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Original Article

miR-96 plays an oncogenic role by targeting FOXO1 in osteosarcoma

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Abstract: *Objective*: To explore the difference in miR-96 expression in human osteosarcoma tissue and cells, as well as its molecular mechanisms in regulating the malignant phenotype of Osteosarcoma. *Methods*: MiR-96 expression in human osteosarcoma tissue and cells was detected using quantitative reverse transcription polymerase chain reaction (qRT-PCR), and the correlation of miR-96 expression with the clinicopathological features of patients was analyzed. MiR-96 inhibitor was transfected *in vitro* into human osteosarcoma cells MG-63, and changes in miR-96 expression in the cells after transfection were detected using qRT-PCR. Meanwhile, changes in cell proliferation and apoptosis after transfection were determined using CCK-8 assay and flow cytometry. Changes in PCDA and caspase-3 protein expression after transfection were detected using Western blotting. Bioinformatics was used to predict the targeting genes of miR-96. Double luciferase reporter gene assay combined with qRT-PCR and Western blotting were used to validate the targeting regulation of miR-96 on FOXO1. In addition, the effect of miR-96 on osteosarcoma xenograft in the nude mouse was detected through the nude mouse subcutaneous tumorigenicity assay. *Results*: MiR-96 showed aberrantly high expression in human osteosarcoma cells MG-63, Saos-2 and U20S, as well as osteosarcoma tissue. Further statistical analysis suggested that miR-96 expression in osteosarcoma tissue was closely related to the Enneking stage and distant metastasis. FOXO1 is a direct target gene of miR-96. Moreover, miR-96 expression level was remarkably reduced in MG-63 cells after transfection with miR-96 inhibitor, while both FOXO1 mRNA and protein expression levels were notably increased, cell proliferation capacity and PCDA protein expression level were markedly lowered, and cell apoptosis rate and caspase-3 protein expression level were evidently enhanced. Furthermore, nude mouse osteosarcoma xenograft growth results revealed that xenograft in MG-63 cells transfected with miR-96 inhibitor was outstandingly reduced in terms of volume and weight. *Conclusion*: MiR-96 shows aberrantly high expression in osteosarcoma tissue and cells. Down-regulating miR-96 expression can apparently suppress osteosarcoma cell proliferation and tumorigenic ability, and enhance its apoptosis, the mechanism of which may be related to the target regulation of FOXO1.

Keywords: Osteosarcoma, miR-96, FOXO1, proliferation, apoptosis

Introduction

Osteosarcoma is a primary malignant bone tumor frequently seen in adolescents, which is associated with high malignant grade, high possibilitie of recurrence and metastasis, and poor prognosis [1]. The 5-year survival rate for osteosarcoma patients is only 60%-75% even though they have received the standard therapeutic scheme of surgery combined with neoadjuvant chemotherapy. Moreover, the prognosis for patients with lung metastasis is even more dismal [2, 3]. The molecular mechanisms for the pathogenesis and metastasis of osteosarcoma remain incompletely clear. Treatment for osteosarcoma is trapped in a bottleneck period in recent years, and the 5-year survival rate for patients has not been remarkably improved.

MicroRNA (miRNA) is a class of single-strand non-coding small RNA constituted by 20-25 nucleotides, which can bind with the 3' untranslated region (3'UTR) of a target gene, thus inhibiting its expression. Meanwhile, it is involved in multiple important physiological processes, such as cell differentiation, proliferation and programmed cell death [4]. MiR-96 is one of the important members of the miR-183 family (miR-183, miR-96 and miR-182). Research finds

Table 1. Associations of miR-96 expression with clinicopathological characteristics of patients (n = 60)

