ORIGINAL RESEARCH

miR-596 suppresses the expression of Survivin and enhances the sensitivity of osteosarcoma cells to the molecular targeting agent anlotinib

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Background: Osteosarcoma (OSA), the most common primary bone malignancy, is characterized by a wide spectrum of complicated pathologies and frequent distal metastasis and causes death in adolescents and young adults worldwide. Antitumor drug treatment strategies include various cytotoxic chemotherapy drugs, while molecular targeted therapy for OSA is currently less used. The present work revealed the role played by the miR-596/Survivin axis in affecting the sensitivity of OSA cells to anlotinib, a novel molecular targeting agent.

Methods: By virtual screening, we found that miR-596 might target Survivin by using an online tool (miRDB). RNA levels of miR-596 and Survivin in clinical specimens were examined with qPCR. The effect of miR-596 on anlotinib's antitumor effect was examined with MTT experiments, the subcutaneous tumor model, or the intramuscular tumor model.

Results: Overexpression of miR-596 via lentiviral particles repressed the protein level of Survivin in U2OS cells. Transfection of miR-596 enhanced the antitumor effect of anlotinib on U2OS cells or five cell lines derived from OSA patients.

Conclusion: miR-596 targets Survivin and enhances the antitumor effect of anlotinib on OSA cells.

Keywords: osteosarcoma cell, microRNAs, Survivin, molecular targeting agents, anlotinib

Introduction

Osteosarcoma (OSA), which is considered as the most common bone malignancy, is characterized by complicated pathologies and sometimes distal metastasis.[1,2] Patients suffering from localized/primary OSA often have a good (about 60–80%) 5-year survival rate, whereas the 5-year survival rate is decreased to about 15–30% in patients with metastatic or recurrent OSA.[3,4] Consequently, chemotherapeutic treatments combined with surgical resection have been widely used to treat OSA, while molecular-targeted therapy for OSA is currently less used. Although the biology and genetics of OSA have gained attention, the clinical outcomes of OSA patients have not yet significantly improved.[3] It has been reported that the occurrence and progress of malignant/metastatic OSA are often driven by genetic or pathological alterations.[5] Increasing evidences have confirmed that the inhibition of angiogenesis process could decelerate the progress or metastasis of OSA.[5] Therefore, angiogenesis inhibitors could be used to treat advanced OSA.[5]

Anlotinib is an orally available, highly potent multitargeting protein-kinase inhibitor that could block the activation of some receptor tyrosine protein kinase

(RTKs), eg, VEGFR2 (vascular endothelial growth factor receptor 2), platelet-derived growth factor receptors α/β (PDGFR α/β), Ret, c-Kit, c-FMS, or discoidin domain receptor 1 (DDR1).[6,7] It has been reported that anlotinib revealed an antitumor effect when used in clinical trials in a variety of human solid tumors, for example non–small-cell lung cancer (NSCLC), hepatocarcinoma (HCC), gastric cancer, renal carcinoma (RC), or soft tissue sarcoma.[8–10] In 2018, anlotinib was approved by the China Food and Drug Administration (CFDA) for the clinical application of NSCLC treatment.[8–10] Therefore, demonstrating the therapeutic effects of anlotinib on OSA cells not only helps to deepen our understanding of anlotinib but also provides more options for the diagnosis and treatment of OSA.

Survivin is a key regulator of cellular survival and injury response. In malignant human cells, Survivin enhances cell survival and decreases apoptosis in response to cellular injury, eg, ion radiation or antitumor agents.[11–13] It has been reported that Survivin could be involved in antitumor agents' resistance.[14] Thus, targeting Survivin is a potential approach to more effective antitumor treatment. MicroRNAs (miRNAs), which have emerged as post-transcriptional modulators of target genes, are endogenous small noncoding RNAs that have been found to have critical roles in functioning as tumor suppressors.[15–18] In the current study, we demonstrated that Survivin was correlated with the prognosis of overall survival (OS) or progression-free survival (PFS) of OSA patients. We also demonstrated that miR-596 enhanced the antitumor effect of anlotinib by targeting Survivin. Therefore, targeting Survivin by miR-596 is a promising approach to achieve effective molecular targeting therapies in OSA treatment.

Materials and methods
Patients and specimens
The collection of clinical specimens and protocols of this work were approved by the Ethics Committee of Yantaishan Hospital. The collection and usage of clinical specimens were with written informed consent from patients, and all protocols or experiments were conducted in accordance with the Declaration of Helsinki. Total RNA samples, extracted from a cohort including 74 tumor and adjacent nontumor tissues and evaluated by accepted pathological and radiological criteria, were conserved in our laboratory and used in this study. The baseline information of the cohort was shown as Table S1. The

prognoses of patients were determined as OS or PFS. The data of TCGA database were also searched following the methods provided by Cao et al (2019) and Fan et al (2019).[19,20]

Cell lines and reagents
U2OS, a typical OSA cell line, was purchased from the cell resources center of the Chinese Academy of Medical Sciences (Beijing, China). Five patient-derived cell lines (PDCs) were separated from the clinical specimens obtained from five patients with OSA during surgery as part of normal medical care. The lentivirus particles of pri-miR-596 or Survivin with a mutation of miR-596 targeted sequences located in 3′-UTR were constructed and purchased (Vigene Corporation, Jinan City, Shandong Province, China). The wild-type sequence or sequence with mutated miR-596-binding site of Survivin's 3′UTR (1921–2100) was obtained by chemical synthesis and cloned into pGL4.26 plasmids to construct luciferase reporters by Vigene Corporation. The luciferase containing wild-type sequence of Survivin's 3′UTR with miR-596-binding site was named as Luc, whereas the luciferase containing sequence of Survivin's 3′UTR with mutated miR-596-binding site was named as Luc[Mut]. The inhibitor of the miR-596 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The antitumor agents doxorubicin (Selleck, TX, USA), cisplatin (Selleck), methotrexate (Selleck), or anlotinib (Selleck) were dissolved in DMSO and conserved in an −80°C condition.

Quantitative polymerase chain reaction
Total RNA samples were extracted from clinical specimens, and qPCR experiments were performed, following protocols provided by the manufacturer (Applied Biosystems, Thermo Fisher Scientific,) and methods described by Ji et al (2017) and Liang et al (2017).[3,15] The expression of Survivin was examined by qPCR with the following primers: Survivin (birc5), forward sequence, 5′-CCACTGAG AACGAGCCA GACTT-3′; reverse sequence 5′-GTATTA CAGGCGTAAGCCACCG-3′; loading control (gapdh), forward sequence, 5′-GTCTCCTCTGACTTCAACAGCG-3′; reverse sequence, 5′-ACCACCCTGTTGCTGTAGCCA A-3′.

