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Downregulation of long non-coding RNA DBH-AS1 inhibits osteosarcoma progression by PI3K-AKT signaling pathways and indicates good prognosis

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Abstract. – OBJECTIVE: Long non-coding RNA DBH-AS1 (DBH-AS1) has emerged as a novel regulator in cancer initiation and progression of several tumors. However, the expression of DBH-AS1 in osteosarcoma and its effect on the tumorigenesis of osteosarcoma are unclear. The purpose of this study was to determine the role of DBH-AS1 in osteosarcoma progression.

PATIENTS AND METHODS: The expression level of DBH-AS1 in 119 pairs of osteosarcoma tissues and five cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The association of DBH-AS1 expression with clinicopathological factors and prognosis was also analyzed. Cell proliferation was measured by Cell Counting Kit-8 (CCK-8), EdU and cell colony formation assays and apoptosis in MG63 and U2OS cells was examined by flow cytometry. Following that, transwell invasion and wound-healing assays were used to explore cell migration and invasion, respectively. The expression of the PI3K/Akt pathway-related proteins was examined by Western blot analysis.

RESULTS: We observed that DBH-AS1 was distinctly overexpressed in osteosarcoma tissue and cells, and associated with lymph node status and metastasis status. Osteosarcoma patients with a higher DBH-AS1 expression showed significantly poorer overall survival than those with lower DBH-AS1 expression. Multivariate analysis demonstrated that high DBH-AS1 expression was an independent poor prognostic factor for osteosarcoma patients. Functional assays revealed that knockdown of DBH-AS1 inhibited cell proliferation, migration and invasion, while promoted apoptosis in osteosarcoma. Moreover, suppression of DBH-AS1 could inhibit the activation of the PI3K/Akt pathway, which was demonstrated by examining the expression levels of p-PI3K and p-Akt.

CONCLUSIONS: Our data first reported that DBH-AS1 may act as an oncogenic lncRNA by modulating the PI3K/Akt pathway in osteosarcoma, which may serve as a candidate prognostic biomarker and target for new therapies in osteosarcoma.

Key Words
LncRNA DBH-AS1, Osteosarcoma, Biomarker, PI3K/Akt pathway, Metastasis.

Introduction

Osteosarcoma, which arises from primitive mesenchymal bone-forming cells, is a high-grade malignant bone tumor that frequently occurs in children and adolescents, which has a particular characteristic for frequent pulmonary metastasis and tissues invasion[1,2]. The common presentation includes the onset of pain and swelling in the affected bone, which wakes the patient from sleep, of patients under insalubrity conditions[3]. Up to date, with the development of multiagent neoadjuvant chemotherapy and surgery, the patients with osteosarcoma have a 40% increase in five-year overall survival[4,5]. However, the survival rate is poor for subjects with metastatic osteosarcoma and about 30% of all osteosarcoma patients showed recurrence or metastasis[6,7]. Thus, it is vital to understand the molecular mechanisms involved in osteosarcoma metastasis and identify novel biomarkers and effective therapeutic strategies for osteosarcoma. Long non-coding RNAs (lncRNAs), which were first described by Brockdorff et al[8] in 1992, are a new class of non-coding RNAs with lengths ranging from 200 bp to 100 kbp[9,10]. They were once regarded as transcriptional "noise" due to their lack of an open read-

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ing frame of significant length and the capability of coding proteins[11]. Emerging evidence showed that several functional lncRNAs display critical roles in a diverse range of cellular functions such as autophagy, differentiation development, adhesion and cells cycles[12,13]. Recently, some functional lncRNAs which were frequently abnormally expressed in patients was shown to contribute to various human diseases including cancers via the modulation of genes levels by chromatin remodeling, DNA or histone protein modification[14,15]. More and more attention focused on the dysregulated expression and critical regulatory functions of lncRNAs in tumors, which suggested that lncRNAs may be used as potential biomarkers and therapeutic targets[16-18]. Although a large number of lncRNAs have been functionally characterized, many lncRNAs remain to be elucidated. Long non-coding RNA DBH-AS1 (DBH-AS1), transcribed from chromosome 9q34, was a newly identified lncRNA. Previously, it was found that the expression levels of DBH-AS1 were distinctly increased in HepG2 cells which were analyzed using microarray analysis[19]. Then, the overexpression of DBH-AS1 was also reported in colorectal cancer[20]. On the other hand, the oncogenic roles of DBH-AS1 were confirmed in hepatocellular carcinoma using gain-function and lost-function assays[21,22]. Up to date, to our best knowledge, the expression profiles of DBH-AS1 in osteosarcoma remain largely unclear, and the potential effects of DBH-AS1 in this tumor have not been investigated.

