Knockdown of DDX46 Inhibits the Invasion and Tumorigenesis in Osteosarcoma Cells

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DDX46, a member of the DEAD-box (DDX) helicase family, is involved in the development of several tumors. However, the exact role of DDX46 in osteosarcoma and the underlying mechanisms in tumorigenesis remain poorly understood. Thus, in the present study, we explored the role of DDX46 in osteosarcoma and the underlying mechanisms. Our results demonstrated that the expression levels of DDX46 in both mRNA and protein were greatly elevated in human osteosarcoma tissues and cell lines. Knockdown of DDX46 obviously inhibited osteosarcoma cell proliferation and tumor growth in vivo. In addition, knockdown of DDX46 also significantly suppressed migration and invasion in osteosarcoma cells. Furthermore, knockdown of DDX46 substantially downregulated the phosphorylation levels of PI3K and Akt in SaOS2 cells. In summary, the present results have revealed that DDX46 plays an important role in osteosarcoma growth and metastasis. Knockdown of DDX46 inhibited osteosarcoma cell proliferation, migration, and invasion in vitro and tumor growth in vivo. Therefore, DDX46 may be a potential therapeutic target for the treatment of osteosarcoma.

Key words: DDX46; Osteosarcoma; Metastasis; PI3K/Akt pathway

INTRODUCTION

Osteosarcoma is the most prevalent primary malignant bone tumor and mainly affects children and young adults[1]. Despite very aggressive treatments including surgery, multiagent chemotherapy, and radiotherapy[2,3], the 5-year survival rate of osteosarcoma patients remains poor, and most of them die of pulmonary metastases eventually[4,5]. Therefore, there is an urgent need to explore the molecular mechanisms of osteosarcoma progression in order to find a treatment for osteosarcoma.

DEAD-box (DDX) RNA helicases play a critical role in all aspects of RNA metabolism such as pre-mRNA splicing, rRNA biogenesis, and transcription[6]. DDX46 belongs to the DDX helicase family. Previous studies have shown that DDX46 plays a role in pre-mRNA splicing in vitro before or during prespliceosome assembly[7,8]. It was reported that DDX46 is required for the development of digestive organs and the brain, mainly via regulating pre-mRNA splicing[9]. In addition, DDX46 is involved in the development of several tumors[10–12]. Li et al. reported that the expression of DDX46 was greatly upregulated in esophageal squamous cell carcinoma (ESCC) tissues and cells compared with normal tissues and cells, and knockdown of DDX46 significantly suppressed ESCC cell proliferation[10]. However, the exact role of DDX46 in osteosarcoma and the underlying mechanisms in tumorigenesis remain poorly understood. Thus, we explored the role of DDX46 in osteosarcoma and the underlying mechanisms. We showed that knockdown of DDX46 inhibited osteosarcoma cell proliferation, migration, and invasion in vitro and tumor growth in vivo. Therefore, DDX46 may represent a potential therapeutic target for the treatment of osteosarcoma.

MATERIALS AND METHODS

Tissue Specimens

Fresh osteosarcoma tissue specimens and their matched adjacent normal bone samples were collected from 13 patients who underwent surgery at the Department of Orthopedics, Huaihe Hospital of Henan University (P.R. China) between June 2014 and September 2015. All specimens were preserved in liquid nitrogen immediately. This study was approved by the ethics committee of the Huaihe Hospital of Henan University, and all patients provided informed consent.

Cell Culture

Three human osteosarcoma cell lines (U2OS, SaOS2, and MG63) and the human osteoblastic cell line (hFOB1.19)

were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) with 10% (v/v) fetal bovine serum (FBS; Gibco) and 100 U/ml streptomycin and penicillin (Gibco) at 37°C in a 5% CO_2 humidified atmosphere.