Luciferase experiments
Cells were transfected with control miRNA + Luc, miR-596 + Luc, miR-596 + inhibitor + Luc or miR-596 + LucMut. Then, cells were harvested for luciferase experiments.

Luciferase experiments were performed by using a luciferase examination kit (Promega Corporation, Madison, WI, USA) following the instructions provided by the manufacturer and methods described by Ma et al (2016) and Wang et al (2019).[21,22] The relative luciferase activation was calculated as: [(luciferase activation of control group/β-galactosidase activation of control group)/(luciferase activation of administration group/β-galactosidase administration of control group)] × 100%. Results were shown as mean ± SD of relative luciferase activation.

Cell culture and survival examination

Cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA), with the addition of 10% FBS at 37°C with a 5% CO_2 condition. Cells were seeded into a 96-well plate (Corning, Inc.) and incubated with the indicated concentrations of antitumor agents diluted by sorafenib DMSO solution (Table S1). After 48 hrs of treatment, cells were harvested for MTT experiments, following the methods described by Feng et al (2018).[23] The inhibition rates of antitumor agents on cells were calculated, following the methods provided by Feng et al (2018).[19] The half-maximal inhibitory concentration values (IC_{50} values) were calculated based on inhibition rates, following the methods described by Guan et al (2017) and Li et al (2018).[24,25]

Antibodies and Western blot

Antibodies to Survivin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Abcam (Cambridge, UK). OSA cells transfected with control, miR-596, miR-596 + Survivin[Mut], or miR-596 + inhibitor were harvested 48 hrs following transfection, and total protein samples were extracted for Western blot experiments. Western blots were performed following a standard method. In brief, total protein samples extracted from OSA cells were separated using SDS-PAGE. Following electrophoresis, protein samples in SDS-PAGE gels were transferred onto polyvinylidene fluoride (PVDF) membranes and blocked, using 5% BSA diluted in tris-buffered saline with Tween 20 (TBST buffer). Membranes were subsequently incubated with the relevant primary and secondary antibodies before detection by chemiluminescence.

Animal experiments

The Ethics Committee of Yantai Hospital approved the methods of animal experiments in this study. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and its associated guidelines.[26,27] Cells (U2OS or PDC) were used to form PDX (patient-derived tumor xenograft) and were cultured (2×10^7 cells per inoculation point) and seeded into the subcutaneous position of nude mice (T-cell deficiency mice) aged 4–6 weeks to form subcutaneous tumor tissues. After 5–6 days, the mice received antitumor agents (anlotinib) once per 2 days. After 4–5 weeks of treatment, the mice were harvested, and subcutaneous tumors were collected.

Tumor weights were measured by the precision balance. Tumor volumes were measured by tumor width × tumor width × tumor length/2.[28,29] The inhibition rates of anlotinib on OSA cells were calculated based on tumor volumes or tumor weights. The formulas used to calculate inhibition rates are as follows: 1) (control group's tumor volumes – anlotinib group's tumor volume)/(control group's tumor volumes) and 2) (control group's tumor weight – anlotinib group's tumor volume)/(control group's tumor volumes). The IC_{50} values of anlotinib on OSA cells' subcutaneous growth were calculated based on the inhibition rates.

Next, cells were mixed with biological-medical gel (Cai-Hong-Yi-Xue-She-Bei, Kunming, China) and injected into muscle tissue located in the legs of nude mice (5×10^5 cells per site). After 4–6 weeks' growth, mice were injected with F-FDG,[18] received MicroPET, and were examined following the methods described by Xu et al (2013).[30] Next, the mice were harvested, and the muscle tissue was collected for H&E staining.[31] Images of the results from MicroPET or H&E staining were quantitatively analyzed via Image J software, according to previously detailed methods.[32,33] The inhibition rate of each group was calculated following the methods provided by Wei et al (2019).[34]

Statistical analysis

Statistical analysis was performed by Bonferroni's correction with or without two-way ANOVA methods using SPSS (Version No. 8.0; IBM Corporation, Armonk, NY, USA). The IC_{50} values of anlotinib on OSA cells were calculated by Origin Software, version 8.5 (OriginLab, Northampton, MA, USA). A *P*-value of <0.05 was considered statistically significant between the results from two groups.

Results

The expression of miR-596/Survivin and correlations with clinical parameters in OSA patients

Survivin was a potential target of miR-596; the italicized font in Figure 1A indicates the potential targeted sequence within PXR mRNA's 3′-UTR (Figure 1). Next, U2OS cells infected with control, miR-596, miR-596 + Survivin^Mut, or miR-596 + inhibitor were harvested for Western blot 48 hrs after transfection. Results showed that compared to the control group, miR-596 significantly decreased the expression of Survivin (Figure 1B and C) but not Survivin^Mut, which contains a mutated sequence

of the miR-596-binding site (Figure 1A–C). Moreover, transfection of miR-596's inhibitor almost blocked the effect of miR-596 on Survivin's expression. To further examine the specificity of miR-596 on Survivin's expression, luciferase reporters were used. As shown in Supplemental Figure 1, miR-596 inhibited the activation of luciferase reporters containing 3`UTR sequences, including the miR-596-targeting site. Transcription of the miR-596 inhibitor or mutated vectors blocked the effect of miR-596 on luciferase reporters' activation (Figure S1).