Clinicopathological features	miR-96 expression		χ^2 value	*P* value
	Low	High		
Age (year)			0.805	0.370
> 20	9	7		
≤ 20	19	25		
Gender			0.954	0.329
Male	20	19		
Female	8	13		
Tumor size (cm)			1.409	0.235
> 5	18	25		
≤ 5	10	7		
Tumor location			1.339	0.247
Limb	25	25		
Other	3	7		
Enneking stage			7.037	0.008
I~IIA	12	4		
IIB~III	16	28		
Distant metastasis			7.999	0.005
Yes	5	17		
No	23	15		

that, miR-96 expression dysregulation can be found in multiple malignant tumors, such as breast cancer [5], bladder cancer [6] and esophageal carcinoma [7], and this participates in the genesis and development of malignant tumors. However, research has not been reported regarding its expression in osteosarcoma, as well as its effect on the malignant phenotype in osteosarcoma cells. The current study aimed to observe the differences in miR-96 expression in human osteosarcoma tissues and cells and to further explore the molecular mechanism, which could provide new references for the pathogenesis and targeted therapy of osteosarcoma.

Material and methods

Clinical specimens

Sixty osteosarcoma samples were collected from surgical resection in Jining NO.1 people's Hospital from January 2012 to January 2017. Additionally, 20 cases of surgically removed para-tumor tissues (5 cm away from the tumor edge) at the same period and were selected as controls. All specimens were placed in liquid nitrogen within 30 min of being excised and preserved in a freezer at -80°C degrees for use. The clinical features of patients are shown in **Table 1**. All patients did not undergo chemotherapy, radiotherapy or other antitumor treatment preoperatively, and they had no malignant tumors in other organs. All specimens were re-checked by 2 pathologists to confirm the diagnosis. This study was approved by the Medical Ethical Committee of the hospital. All patients signed the informed consent.

Cells

Human osteosarcoma cell lines MG-63, Saos-2 and U20S, as well as human osteoblasts hFOB1.19 were purchased from ATCC (USA). MG-63, Saos-2 and U20S cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), while the hFOB1.19 cells were cultivated in DMEM/F12 medium containing 10% FBS. All cells were cultured in a constant temperature incubator at 5% CO_2, 37°C degrees and saturated humidity. The medium was replaced regularly, and conventional passaging was conducted in the presence of 75%-80% cell fusion.

Experimental animals

Twenty SPF grade BALB/c male nude mice at the age of 4-6 weeks with a body weight of 15-20 g were purchased from Beijing Vital River Animal Experiment Center. All animal experimental procedures in this study conformed to animal ethical standards.

qRT-PCR

Tissue or cell total RNA was extracted using Trizol reagent (BD, USA), the purity and concentration of the extracted RNA were detected, and RNA was transcribed into cDNA using the reverse transcription kit (TaKaRa, Japan). All primers were designed and synthesized by Guangzhou Ruibo Biotechnology Company (China). cDNA was used as the template, and quantitative PCR detection was conducted using the PRISM 7000 quantitative PCR machine in accordance with the qRT-PCR kit (TaKaRa, Japan) instruction, with U6/GAPDH as the internal reference. The melting curve was plotted upon the

completion of PCR amplification, and relative quantitative analysis was performed for the target gene expression using the $2^{-\Delta\Delta Ct}$ method to calculate the mRNA expression levels of miR-96 and FOXO1.

Cell transfection

MG-63 cells at exponential phase were divided into miR-96 inhibitor group and inhibitor-NC group, and were transfected with miR-96 inhibitor (GenePharma, China) and inhibitor-NC (GenePharma, China), respectively, using Lipofectamine™2000 (Invitrogen, USA). The transfection efficiency was detected using qRT-PCR, and fresh culture medium was replaced 6h after transfection.

CCK-8 assay

MG-63 cells 24 h after transfection were inoculated into the 96-well culture plate at the density of 5×10^3 cells/well (100 μl/well). 10 μl CCK-8 solution (Beyotime Biological Company, China) was added at different time points after cell adherence and incubated at 37°C degrees and 5% CO_2 for 2 h, and the optical density (OD) value was determined using the Microplate Reader at the wavelength of 450 nm. Three duplicates were set for each well, and the blank control group was also set.