Patients and Methods

Patients and Tissue Specimens

A total of 119 patients diagnosed as osteosarcoma were enrolled in this study and the paired tumor tissues, as well as adjacent normal tissue samples, were collected from the Songshan Hospital of Qingdao University Medical College between May 2008 and August 2011. The pathology Department of the Songshan Hospital of Qingdao University Medical College had performed the histologic diagnoses. After surgical resection, the tissue specimens were immediately frozen using liquid nitrogen and stored at -80°C. None of the experimental subjects received any anti-tumor treatment prior to operation. Written informed consent was obtained from all the subjects and this study was approved by the Ethics Committee of Songshan Hospital of Qingdao University Medical College. The patients' clinical information was listed in Table I.

Cell Lines and Cell Transfection

Four osteosarcoma cell lines: U2OS, Saos-2, HOS and MG63 were obtained from BeNa Culture Collection (Chaoyang, Beijing, China). The osteoblast hFOB1.19 cell line was obtained from TongPai Technology Co., Ltd. (Fengxian, Shanghai, China). The cells were cultured using Roswell Park Memorial Institute-1640 medium (RPMI-1640; Cyagen, Guangzhou, Guangdong, China) supplemented with antibiotics (1%) and 10% of fetal bovine serum (FBS; Gibco, Grand Island, NY,

Table I. Clinical correlation between DBH-AS1 expression and other clinicopathological features in osteosarcoma.

Clinicopathological features	Total no. of patients	DBH-AS1 expression		*p*-value
		High	Low	
Age (years)				0.648
≤60	58	28	30	
>60	61	32	29	
Sex				0.652
Men	67	35	32	
Women	52	25	27	
Tumor size				0.215
≤5 cm	72	33	39	
>5 cm	47	27	20	
Lymph node status				0.011
Negative	84	36	48	
Positive	35	24	11	
Metastasis status				0.017
Negative	85	37	48	
Positive	34	23	11	

USA). The cells were maintained under a condition of 5% CO_2 at 37°C. For cell transfection, we employed a Torpedo siRNA transfection reagent (Kosters, Guangzhou, Guangdong, China). After the cells grown to 60-70% cell confluence, 10 μl of siRNA transfection reagent was mixed with 0.5 μg indicated small interfering RNA (siRNA). After incubation for 20 min, the mixture was added into each well. The siRNAs against DBH-AS1 (siRNA-1 and siRNA-2), as well as negative control siRNAs (si-NC), were synthesized from IBS Biotechnology Co., Ltd. (Songjiang, Shanghai, China).

Reverse Transcription Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNAs from relevant tissue samples or cells were isolated according to the standard TRIzol reagent (Invitrogen, Carlsbad, CA, USA) extraction protocols. The cDNA synthesis was carried out using a cDNA synthesis master mix kit (Simgen, Hangzhou, Zhejiang, China). Then, the qPCR was conducted using a qPCR Premix (SYBR Green) kit on a Bio-Rad CFX96 Real Time-Polymerase Chain Reaction apparatus (RT-PCR; Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The primers for DBH-AS1 and GAPDH were listed in Table II. The qPCR reaction conditions were: 94°C for 25 s, followed by 38 cycles of 94°C for 5 s, 60°C for 30 s, and 72°C for 30 s. The relative expression of DBH-AS1 was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Cells were washed using ice-cold Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and radioimmunoprecipitation assay lysis buffer (RIPA; Beyotime, Shanghai, China) was added into the cells. Then, a Bio-Rad BCA assay kit (Bio-Rad, Hercules, CA, USA) was applied to measure the protein concentration. Subsequently, 5×SDS loading buffer was mixed with the proteins (30 μg per lane) and the mixture was added into each well of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (10%). The proteins were separated and subsequently transferred to polyvinylidene difluoride membranes (PVDF). The membranes were then incubated for 1 h with 5% bovine serum albumin (BSA) at room temperature, followed by being probed with primary antibodies, which were all purchased from Cell Signaling Technology (Danvers, MA, USA), against phosphorylated-PI3K (p-PI3K), phosphorylated-AKT (p-AKT), PI3K, AKT and GAPDH. After incubation for 12 h at 4 °C, the corresponding secondary antibodies and enhancedchemiluminescence assay kit (ECL; GE Healthcare, Waukesha, WI, USA) were employed to visualize the target proteins.

Table II. The primer sequences included in this study.

Name	Primer sequences (5'-3')
DBH-AS1: forward	CGTCCACTCGTCTGTTCACT
DBH-AS1: reverse	TAACACCCCATCCGCTTGT
GAPDH: forward	CAATGACCCCTTCATTGACC
GAPDH: reverse	GACAAGCTTCCCGTTCTCAG

Cell Viability Assays

The cell viability was determined by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assays. In brief, the DBH-AS1 siRNAs or si-NC treated cells were collected and planted into 96-well plates (3000 cells/well). At 48 h, 72 h and 96 h post-plantation, the cells (each well) was added with 15 μl of CCK-8 solution and incubated for 2-3 h at 37°C. Then, the cellular proliferation was evaluated by measuring the absorbance at 450 nm on an Elx800 microplate reader apparatus (BioTek Instruments, Winooski, VT, USA).

