# Downregulation of microRNA-660 inhibits cell proliferation and invasion in osteosarcoma by directly targeting forkhead box O1

PENG ZHANG<sup>1\*</sup>, HAIFENG GAO<sup>2\*</sup>, QING LI<sup>3</sup>, XINLEI CHEN<sup>4</sup> and XIFA  $WU^4$ 

<sup>1</sup>Department of Neurosurgery, Fujian Sanbo Funeng Brain Hospital, Fuzhou, Fujian 350001; Departments of <sup>2</sup>Oncology and <sup>3</sup>Pathology, Dongying People's Hospital, Dongying, Shandong 257000; <sup>4</sup>Department of Orthopedics, Central Hospital of Zibo, Zibo, Shandong 255022, P.R. China

Received February 14, 2018; Accepted May 22, 2018

DOI: 10.3892/mmr.2018.9165

Abstract. The abnormal expression of microRNAs (miRNAs/miRs) has been observed in osteosarcoma (OS), and these differently expressed miRNAs contribute to the occurrence and development of OS by regulating various biological behaviours. Therefore, a comprehensive understanding of the detailed roles of aberrantly expressed miRNAs in OS progression may be favourable to the identification of promising therapeutic strategies for the treatment of patients with this malignancy. The present study demonstrated that miR-660-5p (miR-660) expression was significantly upregulated in OS tissues and cell lines compared with that in normal adjacent tissues and normal human osteoblast hFOB1.19, respectively. miR-660 downregulation led to a significant decrease in the proliferation and invasion of OS cells. Forkhead box O1 (FOXO1) was predicted as a potential target of miR-660. The subsequent luciferase reporter assay indicated that miR-660 directly binds to the 3'-untranslated region of FOXO1. Furthermore, miR-660 inhibition increased the FOXO1 expression in OS cells at mRNA and protein levels. Moreover, FOXO1 was downregulated in OS tissues and this downregulation was negatively correlated with miR-660 levels. Besides, rescue experiments demonstrated that FOXO1 knockdown abolished the effects of miR-660 knockdown on OS cell proliferation and invasion. These results suggest that miR-660 may serve oncogenic roles in OS by directly targeting FOXO1. Targeting miR-660 may be an effective candidate for the treatment of patients with OS.

*Correspondence to:* Professor Xifa Wu, Department of Orthopedics, Central Hospital of Zibo, 54 Gongqingtuan West Road, Zibo, Shandong 255022, P.R. China E-mail: wuxifa\_zibo@163.com

\*Contributed equally

## Introduction

Osteosarcoma (OS), the most common type of bone tumour, occurs in regions with active bone growth and repair (1). OS commonly affects children and adolescents and accounts for approximately 5% of all cases of childhood cancer (2). Patients with OS are subjected to surgical resection as a primary therapeutic method, in addition to chemotherapy or radiotherapy (3). Despite marked development in the diagnosis and therapy, the therapeutic outcome for patients with OS remains unfavourable (4). The 5-year survival rate of patients with OS in early stages is approximately 65-75%, and it decreases to less than 30% for patients with local or distant metastasis (5). The most frequent cause of death for patients with OS is metastasis, especially pulmonary metastasis (6). Therefore, the detailed genetic mechanisms of OS should be elucidated to promote the development of novel therapeutic targets or drug candidates for the treatment of patients with this aggressive malignant tumour.

MicroRNAs (miRNAs) are an abundant group of noncoding, single strand and short RNAs transcribed from nonprotein-coding genes or introns (7). miRNAs are endogenously expressed in animal and plant cells and implicated in gene regulation by directly binding to the 3'-untranslated regions (3'-UTRs) of their genes in a sequence-specific manner, thereby inducing mRNA degradation and translational suppression; consequently, protein expression is inhibited (8). Single miRNA can modulate numerous mRNAs, indicating that miRNAs may participate in various life processes, such as development, cell proliferation, differentiation and metabolism (9). miRNAs are dysregulated in a variety of human malignancies, including OS (10-12). Dysregulation of miRNAs may play tumor suppressive roles or oncogenic roles in human cancers, which decrease the expression of oncogenes and tumor suppressors, respectively (13). Hence, novel miRNAs associated with OS formation and progression should be investigated to promote the understanding of OS pathogenesis so that novel effective therapeutic strategies may be developed.