Flow cytometry

MG-63 cells 24 h after transfection were digested with trypsin, washed with PBS twice, and collected into the centrifuge tube. Binding buffer was added to prepare the cell suspension at the concentration of 1×10^6 cells/ml. The cell suspension was placed into the flow tube, and Annexin V-FITC (BD Biosciences, USA) and PI (BD Biosciences, USA) were added in succession, followed by staining in the dark at room temperature for 15 min. Flow cytometry was then adopted to calculate the cell apoptosis rate.

Dual-luciferase reporter assay

The wild type and mutant reporter gene plasmids were synthesized by GeneCopoeia Company (Guangzhou, China). The PCR amplification product of FOXO1 3'UTR was connected to the Xba I enzymatic digestion site of pGL3 plasmid to construct the wide-type pGL3-FOXO1-wtUTR reporter gene plasmid. Site-specific mutagenesis was conducted in the basic group of FOXO1 3'UTR, and the new plasmid obtained after mutation was the mutant pGL3-FOXO1-mutUTR reporter gene plasmid. The pGL3-FOXO1-wtUTR or pGL3-FOXO1-mutUTR plasmids were transfected into MG-63 cells, using Lipofectamine™ 2000 transfection reagent according to the liposome transfection method, followed by transfection with miR-96 inhibitor or inhibitor-NC. The solution was replaced 6 h after transfection, cells in each group were lysed 24 h later, and the luciferase activity values (firefly luciferase and Ranilla luciferase) in each group were detected according to the Dual-luciferase activity detection kit (Promega). The relative luciferase activity = firefly luciferase activity value/ranilla luciferase activity value.

Western blotting

MG-63 cells were collected and rinsed with PBS 3 times. The protein lysis buffer was added to extract the cell total protein, and the protein concentration was determined using Bradford method. Sixtyμg total protein sample was loaded, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transfered onto the PVDF membrane. Blocking buffer containing 5% skim milk powder was added at room temperature for 2 h. Subsequently, primary antibody FOXO1 (Santa Cruz, USA), PCNA (Cell Signal Technology, USA) and caspase-3 (Cell Signal Technology, USA) was added to incubate at 4°C degrees overnight. The membrane was washed on the following day, followed by addition of secondary antibody (Santa Cruz, USA) to incubate at room temperature for 2 h, followed by exposure, and developing.

Nude mouse subcutaneous tumorigenesis assay

MG-63 cells transfected with miR-96 inhibitor or inhibitor-NC were collected, and the cell density was adjusted to 2×10^8 cells/ml. Twenty nude mice were randomly divided into miR-96 inhibitor and inhibitor-NC groups, with 10 in each group. 0.1 ml miR-96 inhibitor-transfected MG-63 cell suspension was injected subcutaneously into the right hind leg of mice in the miR-96 inhibitor group, while 0.1 ml inhibitor-NC-transfected MG-63 cell suspension was injected subcutaneously into the right hind leg of mice in the inhibitor-NC group. Afterwards,

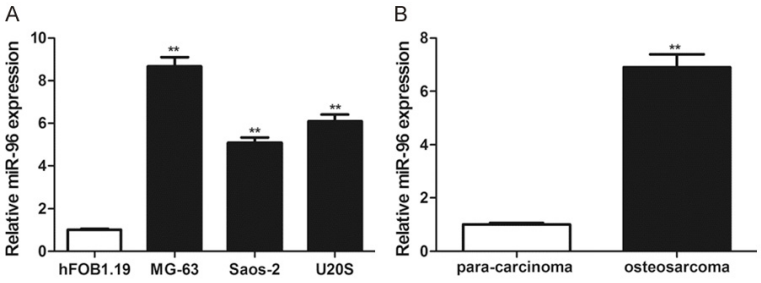

Figure 1. Expression of miR-96 in osteosarcoma cells and tissues. A. miR-96 expression levels in human osteosarcoma cells MG-63, Saos-2 and U20S were remarkably higher than that in human osteoblast hFOB1.19 as measured by using qRT-PCR. **, $P < 0.01$ compared with hFOB1.19. B. miR-96 expression in osteosarcoma tissues was notably higher than that in para-tumor tissues as measured by using qRT-PCR. **, $P < 0.01$ compared with para-tumor tissues.

the growth of the subcutaneous tumors in nude mice was observed daily, and tumor volumes in the nude mice of each group were measured every 5 days. All nude mice were sacrificed 20 days later to weigh the tumor weight. Tumor volume was calculated according to the formula $V (mm^3) = 1/2 \times L \times W^2$, where L stood for tumor length and W represented tumor width.