Short Hairpin RNA-Mediated Knockdown of DDX46 and Cell Transfection

The short hairpin RNA sequence targeting DDX46 (sh-DDX46; 5′-CATCCAAACCCAAGCTATT-3′) and nonsilencing control sequence (sh-NC; 5′-TTCTCCGA ACGTGTCACGT-3′) were designed and synthesized by GeneChem (Shanghai, P.R. China). SaOS2 cells were seeded in each well of a 24-well microplate, grown for 24 h to reach 30%–50% confluence, and then transfected with sh-DDX46 or sh-NC using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from human osteosarcoma samples or cells using TRIzol reagent (Abcam, Cambridge, UK). About 5 μg of total RNA for each sample was reverse transcribed into first-strand cDNA for qRT-PCR analysis. qRT-PCRs were performed on the Bio-Rad iQ5 Real-Time thermal cyclers using SYBRH Premix Ex Taq™ II kit (Takara, Dalian, P.R. China). The PCR primers for DDX46 were 5′-AAAATGGCG AGAAGAGCAACG-3′ (forward) and 5′-CATCATCGT CCTCTAAACTCCAC-3′ (reverse) and for β-actin were 5′-TTAGTTGCGTTACACCCTTTC-3′ (forward) and 5′-ACCTTCACCGTTCCAGTTT-3′ (reverse). β-Actin was used as the internal reference gene. The relative expression levels were calculated using the comparative threshold cycle (Ct) method [relative gene expression = $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$], and the target gene was normalized to the internal reference gene.

Western Blot

Human osteosarcoma tissues or cells were homogenized and lysed with RIPA lysis buffer. The protein concentration was then determined using the Bradford method. A total of 30 μg of protein was separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). Then the membrane was blocked with 2% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature, followed by incubation with primary antibodies (DDX46, E-cadherin, N-cadherin, vimentin, PI3K, p-PI3K, Akt, p-Akt, and GAPDH; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Subsequently, the membrane was incubated with

horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). The target protein was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 1–4 days after transfection. At each time point, 10 μl of WST-1 substrate was added to each well and incubated for 4 h at 37°C. After incubating, the absorbance at 450 nm was measured by an enzyme-linked immunosorbent assay plate reader.

Transwell Migration and Invasion Assays

For the migration assay, 5×10^4 cells/well transfected with sh-DDX46 or sh-NC were plated into the top chambers of the insert. For the invasion assay, infected cells were plated into the top chambers of the insert precoated with Matrigel (BD Biosciences, Bedford, MA, USA). In both assays, 500 μl of DMEM with 10% FBS was added into the lower compartment. After incubating for 24 h at 37°C, the cells remaining on the upper chamber were removed with cotton swabs. The cells on the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and then counted under a light microscope (magnification: 100×).

Xenografted Tumor Model

Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Huaihe Hospital of Henan University. Female Balb/c nude mice (4–5 weeks of age, 18–22 g) were purchased from the Laboratory Animal of the Huaihe Hospital of Henan University of Henan University (P.R. China). Mice were housed under standard conditions of room temperature, humidity, and dark–light cycles in pathogen-free cages with free access to water and food. SaOS2 cells (1×10^6 cells/0.1 ml) transfected with sh-DDX46 or sh-NC were suspended in PBS (0.1 ml) and injected subcutaneously into the flank of nude mice ($n = 6$ per group). Tumor size was measured every 5 days; length and width measurements were obtained with calipers, and tumor volumes were calculated by the formula: $V = 1/2$ (width^2 × length). Twenty days after injection, the animals were sacrificed, and tumors were excised and weighed.

