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RESEARCH PAPER

Circular RNA 0001785 regulates the pathogenesis of osteosarcoma as a ceRNA by sponging miR-1200 to upregulate HOXB2

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ABSTRACT

Circular RNAs (circRNAs) are recently emerged to be promising therapeutic targets of tumors. Osteosarcoma is the most prevalent primary bone tumor and the third most prevalent cancer in children and adolescents. This study firstly analyzed circRNA microarray of osteosarcoma and selected circ-0001785 as the study object. We aimed to comprehensively investigate the expression pattern and biological function of circ-0001785 in the progression of osteosarcoma. Relative levels of circ-0001785 and miR-1200 in the normal human osteoblast cell line and osteosarcoma cell lines were determined. Bioinformatics analyses predicted the binding relationship between miR-1200 to HOXB2 and circ-0001785, while dual-luciferase reporter gene assay further verified this relationship. Flow cytometry and EdU assay were used for evaluating the regulatory effects of circ-0001785/miR-1200/HOXB2 axis on osteosarcoma cells. Consistent with the microarray analysis, circ-0001785 was highly expressed in osteosarcoma cell lines. Knockdown of circ-0001785 attenuated proliferative ability, but induced the apoptosis of osteosarcoma cells. Furthermore, we confirmed that circ-0001785 competitively bound to miR-1200, thus up-regulating its target gene HOXB2. Western blot analyses revealed that circ-0001785 regulated the PI3K/Akt signaling and Bcl-2 family pathway in osteosarcoma. In conclusion, circ-0001785 regulates the pathogenesis of osteosarcoma by sponging miR-1200 to up-regulate HOXB2 expression.

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Osteosarcoma; circRNA; competing endogenous RNAs (ceRNA); proliferation; apoptosis

Introduction

Osteosarcoma is malignant and mainly affects the long bones as well as other human bones [1]. With bimodal distribution, its peaks locate in twenties and late adulthood [2]. Osteosarcomas account for 3%-6% and about 1% cancers in childhood and in adults, respectively [3]. Risk factors for osteosarcoma include medical history of chemotherapy or radiation, Paget disease or other benign bone lesions, and genetic conditions, including Bloom and Werner syndromes, Li-Fraumeni syndrome, hereditary retinoblastoma and Rothmund–Thomson syndrome [4,5]. However, the overall pathogenesis of osteosarcoma remains unclear, with high mortality and poor prognosis [6]. Therefore, it is of great clinical significance to develop effective targets for early diagnosis and treatment of osteosarcoma.

CircRNAs are involved in the pathogenesis of tumors as a hot topic in recent clinical researches. CircRNA forms a covalently closed loop-structure by specific splicing and is identified as the major subtype of gene transcription, which differs from the linear RNA terminated by 5′ hat and 3′ poly (A) [7]. Stably expressed circRNAs almost present in all types of species [8] and are not affected by RNA exonuclease [9]. Functional circRNAs have been shown to serve as RNA-binding protein sequestering agents and cytoplasmic microRNA sponges as well as nuclear transcriptional regulators, indicating the correlation of circular RNAs in the regulatory networks governing gene expression as participants [10,11]. Through the ceRNA network, circRNA is related to the development of many diseases, such as cardiovascular disease [11], breast cancer [12], bladder carcinoma [13], and coronary artery disease [14].

This study verified the highly expressed circ-0001785 in osteosarcoma cell lines. As a ceRNA, circ-0001785 regulated the pathogenesis of osteosarcoma by sponging miR-1200, thus up-regulating HOXB2 and further mediating cellular behaviors of osteosarcoma cells. We believed that circ-0001785

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may be utilized as a novel hallmark for early diagnosis and treatment of osteosarcoma.

Results

Circ-0001785 was highly expressed in osteosarcoma cells

Through analyzing the GSE96964 microarray, circ-0001785 was found to be highly expressed in osteosarcoma cell lines. We identified differentially expressed circRNAs based on the criteria of fold change > 2 and P value <0.05. The differentially expressed circRNAs including circ-0001785 were identified in osteosarcoma cell lines (Figure 1(a)). Subsequently, our results identically confirmed the upregulation of circ-0001785 in osteosarcoma cells relative to osteoblast cells (Figure 1(b,c)). In particular, HOS and U2OS cells selected for subsequent experiments had the highest and lowest expression