Statistical methods

All data were analyzed using SPSS 19.0 software. Measurement data were expressed as ($\overline{x} \pm s$) and analyzed using one-way analysis of variance or t test. Enumeration data were compared using chi-square test. All experiments were repeated 3 times. Difference of $P < 0.05$ was deemed as statistically significant.

Results

Increased expression of miR-96 in osteosarcoma tissues and cells

As was shown in **Figure 1A**, miR-96 expression levels in human osteosarcoma cells MG-63, Saos-2 and U20S were remarkably higher than that in human osteoblast hFOB1.19 ($P < 0.05$). **Figure 1B** suggested that miR-96 expression in osteosarcoma tissues was notably higher than that in para-tumor tissues ($P < 0.01$).

miR-96 is closely related to Enneking classification and distant metastasis of patients

The mean miR-96 expression level in osteosarcoma tissues was used as the threshold, and the patients were divided into high expression group (n = 32) and low expression group (n = 28). As shown in **Table 1**, miR-96 expression level was closely correlated with Enneking classification and distant metastasis ($P < 0.01$), but not with the age, sex, tumor size nor tumor site of patients ($P > 0.05$).

FOXO1 is a direct target gene of miR-96

The results are shown in **Figure 2A**, where there were complementary binding sites to the seed sequence of miR-96 in the 3'-UTR of FOXO1. Results of dual-luciferase reporter gene assay are shown in **Figure 2B**, the luciferase activities in the wtUTR group co-transfected with miR-96 inhibitor and pGL3- FOXO1-wtUTR plasmids were remarkably reduced ($P < 0.05$), while those in mutUTR group co-transfected with miR-96 inhibitor and pGL3-FOXO1-mutUTR plasmids showed no obvious change ($P > 0.05$), suggesting that miR-96 could specifically bind with the FOXO1 3'UTR.

The expression of miR-96 decreases and the expression of FOXO1 mRNA and protein increases in MG-63 cells after transfection with miR-96 inhibitor

Figure 3A suggested that, miR-96 expression level in MG-63 cells of the miR-96 inhibitor group was markedly lower than that in inhibitor-NC group ($P < 0.01$), verifying successful transfection and high transfection efficiency. As is shown in **Figure 3B-D**, the mRNA and protein expression levels of FOXO1 in MG-63 cells of the miR-96 inhibitor group were apparently higher than those in inhibitor-NC group ($P < 0.01$).

Down-regulating miR-96 inhibits the proliferation and promotes apoptosis of MG-63 cells

Results of the CCK assay are shown in **Figure 4A**, which indicated that the cell proliferation capacity in the miR-96 inhibitor group was outstandingly lower than that in the inhibitor-NC group ($P < 0.05$). Results of flow cytometry are presented in **Figure 4B** and **4C**, which suggested that the cell apoptosis rate in the miR-96 inhibitor group was apparently higher than that in the inhibitor-NC group ($P < 0.01$). Results of

