-196a in MG63 and U2OS cells transfected with miR-196a mimic or its appropriate negative control (miR-NC) by real-time PCR. All data are presented as mean ± SEM, n=6. $^{*}P<0.05$, $^{**}P<0.01$ vs. hFOB1.19; $^{\#\#}P<0.01$ vs. miR-NC.

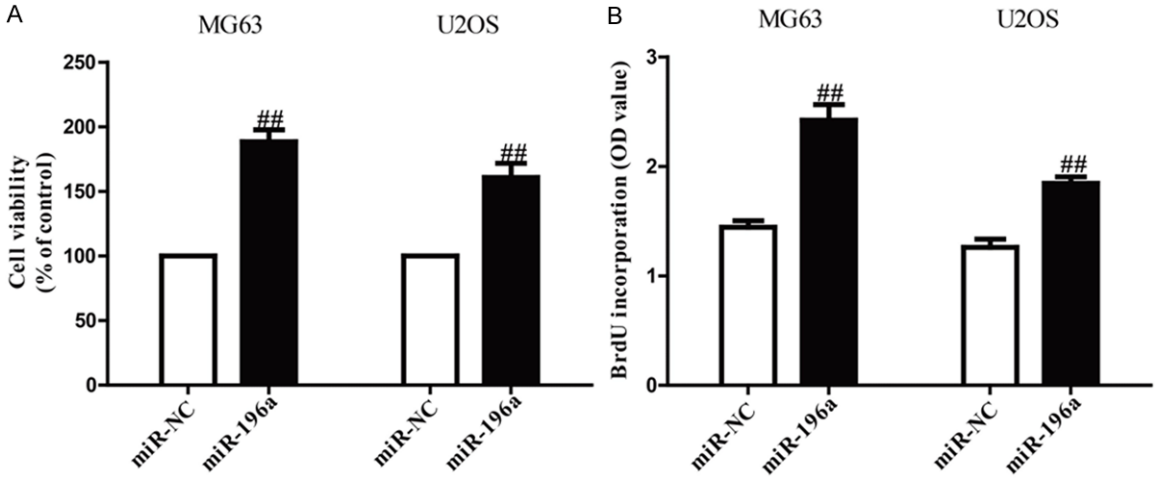

Figure 2. Effects of miR-196a transfection on cell viability and proliferation in MG63 and U2OS cells. MG63 and U2OS cells were transfected with miR-196a or miR-NC. A. Cell viability was assessed by MTT assay. B. Cell proliferation was assessed by BrdU-ELISA assay. All data are presented as mean ± SEM, n=6. $^{\#\#}P<0.01$ vs. miR-NC.

Corp., USA). The densitometry of the bands was quantified using the Image J 1.38X software (USA).

Caspase-3 activity assay

A fluorescent assay kit (Nanjing KeyGen Biotech, China) was used to detect caspase-3 activity according to the manufacturer's instructions. In brief, MG63 and U2OS cells were harvested after transfection of miR-196a or miR-NC, and washed with ice-cold PBS. Cells were lyzed in the lysis buffer, and then centrifuged. After that, the supernatants were collected. Equal amounts of protein samples were reacted with the synthetic fluorescent substrates at 37°C for 4 h and the reactions were read at 405 nm in a microplate reader (Bio-Rad, USA). Fold-increase in caspase-3 activity was determined with values obtained from the treatment samples divided by those from the controls.

Cell cycle analysis

The MG63 and U2OS cells were transfected with miR-196a or miR-NC for 24 h. Then, both