miR-660-5p (miR-660) has been reported to be dysregulated in several human cancer types, such as breast cancer (14), chronic myeloid leukaemia (15) and Hodgkin lymphoma (16). However, the expression pattern, detailed roles and underlying molecular mechanism of miR-660 in OS remain largely

*Key words:* osteosarcoma, microRNA-660, forkhead box O1, proliferation, invasion

unknown. In our study, miR-660 expression was determined in OS tissues and cell lines, and the effects of miR-660 on the proliferation and invasion of OS cells were examined. The regulatory mechanism of the oncogenic roles of miR-660 in OS was also investigated. Our study may provide novel insights into the pathogenesis and development of OS and may be beneficial to the identification of therapeutic target for patients with OS.

# Materials and methods

Patients and tissue specimens. A total of 26 human OS tissues and corresponding normal adjacent tissues (NATs) were obtained from patients who suffered from OS and underwent surgical resection at Central Hospital of Zibo (Zibo, China) between February 2013 and March 2017. None of the patients were treated with chemotherapy or radiotherapy before the surgery was performed. All of the tissues were immediately frozen in liquid nitrogen and stored in a super cold refrigerator at -80°C. The approval for this study was obtained from the Ethic Committee of Central Hospital of Zibo. Written informed consent was also provided by the patients with OS enrolled in this research.

Cell culture and transfection. Human OS cell lines (MG-63, HOS, Saos-2, and U2OS) and a normal human osteoblast hFOB1.19 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units of penicillin/ml and 100 ng of streptomycin/ml (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and maintained at 37°C in a humidified atmosphere with 5%  $CO_2$ .

miR-660 inhibitor and negative control miRNA inhibitor (NC inhibitor) were provided by GenePharma (Shanghai, China). Small interfering RNA (siRNA) against the expression of Forkhead box O1 (FOXO1 siRNA) and NC siRNA were produced by Ribobio (Guangzhou, China). Cells were plated into 6-well culture plates and transfected with miRNA inhibitor or siRNA when the cells were grown to approximately 60-70% confluence by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. At 6-8 h post-transfection, the culture medium was removed, and fresh RPMI-1640 medium containing 10% FBS was added to each well.

Reverse transcription-quantitative polymerase chain reaction (*RT-qPCR*). Total RNA was isolated from tissue specimens or culture cells by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocol. The miR-660 expression level was detected by utilizing a One-Step SYBR<sup>®</sup> PrimeScript<sup>TM</sup> miRNA RT-PCR kit (Takara Bio, Dalian, China) with U6 snRNA as an internal reference. To quantify the mRNA level of FOXO1, we conducted a reverse transcription with a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) and quantitative PCR with a SYBR Premix Ex  $Taq^{TM}$  kit (Takara Biotechnology Co., Ltd.). GAPDH served as an internal control for the mRNA expression of FOXO1. Relative gene expression was analysed using the  $2^{-\Delta\Delta Cq}$  method (17).

Cell Counting Kit-8 (CCK-8) assay. At 24 h post-transfection, the transfected cells were inoculated into 96-well plates at a density of 3,000 cells/well. In our research, four time points after inoculation (0, 24, 48 and 72 h) were chosen, and CCK-8 assays were carried out at each time point. A total of 10  $\mu$ l CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well. After the cells were incubated at 37°C for 2 h, the absorbance at 450 nm was determined with a SpectraMax Microplate<sup>®</sup> Spectrophotometer (Molecular Devices LLC, Sunnyvale, CA, USA).

Transwell invasion assay. After 24 h of transfection, the transfected cells were collected and suspended in FBS-free RPMI-1640 medium. A total of  $1\times10^5$  cells were seeded on the upper surface of Matrigel-coated Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA). RPMI-1640 medium (600  $\mu$ l) containing 20% FBS was added to the lower chambers. The chambers were incubated at 37°C with 5% CO<sub>2</sub> for 24 h. The non-invasive cells that remained on the upper surface of the chambers were removed with a cotton swab. The invasive cells on the lower surface of the chamber were fixed with 4% paraformaldehyde, stained with 0.05% crystal violet and photographed under an inverted microscope (Olympus Corporation, Tokyo, Japan). The number of invasive cells was counted in five randomly selected visual fields (magnification, 200x) from each Transwell chamber.