EdU Assays

EdU (5-Ethynyl-2'-deoxyuridine) assays were also utilized to measure the proliferation of MG63 and U2OS cells using an EdU assay kit (RiboBio, Guangzhou, Guangdong, China). Briefly, MG63 or U2OS cells were transfected with siRNA-1, siRNA-2 or si-NC, respectively. Then, the cells were collected and re-plated into 48-well plates (2000 cells per well). After attachment, the cells were incubated with EdU reagent (100 μl, 50 μM) for about 2 h. After being fixed with paraformaldehyde (4%), the cells were incubated with Apollo Buffer, and subsequently stained with DAPI. Finally, an IX71 fluorescence microscope (Olympus, Tokyo, Japan) was applied to acquire the images.

Cell Colony Formation Assays

An equal number (800 cells per well) of DBH-AS1 siRNAs or si-NC transfected MG63 or U2OS cells was planted in 6-well plates, and the cells were maintained in RPMI-1640 medium (with 10% FBS) for more than 14 days. When the cell

colonies were visible, a crystal violet solution (0.1%; HuaMai, Fangshan, Beijing, China) was applied to stain the cell colonies. After rinsing with PBS three times, the cell colonies were observed using an LWD200-37T microscope (CeWei, Xi'an, China).

Flow Cytometry Analysis

The apoptosis of MG63 and U2OS cells after various treatment was assessed using an apoptosis detection kit (BeiNuo, Shanghai, China). In short, MG63 or U2OS cells after transfection of DBH-AS1 siRNAs or si-NC were collected and treated by Annexin V-FITC/PI (fluorescein isothiocyanate/Propidium Iodide; Vazyme, Nanjing, China) double staining methods. After incubation for 15-20 min in the dark, the cells were washed using ice-cold PBS and subsequently analyzed by a BD FACSVerse flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Caspase 3 and Caspase 9 Determination

We employed a Beyotime caspase-3/9 activity assay kit (Haimen, Jiangsu, China) to determine the activity of caspase 3 and caspase 9. Briefly, the collected MG63 and U2OS cells were lysed by the lysis buffer provided in the assay kit. Subsequently, we applied a 5810R centrifuge (Eppendorf, Hamburg, Germany) to collect the supernatant of the cell lysates (12000 g/min; 15 min). Finally, the Ac-DEVD-pNA (2mM) was added into the cell lysates and an HBS-1096C Pro microplate reader (DeTie, Nanjing, China) was utilized to determine the absorbance of 405 nm.

Wound Healing Assays

The DBH-AS1 siRNAs or si-NC transfected cells were digested using 0.25% trypsin and adjusted the cellular density of 2×10^5 per ml. Subsequently, 1 ml cells (per well) were added into 12-well plates and the cells were maintained at 37°C with 5% CO_2 until 100% cell confluence. Then, a 200 μl micropipette was applied to vertically scratch in the 12-well plates and the wounded areas were created. After rinsing with PBS twice, the wounded areas were photographed by a microscope (LWD200-37T, CeWei, Xi'an, China).

Transwell Invasion Assays

After MG63 or U2OS cells were transfected with DBH-AS1 siRNAs or si-NC, the cells were collected and 1×10^5 cells (per well; without serum) were added into the Matrigel-coated upper chambers of Corning transwell (pore size: 8 μm; Unique, Chaoyang, Beijing, China). Then, as a chemoattractant, 600 μl of the medium (containing 10% FBS) was added into the lower chambers. After incubation for 24 h, crystal violet solution (0.1%; HuaMai, Beijing, China) was applied to stain the invaded cells. The cells were washed using PBS three times and an LWD200-37T microscope (CeWei, Xi'an, China) was applied to observe and photograph the invaded cells.

Statistical Analysis

SPSS 20.0 statistics software (SPSS Inc., Chicago, IL, USA) was utilized to determine statistical differences by the use of Student's *t*-test. The multi-group comparison was performed using one-way analysis of variance. The paired comparison was performed by SNK approach. The chi-square test was applied to the examination of the relationship between DBH-AS1 expression levels and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method with the log-rank test. Multivariate survival analysis was performed for all parameters that were significant in the univariate analyses using the Cox regression model. All tests were two-tailed and the results with $p0<0.05$ were considered statistically significant.

Results

The Expression of DBH-AS1 Was Upregulated in Osteosarcoma Patients

To explore whether the expression of DBH-AS1 differed between osteosarcoma and matched normal bone tissue, we collected 119 paired osteosarcoma tissues and matched normal bone tissues and analyzed its expression by RT-PCR. As shown in Figure 1A, the results showed that the relative expression levels of DBH-AS1 in osteosarcoma tissues were significantly higher than that in matched noncancer bone tissues ($p<0.01$). In addition, we found that patients with advanced clinical stages displayed a higher level of DBH-AS1 (Figure 1B). Subsequently, the basal levels of DBH-AS1 in four osteosarcoma cell lines were also determined by the Real Time-PCR analysis. It showed that DBH-AS1 expression was distinctly upregulated in four osteosarcoma cell lines compared to that in the osteoblast hFOB1.19 cell line, as shown in Figure 1C. Overall, our results suggested that DBH-AS1 was upregulated in osteosarcoma.