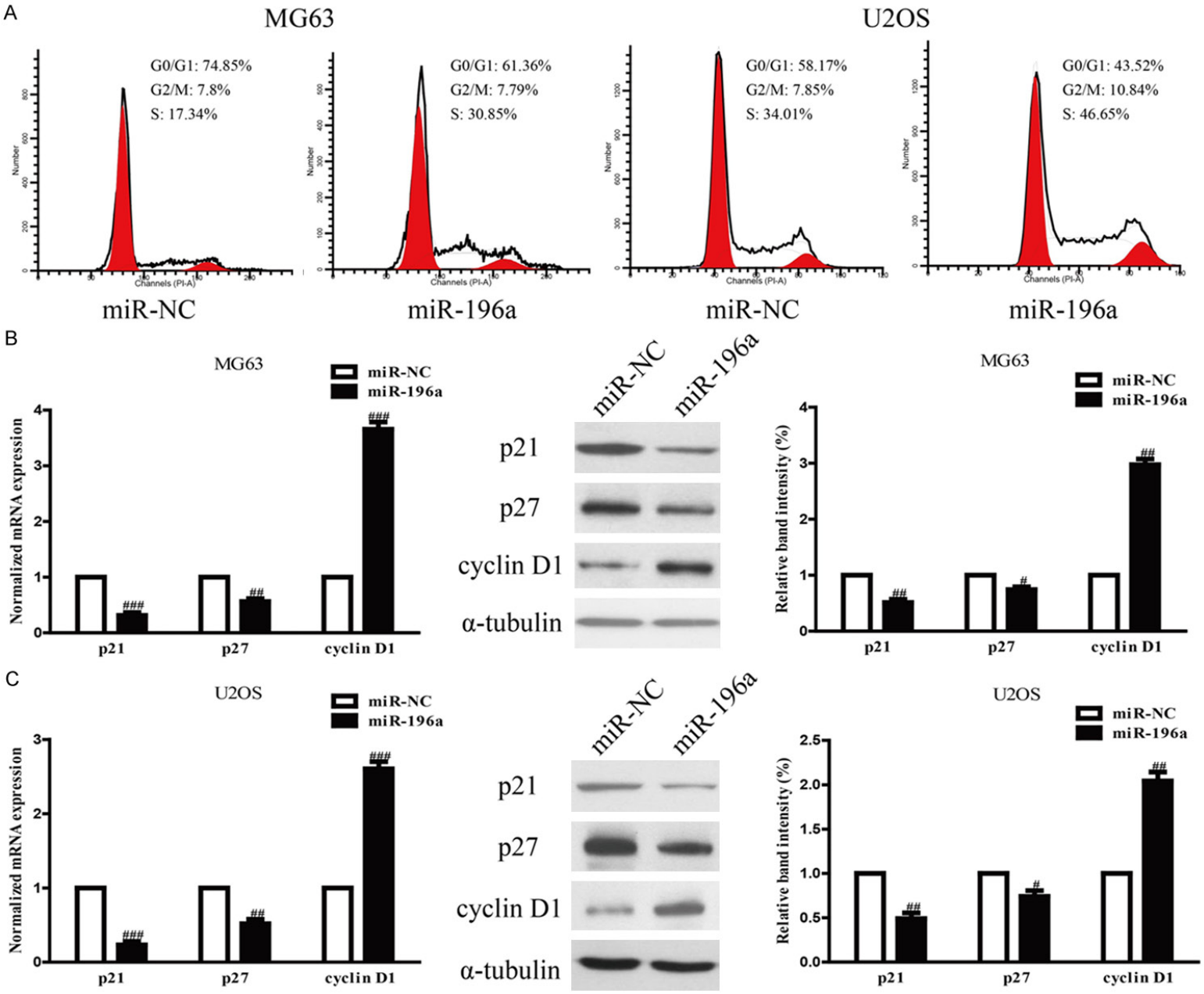
A
MG63
U2OS
G0/G1: 74.85%
G2/M: 7.8%
S: 17.34%
G0/G1: 61.36%
G2/M: 7.79%
S: 30.85%
G0/G1: 58.17%
G2/M: 7.85%
S: 34.01%
G0/G1: 43.52%
G2/M: 10.84%
S: 46.65%
Number
Channels (PI-A)
miR-NC
miR-196a
miR-NC
miR-196a
B
MG63
miR-NC
miR-196a
Normalized mRNA expression
p21
p27
cyclin D1
miR-NC
miR-196a
p21
p27
cyclin D1
α-tubulin
MG63
miR-NC
miR-196a
Relative band intensity (%)
p21
p27
cyclin D1
C
U2OS
miR-NC
miR-196a
Normalized mRNA expression
p21
p27
cyclin D1
miR-NC
miR-196a
p21
p27
cyclin D1
α-tubulin
U2OS
miR-NC
miR-196a
Relative band intensity (%)
p21
p27
cyclin D1

Figure 3. Effects of miR-196a transfection on cell cycle and cell cycle-related proteins in MG63 and U2OS cells. MG63 and U2OS cells were transfected with miR-196a or miR-NC. A. Cell cycle was detected by flow cytometry. B. The mRNA levels and C. the protein expressions of p21, p27 and cyclin D1 were determined respectively by real time-PCR and Western Blot using α-tubulin as an internal control. All data are presented as mean ± SEM, n=6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. miR-NC.

cells were collected by trypsinization, washed with ice-cold PBS, and fixed in ice-cold 70% methanol by incubating them for 1 h at 4°C. The cells were then centrifuged, suspended in PBS, and incubated with RNase for 30 min at 37°C. The cells were then stained with propidium iodide for 1 h, and analyzed by FACScan flow cytometer and CellQuest 3.3 software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Data for each study parameter from each group were presented as mean ± standard error of the mean (S.E.M.). Data from each group were statistically analyzed by a two-tailed Student's t test or one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.05$.

Results

The expression of miR-196a was increased in osteosarcoma (OS) cell lines

To determine the levels of miR-196a in OS cells, four osteosarcoma cell lines (MG63, HOS, Sa-OS-2, and U2OS) and a human normal osteoblastic cell line (hFOB1.19) were used to detect the level of miR-196a by real time-PCR. Our results demonstrated that the level of miR-196a was significantly increased in all four OS cell lines compared to that in human normal osteoblastic cell line hFOB1.19, as shown in **Figure 1A**. Among these OS cell lines, MG63 and U2OS cells were used to study further. In addition, the results from real time-PCR analysis showed that miR-196a displayed evident up-regulation in miR-196a group compared to miR-NC group ($P<0.01$). These results confirmed that we effectively overexpressed miR-196a expression in MG63 and U2OS cells (**Figure 1B**).