Prediction of miR-660 target genes and luciferase reporter assay. The putative targets of miR-660 were predicted using TargetScan (http://www.targetscan.org) and Pictar (http://www.pictar.org/).

The FOXO1 3'-UTR containing wild-type (Wt) and mutant-type (Mut) predicted miR-660 binding sequences was generated by GenePharma, cloned into the pGL3 plasmid (Promega Corporation, Madison, WI, USA), and named as pGL3-FOXO1-Wt-3'-UTR and pGL3-FOXO1-Mut-3'-UTR, respectively. Cells were seeded into 24-well plates and cotransfected using Lipofectamine 2000 with miR-660 inhibitor or NC inhibitor, and pGL3-FOXO1-Wt-3'-UTR or pGL3-FOXO1-Mut-3'-UTR. 48 h after transfection, luciferase activities were detected using the Dual-Luciferase Reporter Assay System (Promega Corporation). The firefly luciferase activity was normalized to Renilla luciferase activity.

Western blotting analysis. Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Inc., Shanghai, China) supplemented with 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 1 mg/ml aprotinin (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The concentration of total protein was quantified using the BCA Protein Assay kit (Beyotime Institute of Biotechnology, Inc.). Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis before being transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Subsequent to block with 5% non-fat milk for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-human FOXO1 monoclonal antibody (ab52857; Abcam, Cambridge, UK) and rabbit anti-human GAPDH monoclonal antibody



Figure 1. miR-660 expression is upregulated in OS tissues and cell lines. (A) The miR-660 expression level was determined in 26 pairs of OS tissues and corresponding NATs. \*P<0.05 vs. NATs. (B) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the miR-660 expression in four OS cell lines and a normal human osteoblast hFOB1.19. \*P<0.05 vs. hFOB1.19. miR, microRNA; OS, osteosarcoma; NATs, normal adjacent tissues.

(ab128915; Abcam). Afterwards, the membranes were washed thrice with Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated with Goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibodies (ab205718; Abcam) followed by visualization using an enhanced chemiluminescence system (EMD Millipore). GAPDH served as a normalization control.

Statistical analysis. Statistical analysis was performed using Statistical Package for Social Sciences version 19.0 (IBM Corp., Armonk, NY, USA). Data were shown as mean ± standard deviation and analysed with Student's t-test or one-way ANOVA plus multiple comparisons combined with Tukey's post hoc test. The relationship between miR-660 and FOXO1 mRNA in OS tissues was examined through Spearman's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

#### Results

miR-660 expression is frequently upregulated in OS tissues and cell lines. To evaluate the miR-660 expression level in OS, RT-qPCR was performed to measure the miR-660 expression in 26 pairs of OS tissues and the corresponding NATs. The results showed that the miR-660 expression level was significantly higher in the OS tissues than that in the NATs (P<0.05; Fig. 1A). We next sought to examine whether miR-660 upregulation also occurred in OS cell lines. miR-660 was overexpressed in all of the four human OS cell lines, namely, MG-63, HOS, Saos-2 and U2OS, compared with that in the normal human osteoblast hFOB1.19 (P<0.05; Fig. 1B). These results suggested that miR-660 is upregulated in OS tissues and cell lines.

miR-660 inhibition restricts the proliferative and invasive abilities of OS cells. To illustrate the biological roles of miR-660 in the development of OS, miR-660 inhibitor was introduced to MG-63 and Saos-2 cells to decrease the endogenous miR-660 level. The transfection efficiency was evaluated through RT-qPCR, and the results demonstrated that miR-660 was markedly downregulated in miR-660 inhibitor-transfected MG-63 and Saos-2 cells (P<0.05; Fig. 2A). The transfected MG-63 and Saos-2 cells were tested to examine their proliferation rate through the CCK-8 assay. The CCK-8 assay revealed that miR-660 downregulation reduced the proliferation of MG-63 and Saos-2 cells compared with that in the NC inhibitor groups (P<0.05; Fig. 2B). Transwell invasion assay was performed to determine the effect of miR-660 inhibition on OS cell invasion. In Fig. 2C, the invasion abilities of the MG-63 and Saos-2 cells transfected with miR-660 inhibitor were significantly lower than those of the cells transfected with NC inhibitor (P<0.05; Fig. 2C). These results suggested that miR-660 may play an oncogenic role in OS progression.