Next, the expression of Survivin or miR-596 in clinical specimens was examined. As shown in Figure 2A and B, the expression of Survivin is much higher in OSA
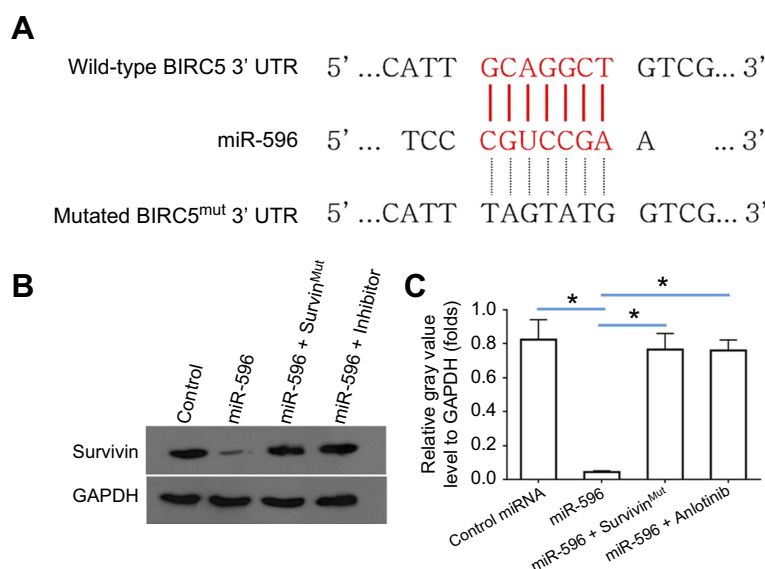
Figure 1 miR-596 targets Survivin's 3′-UTR. (**A**) The bold, italicized type image indicates the potential miR-596-binding sites or mutated sites located in Survivin's 3′-UTR. (**B**) U2OS cells, which were transfected with control (control miRNA), miR-596, miR-596 + Survivin^Mut, or miR-596c + its inhibitor, were harvested for Western blot experiments. The protein level of Survivin or GAPDH in U2OS cells was examined by their antibodies, and GAPDH was chosen as the loading control. Results were shown as images from Western blot (**B**) or quantitative analysis results (**C**). *$P<0.05$.
Abbreviations: miR, microRNA; Mut, mutation.
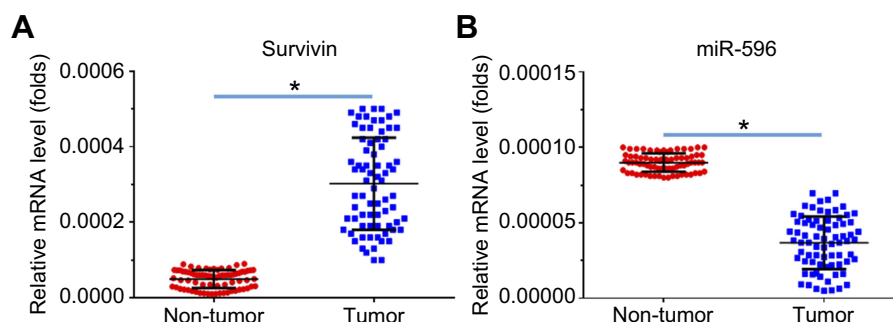
Figure 2 Expression of Survivin and miR-596 in clinical specimens. RNA levels of Survivin (**A**) or miR-596 were examined in 74 paired nontumor/tumor clinical tissues. *$P<0.05$ versus nontumor group with tumor group.
**Abbreviation:** miR, microRNA.

specimens than in paired nontumor specimens, whereas the expression of miR-596 is much lower in OSA specimens than in paired nontumor specimens. To further investigate the clinical significance of miR-596/Survivin, the relationship between Survivin or miR-596 levels and prognoses of patients was examined. As shown in Figure 3 and Tables 1 and 2, Kaplan–Meier survival analysis indicated that patients with high levels of Survivin had a poorer OS and PFS than those with low Survivin levels, whereas patients with high levels of miR-596 showed better OS and PFS than those with low miR-596 levels. Moreover, results shown in Figure 4 further confirm that the expression of Survivin is negatively correlated with miR-596 in

OSA specimens. Therefore, miR-596 could repress the expression of Survivin by targeting Survivin mRNA's 3′-UTR directly.

miR-596 enhances the sensitivity of OSA cells to anlotinib

MTT assays were performed to determine whether miR-596 enhances the in vitro antitumor effect of anlotinib on OSA cells. As shown in Figure 5 and Table 3, anlotinib inhibited the survival of U2OS cells. Transfection of miR-596 enhanced the sensitivity of OSA cells to anlotinib, and the IC_{50} values of anlotinib were, respectively, decreased (Table 3). The effects of anlotinib treatment on
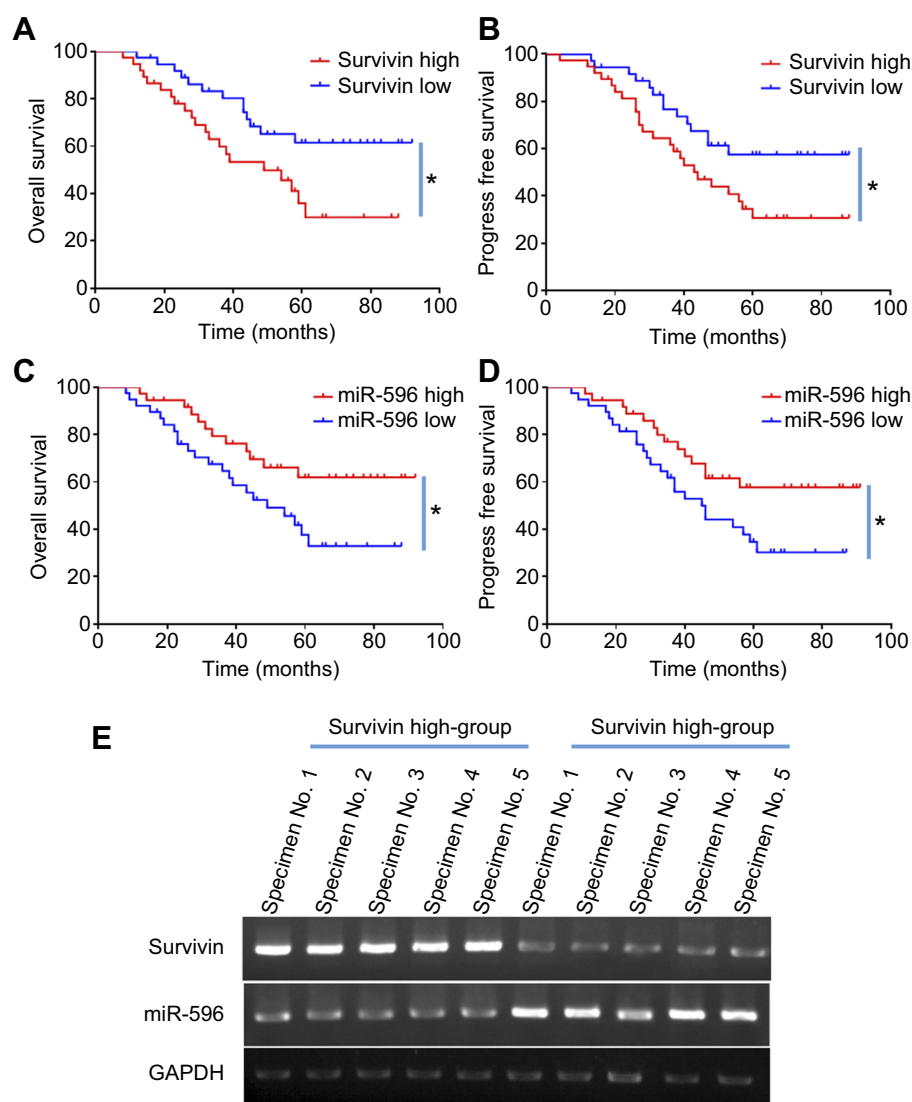
Figure 3 Expression of Survivin/miR-596 and correlation with patients' prognosis (**A–B**). Kaplan–Meier survival curves and log-rank tests were used to compare the OS (**A**) or PFS (**B**) of low-Survivin group and high-Survivin group patients (**C-D**). Kaplan–Meier survival curves and log-rank tests were used to compare the OS (**C**) or PFS (**D**) of low-miR-596 group and high-miR-596 group patients. (**E**) The representative image of clinical specimens by qPCR and DNA electrophoresis. *$P<0.05$.
Abbreviations: miR, microRNA; OS, overall survival; PFS, progression-free survival.