Statistical Analysis

All statistical analyses were conducted using the SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA), and the data are expressed as means ± SD. Statistical significance was analyzed with the Student's t-test for comparison of two groups or one-way ANOVA for multiple comparisons. A value of $p < 0.05$ was considered to indicate statistical significance.
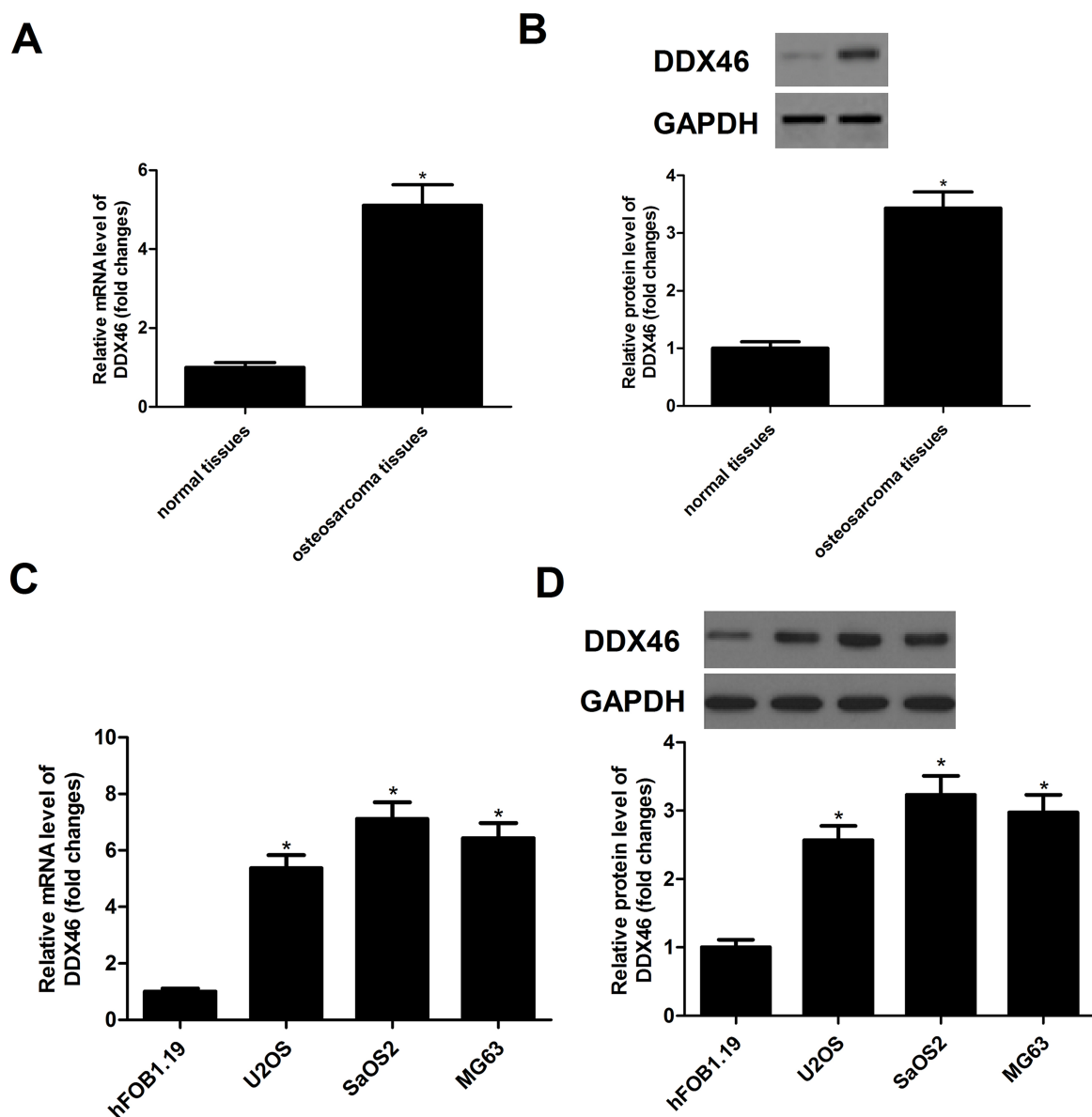
RESULTS

DDX46 Was Highly Expressed in Human Osteosarcoma Tissues and Cell Lines

First, we examined the expression of DDX46 in human osteosarcoma tissues using qRT-PCR and Western blotting. The results indicated that DDX46 expression levels in both mRNA and protein were significantly higher in human osteosarcoma tissues than in normal bone tissues (Fig. 1A and B). Consistent with observations from samples, we observed that the three human osteosarcoma cell lines displayed higher expression levels than the normal human osteoblastic cell line (Fig. 1C and D).

Knockdown of DDX46 Inhibited the Proliferation of Osteosarcoma Cells

To further investigate the effect of DDX46 on osteosarcoma cell proliferation, we used shRNA-mediated inhibition of DDX46 in SaOS2 cells. shRNA transduction significantly decreased DDX46 expression in SaOS2 cells compared to the sh-NC group, as shown by qRT-

Figure 1. DDX46 was highly expressed in human osteosarcoma tissues and cell lines. (A) Quantification of DDX46 mRNA in human osteosarcoma tissues using qRT-PCR analysis. (B) Detection of DDX46 protein in human osteosarcoma tissues by Western blotting analysis. *$p < 0.05$ versus control group denotes a significant difference. (C) Quantification of DDX46 mRNA in human osteosarcoma cell lines using qRT-PCR analysis. (D) Detection of DDX46 protein in human osteosarcoma cell lines by Western blotting analysis. *$p < 0.05$ versus hFOB1.19 group denotes a significant difference.