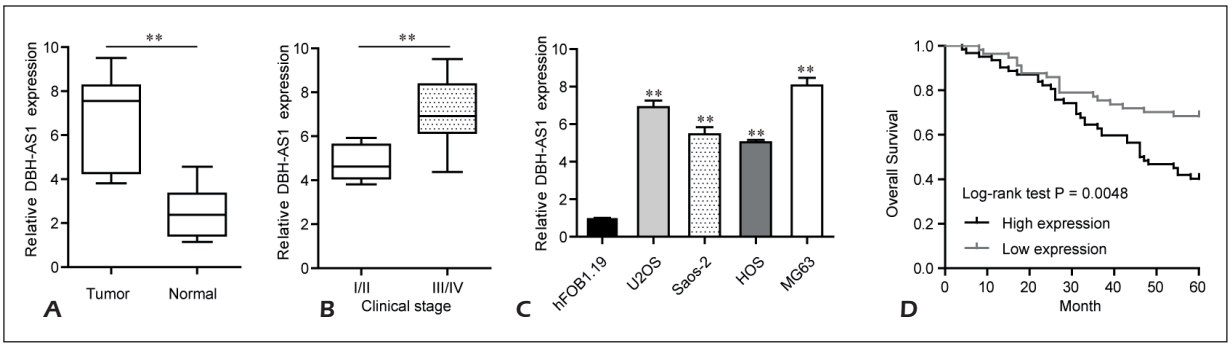

Figure 1. DBH-AS1 was highly expressed in osteosarcoma and associated with poor prognosis. **A**, Expression of DBH-AS1 in osteosarcoma and normal adjacent bone tissues. **B**, The osteosarcoma patients with advanced stages showed a higher level of DBH-AS1. **C**, The relative expression of DBH-AS1 in four osteosarcoma cell lines and Hfob1.19 was measured using qRT-PCR. **D**, DBH-AS1 expression levels were divided into high-expression and low-expression group. High-expression of DBH-AS1 indicated the poor prognosis of osteosarcoma patients. *p<0.05, **p<0.01.

Prognostic Potential of DBH-AS1 Levels in Osteosarcoma

To study the clinical significance of DBH-AS1 in osteosarcoma, based on the median DBH-AS1 level, all 119 osteosarcoma patients were divided into two subgroups (High and Low). The relationships between DBH-AS1 expression levels and clinicopathological features were shown in Table I. The results of statistical analysis using chi-square test indicated that higher DBH-AS1 expression was markedly positive associated with lymph node status (p=0.011) and metastasis status (p=0.017). However, there was no association between DBH-AS1 expression and other clinical factors, such as age, sex and tumor size (all p>0.05). To further explore the potential prognostic value of DBH-AS1 in osteosarcoma, we performed a 5-year follow-up. During the follow-up, the median follow-up time was 46.5±12.1 months. The results of the Kaplan-Meier analysis indicated that patients with higher levels of DBH-AS1 had shorter overall survival than those with low levels (Figure 1D, p=0.0048). Afterward, the results of the univariate analysis revealed that lymph node status, metastasis status and DBH-AS1 expression were associated with overall survival of osteosarcoma patients (Table III). Moreover, multivariate analysis, used to analyze parameters with significance based on univariate analysis, confirmed that DBH-AS1 expression (HR=2.944, 95% CI: 1.218-4.372, p=0.009) remained an independent prognostic biomarker for prediction of the overall survival in osteosarcoma.

Repressing Expression of DBH-AS1 Inhibited the Proliferation of Osteosarcoma Cells and Accelerated Cell Apoptosis

Since DBH-AS1 was distinctly overexpressed in osteosarcoma, we next performed functional studies using siRNAs specific targeting DBH-AS1 (siRNA-1, siRNA-2) to study the functional effects of DBH-AS1 in osteosarcoma cells. First, the siRNAs targeting DBH-AS1 and negative control siRNAs (si-NC) were separately transfected into MG63 or U2OS cells. Then, the qRT-PCR analysis was carried out to detect the knockdown efficiency of DBH-AS1 siRNAs and the results confirmed that transfection of DBH-AS1 siRNAs was capable of

Table III. Multivariate analysis of overall survival and disease-free survival in ESCC patients.