FOXO1 is a direct target gene of miR-660 in OS cells. Bioinformatics analysis was performed to predict the potential targets of miR-660 and to explore the mechanisms underlying the oncogenic roles of miR-660 in OS. A putative binding site for miR-660 was observed in the 3'-UTR of FOXO1 (Fig. 3A). FOXO1 was selected for further experimental confirmation because FOXO1 played tumour suppressive roles in the onset and progression of OS (18-20). Luciferase reporter assay was subsequently carried out in the MG-63 and Saos-2 cells to confirm this prediction. miR-660 inhibition led to a significant increase in the luciferase activities of pGL3-FOXO1-Wt-3'-UTR in the MG-63 and Saos-2 cells (P<0.05), but no significant change was occurred in the pGL3-FOXO1-Mut-3'-UTR group (Fig. 3B). RT-qPCR and Western blot analysis confirmed that the mRNA (P<0.05; Fig. 3C) and protein (P<0.05; Fig. 3D) expression levels of FOXO1 were upregulated in the MG-63 and Saos-2 cells transfected with miR-660 inhibitor. These results demonstrated that FOXO1 is a direct target of miR-660 in OS.

FOXO1 is downregulated in OS tissues and negatively correlated with miR-660 expression. Considering that FOXO1 is identified as a direct target of miR-660, we next sought to further examine the relationship between miR-660 and FOXO1 in OS. Firstly, RT-qPCR was conducted to detect the mRNA level of FOXO1 in 26 pairs of OS tissues and NATs.



Figure 2. miR-660 downregulation inhibits the proliferation and invasion of MG-63 and Saos-2 cells. (A) The miR-660 expression in MG-63 and Saos-2 cells transfected with miR-660 inhibitor or NC inhibitor was evaluated through Reverse transcription-quantitative polymerase chain reaction. \*P<0.05 vs. NC inhibitor. (B) The proliferative abilities of MG-63 and Saos-2 cells transfected with miR-660 inhibitor or NC inhibitor were assessed through a CCK-8 assay. \*P<0.05 vs. NC inhibitor. (C) The invasion capacities of MG-63 and Saos-2 cells transfected with miR-660 inhibitor or NC inhibitor were detected with a Transwell invasion assay (magnification, x200). \*P<0.05 vs. NC inhibitor. miR, microRNA; NC, negative control; CCK-8, Cell Counting Kit-8.



Figure 3. FOXO1 is identified as a direct target of miR-660 in OS. (A) Wt and Mut binding sites for miR-660 in the 3'-UTR of FOXO1. (B) MG-63 and Saos-2 cells were co-transfected with pGL3-FOXO1-Wt-3'-UTR or pGL3-FOXO1-Mut-3'-UTR and miR-660 inhibitor or NC inhibitor. Luciferase activities were detected at 48 h post-transfection. \*P<0.05 vs. NC inhibitor. (C and D) MG-63 and Saos-2 cells were transfected with miR-660 inhibitor or NC inhibitor. After transfection was conducted, (C) reverse transcription-quantitative polymerase chain reaction and (D) western blot analysis were performed to measure the mRNA and protein levels of FOXO1. \*P<0.05 vs. NC inhibitor. FOXO1, forkhead box O1; NC, negative control; Wt, wild-type; Mut, mutant; 3'-UTR, 3'-untranslated region.



Figure 4. FOXO1 is downregulated in OS tissues and inversely correlated with miR-660 expression. (A) The mRNA and (B) protein expression levels of FOXO1 were detected in OS tissues and NATs through Reverse transcription-quantitative polymerase chain reaction and western blot analysis. \*P<0.05 vs. NATs. (C) The relationship between the expression levels of miR-660 and FOXO1 mRNA in OS tissues was examined through Spearman's correlation analysis. r=-0.5476, P=0.0038. FOXO1, forkhead box O1; OS, osteosarcoma; NATs, normal adjacent tissues.

The mRNA expression level of FOXO1 was weakly expressed in the OS tissues in comparison with that in the NATs (Fig. 4A, P<0.05). Secondly, the protein expression of FOXO1 was determined in several pairs of OS tissues and NATs. In Fig. 4B, the FOXO1 protein was downregulated in the OS tissues relative to that in the NATs. An inverse association between miR-660 and FOXO1 mRNA was also validated in the OS tissues (r=-0.5476, P=0.0038; Fig. 4C).