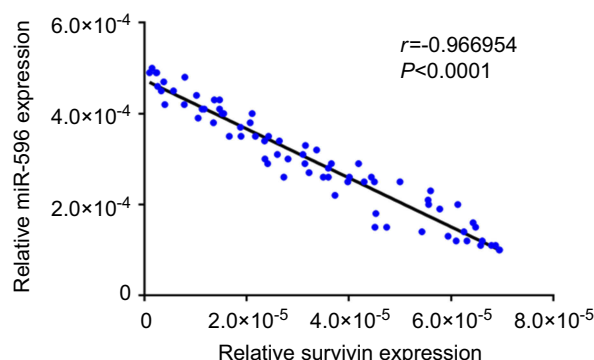**Table 1** miR-596 expression and clinical outcome of osteosarcoma patients

	miR-596 mRNA expression		P
	High (n=37)	Low (n=37)	
PFS	66.8 56.8–76.8 (M)	50.3 41.2–59.4 (M)	0.041
OS	70.2 60.2–80.2 (M)	52.7 42.8–62.0 (M)	0.036

Abbreviations: PFS, progress free survival; OS, overall survival; M, months.

Table 2 Survivin expression and clinical outcome of osteosarcoma patients

	Survivin mRNA expression		P
	High (n=37)	Low (n=37)	
PFS	50.5 41.2–59.7 (M)	65.3 56.0–74.6 (M)	0.035
OS	51.1 41.2–61.0 (M)	70.3 60.7–79.9 (M)	0.022

Abbreviations: PFS, progress free survival; OS, overall survival; M, months.

Figure 5 miR-596 enhances the antitumor effect of anlotinib on cultured U2OS cells. U2OS cells transfected with control miRNA or miR-596 were treated with the indicated concentration of anlotinib. Cells were harvested for MTT experiments, and the inhibition rates of anlotinib were calculated by OD490 nm. *$P<0.05$. **Abbreviation:** miR, microRNA.

Transfection of miR-596 enhanced the antitumor effect of anlotinib on OSA cells' subcutaneous growth, and the IC_{50} values of anlotinib on OSA cells' subcutaneous growth were, respectively, decreased. The effect of anlotinib treatment on the PDX model, formed by PDC cell lines from the five patients, is shown in Table 4. Anlotinib inhibited the survival of PDC cells in a dose-dependent manner. To further examine the antitumor effect of anlotinib on OSA cells, U2OS cells were injected into the muscle tissues of nude mice. As shown in Figures 7 and 8, U2OS cells can form tumor lesions in the muscle tissues of nude mice. Anlotinib inhibited the growth of U2OS cells in nude mice muscle tissues, and miR-596 enhanced the antitumor effects of anlotinib on U2OS cells.

Moreover, the specificity of miR-596 on anlotinib-inhibiting OSA cell survival or growth was examined. As shown in Figures 7–9, and Table 1, Survivin^{Mut}-expressed cells and tumors could not be affected by miR-596. Therefore, miR-596 enhances the sensitivity of OSA cells to anlotinib.

Figure 4 Correlation of RNA levels of miR-596 and Survivin in clinical tumor specimens from osteosarcoma patients. The relationship between the RNA levels of miR-596 and Survivin was assessed by Spearman's rank correlation analysis. The symbols represent individual samples (a total of 74 specimens). **Abbreviation:** miR, microRNA.

OSA cell lines from the five patients (PDCs) are also shown in Table 3. Anlotinib inhibited the survival of the OSA cells (PDCs) in a dose-dependent manner, and the IC_{50} values of anlotinib on PDCs' survival were, respectively, decreased.

Next, the effect of miR-596 and anlotinib coadministration on OSA cells was examined in a subcutaneous model. As shown in Figure 6 and Table 4, anlotinib inhibited the subcutaneous growth of U2OS cells in nude mice.

miR-596 enhances the sensitivity of OSA cells to cytotoxic chemotherapies

To further determine whether miR-596 enhances the in vitro antitumor effect of other antitumor agents, MTT assays were performed. As shown below, the cytotoxic chemotherapies doxorubicin (Table 5), cisplatin (Table 6), or methotrexate (Table 7) inhibited the survival of U2OS cells in a dose-dependent manner. Transfection of miR-596 enhanced the sensitivity of OSA to other antitumor agents, and the IC_{50} values of anlotinib were, respectively, decreased. Therefore, miR-596 enhances the sensitivity of OSA cells to cytotoxic chemotherapies. The

Table 3 The IC$_{50}$ values of anlotinib on osteosarcoma cell lines

Cell lines	Control	miR-596	miR-596 + Survivin^{Mut}	miR-596 + inhibitor
	IC$_{50}$ values (µmol/L) of anlotinib			
U2OS	0.66±0.11	0.21±0.02	0.58±0.15	0.49±0.08
PDC-1	0.75±0.22	0.15±0.01	0.70±0.20	0.55±0.10
PDC-2	0.80±0.25	0.28±0.05	0.78±0.23	0.65±0.13
PDC-3	0.65±0.30	0.10±0.03	0.67±0.33	0.56±0.15
PDC-4	0.71±0.33	0.20±0.04	0.53±0.25	0.60±0.07
PDC-5	0.75±0.10	0.35±0.02	0.72±0.24	0.63±0.14