PCR analysis (Fig. 2A) and Western blotting analysis (Fig. 2B). Then we performed the WST-1 assay to investigate the effect of DDX46 on osteosarcoma cell proliferation. As expected, cell proliferation was significantly suppressed by sh-DDX46 in SaOS2 cells, compared with the sh-NC group (Fig. 2C).

Knockdown of DDX46 Inhibited the Migration and Invasion of Osteosarcoma Cells

We examined the effects of DDX46 on cell migration and invasion using the Transwell migration assay and the Matrigel invasion assay, respectively. Knockdown of DDX46 obviously suppressed the migrative ability of SaOS2 cells, compared with the sh-NC group (Fig. 3A). Similarly, we found that knockdown of DDX46 could suppress the invasive ability of SaOS2 cells (Fig. 3B). In addition, we evaluated the effect of DDX46 on the expression levels of EMT-related markers by Western blotting. The results showed that the protein expression level of E-cadherin was dramatically upregulated in the DDX46-knockdown SaOS2 cells compared with the sh-NC group, while the protein expression levels of N-cadherin and vimentin were downregulated (Fig. 3C).

Knockdown of DDX46 Inhibited the Growth of Osteosarcoma In Vivo

To further examine the effects of DDX46 on tumor growth in vivo, SaOS2 cells stably expressing sh-DDX46 or sh-NC were injected subcutaneously into the flank of nude mice. The tumor volumes formed by DDX46-knockdown SaOS2 cells were smaller than control tumors (Fig. 4A). The average tumor weight was also significantly decreased in DDX46-silencing tumors compared to the controls (Fig. 4B).

Knockdown of DDX46 Inhibited the Activation of the PI3K/Akt Pathway in Osteosarcoma Cells

Various studies have demonstrated that the PI3K/Akt pathway plays a critical role in the development of tumors. Thus, we investigated the effect of DDX46 on the expression of certain molecules involved in the PI3K/Akt signaling pathway in SaOS2 cells. Knockdown of DDX46 substantially downregulated the phosphorylation levels of PI3K and Akt in SaOS2 cells, compared with the sh-NC group (Fig. 5A). Furthermore, we examined the effects of the Akt inhibitor (Wortmannin) on DDX46-mediated proliferation and invasion of SaOS2 cells. The results indicated that Wortmannin significantly enhanced the inhibitory effects of sh-DDX46 on SaOS2 cell proliferation (Fig. 5B) and invasion (Fig. 5C).
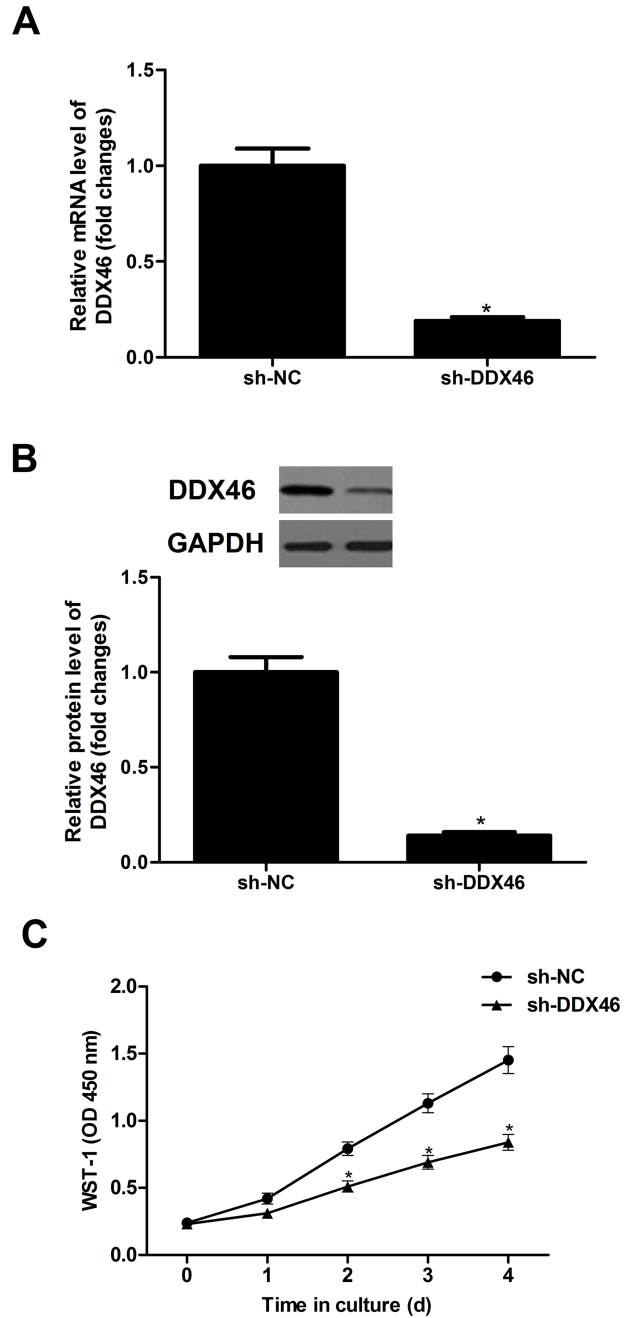
Figure 2. Knockdown of DDX46 inhibited the proliferation of osteosarcoma cells. SaOS2 cells were infected with sh-DDX46 or sh-NC for 24 h. (A) mRNA expression level of DDX46 in SaOS2 cells. (B) Protein expression level of DDX46 in SaOS2 cells. (C) Cell proliferation was measured using the WST-1 assay. $*p < 0.05$ versus sh-NC group denotes a significant difference.