Variables	Univariate analyses			Multivariate analyses		
	HR	95% CI	p	HR	95% CI	p
Age (≤ 60/> 60)	1.477	0.673-2.321	0.332	–	–	–
Sex (Men/Women)	1.324	0.487-1.984	0.139	–	–	–
Tumor size (≤ 5 cm/> 5 cm)	1.894	0.784-2.341	0.093	–	–	–
Lymph node status (Negative/Positive)	3.246	1.437-4.775	0.007	2.875	1.238-4.168	0.016
Metastasis status (Negative/Positive	3.174	1.388-4.436	0.011	2.675	1.185-3.889	0.018
DBH-AS1 expression (High/Low)	3.457	1.582-5.447	0.001	2.944	1.218-4.372	0.009

suppressing the expression of DBH-AS1 (Figure 2A). Subsequently, we conducted CCK-8 assays and the results suggested that suppression of DBH-AS1 markedly reduced the cellular viability in MG63 and U2OS cells (Figure 2B). Furthermore, the EdU assays were performed and the data suggested that the proliferative rates of both MG63 and U2OS cells transfected with DBH-AS1 siRNAs were remarkably suppressed (Figure 2C). Additionally, cell colony formation assays were employed to evaluate the tumorigenicity of ovarian cancer cells after transfection with DBH-AS1 siRNAs. The results confirmed

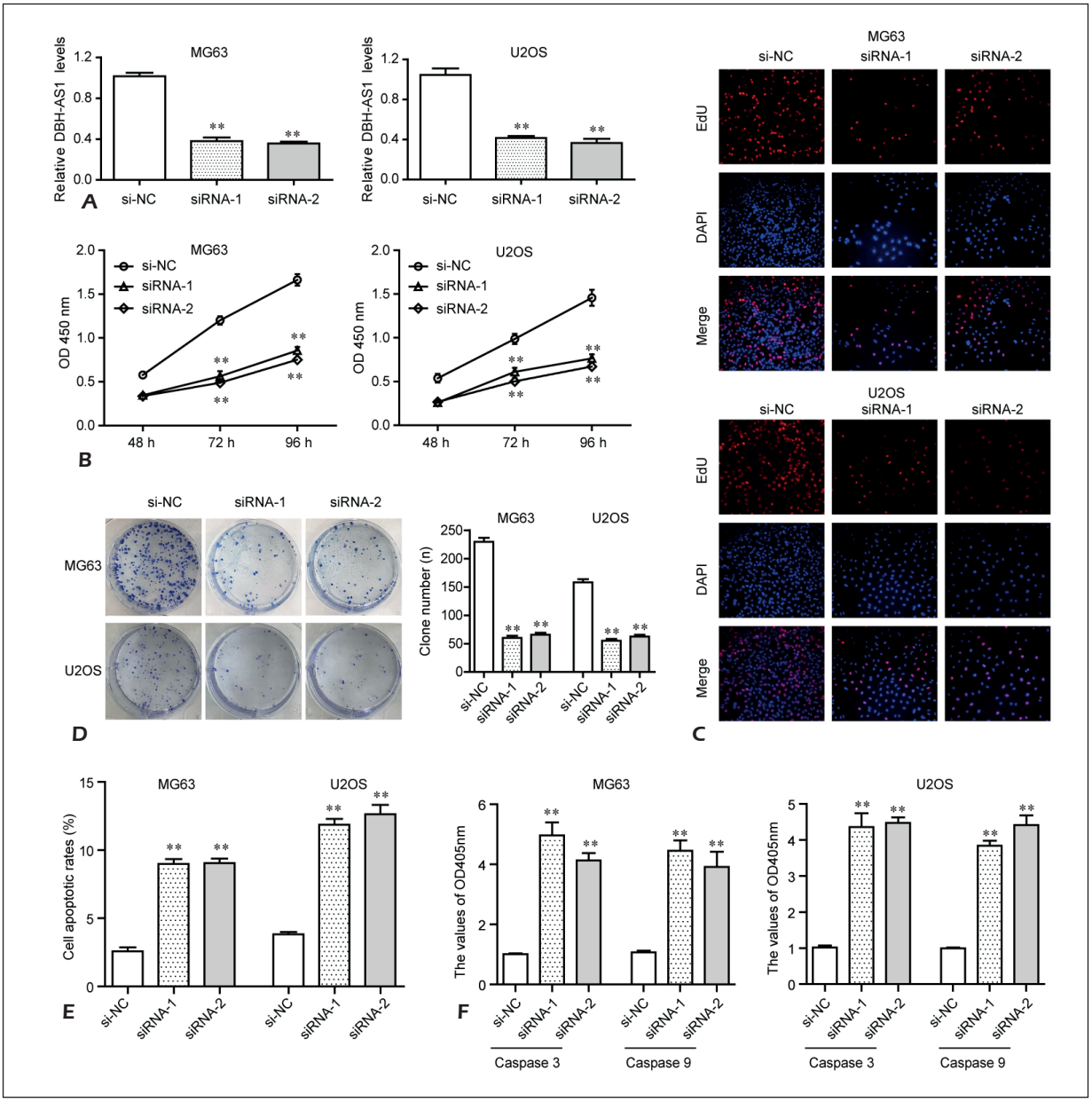

Figure 2. The influence of DBH-AS1 on the proliferation and apoptosis of MG63 and U2OS cells. **A**, Expression levels of DBH-AS1 in MG63 and U2OS cells transfected with DBH-AS1 siRNAs (siRNA-1, siRNA-2) or negative control siRNAs (si-NC). **B**, The cell proliferation of MG63 and U2OS cells were determined by CCK-8 assays. **C**, Transfection of DBH-AS1 siRNAs reduced the proliferative MG63 and U2OS cells using EdU assays. Nuclei double-labeled with EdU (red) and DAPI (blue) were considered positive cells. **D**, Knockdown of DBH-AS1 decreased the cell colony number of MG63 and U2OS cells. **E**, Flow cytometry detected the cell apoptosis of MG63 and U2OS cells after transfection of DBH-AS1 siRNAs. **F**, The activity of caspase 3 and caspase 9 in MG63 and U2OS cells was examined, and transfection of DBH-AS1 siRNAs increased the activity of caspase 3 as well as caspase 9. *p<0.05, **p<0.01.