Overexpression of miR-196a promoted cell proliferation in both MG63 and U2OS cells

To examine the role of miR-196a in proliferation of OS cells, MG-63 and U2OS cells were transfected with miR-196a or miR-NC. MTT assay demonstrated that transfection of miR-196a evidently increased the viabilities of MG-63 and U2OS cells (**Figure 2A**). Besides, we also observed promoted cell proliferation in miR-196a mimic-transfected cells, as assessed by the Brdu-ELISA assay (**Figure 2B**), showing a positive correlation with the MTT assays. These results indicated that miR-196a overexpression had available proliferation promotion effect in both MG-63 and U2OS cells.

Up-regulation of miR-196a accelerated cell cycle and modulated cell cycle-related proteins in OS cells

Because miR-196a mimic evidently promoted cell proliferation in both MG-63 and U2OS cells, we assumed that miR-196a could accelerate the cell cycle of OS cells. We confirmed this hypothesis by flow cytometry. Our results showed that overexpression of miR-196a drastically decreased the percentage of cells in the G1/G0 peak and increased the percentage of cells in the S peak in both MG-63 and U2OS cells compared with cells transfected with miR-NC (**Figure 3A**). Therefore, transfection of miR-196a mimic may increase the proliferation of osteosarcoma cells by promoting the G1/S cell cycle transition. However, we further investigated whether the cyclin-dependent kinase (CDK) inhibitors p21, p27 or the CDK regulator cyclin D1 could be regulated by miR-196a. To demonstrate this hypothesis, we examined the effects of miR-196a transfection on the expressions of p21 and p27 and cyclin D1, which are known as critical molecules involved in cell cycle arrest. As shown in **Figure 3**, the mRNA levels and protein expressions of p21 and p27 were down-regulated in MG-63 and U2OS cells transfected with miR-196a (**Figure 3B**). Besides, cyclin D1 is a nuclear protein required for cell cycle regulation in the G1 phase of proliferating cells, while protein expression and mRNA level of cyclin D1 were markedly upregulated in miR-196a-transfected cells compared with cells transfected with miR-NC (**Figure 3B**). These results indicated that transfection of miR-196a accelerated cell growth in MG-63 and U2OS cells by regulating expression of cell cycle-related proteins.