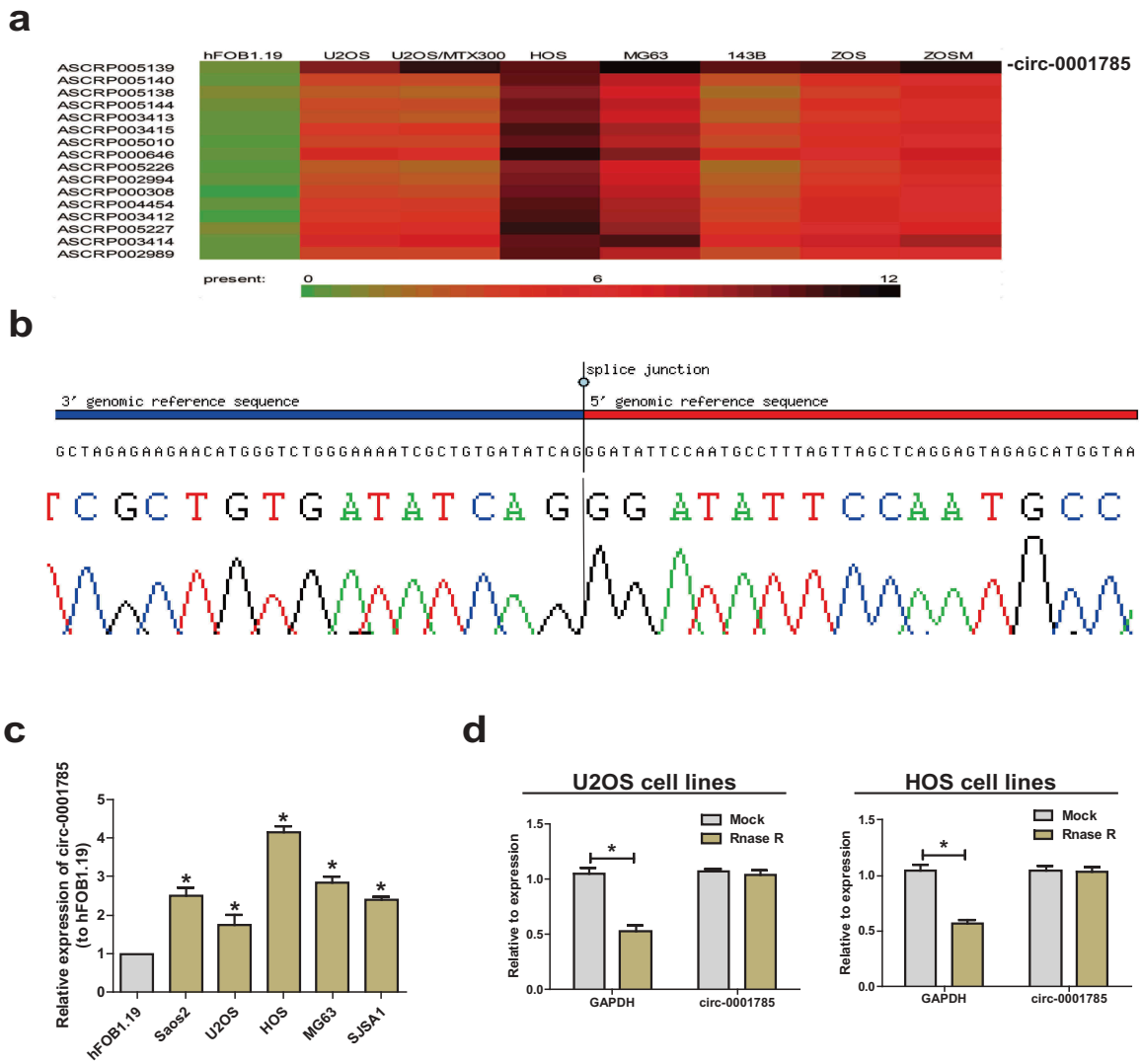

Figure 1. Characteristics and expression of circ-0001785 in osteosarcoma cells.

Microarray for analysis was GSE96964 from platform GPL19978 containing t even human osteosarcoma cell lines (U2OS, U2OS/MTX300, HOS, MG63, 143B, ZOS, ZOSM) and the human osteoblast hFOB1.19. Differentially expressed circRNAs were identified based on the criteria of fold change more than 2 and P value less than 0.05. (a) Heatmap showed differentially expressed circRNAs with most significance between osteosarcoma cell line and hFOB1.19 cell line. Circ-0001785 expression was up-regulated in osteosarcoma cells. (b) Sequence of circ-0001785 in circBase (upper panel) consistent with that in Sanger sequencing (lower panel). (c) Expression level of circ-0001785 in osteosarcoma cell lines (Saos2, MG63, HOS, U2OS, SJSA1) and the normal human osteoblast cell line (hFOB1.19) *via* qRT-PCR. (d) Resistance of Circ-0001785 in osteosarcoma cells to RNase R digestion. *P< 0.05, results shown as mean ± SD.

of circ-0001785. Due to its resistance to RNase R digestion, we confirmed the circRNA characteristics of circ-0001785 (Figure 1(d)).

Down-regulation of circ-0001785 inhibited proliferation and promoted apoptosis in osteosarcoma cells

To investigate the biological effect of circ-0001785 in osteosarcoma cells, siRNA circ-0001785 and circ-0001785 overexpression plasmids were used for the transfection of HOS and U2OS cells. QRT-PCR first verified its transfection efficacy (Figure 2(a)). EdU assay revealed that circ-0001785 knockdown markedly suppressed the proliferative rate of osteosarcoma cells than controls (Figure 2(b)). Flow cytometry indicated that silence of circ-0001785 mildlyinduced the apoptosis of osteosarcoma cells and the result is statistically significant (Figure 2(c)). Protein levels of apoptotic-related genes were determined. The results showed that the activity of cleaved caspase-9 was upregulated by the transfection with siRNA circ-0001785, indicating the induced apoptosis (Figure 2 (d,e)).

Circ-0001785 promotes osteosarcoma cell proliferation in vivo

To explore the role of circ-0001785 in osteosarcoma tumor growth in vivo, U2OS cells transfected with negative control or si-circ-0001785 were subcutaneously injected into nude mice. Our results showed that down-regulation of circ-0001785 decreased the tumor volume (Figure 3(a)) and tumor weight (Figure 3(b)) after the four-week intratumorally injection. Furthermore, immunohistochemistry demonstrated that mice treated with si-circ-0001785 appeared to have lower level of PCNA, the proliferation-specific gene (Figure 3(c)). Interestingly, after removing the lung tissue of nude mice, we found that the