FOXO1 knockdown abolishes the functions of miR-660 in OS cells. On the basis of our findings described above, we hypothesised that FOXO1 mediated the oncogenic roles of miR-660 in OS cells. A series of rescue experiments was conducted to test this hypothesis. Firstly, we transfected MG-63 and Saos-2 cells with NC siRNA or FOXO1 siRNA and detected the protein level of FOXO1. As shown in Fig. 5A, FOXO1 expression was efficiently knocked down in MG-63 and Saos-2 cells after transfection with FOXO1 siRNA (P<0.05). Next, MG-63 and Saos-2 cells were transfected with miR-660 inhibitor and FOXO1 siRNA or NC siRNA. Western blot analysis indicated that the FOXO1 protein expression was restored by FOXO1 siRNA co-transfection in MG-63 and Saos-2 cells (P<0.05; Fig. 5B and C). Furthermore, CCK-8 and Transwell invasion assays revealed that FOXO1 knockdown could abrogate the effects of miR-660 inhibition on the proliferation (P<0.05; Fig. 5D) and invasion (P<0.05; Fig. 5E and F) of MG-63 and Saos-2 cells. Thus, our data evidently demonstrated that miR-660 probably performs an oncogenic function in OS at least partially by regulating FOXO1 expression.

# Discussion

The abnormal expression of miRNAs has been observed in OS, and these differently expressed miRNAs contribute to the occurrence and development of OS by regulating various biological behaviours, such as cell proliferation, apoptosis, cycle, migration, invasion and metastasis (21-23). Therefore, an comprehensive understanding of the detailed roles of aberrantly expressed miRNAs in OS progression may be favourable to the identification of promising therapeutic strategies for the treatment of patients with OS. In our current study, miR-660

was upregulated in OS tissues and cell lines compared with that in NATs and normal human osteoblast hFOB1.19, respectively. miR-660 downregulation prohibited cell proliferative and invasive abilities in OS. FOXO1 was identified as a direct target of miR-660 in OS. FOXO1 was downregulated in OS tissues, and this downregulation was inversely correlated with the miR-660 expression level. Moreover, FOXO1 knockdown abrogated the oncogenic effects of miR-660 on the proliferation and invasion of OS cells. Based on these data, our conclusion was that targeting miR-660 may be an effective therapeutic target to inhibit the growth and metastasis of OS.

miR-660 has been reported to be upregulated in breast cancer tissues and cell lines (14). The overall survival of patients with breast cancer and with a high miR-660 level is shorter than that of patients with low miR-660 (24). miR-660 knockdown attenuates cell proliferation, migration and invasion, promotes apoptosis and induces G1 arrest in the cell cycle (14). Salati found that miR-660 expression is highly expressed in chronic myeloid leukaemia. Ectopic miR-660 expression can protect chronic myeloid leukaemia cells from apoptosis caused by tyrosine kinase inhibitors and lead to resistance to tyrosine kinase inhibitors (15). Aberrantly overexpressed miR-660 is also observed in classical Hodgkin lymphoma (16). However, miR-660 is downregulated in lung cancer tissues, cell lines and plasma. Restoration miR-660 expression plays tumour suppressive roles in lung cancer progression by inhibiting cell growth and metastasis in vitro and in vivo and by promoting cell apoptosis in vitro (25,26). These conflicting studies suggested that the expression patterns and roles of miR-660 in human malignancy exhibit tissue specificity and may be developed as a potential therapeutic target for anticancer therapy.

Several miR-660 targets, including TFCP2 (14) in breast cancer, TET2 (15), EPAS1 (15) in chronic myeloid leukaemia and MDM2 (25) in lung cancer, have been identified. In our study, FOXO1 was demonstrated to be a direct target of miR-660 in OS. FOXO1, a member of the Forkhead box (FOX) family, is a transcription factor and underexpressed in various human cancers, such as gastric cancer (27), breast cancer (28), lung cancer (29) and prostate cancer (30). FOXO1 expression is closely related to clinicopathological features and prognosis in human cancers.