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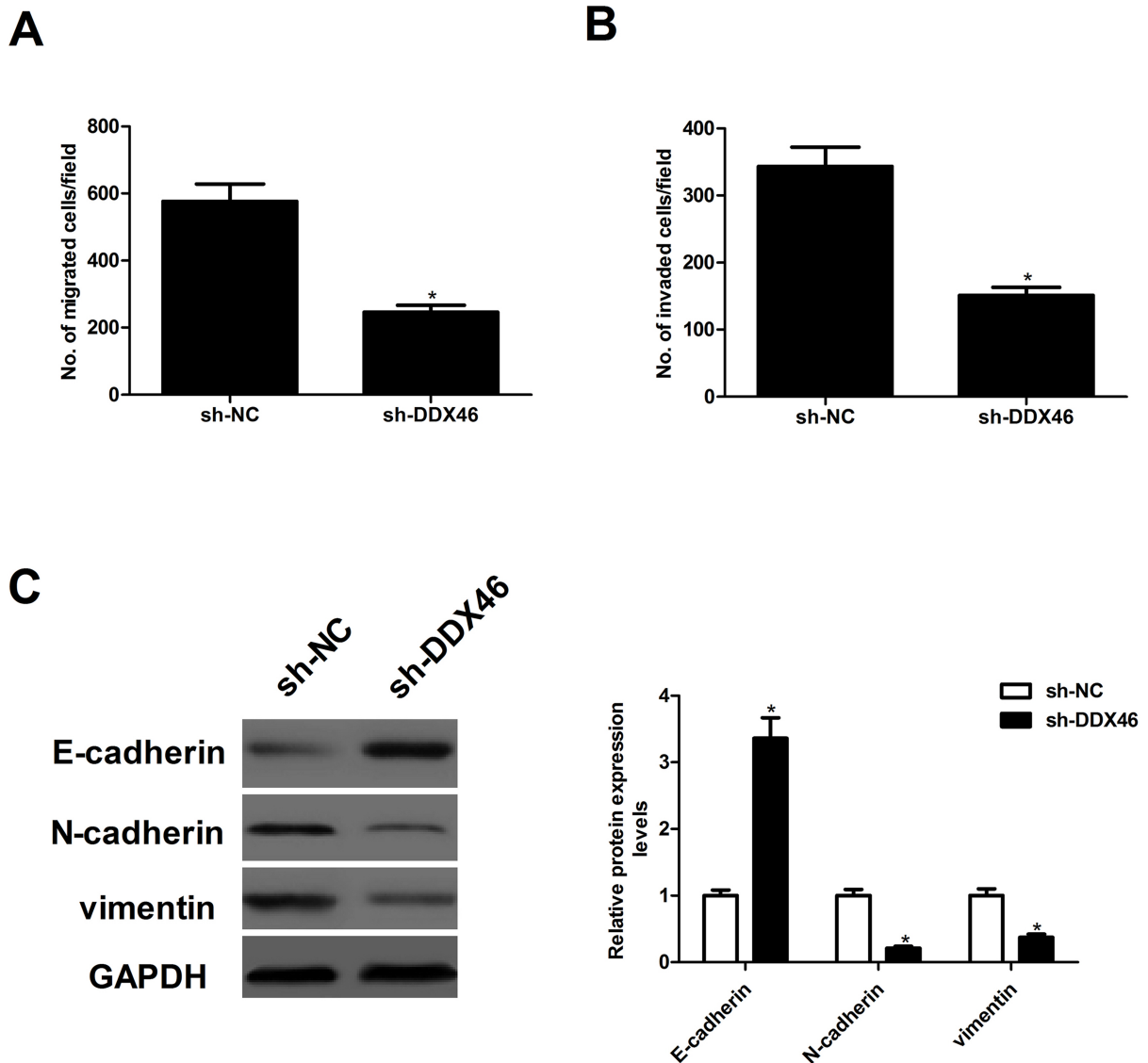
Figure 3. Knockdown of DDX46 inhibited the migration and invasion of osteosarcoma cells. SaOS2 cells were infected with sh-DDX46 or sh-NC for 24 h. (A) Cell migration was evaluated by the Transwell migration assay. (B) Cell invasion was measured using the Matrigel invasion assay. (C) Western blotting was used to measure the protein levels of E-cadherin, N-cadherin, and vimentin in SaOS2 cells. Protein expression was analyzed using the BandScan 5.0 software and normalized to GAPDH. *$p < 0.05$ versus sh-NC group denotes a significant difference.