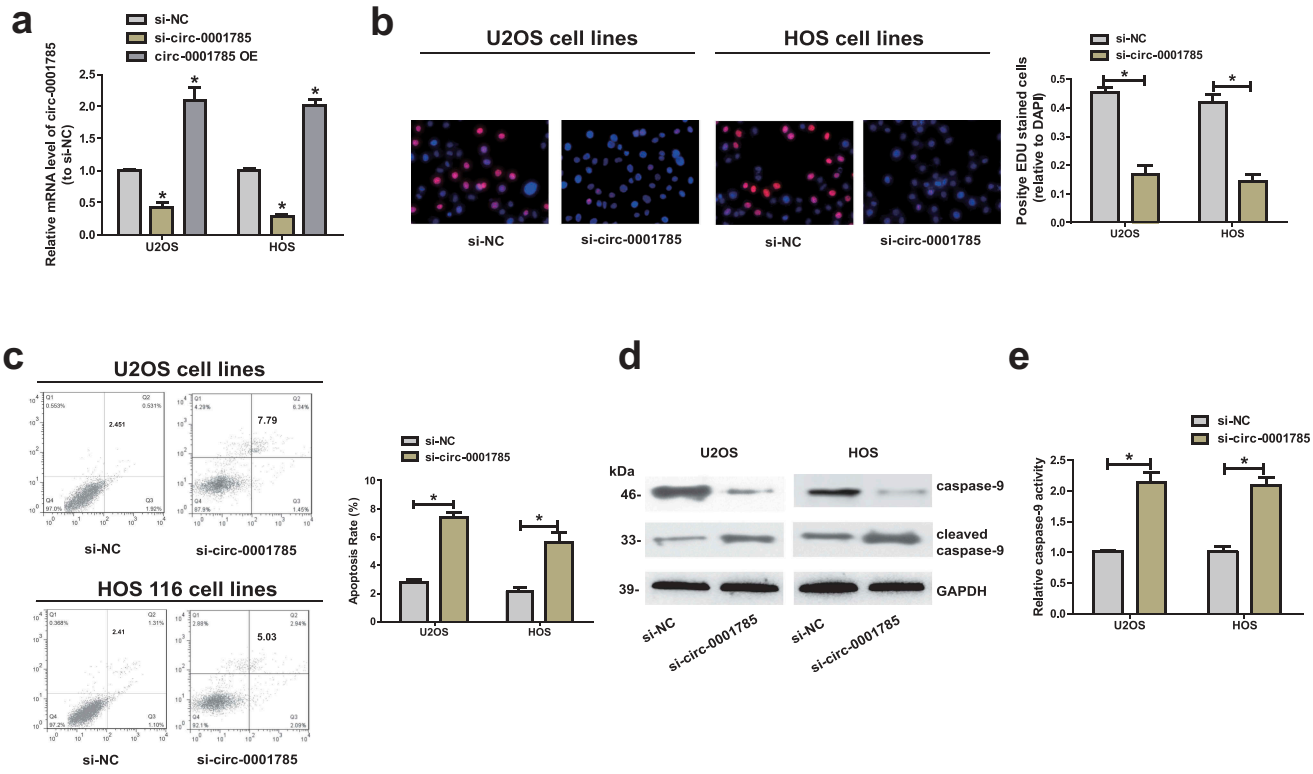

Figure 2. Circ-0001785 promoted proliferation and inhibited apoptosis of osteosarcoma cells.

(a) Expression level of circ-0001785 in osteosarcoma cells transfected with circ-0001785 mimics, siRNA circ-0001785 or negative control (si-NC) detected by qRT-PCR. (b) EdU assay for determining the proliferation of osteosarcoma cells after transfection with siRNA circ-0001785 or si-NC. (c) Flow cytometry for determining the apoptosis of osteosarcoma cells after transfection with siRNA circ-0001785 or si-NC. (d, e) Caspase-9 activity in osteosarcoma cells after transfection with siRNA circ-0001785 or si-NC. *P< 0.05, results shown as mean ± SD.

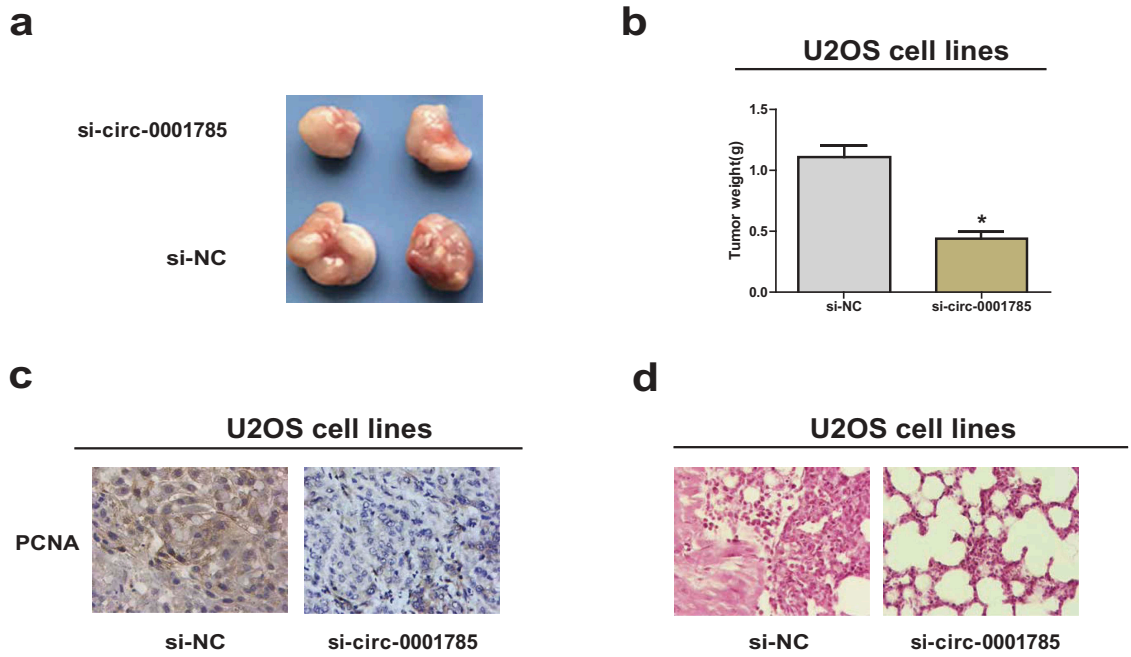

Figure 3. Circ-0001785 promotes osteosarcoma cell proliferation in vivo.
(a) Representative images of xenografts tumor in nude mice. (b) Tumor weight was monitored. (c) Representative images of IHC stained PCNA is shown. (d) Representative images of HE stained Lung tissues are shown.

destruction of lung tissue was more pronounced in control group compared to the si-CIRC-0001785 group (Figure 3(d)).