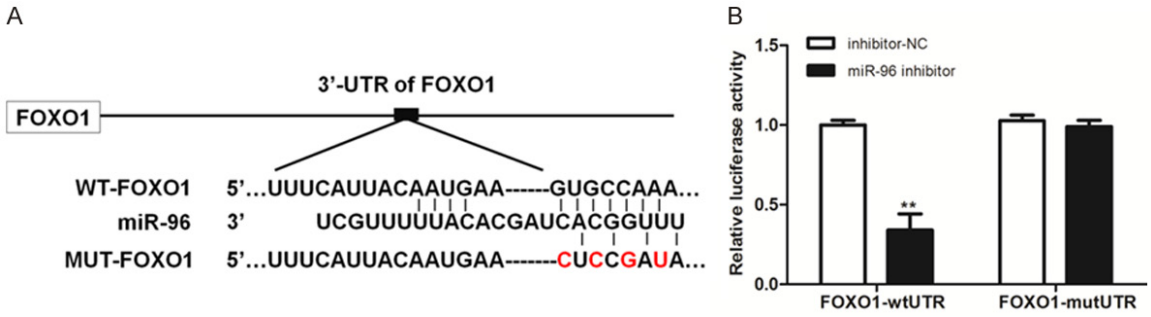

Figure 2. miR-96 directly targets FOXO1. A. Bioinformatics predicted interactions of miR-96 and their binding sites at the 3'UTR of FOXO1. B. MG-63 cells were co-transfected with miR-96 inhibitor with pGL3- FOXO1-wtUTR or pGL3-FOXO1-mutUTR. Luciferase activity was assayed. **, $P < 0.01$ compared with inhibitor-NC group.

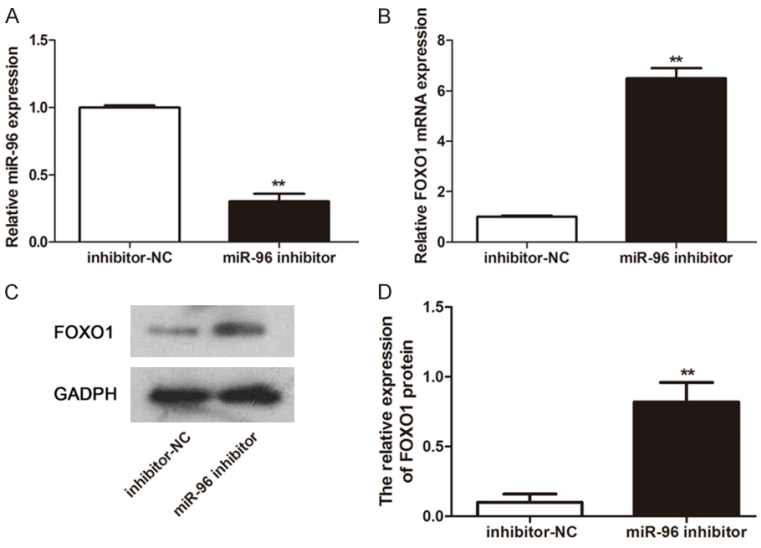

Figure 3. miR-96, FOXO1 mRNA and protein expression in MG-63 cells after transfection with miR-96 inhibitor. A. miR-96 expression level in miR-96 inhibitor group was markedly lower than that in inhibitor-NC group as measured by using qRT-PCR. **, $P < 0.01$ compared with inhibitor-NC group. B-D. FOXO1 mRNA and protein expression levels in miR-96 inhibitor group were apparently higher than those in inhibitor-NC group as measured by using qRT-PCR and Western blotting.

Western blotting are shown in **Figure 4D-F**, compared with the inhibitor-NC group, the expression of PDNA protein related to proliferation in MG-63 cells of miR-96 inhibitor group was significantly decreased ($P < 0.05$), while the expression of Caspase-3 protein related to apoptosis was significantly increased ($P < 0.05$).

Down-regulation of miR-96 inhibits the growth of xenogeneic osteosarcoma in nude mice

Results of the subcutaneous tumorigenicity assay in the nude mice suggested that all tumors in the right hind leg of mice had grown. Meanwhile, round or oval off-white solid tumors could be seen in autopsy, along with nodular projections on the surface and fish-like section, yielding the tumor formation rate of 100%. Growth of nude mouse tumors suggested that tumors in nude mice in both the miR-96 inhibitor group and the inhibitor-NC group had gradually increased, but the tumor volume in the miR-96 inhibitor group was markedly smaller than that in inhibitor-NC group ($P < 0.01$, **Figure 5A**). Moreover, the tumor weight in the miR-96 inhibitor group was evidently lower than that in the inhibitor-NC group ($P < 0.01$, **Figure 5B**).