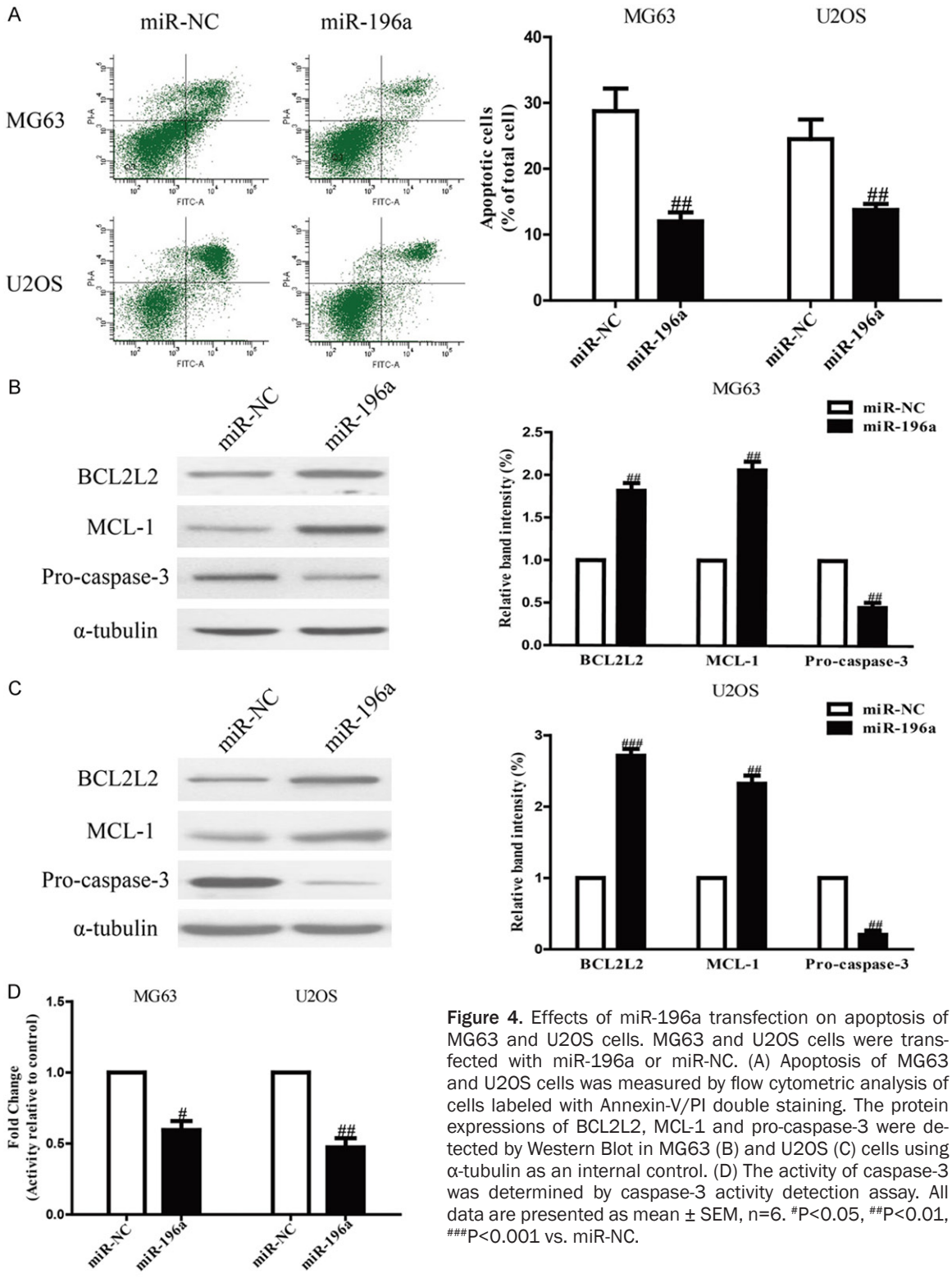

Figure 4. Effects of miR-196a transfection on apoptosis of MG63 and U2OS cells. MG63 and U2OS cells were transfected with miR-196a or miR-NC. (A) Apoptosis of MG63 and U2OS cells was measured by flow cytometric analysis of cells labeled with Annexin-V/PI double staining. The protein expressions of BCL2L2, MCL-1 and pro-caspase-3 were detected by Western Blot in MG63 (B) and U2OS (C) cells using α-tubulin as an internal control. (D) The activity of caspase-3 was determined by caspase-3 activity detection assay. All data are presented as mean ± SEM, n=6. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. miR-NC.

Transfection of miR-196a negatively regulated apoptosis in OS cells

In order to explore whether anti-apoptosis took part in miR-196a mimic-induced cell proliferation, the total apoptosis rates of MG-63 and U2OS cells were detected by flow cytometric analysis of cells labeled with Annexin-V/PI double staining. As shown in **Figure 4A** and **4B**, flow cytometry analysis showed that the number of