Circ-0001785 regulated Bcl-2 family and PI3k/Akt/mTOR pathway

Since Bcl-2 family and PI3K/Akt/mTOR pathway were vital apoptotic-regulators [15–17], we evaluated the regulatory influence of circ-0001785 on them. Silence of circ-0001785 showed marked downregulation effect on anti-apoptotic genes in Bcl-2 family (Bcl-W, A1 and Bcl-2) and converse upregulation effect on pro-apoptotic gene Bad (Figure 4(a)). Meanwhile, p-PI3K, p-mTOR and p-Akt were downregulated by the transfection with siRNA circ-0001785 in osteosarcoma cells whereas total levels of PI3K, Akt and mTOR remained unchanged (Figure 4(b)). These results indicated that circ-0001785 may affect the apoptosis of osteosarcoma cells *via* inhibiting PI3K/Akt/mTOR pathway and regulating Bcl-2 family.

Circ-0001785 directly bound to miR-1200

CircRNAs functioned as competing endogenous RNAs for sponging corresponding miRNAs, thereafter regulating their biological functions [18,19]. Here, bioinformatics analysis was used to predict potential binding sites between miR-1200 and circ-0001785, while dual-luciferase reporter gene assay was applied for further verification. The data showed lower luciferase intensity in HOS and U2OS cells cotransfected with wild-type circ-0001785 and miR-1200 mimics. However, we did not observe an obvious change in luciferase intensity after co-transfection with miR-1200 mimics and mutant-type circ-0001785 (Figure 5(a,b)). A negative correlation was identified between circ-0001785 and miR-1200 as overexpressed circ-0001785 downregulated miR-1200, whereas circ-0001785 knockdown upregulated miR-1200 (Figure 5(c)). Moreover, miR-1200 was lowly expressed in osteosarcoma cells (Figure 5(d)). Taken together, these data indicated that miR-1200 could directly bind to circ-0001785 in osteosarcoma cells.

Circ-0001785 regulated HOXB2 expression *via* inhibiting miR-1200

To date, the biological function of miR-1200 is rarely reported. We first predicted the potential downstream of miR-1200 on miRDB and Targetscan. HOXB2 was screened as a potential target (Figure 6(a)). Dual-luciferase reporter gene assay further revealed

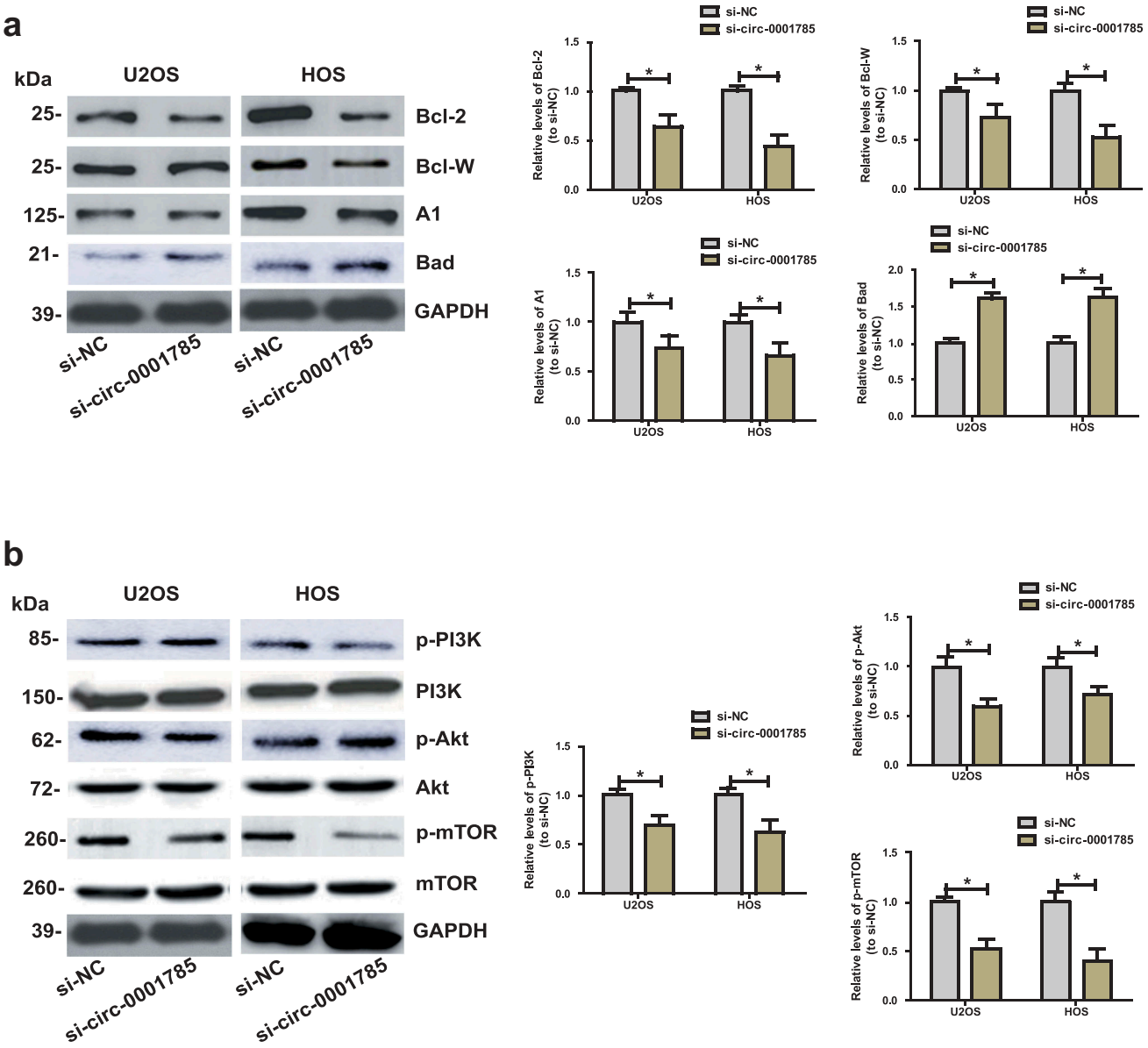

Figure 4. Circ-0001785 regulated Bcl-2 family and PI3K/Akt/mTOR pathway.