that the cellular colony formation number was notably decreased following DBH-AS1 depletion in MG63 and U2OS cells (Figure 2D). Moreover, the influence of DBH-AS1 on cell apoptosis was also determined using flow cytometric analysis and the data validated that the proportion of apoptotic cells in DBH-AS1 siRNAs-transfected groups was markedly elevated (Figure 2E). In addition, the activity of apoptosis relevant molecules was examined in DBH-AS1 siRNAs-transfected MG63 and U2OS cells using caspase 3/caspase 9 activity detection assays. These results indicated that depression of DBH-AS1 significantly elevated the activity of caspase 3 and caspase 9 (Figure 2F). These findings indicated that DBH-AS1 play an important role in the modulation of growth of osteosarcoma.

Knockdown of DBH-AS1 Depressed Metastasis of Osteosarcoma Cells

To further explore the effects of DBH-AS1 in regulating the metastatic potentials of osteosarcoma cells, we performed wound healing assays and transwell assays using MG63 and U2OS cells. The results demonstrated that the migratory ability of MG63 cells transfected with DBH-AS1 siRNAs markedly slowed down at 48 h (Figure 3A). Similarly, the migration of DBH-AS1 siRNAs-transfected U2OS cells was also decreased when compared with the controls (Figure 3B). In addition, the results from the transwell assays showed that the invasive cell number of DBH-AS1 siRNAs-transfected MG63 cells was remarkably reduced, which suggested that knockdown of DBH-AS1 dramatically inhibited the invasive capacity of MG63 cells (Figure 3C). Similar results were also observed in U2OS cells transfected with DBH-AS1 siRNAs (Figure 3D). Therefore, these data demonstrated that DBH-AS1 functioned as a positive regulator in modulating the metastasis of osteosarcoma.

DBH-AS1 Modulated the PI3K/AKT Signaling in Osteosarcoma Cells

Next, we attempted to uncover the detail molecular mechanisms by which DBH-AS1 exerted its oncogenic functions in osteosarcoma. Evidence had certified that lncRNAs exerted their