DISCUSSION

In general, we have demonstrated that the expression levels of DDX46 in both mRNA and protein were greatly elevated in human osteosarcoma tissues and cell lines. Knockdown of DDX46 obviously inhibited osteosarcoma cell proliferation and tumor growth in vivo. In addition, knockdown of DDX46 significantly suppressed migration and invasion in osteosarcoma cells. Furthermore, knockdown of DDX46 substantially downregulated the phosphorylation levels of PI3K and Akt in SaOS2 cells.

DDX46 has been reported to be involved in cell growth, metastasis, and apoptosis in certain cancers. A previous study by Li et al. confirmed that DDX46 protein expression was strongly increased in colorectal

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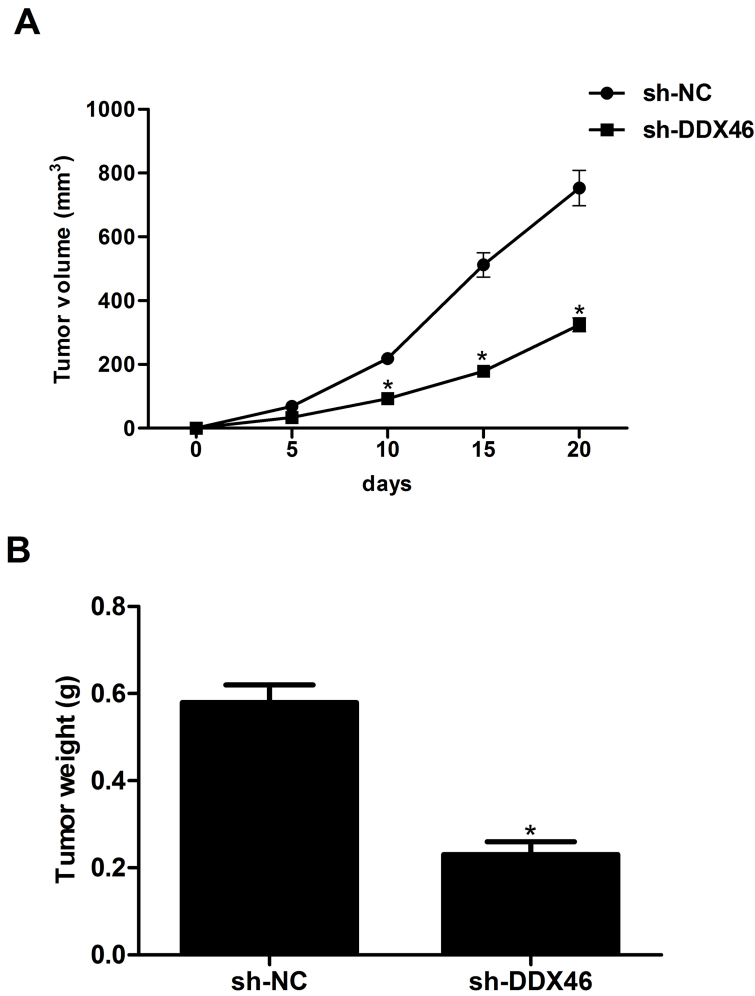
Figure 4. Knockdown of DDX46 inhibited the growth of osteosarcoma in vivo. SaOS2 cells stably expressing sh-DDX46 or sh-NC were injected subcutaneously into the flank of nude mice. (A) The tumor volume was monitored every 5 days. (B) Twenty days after injection, the animals were sacrificed, and tumors were excised and weighed. *$p<0.05$ versus sh-NC group denotes a significant difference.