(a) Western blot analyses of Bcl-W, Bcl-2, A1 and Bad in osteosarcoma cells transfected with siRNA circ-0001785 or si-NC. (b) Western blot analyses of p-PI3K, PI3K, p-Akt, Akt, p-mTOR, and mTOR in osteosarcoma cells transfected with siRNA circ-0001785 or si-NC. *P< 0.05, results shown as mean ± SD.

a reduction in luciferase intensity after co-transfection with miR-1200 mimics and wild-type HOXB2. In comparison, luciferase intensity remained unchanged in those co-transfected with mutant-type HOXB2 and miR-1200 mimics (Figure 6(b)). Next, both mRNA and protein level of HOXB2 were determined in osteosarcoma cells transfected with miR-1200 mimics, NC, si-circ-0001785 or circ-0001785 overexpression plasmid, respectively. HOXB2 was markedly down-regulated by circ-0001785 knockdown or miR-1200 overexpression. Conversely, HOXB2 expression was up-regulated after circ-0001785 overexpression (Figure 6(c,d)). QRT-PCR data indicated marked up-regulation of HOXB2 expression in osteosarcoma cells relative to hFOB1.19 cells (Figure 6(e)). Taken together, these findings demonstrated that circ-0001785 regulated HOXB2 expression *via* inhibiting miR-1200 in osteosarcoma cells.

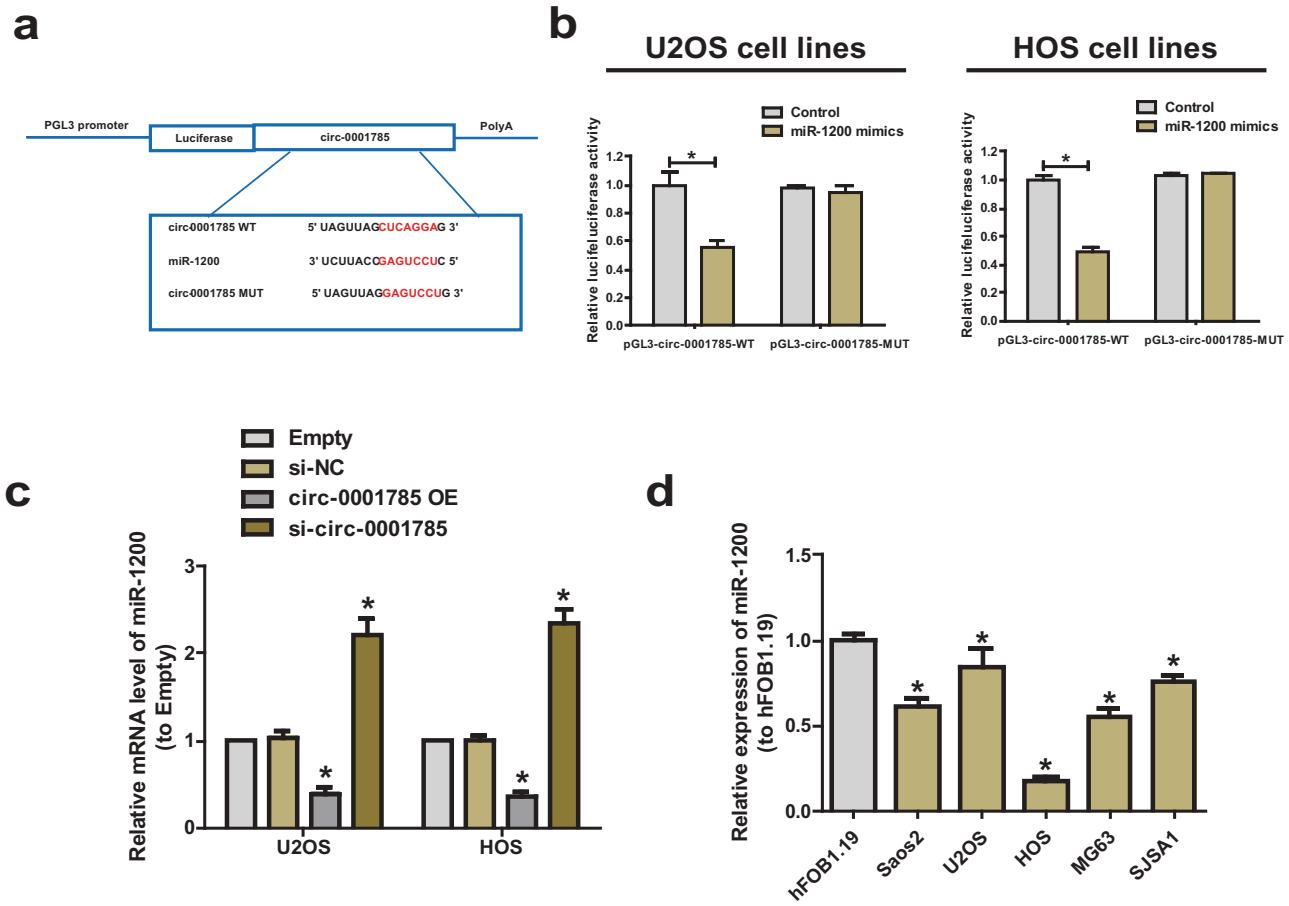

Figure 5. Circ-0001785 directly bound to miR-1200 in osteosarcoma cells.

(a) Potential binding sites between circ-0001785 and miR-1200. (b) Dual-luciferase reporter gene assay in osteosarcoma cells co-transfected with wild-type/mutant-type circ-0001785 and miR-1200 mimics/negative control. (c) MiR-1200 level in osteosarcoma cells transfected with si-NC, siRNA circ-0001785 or circ-0001785 overexpression plasmid *via* qRT-PCR. (d) Expression level of miR-1200 in osteosarcoma cell lines (MG63, Saos2, U2OS, HOS, SJSA1) and the normal human osteoblast cell line (hFOB1.19) *via* qRT-PCR. *$P< 0.05$, results shown as mean ± SD.