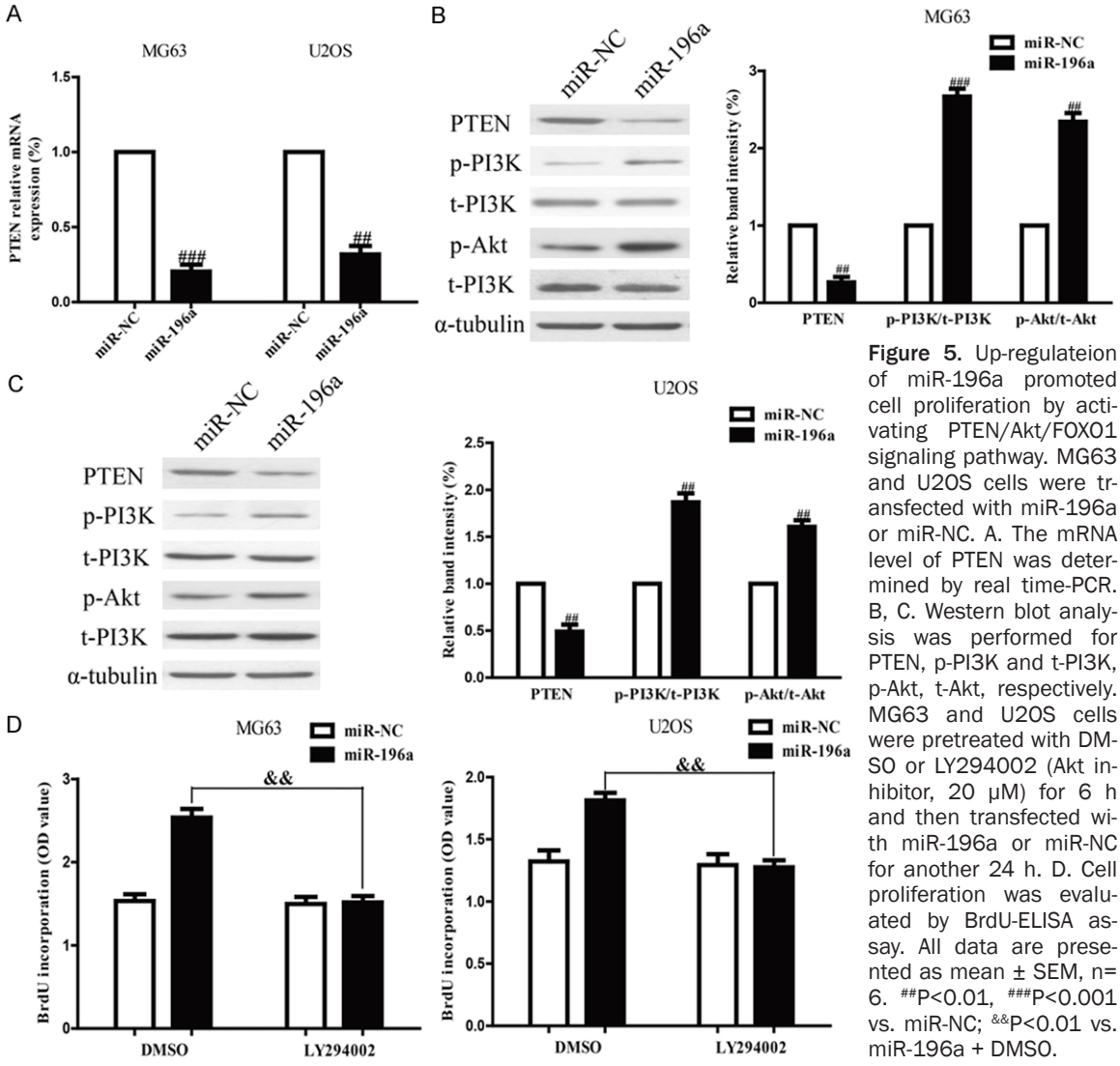

Figure 5. Up-regulateion of miR-196a promoted cell proliferation by activating PTEN/Akt/FOX01 signaling pathway. MG63 and U2OS cells were transfected with miR-196a or miR-NC. A. The mRNA level of PTEN was determined by real time-PCR. B, C. Western blot analysis was performed for PTEN, p-PI3K and t-PI3K, p-Akt, t-Akt, respectively. MG63 and U2OS cells were pretreated with DMSO or LY294002 (Akt inhibitor, 20 μM) for 6 h and then transfected with miR-196a or miR-NC for another 24 h. D. Cell proliferation was evaluated by BrdU-ELISA assay. All data are presented as mean ± SEM, n= 6. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. miR-NC; $^{\&\&}P<0.01$ vs. miR-196a + DMSO.

apoptotic MG-63 and U2OS cells was significantly lower in miR-196a mimic group than that in miR-NC group. In order to investigate the molecular mechanism of anti-apoptosis, we further determined the expressions of apoptosis-related proteins including BCL2L2, MCL-1 and procaspase-3. Western blotting showed that miR-196a overexpression up-regulated the expression of procaspase-3 in both MG-63 and U2OS cells (**Figure 4B**, **4C**). These results were further determined by caspase activity assay showing that activity of caspase-3 was significantly inhibited by transfection of miR-196a (**Figure 4D**). Up-regulation of miR-196a also increased anti-apoptotic protein BCL2L2 and MCL-1 expressions (**Figure 4B**, **4C**). Taken together, these apoptosis results suggested that miR-196a might negatively regulate apoptosis of MG-63 and U2OS cells.

MiR-196a overexpression promoted proliferation of OS cells through PTEN/PI3K/Akt pathway

To further investigate the molecular mechanism of miR-196a on the proliferation in OS cells, we determined the effect of miR-196a on the PTEN/PI3K/Akt pathway. Twenty-four hours after miR-196a transfection, the expressions of PTEN and phosphorylation of PI3K and Akt in both MG63 and U2OS cells were determined by Western blotting. In both OS cells, overexpression of miR-196a decreased the mRNA level and protein expression of PTEN that is a tumor suppressor and a major negative regulator of the PI3K/Akt pathway (**Figure 5A-C**). It has been reported that down-regulation of PTEN expression was contributing to increased PI3K and Akt activation, and the subsequent growth

and survival in various tumors. Our results showed that miR-196a transfection remarkably increased PI3K and Akt phosphorylation in MG63 and U2OS cells (**Figure 5B, 5C**). However, miR-196a had no effect on total PI3K and Akt expressions. Moreover, in the cell proliferation assay, the proliferation promotion effect of miR-196a overexpression was largely blocked by pretreatment with LY294002, an Akt inhibitor (**Figure 5D**). Collectively, these results suggested that transfection of miR-196a promoted proliferation in both MG63 and U2OS cells by regulating PTEN/PI3K/Akt pathway.

Discussion

Emerging reports have demonstrated that miRNAs take part in the development and progression of various cancers including OS via regulation of expression of multiple target genes related to the development and progression. Therefore, identification of specific miRNAs and their targets related to tumorigenesis would supply valuable insight for the diagnosis and treatment of patients with human malignancies. MiRNA profiling studies suggest that miR-196a is overexpressed in various types of cancers, such as breast, gastric, pancreatic, colorectal and cervical cancer [23-27]. Intriguingly, increasing reports confirm that miR-196a contributes to the development and progression of cancers.