cancer (CRC) tissues compared to adjacent tissues, and downregulation of DDX46 markedly suppressed CRC cell proliferation[12]. However, the function and roles of DDX46 in human osteosarcoma are still undefined. Herein we found that the expression of DDX46 levels in both mRNA and protein was greatly elevated in human osteosarcoma tissues and cell lines. In addition, knockdown of DDX46 obviously inhibited osteosarcoma cell proliferation and tumor growth in vivo, implicating that DDX46 may function as an oncogene in the development and progression of osteosarcoma.

Cancer cell migration and invasion are the critical steps for tumor metastasis. EMT has received considerable attention as a conceptual paradigm for explaining metastatic and invasive behavior during cancer progression[13].

A growing body of evidence indicates that several EMT-related molecules, such as E-cadherin, N-cadherin, TWIST, and SNAIL, are implicated in complex pathogenesis of osteosarcoma[14–16]. Herein we observed that knockdown of DDX46 significantly suppressed migration and invasion in osteosarcoma cells. In addition, knockdown of DDX46 upregulated the protein expression level of E-cadherin and downregulated the protein expression levels of N-cadherin and vimentin in SaOS2 cells. These data suggest that knockdown of DDX46 inhibited osteosarcoma cell migration and invasion via suppressing the EMT process.

The PI3K/Akt signaling pathway plays a critical regulatory role in tumorigenesis by regulating cell proliferation, cell cycle progression, metastasis, and the EMT process, as well as drug resistance[17,18]. Compelling