Discussions

Osteosarcoma derives from the mesenchymal tissue, which is characterized by the production of fusiform matrix cells of osteoid tissue. Osteosarcoma is associated with extremely high malignant grade and poor prognosis. In recent years, chemotherapy technology and surgical techniques have been greatly improved; however, the therapeutic effect of osteosarcoma remains unsatisfactory. Moreover, the prognosis for osteosarcoma is dismal. Therefore, it is of great significance to investigate the genesis and development mechanisms of osteosarcoma. Treatment at the gene level has become the hotspot of osteosarcoma research, and

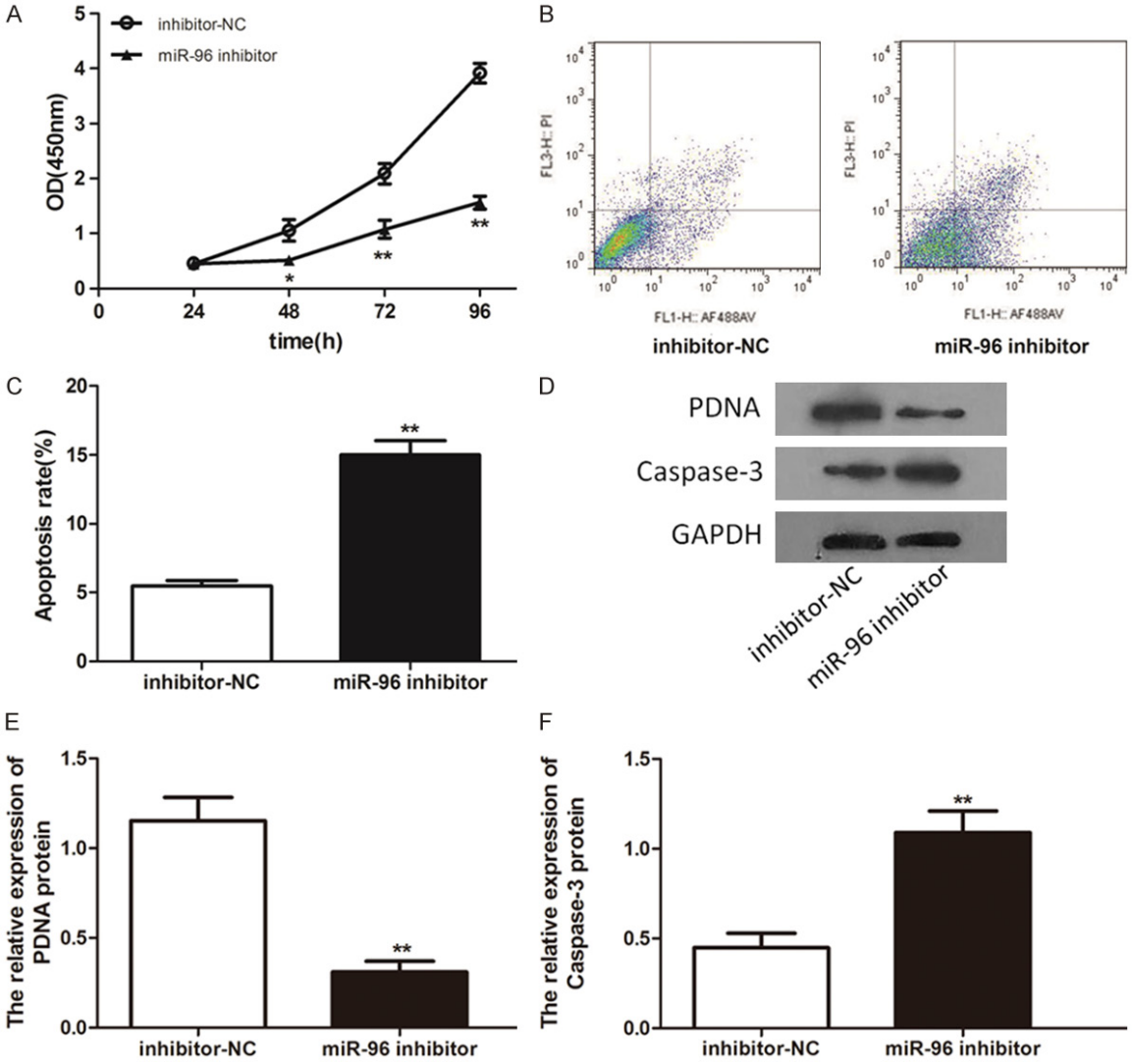

Figure 4. Effect of down-regulating miR-96 on the proliferation and apoptosis of MG-63 cells. A. The cell proliferation capacity in miR-96 inhibitor group was outstandingly lower than that in inhibitor-NC group as measured by using CCK-8 assay. *, $P < 0.05$ and **, $P < 0.01$ compared with inhibitor-NC group. B and C. The cell apoptosis rate in miR-96 inhibitor group was apparently higher than that in inhibitor-NC group as measured by using flow cytometry. **, $P < 0.01$ compared with inhibitor-NC group. D-F. PDNA protein expression levels in miR-96 inhibitor group were apparently lower than those in inhibitor-NC group as measured by using Western blotting. Caspase-3 protein expression levels in miR-96 inhibitor group were obviously higher than those in inhibitor-NC group as measured by using Western blotting.