Knockdown of HOXB2 induced apoptosis in osteosarcoma cells

The biological effect of HOXB2 in osteosarcoma cells was specifically analyzed. Firstly, miR-1200 expression was sufficiently down-regulated in HOS and U2OS cells by transfection with miR-1200 inhibitor. Conversely, miR-1200 expression was up-regulated after transfection with circ-0001785 mimics (Figure 7(a)). Then transfection with siRNA HOXB2 sufficiently downregulated HOXB2 expression in HOS and U2OS cells (Figure 7(b)). The same results were obtained by Western blotting (Figure 7(c)). Interestingly, we observed a similar influence of HOXB2 on osteosarcoma cell apoptosis as circ-0001785. Silence of HOXB2 remarkably induced apoptosis, miR-1200 could reverse the regulatory effect of circ-0001785 on osteosarcoma cell apoptosis (Figure 7(d)). Furthermore, the determination of caspase-9 activity yielded similar trends as flow cytometry indicated (Figure 7(e,f)). To sum up, these findings suggested that circ-0001785 exerted an oncogenic role in osteosarcoma *via* the circ-0001785/miR-1200/HOXB2 axis.

Discussion

As the most common primary bone malignancy in pediatric population [20], the pathogenesis of osteosarcoma has been extensively studied in recent years

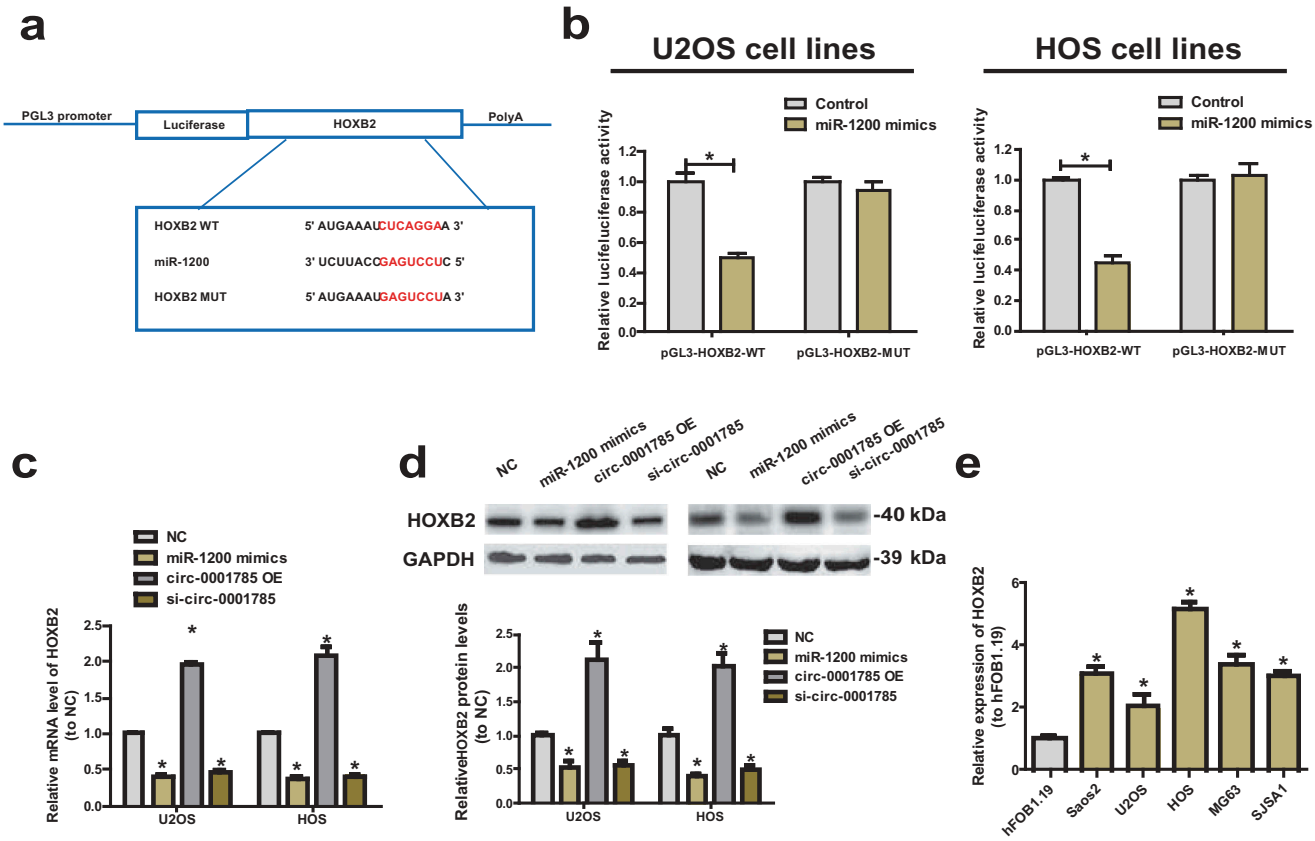

Figure 6. Circ-0001785 upregulated HOXB2 *via* sponging miR-1200 in osteosarcoma cells.

(a) Potential binding sites between HOXB2 and miR-1200. (b) Dual-luciferase reporter gene assay in osteosarcoma cells co-transfected with wild-type/mutant-type HOXB2 and miR-1200 mimics/negative control. (c, d) mRNA and protein level of HOXB2 in osteosarcoma cells transfected with miR-1200 mimics, NC, siRNA circ-0001785 or pcDNA circ-0001785. (e) Expression level of HOXB2 in osteosarcoma cell lines (HOS, Saos2, MG63, SJSA1, U2OS) and the normal human osteoblast cell line (hFOB1.19) *via* qRT-PCR. *P< 0.05, results shown as mean ± SD.