In this paper, our results demonstrated that miR-196a showed higher expression levels in osteosarcoma cell lines (MG63, HOS, SaOS-2, and U2OS) compared to a human normal osteoblastic cell line hFOB1.19. The results of miR-196a expressions in osteosarcoma cell lines in this paper was consistent with that in osteosarcoma tissues, which displayed that miR-196a evidently over-expressed in osteosarcoma tissues [28]. Altogether, our results indicated that miR-196a acts as an oncogene playing an important role in the development and progression of OS. Therefore, we speculated that miR-196a might positively regulate tumor cell proliferation. In order to investigate the function of miR-196a in osteosarcoma cells, the expression of miR-196a over-expressed in both MG63 and U2OS cells. The results of MTT assay and Brdu-ELISA assay showed that viabilities and proliferation of MG63 and U2OS cells were significantly higher in cells transfected with miR-196a than those in cells transfected with miR-NC, which indicated that miR-196a could improve MG63 and U2OS cells viabilities and proliferation. The results of cell cycle assay by flow cytometry showed that miR-196a mimic could promote cell cycle progression of MG63 and U2OS cells, which was potentially due to up-regulation of growth-promoting factors such as cyclin D1 or down-regulation of growth-inhibitory factors such as $p21^{CIP1}$ and $p27^{KIP1}$ in the downstream of the miR-196a target genes. The p21 and p27 proteins, two cyclin-dependent kinase inhibitors, are key regulators of the cell growth, which promote cell cycle arrest primarily at the G1/S transition of the cell cycle [29]. Hence, p21 and p27 were determined in our study. The results showed that the expressions of p21 and p27 were decreased in MG-63 and U2OS cells transfected with miR-196a, which suggested that the overexpression of miR-196a might be a major event in cancer pathogenesis, partly due to its ability to down-regulate p21 and p27, leading to promoting cell cycle progression. Besides, cyclin D1 is a growth-promoting factor required for cell cycle regulation in the G1 phase of proliferating cells. Our results showed that up-regulation of miR-196a significantly increased the expression of cyclin D1 in MG-63 and U2OS cells. However, further study is required to elucidate the exact mechanism. Our findings also exhibited that up-regulation of miR-196a could suppress apoptosis of MG63 and U2OS cells, which was consistent with that in pancreatic cancer cell, which showed that overexpression of miR-196a could inhibit the apoptosis of Panc-1 cells [30]. It has been reported that down-regulation of anti-apoptotic proteins BCL2L2 (Bcl-w) and MCL-1 may contribute to increasing apoptosis of OS cells [31]. Both BCL2L2 and MCL-1, two members of the Bcl-2 family, can improve cells survival. BCL2L2 can form complexes of Bax/Bcl-w and Bak/Bcl-w by binding to pro-apoptotic proteins such as Bax and Bak. The decreased expression of Bcl-w activates Bax and Bak, and then induces apoptosis [32, 33]. Moreover, MCL-1 can inactivate the pro-apoptotic protein Bak, and then combine with Bak via the BH3 domain to form a heterodimer. Then Bak is inactivated, resulting in inhibiting the activation of caspase cascade to promote cell viability [34]. Therefore, we examined the expressions of BCL2L2, MCL-1and procaspase-3, and the activity of caspase-3. We found that expressions of BCL2L2, MCL-1 and procaspase-3 were up-regulated and the activity of caspase-3

was inhibited in MG63 and U2OS cells after transfection of miR-196a. Further, the above results suggested that miR-196a might promote the growth and proliferation of MG63 and U2OS cells and inhibit the apoptosis of MG63 and U2OS cells. However, the precise mechanisms were still further investigated.

We next demonstrated that PTEN is a direct target of miR-196a in MG63 and U2OS cells. PTEN, a well-established tumor suppressor, is closely related to the development and progression of tumors [35]. PTEN dephosphorylates Phosphatidylinositol 3,4,5-trisphosphate (PIP3), and antagonizes phosphatidylinositol 3-kinase (PI3K) signaling [36]. The PTEN/PI3K/Akt signaling pathway is closely related to tumorigenesis and metastasis [37]. PTEN suppresses growth and motility of cancer cells through inhibiting the PI3K/Akt signaling pathway [38]. PTEN expression is down-regulated in certain malignant tumors, leading to increasing the activation of Akt and promoting cell proliferation, migration, invasion and angiogenesis [39]. Collectively, our findings exhibited that overexpression of miR-196a promoted growth and proliferation of MG63 and U2OS cells, and up-regulation of miR-196a was correlated with down-regulation of PTEN in both OS cells.