effective gene therapy targets are urgently needed. It is currently believed that the genesis of malignant tumors is induced by the imbalance between cell proliferation and apoptosis; meanwhile, blocked cell apoptosis and infinite cell proliferation accounts for an important mechanism of tumor genesis and development [8]. The foundation of tumor biological targeted therapy is to inhibit tumor cell proliferation and induce its apoptosis. In recent years, the role of miRNA in disease has attracted increasing attention with the development of epigenetics. A growing number of studies suggest that miRNA plays a vital regulatory role in osteosarcoma proliferation and apoptosis [9, 10]. Scholars He C et al. [11] discovered that miR-34 could suppress the expression of CDK4/6, cyclin E2, E2F3/5, cMET and bcl-2 in a p53-dependent manner. Moreover, it could also induce cell cycle arrest at stage G1 and jointly regulate osteosarcoma cell proliferation and apoptosis. Scholars Zhao D et al. [12] reported that down-

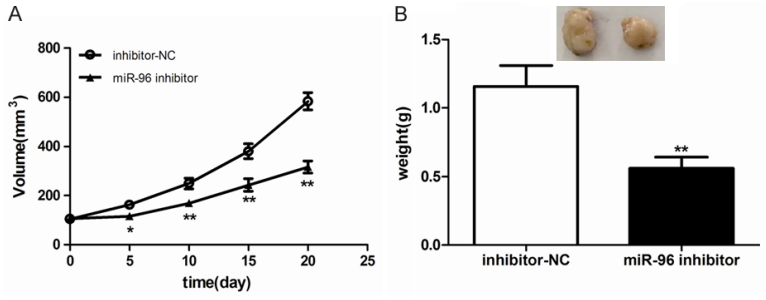

Figure 5. Effect of down-regulating miR-96 on osteosarcoma xenograft growth in nude mice. A. The tumor volume in miR-96 inhibitor group was markedly smaller than that in inhibitor-NC group. *, $P < 0.05$ and **, $P < 0.01$ compared with inhibitor-NC group. B. The tumor weight in miR-96 inhibitor group was evidently lower than that in inhibitor-NC group. **, $P < 0.01$ compared with inhibitor-NC group.

regulating miR-34 expression in osteosarcoma cells could suppress tumor cell proliferation and promote its apoptosis through up-regulating PTEN gene expression. Scholars Thayanithy V et al. [13] verified that miRNAs on the 14q32 site, such as miR-382, miR-134, miR-369-3p and miR-544, were expressed in low quantities in osteosarcoma, while up-regulating the expression of these miRNAs could promote osteosarcoma cell apoptosis.