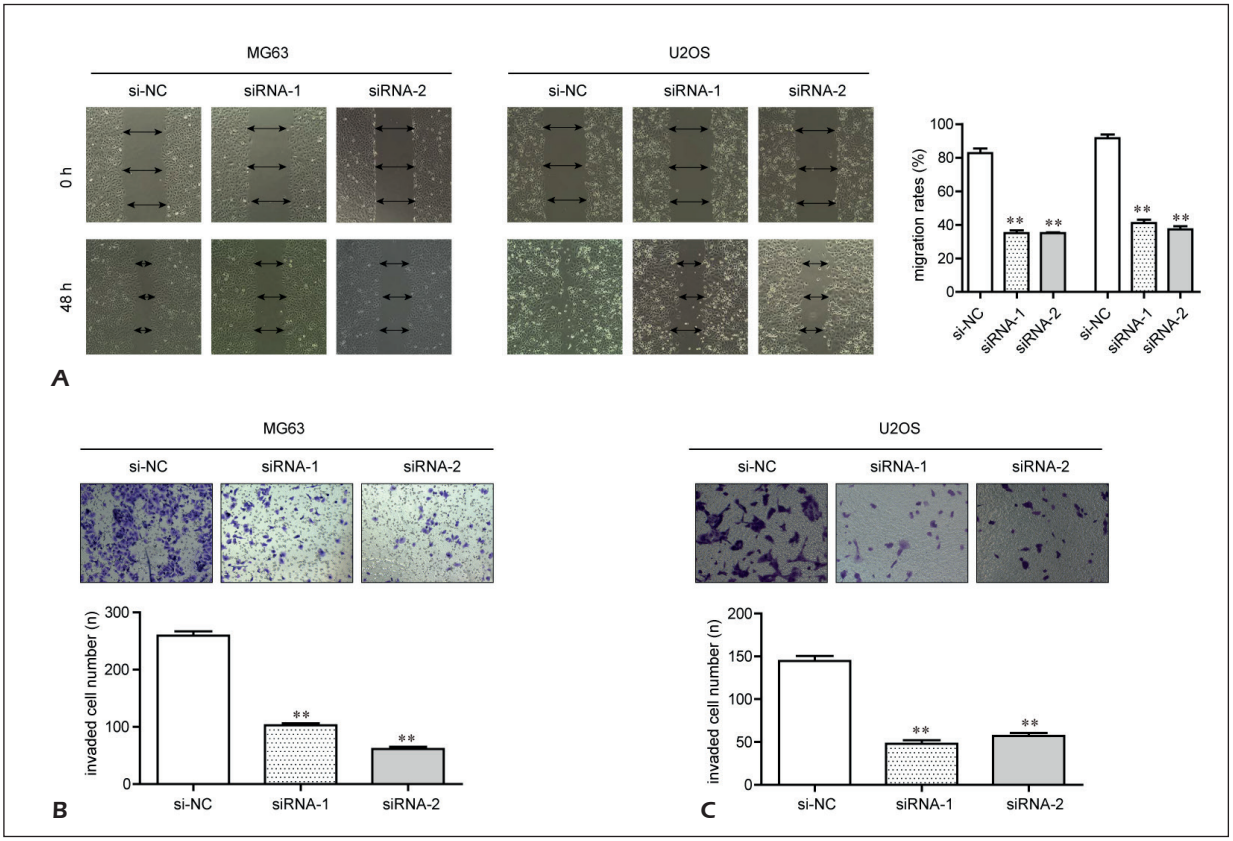

Figure 3. The effects of DBH-AS1 on the metastatic potentials of MG63 and U2OS cells. **A**, Wound healing assays detected the relative migratory rates of MG63 and U2OS cells after transfection of DBH-AS1 siRNAs. **B-C**, The invasive capacity of MG63 and U2OS cells transfected with DBH-AS1 siRNAs was evaluated using transwell invasion assays. *p<0.05, **p<0.01.

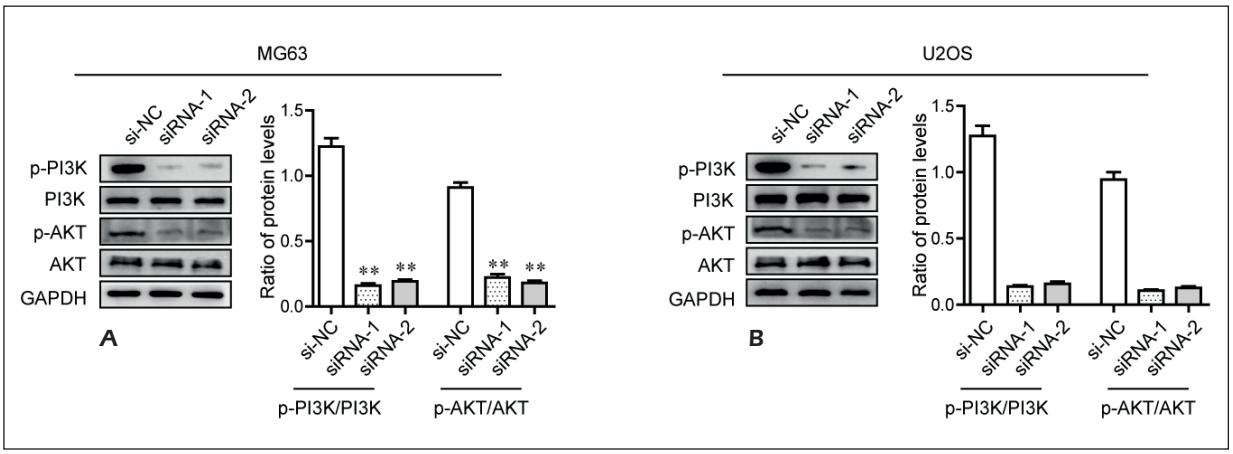

Figure 4. The activity of PI3K/AKT signaling was suppressed in DBH-AS1 deficiency MG63 and U2OS cells. **A**, Western blot analysis detected the protein expression of p-PI3K p-AKT, PI3K and AKT in MG63 cells. The optical density of protein bands was analyzed by Image J (NIH, Bethesda, ML, USA). **B**, The protein expression of p-PI3K p-AKT, PI3K and AKT in U2OS cells transfected with DBH-AS1 siRNAs was measured by Western blot. *p<0.05, **p<0.01.

functions not only via sponging other non-coding RNAs, but also affecting essential signaling pathways. Therefore, we focused on investigating the PI3K/AKT signaling pathway because it was one of the most common dysregulated signaling implicated in osteosarcoma development. Western blot analysis was performed and the data suggested that silence of DBH-AS1 in MG63 cells resulted in significantly decreased protein expression of p-PI3K and p-AKT, while differences were not observed in the levels of PI3K and AKT (Figure 4A). We also observed that the expression of p-PI3K and p-AKT was notable alternation when the DBH-AS1 siRNAs were transfected into U2OS cells (Figure 4B). Therefore, these data indicated that depression of DBH-AS1 led to significant inhibition of the PI3K/AKT signaling in osteosarcoma cells.