In conclusion, we demonstrated that miR-196a mimic promoted proliferation of MG63 and U2OS cells through downregulation of $p21^{/CIP1}$ and $p27^{/KIP1}$ and upregulation of cyclin D1, resulting in accelerating cell cycle progression. Furthermore, our findings confirmed that upregulation of miR-196a had anti-apoptotic function in MG63 and U2OS cells by increasing BCL2L2 and MCL-1 expressions and thereby activating caspase3. However, the possible mechanism was that overexpression of miR-196a promoted proliferation of MG63 and U2OS cells by modulating the PTEN/PI3K/Akt signaling pathway. Our findings indicated that miR-196a has potential roles as a novel treated target for OS therapy.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yong Shang, Department of Orthopedics, Air Force General Hospital of PLA, 30 Fucheng Road, Haidian District, Beijing 100142, People's Republic of China. Tel: +86 10 68410099; Fax: +86 10 68410099; E-mail: shangyongbmb@163.com

References

[1] Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the surveillance, epidemiology, and end results program. Cancer 2009; 115: 1531-1543.

[2] Yan K, Gao J, Yang T, Ma Q, Qiu X, Fan Q, Ma B. MicroRNA-34a inhibits the proliferation and metastasis of osteosarcoma cells both in vitro and in vivo. PLoS One 2012; 7: e33778.

[3] Amankwah EK, Conley AP, Reed DR. Epidemiology and therapies for metastatic sarcoma. Clin Epidemiol 2013; 5: 147-162.

[4] Bacci G, Briccoli A, Rocca M, Ferrari S, Donati D, Longhi A, Bertoni F, Bacchini P, Giacomini S, Forni C, Manfrini M, Galletti S. Neoadjuvant chemotherapy for osteosarcoma of the extremities with metastases at presentation: Recent experience at the Rizzoli Institute in 57 patients treated with cisplatin, doxorubicin, and a high dose of methotrexate and ifosfamide. Ann Oncol 2003; 14: 1126-1134.

[5] Rainusso N, Wang LL, Yustein JT. The adolescent and young adult with cancer: state of the art-bone tumors. Curr Oncol Rep 2013; 15: 296-307.

[6] Wang Z, Cai H, Lin L, Tang M, Cai H. Upregulated expression of microRNA-214 is linked to tumor progression and adverse prognosis in pediatric osteosarcoma. Pediatr Blood Cancer 2014; 61: 206-210.

[7] Zhou W, Shi G, Zhang Q, Wu Q, Li B, Zhang Z. MicroRNA-20b promotes cell growth of breast cancer cells partly via targeting phosphatase and tensin homologue (PTEN). Cell Biosci 2014; 4: 62.

[8] Valeri N, Braconi C, Gasparini P, Murgia C, Lampis A, Paulus-Hock V, Hart JR, Ueno L, Grivennikov SI, Lovat F, Paone A, Cascione L, Sumani KM, Veronese A, Fabbri M, Carasi S, Alder H, Lanza G, Gafa' R, Moyer MP, Ridgway RA, Cordero J, Nuovo GJ, Frankel WL, Rugge M, Fassan M, Groden J, Vogt PK, Karin M, Sansom OJ, Croce CM. MicroRNA-135b promotes cancer progression by acting as a downstream effector of oncogenic pathways in colon cancer. Cancer Cell 2014; 25: 469-483.

[9] Li W, Guo F, Wang P, Hong S, Zhang C. MiR-221/222 confers radioresistance in glioblastoma cells through activating Akt independent of PTEN status. Curr Mol Med 2014; 14: 185-195.

[10] Nian W, Ao X, Wu Y, Huang Y, Shao J, Wang Y, Chen Z, Chen F, Wang D. MiR-223 functions as a potent tumor suppressor of the Lewis lung