MiR-96 is one of the miR-183 family members discovered recently, which locates on human chromosome 7q32.2. Recent research indicates that miR-96 plays different roles in different tumors. In breast cancer cell, miR-96 can promote cell proliferation, migration and invasion; moreover, miR-96 can specifically regulate the expression of PTPN9 protein, thus playing a role as oncogene [14]. On the contrary, in renal carcinoma, miR-96 plays a role as tumor suppressor gene. The invasion capacities of the highly invasive cell line Caki-1 and a cell line with low invasion potential 786-0 have increased after transfection with miR-96 inhibitor; whereas transfection with miR-96 monologue will decrease the invasion capacity [15], suggesting that miR-96 plays distinct roles in different tumors. However, miR-96 expression as well as its role in osteosarcoma remains unclear at present. In this research, we find that miR-96 shows aberrantly high expression in human osteosarcoma cells and tissues, and miR-96 expression is closely related to Enneking classification and distant metastasis, revealing that miR-96 plays a role as oncogene in the genesis and development of osteosarcoma.

To further investigate the effect of miR-96 on osteosarcoma cell proliferation and apoptosis, cells were transfected with miR-96 inhibitor in our experiment *in vitro*, so as to reduce miR-96 expression in osteosarcoma MG-63 cells. At the same time, cell proliferation and apoptosis after down-regulating miR-96 expression are detected through CCK-8 assay and flow cytometry. The results indicated that MG-63 cells with down-regulated miR-96 expression had apparently reduced cell proliferation capacity while markedly enhanced apoptosis rate. Moreover, Western blot results showed that down-regulated miR-96 expression could significantly reduce the expression of PDNA protein related to proliferation and increase the expression of Caspase-3 protein related to apoptosis. Such findings demonstrate that suppressing miR-96 expression can suppress the development of osteosarcoma. Subsequently, we constructed the nude mouse subcutaneous tumor model and discovered that down-regulating miR-96 expression in MG-63 cells can notably reduce the growth velocity of nude mouse xenograft.

The effect of miRNA mainly depends on the biological effect of downstream target genes that it regulates. Scholars Song HM et al. [16] discovered that, in thyroid cancer cell, miR-96 could specifically regulate FOXO1 gene expression, thus regulating cell proliferation, apoptosis and clone formation ability. Furthermore, it is also discovered in tumors such as prostate cancer [17, 18], bladder cancer [19] and breast cancer [20] that, miR-96 can regulate FOXO1 gene to exert its function. To investigate the underlying mechanism by which miR-96 regulates the downstream target genes in osteosarcoma, we detect the changes in expression levels of FOXO1 mRNA and protein in MG-63 cells after down-regulating miR-96 expression through qRT-PCR and Western blotting. The results indicate that FOXO1 mRNA and protein expression levels remarkably increased with the decrease in miR-96 expression level. Moreover, the results of double luciferase report experiments showed that miR-96 could specifically bind with the FOXO1 3'UTR. The above

results showed that the role of miR-96 in osteosarcoma genesis and development is achieved by targeting FOXO1.

FOXO1 is an important member of the fork head transcription factor FOXO family, which can regulate multiple key cellular functions, including cell proliferation, differentiation, apoptosis and angiogenesis [21]. It has been currently verified that FOXO1 can regulate the expression of multiple downstream target genes, thus inhibiting cell growth, regulating cell cycle, promoting apoptosis and fighting against peroxidation damage. In addition, it is involved in the formation process of multiple tumors as a tumor suppressor gene [22]. Plenty of studies have confirmed that FOXO1 is an osteosarcoma inhibiting factor, which plays a vital role in the genesis and development of osteosarcoma [23-25].

In conclusion, miR-96 shows aberrantly high expression in osteosarcoma tissues and cells, while down-regulating miR-96 expression can remarkably suppress the proliferation and tumor formation capacities of osteosarcoma cells and promote its apoptosis. Its mechanism may be related to the specific regulation of FOXO1 gene. Importantly, it is promising to become a potential target of gene therapy for osteosarcoma.

Disclosure of conflict of interest

None.

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