Abbreviation: PDC, patient-derived cells.
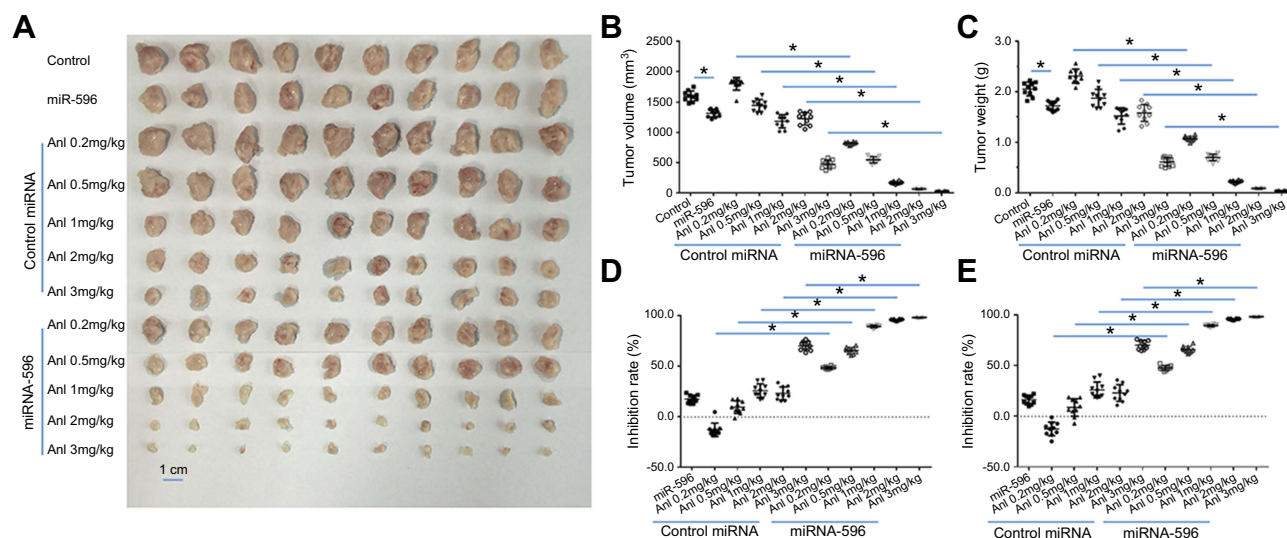
Figure 6 miR-596 enhances the antitumor effect of anlotinib on U2OS cell subcutaneous growth. U2OS cells transfected with control or miR-596 were injected into nude mice to form subcutaneous tumors. Four days to 5 days after injection, mice received orally administrated concentrations of anlotinib once per 2 days. After 10 treatments (about 3 weeks), mice were harvested, and tumor tissues were harvested. Results are shown as images of subcutaneous tumors (**A**), tumor volumes (**B**), tumor weights (**C**), inhibition rates of anlotinib calculated by tumor volumes (**D**), or inhibition rates of anlotinib calculated by tumor weights (**E**). *$P<0.05$.
Abbreviations: miR, microRNA; Mut, mutation; Anl, anlotinib.

Table 4 The IC$_{50}$ values of anlotinib on osteosarcoma cell lines in nude mice

| Cell lines	Control	miR-596
	IC$_{50}$ values (mg/kg) of anlotinib	
U2OS	2.50±0.75	0.33±0.11
PDX-1	2.88±0.55	0.50±0.05
PDX-2	1.97±0.30	0.44±0.10
PDX-3	2.32±0.51	0.76±0.22
PDX-4	1.62±0.29	0.35±0.10
PDX-5	1.98±0.46	0.55±0.25

Abbreviation: PDX, patient-derived tumor xenograft.

effects of anlotinib treatment on the OSA cell lines from the five patients are shown in Table 2. Anlotinib inhibited the survival of OSA cells in a dose-dependent manner, and

miR-596 enhanced the antitumor effect of anlotinib on OSA cells.