Figure 5. FOXO1 knockdown can rescue the effects of miR-660 underexpression on OS cell proliferation and invasion. (A) MG-63 and Saos-2 cells were transfected with NC siRNA or FOXO1 siRNA. Western blot analysis was conducted at 72 h post-transfection to determine FOXO1 protein expression. \*P<0.05 vs. NC siRNA. (B and C) The protein expression of FOXO1 was examined in MG-63 and Saos-2 cells co-transfected with miR-660 inhibitor and FOXO1 siRNA or NC siRNA through western blot analysis. (B) Representative blot and (C) quantification of protein expression. \*P<0.05 vs. NC inhibitor. \*P<0.05 vs. miR-660 inhibitor+FOXO1 siRNA. (D-F) The proliferation and invasion of these cells were determined through (D) CCK-8 and (E and F) Transwell invasion assays (magnification, x200). \*P<0.05 vs. NC inhibitor. \*P<0.05 vs. miR-660 inhibitor+FOXO1 siRNA. FOXO1, forkhead box O1; OS, osteosarcoma; NC, negative control; CCK-8, Cell Counting Kit-8.

For instance, FOXO1 is downregulated in bladder cancer. Decreased FOXO1 expression is significantly correlated with tumour stage and grade. The survival time of patients with bladder cancer and high FOXO1 levels is longer than that of patients with low FOXO1 expression (31,32). FOXO1 plays important roles in carcinogenesis and cancer progression by regulating several biological functions, including cancer cell proliferation, differentiation, apoptosis, cycle, migration, invasion, metastasis and angiogenesis (33-35). These findings suggested that FOXO1 may be an excellent target for human cancer therapy.

FOXO1 expression is generally low in OS tissues and cell lines. Functional analysis revealed that FOXO1 might play tumour suppressive roles in OS oncogenesis by inhibiting cell proliferation, survival, colony formation, migration and invasion (18-20). Previous studies demonstrated that FOXO1 can be targeted by multiple miRNAs in human cancers. For example, miR-374a and miR-135b directly target FOXO1 and promote cell proliferation and invasion in OS (18,36). Furthermore, FOXO1 is directly targeted by miR-196a in hepatocellular carcinoma (37), miR-223 in breast cancer (38), miR-215 in gastric cancer (39) and miR-132 in laryngeal squamous cell carcinoma (40), and therefore to be involved in regulating the tumorigeneis and tumor development of these specific tumor types. Hence, miRNA-based targeted therapy of FOXO1 may be an attractive therapeutic strategy for patients with malignant diseases.

In summary, this study revealed that the miR-660 expression level increased in OS tissues and cell lines, and its downregulation prohibited OS cell proliferation and invasion. FOXO1 was confirmed as a direct target gene of miR-660 in OS. On the basis of these results, we proposed the hypothesis that the inhibition of miR-660 or the restoration of FOXO1 expression might be a novel therapeutic method for patients with OS. However, there are several limitations in our study. We did not analyse the effect of miR-660 inhibition on OS tumor growth *in vivo*. Additionally, we could not conclude that FOXO1 was the primary or only target of miR-660 in OS. In our future research, intensive studies are necessary to overcome these limitations.

# Acknowledgements

Not applicable.

# Funding

No funding was received.

## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## **Authors' contributions**

XW, PZ and HG designed the present study. PZ and HG performed RT-qPCR and Transwell invasion assays. QL and XC conducted the CCK-8 assay, western blot analysis and luciferase reporter assay. XW performed the bioinformatics analysis and statistical analysis. All authors read and approved the final draft.

# Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Central Hospital of Zibo (Zibo, China), and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Central Hospital of Zibo. Written informed consent was obtained from all patients for the use of their clinical tissues.

# **Consent for publication**

Not applicable.

## **Competing interests**

The authors declare that they have no competing interests.