Discussion

Osteosarcoma is the most common primary malignant tumor of bone. The incidence of osteosarcoma increases yearly in China[23]. Early diagnosis and prediction of prognosis of osteosarcoma are very important for doctors to design treatment plan[24,25]. In clinical practice, although several clinicopathological features such as distant metastasis, clinical stage and tumor size have been used as prognostic factors, the sensitivity and specificity are low[26,27]. Up to date, the identification of cancer biomarkers become a new hotspot and the application of cancer biomarkers allows early diagnosis and fast start of therapy and predict the risk of relapse in osteosarcoma patients[25,28]. Growing evidence[29,30] indicated that lncRNAs could be good candidates for cancer biomarkers, including osteosarcoma, and acquired high specificity, high sensitivity and noninvasive characteristics.

In this study, we performed RT-PCR to explore whether DBH-AS1 was abnormally expressed in osteosarcoma, finding that its expression was significantly up-regulated in both osteosarcoma tissues and cell lines. In addition, higher expression of DBH-AS1 in osteosarcoma tissues with advanced stages was observed. Then, it was found that high DBH-AS1 expression was markedly associated with positive lymph node status and metastasis status. Using the Kaplan-Meier model, we demonstrated that osteosarcoma patients with high DBH-AS1 had remarkably shorter overall survival than patients with low DBH-AS1, suggesting that DBH-AS1 may act as a negative regulator in clinical progress of osteosarcoma. More importantly, multivariate Cox analysis showed that DBH-AS1 was an independent prognostic factor of osteosarcoma. Our findings first indicated that DBH-AS1 was distinctly overexpressed in osteosarcoma and may be used as a new diagnostic and prognostic biomarker for osteosarcoma subjects. However, further research on a large number of patients is needed to further confirm our conclusion due to the relatively small number of osteosarcoma patients analyzed in the current study.

As a recent identified tumor-related lncRNA, DBH-AS1 was first reported to be highly expressed

in hepatocellular carcinoma. The functional investigation indicated that DBH-AS1, modulated by HBx protein, could promote hepatocellular carcinoma cells growth by modulating MAPK signaling[21]. In colorectal cancer, DBH-AS1 was reported to be up-regulated and associated with poor prognosis[20]. Up to date, the functional studies and potential mechanism of DBH-AS1 in other tumors have not been performed. In this work, we performed functional assays by down-regulating the expression of DBH-AS1 in MG63 and U2OS cells and found that knockdown of DBH-AS1 distinctly suppressed the proliferation and led to a significant decrease in the number of colonies formed by MG63 and U2OS cells. In addition, we also found that down-regulation of DBH-AS1 promoted apoptosis. Moreover, apoptosis-related factors, including caspase 3 and caspase 9, was detected in DBH-AS1-knockdown cells using Beyotime caspase-3/9 activity assay kit and the results confirmed that down-regulation of DBH-AS1 promoted the activity of caspase 3 and caspase 9. Those results suggested that DBH-AS1 served as a tumor promoter in osteosarcoma by suppressing the activation of caspase-3 and caspase 9. However, the exact mechanism for this change remains to be further studied. On the other hand, the results of wound healing assays and transwell invasion assays indicated that knockdown of DBH-AS1 inhibited the migration and invasion of osteosarcoma cells. Our findings, for the first time, reported that DBH-AS1 functioned as a tumor promoter in the progression of osteosarcoma. The PI3K-AKT signaling pathway is activated by various types of cellular stimuli or toxic insults and participates in the modulation of several cellular progress such as division, translation, proliferation, metabolism and cell cycle[31,32]. As previously referred, the PI3K-AKT signaling pathway and the downstream signaling molecules of PI3K (Akt, mTOR, PDK and ILK), activated in various tumors, mediate the process of EMT and have attracted growing attention as a potential target for the treatment of osteosarcoma[33-35]. The regulatory mechanism involved in the PI3K-AKT signaling pathway is very complex. More and more lncRNAs were reported to display their tumor-promotive or tumor-suppressive roles by modulating PI3K-AKT signaling. For instance, lncRNA HULC was found to inhibit proliferation and metastasis of osteosarcoma via modulation of PI3K-AKT signaling[36]. LncRNA MALAT1 was reported to promote cancer metastasis in osteosarcoma by regulating PI3K-AKT signaling[37]. Thus, we wondered whether DBH-AS1 also displayed similar regulator effects on PI3K-AKT signaling. In this study, we simultaneously evaluated the expression levels of p-PI3K and p-AKT when DBH-AS1 was knocked down in MG63 and U2OS cells. As expected, knockdown of DBH-AS1 distinctly suppressed expression levels of p-PI3K and p-AKT, suggesting that the activity of PI3K-AKT signaling was suppressed. Our results revealed that DBH-AS1 suppressed osteosarcoma cells proliferation and metastasis by regulating PI3K-AKT signaling.

Conclusions

We first provided evidence that DBH-AS1 was upregulated in osteosarcoma and regulated tumor cells proliferation and metastasis. In addition, DBH-AS1 executed its tumor-promotive effects by modulating PI3K-AKT signaling. Thus, modulation of DBH-AS1 may represent novel approaches for the interventional treatment of osteosarcoma.

Conflict of Interests

The authors declare no conflict of interest.

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