[21,22]. With the in-depth researches on DNA, epigenetic regulation of disease progression has been well concerned. Several circRNAs participating in the pathogenesis of osteosarcoma have been observed, which may be used as diagnostic and therapeutic targets for osteosarcoma, including hsa_circ_0001564 [23], hsa-circ-0016347 [24], Hsa_circ_0009910 [25]. In this paper, circ-0001785 was highly expressed in osteosarcoma cells. Knockdown of circ-0001785 markedly suppressed proliferative rate, but induced the apoptosis of osteosarcoma cells. Our results demonstrated the vital function of circ-0001785 in the progression of osteosarcoma.

MiRNAs are small non-coding RNAs (ncRNAs) capable of regulating gene expressions [26]. MiRNAs involve in cell cycle development, cell differentiation, and regulation [27], and are also closely related to many diseases, especially tumors [28,29]. In present study, bioinformatics analysis was used to first predict complementary sequences of target miRNA to circ-0001785 while dual-luciferase reporter gene assay was used for its further verification. qRT-PCR was performed for verifying the low expression of miR-1200 in osteosarcoma cells. With the same detection approach, HOXB2 was predicted as a direct target of miR-1200. A series of functional experiments suggested that circ-0001785 exerted an oncogenic function by sponging miR-1200 to upregulate HOXB2 in osteosarcoma.

HOXB2 is one of the homeobox master development-controlling genes regulating morphogenesis and cell differentiation [30]. Multiple HOXB2-related diseases have been found, such as lung cancer [31], cervical cancer [32], pancreatic cancer [33]. This study verified that downregulated HOXB2 activated caspase-9 and ultimately enhanced apoptotic rate in osteosarcoma cells.

In conclusion, we confirmed the upregulated circ-0001785 in osteosarcoma cells, which exerted an oncogenic role by sponging miR-1200 to

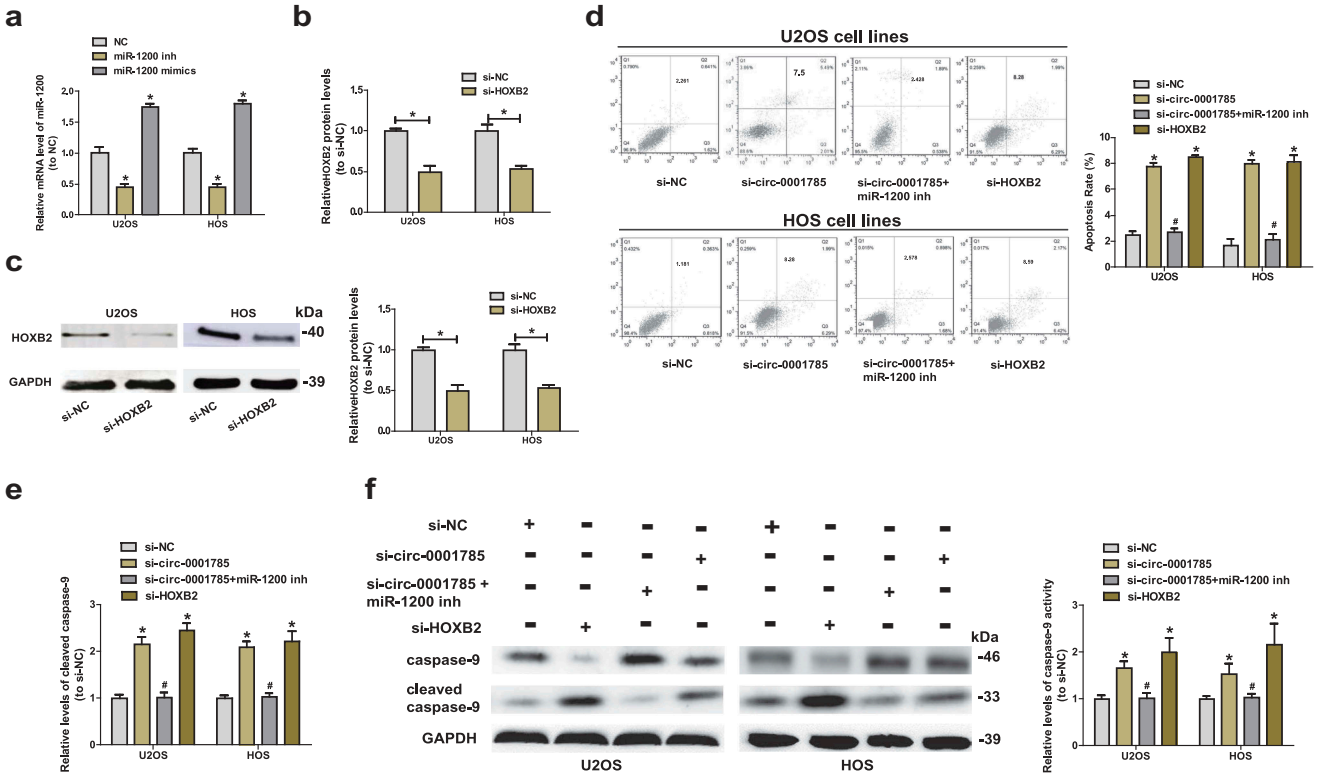

Figure 7. Knockdown of HOXB2 induced apoptosis in osteosarcoma cells.

(a) miR-1200 level in osteosarcoma cells after transfection with siRNA miR-1200, miR-1200 mimics. (b) mRNA level of HOXB2 in osteosarcoma cells transfected with siRNA HOXB2 *via* qRT-PCR. (c) Western blot of HOXB2 in osteosarcoma cells transfected with siRNA HOXB2. (D) Apoptosis in transfected osteosarcoma cells *via* flow cytometry. (e, f) Caspase-9 activity in transfected osteosarcoma cells. *$P < 0.05$ versus control group, $^{\#}$ $P < 0.05$ versus si-circ-0001785 group, results shown as mean ± SD.

upregulate HOXB2 as a ceRNA. We believed that circ-0001785 may be utilized as a therapeutic target for osteosarcoma.