Discussion

Currently, one of the main strategies for molecular targeted therapy of malignant tumors is the oral administration of small-molecule multitarget protein kinase inhibitors.[35,36] These drugs can directly inhibit the RTK and mitogen-activated protein kinase as well as other signaling pathways to inhibit malignant tumors delaying cell proliferation or decelerating tumor tissue growth. Molecular targeting agents also can inhibit tumor cell metastasis, invasion, and tumor angiogenesis by inhibiting RTK, such as in VEGFR.[37–41] Anlotinib is a new molecular targeted drug with intellectual property rights in China and has strong inhibitory effects on VEGFR and
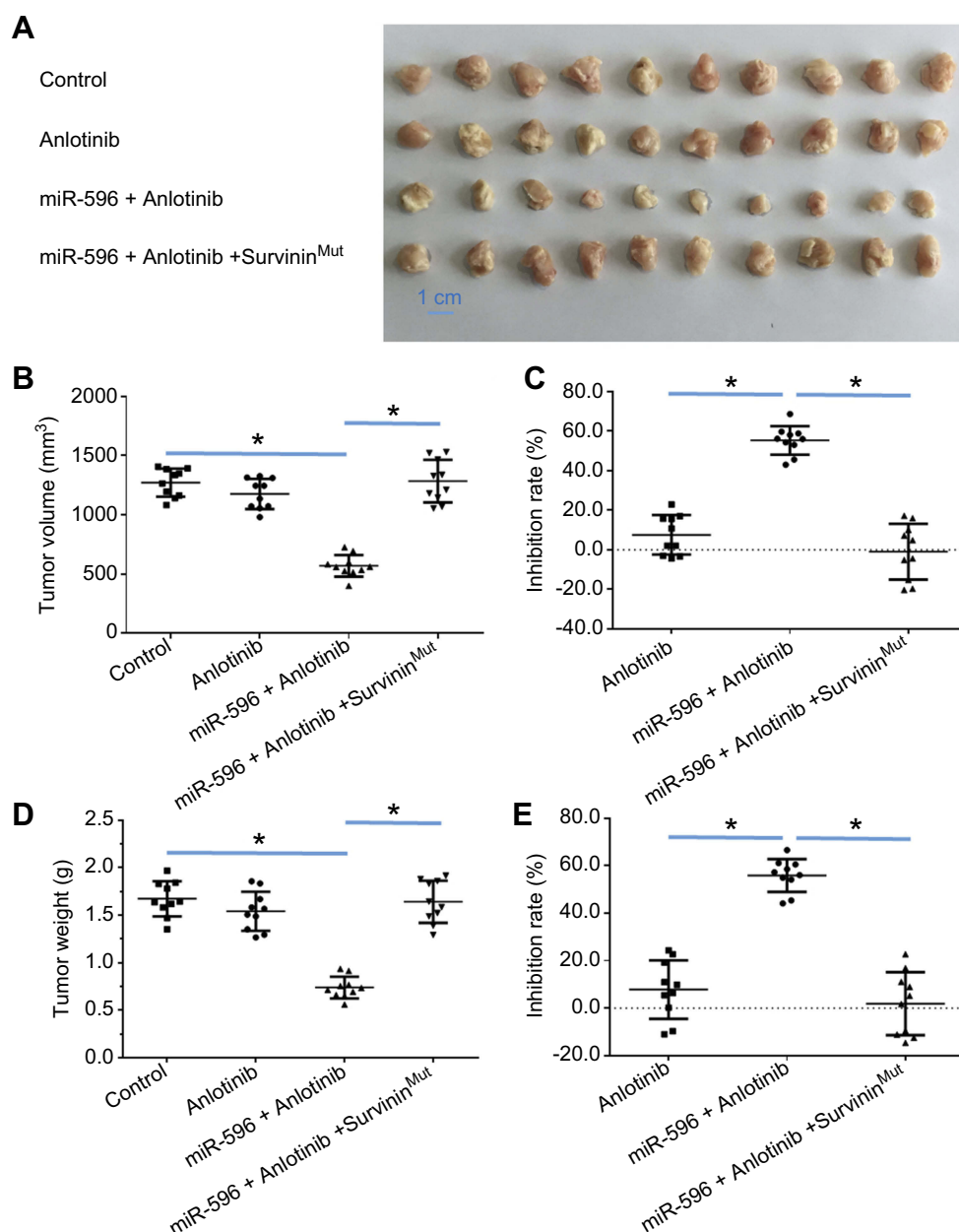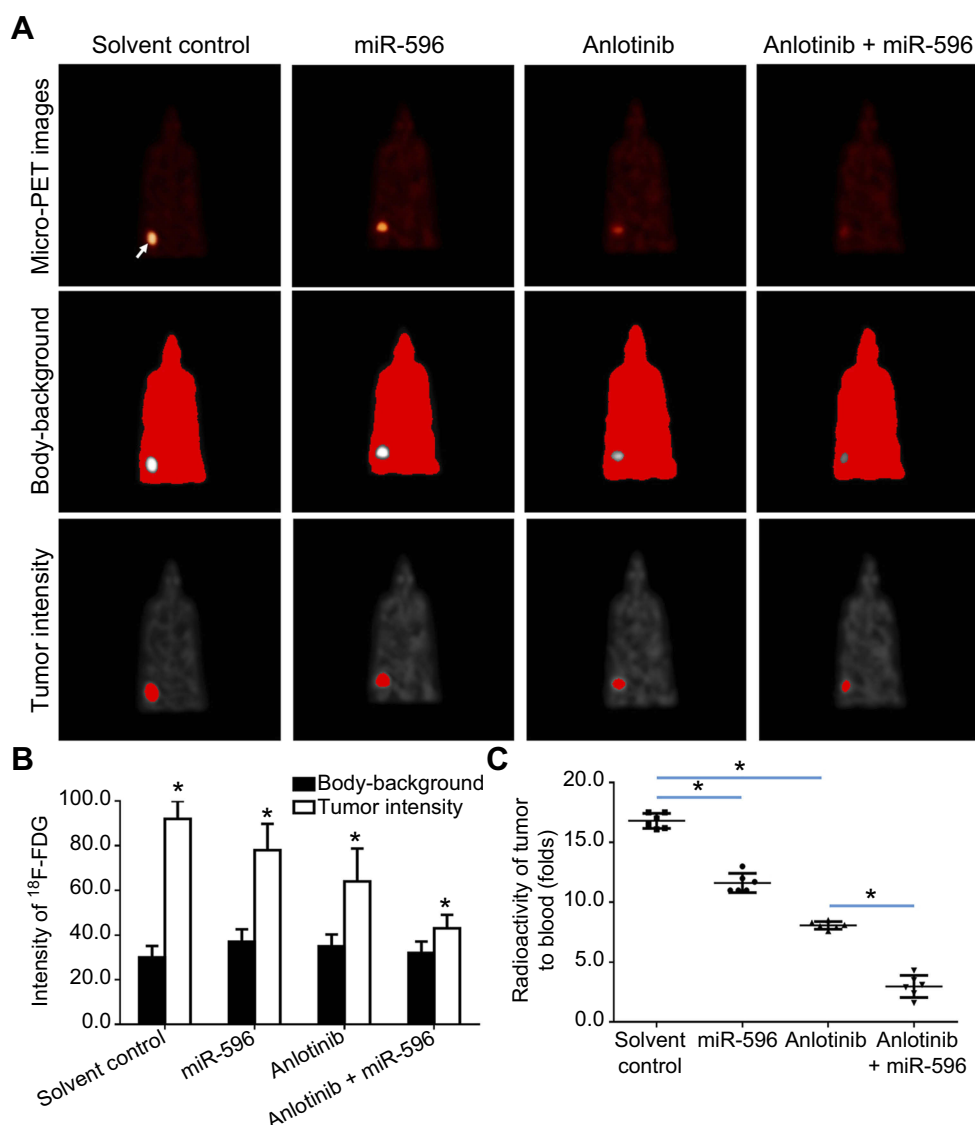
Figure 7 miR-596 enhanced the antitumor activation of anlotinib on U2OS cell subcutaneous growth by targeting Survivin. U2OS cells transfected with control miRNA, miR-596, or miR-596 + Survivin^{Mut} were seeded into nude mice to form subcutaneous tumors. The mice received a 0.5 mg/kg dose of anlotinib (a dose without significant antitumor effect) by oral administration. The results are shown as (**A**) images of subcutaneous tumors, (**B**) tumor volumes, and (**C**) or tumor weight (**D**) tumor weights. The inhibition rate calculated by tumor volume (**E**) is shown. *$P<0.05$.
Abbreviation: miR, microRNA.