carcinoma cell line by targeting insulin-like growth factor-1 receptor and cyclin-dependent kinase 2. Oncol Lett 2013; 6: 359-366.
[11] Wang Y, Zhao W, Fu Q. MiR-335 suppresses migration and invasion by targeting ROCK1 in osteosarcoma cells. Mol Cell Biochem 2013; 384: 105-111.
[12] Huang F, Tang J, Zhuang X, Zhuang Y, Cheng W, Chen W. MiR-196a promotes pancreatic cancer progression by targeting nuclear factor kappa-B-inhibitor alpha. PLoS One 2014; 9: e87897.
[13] Hou T, Ou J, Zhao X, Huang X, Huang Y, Zhang Y. MicroRNA-196a promotes cervical cancer proliferation through the regulation of FOXO1 and p27Kip1. Br J Cancer 2014; 110: 1260-1268.
[14] Liu XH, Lu KH, Wang KM, Sun M, Zhang EB, Yang JS, Yin DD, Liu ZL, Zhou J, Liu ZJ, De W, Wang ZX. MicroRNA-196a promotes non-small cell lung cancer cell proliferation and invasion through targeting HOXA5. BMC Cancer 2012; 12: 348.
[15] Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997; 15: 356-362.
[16] Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate. J Biol Chem 1998; 273: 13375-13378.
[17] Jiang J, Zhang Y, Yu C, Li Z, Pan Y, Sun C. MicroRNA-492 expression promotes the progression of hepatic cancer by targeting PTEN. Cancer Cell Int 2014; 14: 95.
[18] Sun Y, Zhang B, Cheng J, Wu Y, Xing F, Wang Y, Wang Q, Qiu J. MicroRNA-222 promotes the proliferation and migration of cervical cancer cells. Clin Invest Med 2014; 37: E131.
[19] Feng Y, Liu J, Kang Y, He Y, Liang B, Yang P, Yu Z. MiR-19a acts as an oncogenic microRNA and is up-regulated in bladder cancer. J Exp Clin Cancer Res 2014; 33: 67.
[20] Shen L, Chen XD, Zhang YH. MicroRNA-128 promotes proliferation in osteosarcoma cells by downregulating PTEN. Tumour Biol 2014; 35: 2069-2074.
[21] Jianwei Z, Fan L, Xiancheng L, Enzhong B, Shuai L, Can L. MicroRNA 181a improves proliferation and invasion, suppresses apoptosis of osteosarcoma cell. Tumour Biol 2013; 34: 3331-3337.
[22] Gao Y, Luo LH, Li S, Yang C. MiR-17 inhibitor suppressed osteosarcoma tumor growth and metastasis via increasing PTEN expression. Biochem Biophys Res Commun 2014; 444: 230-234.
[23] Sun M, Liu XH, Li JH, Yang JS, Zhang EB, Yin DD, Liu ZL, Zhou J, Ding Y, Li SQ, Wang ZX, Cao XF, De W. MiR-196a is upregulated in gastric cancer and promotes cell proliferation by downregulating p27 (kip1). Mol Cancer Ther 2012; 11: 842-852.
[24] Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, Fyles T, McCready D, Wong D, Gerster K, Waldron L, Jurisica I, Penn LZ, Liu FF. Robust global microRNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest 2009; 89: 597-606.
[25] Wang YX, Zhang XY, Zhang BF, Yang CQ, Chen XM, Gao HJ. Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis. J Dig Dis 2010; 11:50-54.
[26] Bloomston M, Frankel W, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007; 297: 1901-1908.
[27] Papaconstantinou IG, Lykoudis PM, Gazouli M, Manta A, Polymeneas G, Voros D. A review on the role of microRNA in biology, diagnosis, and treatment of pancreatic adenocarcinoma. Pancreas 2012; 41: 671-677.
[28] Zhang C, Yao C, Li H, Wang G, He X. Combined Elevation of microRNA-196a and microRNA-196b in Sera Predicts Unfavorable Prognosis in Patients with Osteosarcomas. Int J Mol Sci 2014; 15: 6544-6555.
[29] Narla G, Friedman SL, Martignetti JA. Kruppel cripples prostate cancer: KLF6 progress and prospects. Am J Pathol 2003; 162: 1047-1052.
[30] Liu M, Du Y, Gao J, Liu J, Kong X, Gong Y, Li Z, Wu H, Chen H. Aberrant expression miR-196a is associated with abnormal apoptosis, invasion, and proliferation of pancreatic cancer cells. Pancreas 2013; 42: 1169-1181.
[31] Zhao H, Li M, Li L, Yang X, Lan G, Zhang Y. MiR-133b is down-regulated in human osteosarcoma and inhibits osteosarcoma cells proliferation, migration and invasion, and promotes apoptosis. PLoS One 2013; 8: e83571.
[32] Yan W, Samson M, Jegou B, Toppari J. Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. Mol Endocrinol 2000; 14: 682-699.
[33] Holmgreen SP, Huang DC, Adams JM, Cory S. Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members. Cell Death Differ 1999; 6: 525-532.

[34] Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DC. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005; 19: 1294-1305.

[35] Li D, Zhang Y, Xie Y, Xiang J, Zhu Y, Yang J. Enhanced tumor suppression by adenoviral PTEN gene therapy combined with cisplatin chemotherapy in small-cell lung cancer. Cancer Gene Ther 2013; 20: 251-259.

[36] Leslie NR, Batty IH, Maccario H, Davidson L, Downes CP. Understanding PTEN regulation: PIP2, polarity and protein stability. Oncogene 2008; 27: 5464-5476.

[37] Castellino RC, Durden DL. Mechanisms of disease: the PI3K-Akt-PTEN signaling node-an intercept point for the control of angiogenesis in brain tumors. Nat Clin Pract Neurol 2007; 3: 682-693.

[38] Zhang G, Zhou H, Xiao H, Liu Z, Tian H, Zhou T. MicroRNA-92a functions as an oncogene in colorectal cancer by targeting PTEN. Dig Dis Sci 2014; 59: 98-107.

[39] Phin S, Moore MW, Cotter PD. Genomic rearrangements of PTEN in prostate cancer. Front Oncol 2013; 3: 240.