#### References

- 1. Valery PC, Laversanne M and Bray F: Bone cancer incidence by morphological subtype: A global assessment. Cancer Causes Control 26: 1127-1139, 2015.
- 2. Meyers PA, Schwartz CL, Krailo M, Kleinerman ES, Betcher D, Bernstein ML, Conrad E, Ferguson W, Gebhardt M, Goorin AM, et al: Osteosarcoma: A randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. J Clin Oncol 23: 2004-2011, 2005.
- 3. Marina N, Gebhardt M, Teot L and Gorlick R: Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 9: 422-441, 2004.
- 4. Geller DS and Gorlick R: Osteosarcoma: A review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol 8: 705-718, 2010.
- 5. Gorlick R, Janeway K, Lessnick S, Randall RL and Marina N; COG Bone Tumor Committee: Children's oncology Group's 2013 blueprint for research: Bone tumors. Pediatr Blood Cancer 60: 1009-1015, 2013.
- 6. Di Fiore R, Drago-Ferrante R, Pentimalli F, Di Marzo D, Forte IM, Carlisi D, De Blasio A, Tesoriere G, Giordano A and Vento R: Let-7d miRNA shows both antioncogenic and oncogenic functions in Osteosarcoma-derived 3AB-OS cancer stem cells. J Cell Physiol 231: 1832-1841, 2016.

- 7. Calin GA and Croce CM: MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857-866, 2006.
- 8. Carrington JC and Ambros V: Role of microRNAs in plant and
- animal development. Science 301: 336-338, 2003.
  Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
- 10. Bartel DP: MicroRNAs: Target recognition and regulatory functions. Cell 136: 215-233, 2009.
- Shukla GC, Singh J and Barik S: MicroRNAs: Processing, 11. maturation, target recognition and regulatory functions. Mol Cell Pharmacol 3: 83-92, 2011.
- 12. Kushlinskii NE, Fridman MV and Braga EA: Molecular mechanisms and microRNAs in osteosarcoma pathogenesis. Biochemistry (Mosc) 81: 315-328, 2016.
- 13. Jansson MD and Lund AH: MicroRNA and cancer. Mol Oncol 6: 590-610, 2012
- 14. Shen Y, Ye YF, Ruan LW, Bao L, Wu MW and Zhou Y: Inhibition of miR-660-5p expression suppresses tumor development and metastasis in human breast cancer. Genet Mol Res: 16, 2017.
- 15. Salati S, Salvestrini V, Carretta C, Genovese E, Rontauroli S, Zini R, Rossi C, Ruberti S, Bianchi E, Barbieri G, et al: Deregulated expression of miR-29a-3p, miR-494-3p and miR-660-5p affects sensitivity to tyrosine kinase inhibitors in CML leukemic stem cells. Oncotarget 8: 49451-49469, 2017.
- 16. Paydas S, Acikalin A, Ergin M, Celik H, Yavuz B and Tanriverdi K: Micro-RNA (miRNA) profile in Hodgkin lymphoma: Association between clinical and pathological variables. Med Oncol 33: 34, 2016.
- 17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001. 18. Pei H, Jin Z, Chen S, Sun X, Yu J and Guo W: MiR-135b promotes
- proliferation and invasion of osteosarcoma cells via targeting FOXO1. Mol Cell Biochem 400: 245-252, 2015.
- 19. Wang J and Sun G: FOXO1-MALAT1-miR-26a-5p feedback loop mediates proliferation and migration in osteosarcoma cells. Oncol Res 25: 1517-1527, 2017.
- 20. Guan H, Tan P, Xie L, Mi B, Fang Z, Li J, Yue J, Liao H and Li F: FOXO1 inhibits osteosarcoma oncogenesis via Wnt/β-catenin pathway suppression. Oncogenesis 4: e166, 2015.
- 21. Kafchinski LA and Jones KB: MicroRNAs in osteosarcomagenesis. Adv Exp Med Biol 804: 119-127, 2014. 22. Gang L, Qun L, Liu WD, Li YS, Xu YZ and Yuan DT:
- MicroRNA-34a promotes cell cycle arrest and apoptosis and Suppresses cell adhesion by targeting DUSP1 in osteosarcoma. Am J Transl Res 9: 5388-5399, 2017.
- 23. Xiao Y, Zhao Q, Du B, Chen HY and Zhou DZ: MicroRNA-187 inhibits growth and metastasis of osteosarcoma by downregulating S100A4. Cancer Invest 36: 1-9, 2018.
- 24. Krishnan P, Ghosh S, Wang B, Li D, Narasimhan A, Berendt R, Graham K, Mackey JR, Kovalchuk O and Damaraju S: Next generation sequencing profiling identifies miR-574-3p and miR-660-5p as potential novel prognostic markers for breast cancer. BMC Genomics 16: 735, 2015.
- 25. Fortunato O, Boeri M, Moro M, Verri C, Mensah M, Conte D, Caleca L, Roz L, Pastorino U and Sozzi G: Mir-660 is downregulated in lung cancer patients and its replacement inhibits lung tumorigenesis by targeting MDM2-p53 interaction. Cell Death Dis 5: e1564, 2014.
- 26. Borzi C, Calzolari L, Centonze G, Milione M, Sozzi G and Fortunato O: mir-660-p53-mir-486 Network: A new key regulatory pathway in lung tumorigenesis. Int J Mol Sci 18: pii: E222, 2017.
- 27. Ko YS, Cho SJ, Park J, Kim Y, Choi YJ, Pyo JS, Jang BG, Park JW, Kim WH and Lee BL: Loss of FOXO1 promotes gastric tumour growth and metastasis through upregulation of human epidermal growth factor receptor 2/neu expression. Br J Cancer 113: 1186-1196, 2015.
- 28. Wu Y, Elshimali Y, Sarkissyan M, Mohamed H, Clayton S and Vadgama JV: Expression of FOXO1 is associated with GATA3 and Annexin-1 and predicts disease-free survival in breast cancer. Am J Cancer Res 2: 104-115, 2012.
- 29. Maekawa T, Maniwa Y, Doi T, Nishio W, Yoshimura M, Ohbayashi C, Hayashi Y and Okita Y: Expression and localization of FOXO1 in non-small cell lung cancer. Oncol Rep 22: 57-64, 2009.
- Zhang H, Pan Y, Zheng L, Choe C, Lindgren B, Jensen ED, Westendorf JJ, Cheng L and Huang H: FOXO1 inhibits Runx2 transcriptional activity and prostate cancer cell migration and invasion. Cancer Res 71: 3257-3267, 2011.