Materials and methods

Cell culture and transfection

The normal human osteoblast cell line (hFOB1.19) and osteosarcoma cell lines (Saos2, U2OS, SJSA1, HOS, MG63) were provided by Shanghai Cell Bank of Chinese Academy of Sciences. DMEM (Gibco BRL, Grand Island, NY, USA) containing 10% FBS (Gibco, Carlsbad, CA) was used for cell culture, with cells maintained at 37°C in a 5% CO_2 incubator.

For transfections, cells at the confluence of 60–80% were transfected with Lipofactamine 2000 for 24 h (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The circ-0001785 overexpressing plasmids, siRNAs against circ-0001785, miR-1200 mimics, and miR-637 inhibitors were all synthesized from GenePharma (Shanghai, China). Detailed sequences were depicted in Table 1.

RNA isolation and quantitative PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied for extracting total RNAs. The abundance of transcript was determined using SYBR PremixEx Taq (Vazyme, Shanghai, China). GenePharma (Shanghai, China) provided the primer construction. All the PCR primers were listed in Table 1.

Rnase r digestion

Five micrograms of total RNAs were cultured with 3U/μg RNase R (Epicentre Biotechnologies, Shanghai, China) at 37°C for 15 min. RNase R digestion was performed in duplicate as previously described.

Table 1. Sequences of primers for qRT-PCR and siRNA related sequence.

Name		Sequence
circ-0001785	Forward	5′- AAGAACATGGGTCTGGGAAA –3′
	Reverse	5′- CCGAGGTCTTTCATTCTTGC –3′
HOXB2	Forward	5′- CGCCAGGATTCACCTTTCCTT –3′
	Reverse	5′- CCCTGTAGGCTAGGGGAGAG –3′
GAPDH	Forward	5′- AGAAGGCTGGGGCTCATTTG –3′
	Reverse	5′- AGGGGCCATCCACAGTCTTC –3′
U6	Forward	5′- CTCGCTTCGGCAGCACA –3′
	Reverse	5′- AACGCTTCACGAATTTGCGT –3′
miR-1200	Forward	5′-ACACTCCAGCTGGGCTCCTGAGCCATTCTG-3′
	Reverse	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAGGCTCA-3′
circ-0001785 siRNA	Sense	5′- TCGCCGGGGAGTCAGGCCCTT –3′
	Antisense	5′- GGGCCTGACTCCCCGGCGATT –3′
miR-1200 mimics	Sense	5′- CTCCTGAGCCATTCTGAGCCTC –3′
	Antisense	5′- CTCCGAGTCTTACCGAGTCCTT –3′
miR-1200 inhibitor	Sense	5′- CTCCGAGTCTTACCGAGTCCTC –3′

Ethynyldeoxyuridine (edu) analysis

For EdU assay, the transfected osteosarcoma cells were treated with 50 μM of EdU (KeyGen Biotech, Nanjing, China) for 2 h at 37°C and then the cultured cells were fixed with 4% paraformaldehyde for 30 min and stained with 1× Apollo reaction cocktail for 30 min before being incubated with 100 μl of Hoechst33342 at 5 μg/ml for 30 min. Nuclei staining was performed by adding DAPI. The percentage of EdU-positive cells was examined using a fluorescent microscope.

Western blot

Proteins were harvested from cell lysis, quantified, and loaded in 12% SDS-PAGE with 50μg protein sample/lane. Subsequently, proteins were transferred onto a PVDF membrane, incubated using primary antibodies at 4°C overnight and then secondary antibodies for 2 h. Finally, membranes were subjected to band exposure using enhanced chemiluminescence system (Thermo Fisher Scientific, USA) and analyzed by Quantity One software (Bio-Rad Laboratories, San Diego, CA, USA). Abcam (Shanghai, China, China) provided the primary antibodies of cleaved caspase-9 (ab2324), caspase-9 (ab219590), Bcl-W (ab38629), Bcl-2 (ab185002), Bad (ab90435), A1 (ab75887), phospho-PI3K (ab138364), Akt (ab179463), phospho-Akt (ab38449), PI3K (ab32089), GAPDH (ab37168), mTOR (ab2732), HOXB2 (ab136856) and phospho-mTOR (ab109268), as well as secondary antibodies.

Caspase-9 activity assay

Cell lysis was incubated with the colorimetric substrates (Ac-DEVD-AMC) in dark at 37°C for 1 h and then subject to absorbance measurement using a microplate reader at the wavelength of 405 nm. Caspase-9 activation kit (R&D Systems, Minneapolis, MN, USA) was applied here.

Apoptosis assay

For the cell apoptosis, annexin-V mixed with PI (KeyGEN, Nanjing, China) was used to stain the treated cells. All assays were conducted and analyzed with a flow cytometer (FACScan; BD Biosciences, USA) equipped with Cell Quest software (BD Biosciences).

Dual-luciferase reporter gene assay

Wild plasmids circ-0001785-WT and HOXB2-WT containing binding sites for miR-637 were integrated into the pGL3 promoter vector. Then, mutant plasmids circ-0001785-MUT and HOXB2-MUT containing reverse sequence of the binding sites for miR-637 were integrated into the pGL3 promoter vector (GenePharma, Shanghai, China). Cell co-transfection was performed by miR-1200 mimics/negative control and wild-type/mutant-type sequences for 48 h. Dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used for finally determining the luciferase intensity.

Animal experiments

The Animal experiments were approved by the Ethics Committee of China Medical University. BALB/c thymic free nude mice (female, 4-6 weeks old) were purchased from Animal center of Shanghai Jiaotong University. The flanks of mice were subcutaneously injected with U2OS cells. Mice were sacrificed 4 weeks after cell injection and tumors were resected for analysis.

Statistical analysis

SPSS 22.0 software was used for data analyses. Data were reported as mean ± SD. Unpaired two-sided *t*-test was performed for analyzing the between-group differences. $P < 0.05$ was considered statistically significant.

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Conflicts of interest

None.

Disclosure statement

No potential conflict of interest was reported by the authors.

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