c-Kit.[8–10] However, these molecularly targeted drugs also face many challenges in clinical use: molecular targeted drugs are expensive and drug resistant.[42] At the same time, the toxic side effects of these drugs cannot be ignored.[43] Therefore, how to achieve more safe and effective molecular targeted therapy and to achieve lower doses of drugs that achieve antitumor effect is an urgent problem to be solved. The results of this study indicate that the use of miR-596 can significantly increase the killing effect of anlotinib on OSA cells, using a lower dose to achieve an equal, or even greater, antitumor effect. This study uses not only existing cell lines but also patient-derived tumor cells, which helps to increase our understanding of anlotinib and also provides an experimental basis for establishing safer, more effective molecular-targeted therapy.
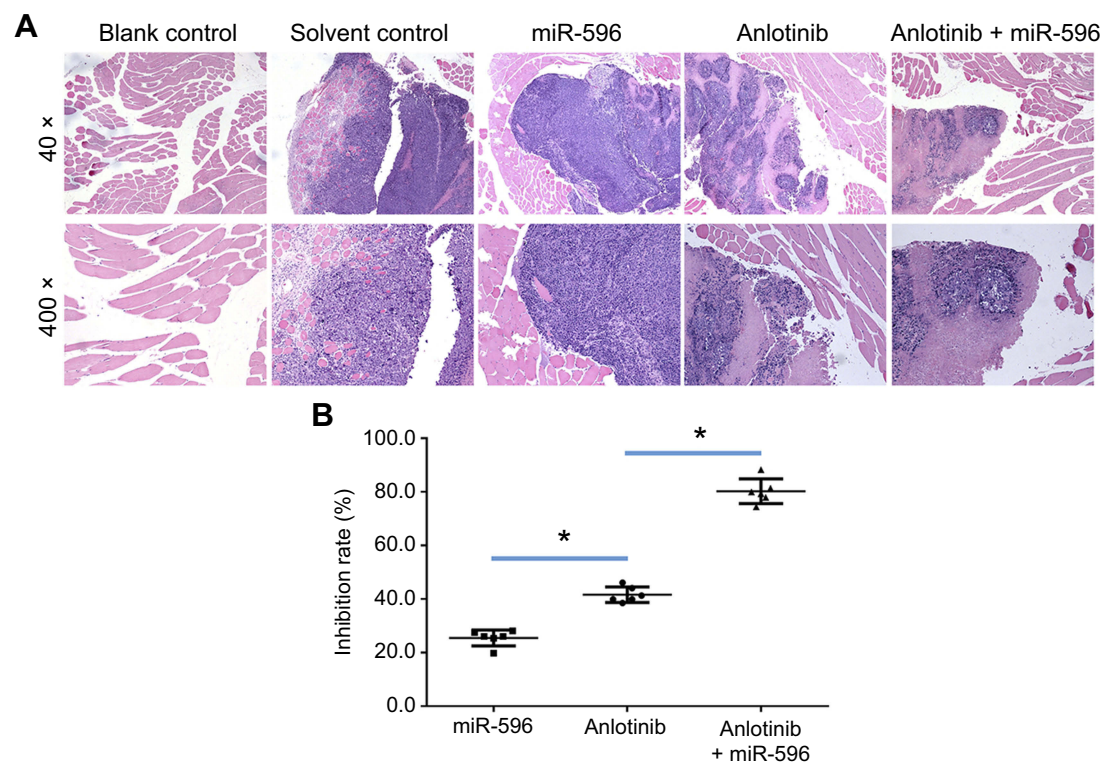
Figure 8 miR-596 enhances the antitumor effect of anlotinib on U2OS cell growth in muscle tissues of mice via ^microPET screening. U2OS cells transfected with control, miR-596, or miR-596 + Survivin^Mut were injected into muscle tissues to form tumor lesions. Four days to 5 days after intramuscular injection of U2OS cells, mice received oral administration of a 1mg/kg dose of anlotinib once per 2 days. After 10 treatments with anlotinib, mice received ^microPET screening. Results were shown as ^microPET images: (**A**) intensity of ^{18}F-FDG of ^microPET images and (**B**) radioactivation of tumor tissues to blood. (**C**) The radio-activation of tumor tissues to blood was shown as quantitative results. *$P<0.05$.
Abbreviations: miR, microRNA; PET, positron emission computed tomography; ^{18}F-FDG, ^{18}F-Fludeoxyglucose.

Survivin, also called baculoviral inhibitor of apoptosis repeat-containing (BIRC5), API4, EPR-1, or baculoviral IAP repeat-containing 5, is a protein that, in humans, is encoded by the BIRC5 gene.[44–46] Survivin is currently found to be the most potent cell survival antiapoptotic regulator.[47,48] Antitumor drugs used in molecular targeted therapy can damage and kill tumor cells, or damage and cause death due to injury factors.[49,50] Survivin can act as a major regulator of the tumor cell damage response and can play a protective role for cells.[51,52] This study found that the expression of Survivin in adjacent tissues was significantly lower than that in cancer tissues, while the trend of miR-596 was

exactly the opposite. At the same time, the use of miR-596 to downregulate the expression of Survivin can upregulate the sensitivity of OSA cells to anlotinib. The use of miR-596 to downregulate the expression of Survivin also upregulates the sensitivity of OSA cells to some cytotoxic chemotherapeutic drugs. Moreover, the BCL-xL, BAX, Casp 9, Casp 3, and p21 are the negative regulators of cancer cell proliferation or cell cycle. It is valuable to examine the expression of these factors and develop the therapeutic strategies based on these factors.[53] Some other pathways, such as Notch pathway, can function as regulating hub that modulates the expression of Survivin, CL-xL, BAX, Casp 9, Casp 3, and p21.[54–57]

Figure 9 miR-596 enhances the antitumor effect of anlotinib on U2OS cell growth in muscle tissues. U2OS cells transfected with control, miR-596, or miR-596 + Survivin[Mut] were injected into muscle tissues to form tumor lesions. Four days to 5 days after intramuscular injection of U2OS cells, mice received oral administration of 1mg/kg dose of anlotinib once per 2 days. After 10 treatments, mice were harvested, and muscle tissues were harvested for H&E staining. Results were shown as (**A**) images from H&E staining and (**B**) inhibition rates of anlotinib on intramuscular growth of U2OS cells. *$P<0.05$.
Abbreviation: miR, microRNA.

Table 5 The IC$_{50}$ values of Doxorubicin on osteosarcoma cell lines

Cell lines	Control	miR-596	miR-596 + Survivin[Mut]	miR-596 + inhibitor
	IC$_{50}$ values (µmol/L) of doxorubicin			
U2OS	0.25±0.04	0.05±0.01	0.19±0.01	0.22±0.04
PDC-1	0.40±0.10	0.06±0.02	0.30±0.05	0.25±0.03
PDC-2	0.35±0.06	0.10±0.02	0.33±0.10	0.26±0.05
PDC-3	0.41±0.12	0.21±0.05	0.36±0.08	0.35±0.02
PDC-4	0.50±0.03	0.08±0.01	0.48±0.05	0.40±0.03
PDC-5	0.15±0.00	0.01±0.00	0.10±0.01	0.12±0.01

Abbreviation: PDCs, patient-deviced cells.