- Kim TH, Jo SW, Lee YS, Kim YJ, Lee SC, Kim WJ and Yun SJ: Forkhead box O-class 1 and forkhead box G1 as prognostic markers for bladder cancer. J Korean Med Sci 24: 468-473, 2009.
- 32. Zhang Y, Jia L, Zhang Y, Ji W and Li H: Higher expression of FOXOs correlates to better prognosis of bladder cancer. Oncotarget 8: 96313-96322, 2017.
- 33. Kim SY, Ko YS, Park J, Choi Y, Park JW, Kim Y, Pyo JS, Yoo YB, Lee JS and Lee BL: Forkhead transcription factor FOXO1 inhibits angiogenesis in gastric cancer in relation to SIRT1. Cancer Res Treat 48: 345-354, 2016.
- 34. Zhao Y, Wang Y and Zhu WG: Applications of post-translational modifications of FoxO family proteins in biological functions. J Mol Cell Biol 3: 276-282, 2011.
- 35. Dong T, Zhang Y, Chen Y, Liu P, An T, Zhang J, Yang H, Zhu W and Yang X: FOXO1 inhibits the invasion and metastasis of hepatocellular carcinoma by reversing ZEB2-induced epithelial-mesenchymal transition. Oncotarget 8: 1703-1713, 2017.

- 36. He W, Feng L, Xia D and Han N: MiR-374a promotes the proliferation of human osteosarcoma by downregulating FOXO1 expression. Int J Clin Exp Med 8: 3482-3489, 2015.
- expression. Int J Clin Exp Med 8: 3482-3489, 2015.
  37. Yang L, Peng F, Qin J, Zhou H and Wang B: Downregulation of microRNA-196a inhibits human liver cancer cell proliferation and invasion by targeting FOXO1. Oncol Rep 38: 2148-2154, 2017.
- Wei YT, Guo DW, Hou XZ and Jiang DQ: miRNA-223 suppresses FOXO1 and functions as a potential tumor marker in breast cancer. Cell Mol Biol (Noisy-le-Grand) 63: 113-118, 2017.
- Zang Y, Wang T, Pan J and Gao F: miR-215 promotes cell migration and invasion of gastric cancer cell lines by targeting FOXO1. Neoplasma 64: 579-587, 2017.
- 40. Lian R, Lu B, Jiao L, Li S, Wang H, Miao W and Yu W: MiR-132 plays an oncogenic role in laryngeal squamous cell carcinoma by targeting FOXO1 and activating the PI3K/AKT pathway. Eur J Pharmacol 792: 1-6, 2016