Table 6 The IC$_{50}$ values of cisplatin on osteosarcoma cell lines

Cell lines	Control	miR-596	miR-596 + Survivin[Mut]	miR-596 + inhibitor
	IC$_{50}$ values (µmol/L) of cisplatin			
U2OS	0.44±0.14	0.11±0.03	0.35±0.04	0.29±0.01
PDC-1	0.60±0.09	0.15±0.01	0.50±0.10	0.46±0.16
PDC-2	0.46±0.04	0.10±0.02	0.43±0.05	0.36±0.04
PDC-3	0.38±0.10	0.10±0.03	0.35±0.07	0.56±0.15
PDC-4	0.59±0.09	0.20±0.04	0.53±0.25	0.60±0.07
PDC-5	0.36±0.04	0.35±0.02	0.33±0.24	0.28±0.07

Abbreviation: PDCs, patient-derived cells.

Table 7 The IC$_{50}$ values of methotrexate on osteosarcoma cell lines

Cell lines	Control	miR-596	miR-596 + SurvivinMut	miR-596 + inhibitor
	IC$_{50}$ values (µmol/L) methotrexate			
U2OS	0.58±0.11	0.12±0.03	0.54±0.06	0.51±0.15
PDC-1	0.45±0.06	0.09±0.02	0.35±0.13	0.40±0.04
PDC-2	0.32±0.22	0.07±0.01	0.25±0.10	0.30±0.06
PDC-3	0.31±0.02	0.16±0.05	0.40±0.20	0.56±0.15
PDC-4	0.45±0.09	0.25±0.04	0.53±0.25	0.35±0.16
PDC-5	0.32±0.08	0.08±0.01	0.25±0.06	0.22±0.05

Abbreviation: PDCs, patient-derived cells.

Therefore, it is valuable to examine the effect of Notch pathway on anlotinib.

Overexpression of miRNA via virus vectors has been considered as a promising approach to inhibit the proliferation or metastasis of cancer cells.[58–64] In the present work, miR-596 was identified in clinical specimens and was prepared as lentivirus particles. Besides miR-596, microRNA-330-5p, microRNA-218, miR-542-3p, or miR-494 could also target Survivin and inhibit the proliferation or metastasis in some other cancers, such as gastric cancer.[65–68] It is valuable to examine the expression of the miRs in clinical tissues. TCGA is a common database; however, there are no data about Survivin-OSA in TCGA. Therefore, we examined the expression level of miR-596 and Survivin in clinical specimens.

Furthermore, subcutaneous tumors are a common research model for the antitumor effects of drugs. This study established a subcutaneous tumor research model to examine the role of anlotinib. However, it is difficult to inspect the invasion and destruction of OSA cells on muscle and other tissues through simple subcutaneous tumors. For this reason, OSA cells were directly injected into the muscle tissue of nude mice for PET imaging in small animals. The growth of OSA cells in the muscle tissues was examined by the pathological analysis of tissues. The quantitative analysis of the images of pathological analysis finally determined the damage of OSA cells in muscle tissues.

Conclusion

miR-596 targets Survivin and enhances the antitumor effect of anlotinib on OSA cells.

Abbreviations

OSA, osteosarcoma; miR, microRNAs; VEGFR2, vascular endothelial growth factor receptor 2; platelet-derived growth factor receptors α/β, PDGFR α/β; discoidin domain receptor 1, DDR1; non–small-cell lung cancer (NSCLC); HCC, hepatocarcinoma; OS, survival; PFS, progression-free survival; RTK, receptor tyrosine protein kinase; MAPK, mitogen-activated protein kinase; BIRC5, baculoviral inhibitor of apoptosis repeat-containing.

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Author contributions

All authors made substantial contributions to the design and conception, acquisition, analysis, or interpretation of data. All authors took part in either drafting or revising the manuscript. All authors also gave final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials
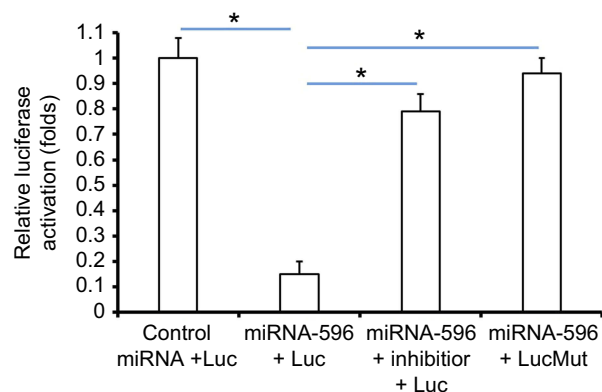
Figure S1 miR-596 inhibited the activation of luciferase reporters containing 3′UTR sequence of Survivin. The wild-type sequence or sequence with mutated miR-596-binding site of Survivin's 3′UTR (1921–2100) was obtained by chemical synthesis and cloned into pGL4.26 plasmids to construct luciferase reporters by Vigene Corporation, Jinan City, Shandong Province, China. The luciferase containing wild-type sequence of Survivin's 3′UTR with miR-596-binding site was named as Luc, whereas the luciferase containing a sequence of Survivin's 3′UTR with mutated miR-596-binding site was named as Luc^{Mut}. Cells were transfected with control miRNA + Luc, miR-596 + Luc, miR-596 + inhibitor + Luc or miR-596 + Luc^{Mut}. Then, cells were harvested for luciferase experiments. Results were shown as mean ± SD of relative luciferase activation. *$P<0.05$.

Table S1 The baseline characters of patients included

Characteristics	n (%)
Total number	74
Gender	
Male	41 (55.4)
Female	33 (44.6)
Tumor size (cm)	
> 7	49 (60.8)
≤ 7	25 (39.2)
TNM stage	
I	32 (43.2)
II	25 (33.7)
III	17 (51.6)
Location	
Distal femur	31 (41.9)
Proximal femur	7 (9.4)
Proximal humerus	13 (17.5)
Proximal tibia	20 (27.0)
Others	3 (4.0)
Relapse	
Yes	11 (14.9)
No	63 (85.1)
Metastasis location	
Lung	21 (28.3)
Others	4 (5.4)
None	49 (66.2)