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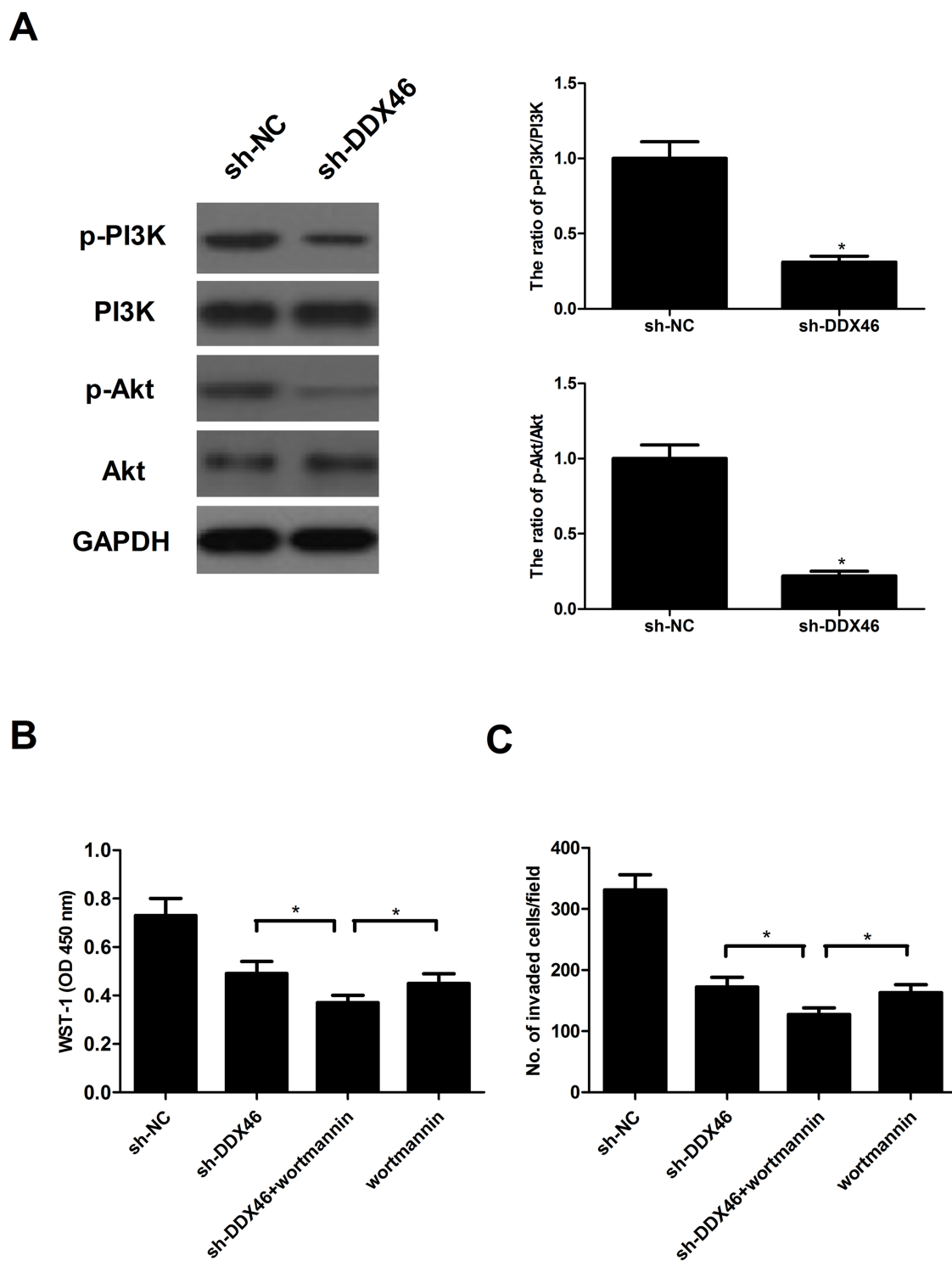
Figure 5. Knockdown of DDX46 inhibited the activation of the PI3K/Akt pathway in osteosarcoma cells. SaOS2 cells were infected with sh-DDX46 or sh-NC for 24 h. (A) Western blotting was used to measure the protein levels of PI3K, p-PI3K, Akt, and p-Akt in SaOS2 cells, and the relative protein expression levels of p-PI3K and p-Akt were quantified. (B) SaOS2 cells were transfected with sh-DDX46 or sh-NC in the presence or absence of the Wortmannin (100 nM) for 24 h. Cell proliferation was examined by the WST-1 assay. (C) Cell invasion was evaluated by the Matrigel invasion assay. $*p < 0.05$.

evidence has emerged to show that this pathway is frequently hyperactivated in osteosarcoma and contributes to the initiation and development of osteosarcoma[19–21]. Akt, a member of the AGC serine–threonine kinase family, is a major signaling molecule downstream of PI3K[22]. The activation of Akt further phosphorylates multiple proteins that regulate various cellular responses, including cell proliferation, metastasis, and the EMT process[23]. So inhibition of the PI3K/Akt signaling pathway represents an attractive potential therapeutic approach for osteosarcoma[24–26]. It was reported that the PI3K-specific inhibitor LY294002 significantly suppressed osteosarcoma cell proliferation, migration, and invasion via downregulation of the activity of the PI3K/Akt signaling pathway[27]. In this study, we found that knockdown of DDX46 substantially downregulated the phosphorylation levels of PI3K and Akt in SaOS2 cells. In addition, we observed that Wortmannin significantly enhanced the inhibitory effects of sh-DDX46 on SaOS2 cell proliferation and invasion. These data suggest that knockdown of DDX46 inhibited metastasis and tumorigenesis in osteosarcoma cells via the inactivation of the PI3K/Akt pathway.

In summary, the present results have revealed that DDX46 may play an important role in osteosarcoma growth and metastasis. Knockdown of DDX46 inhibited osteosarcoma cell proliferation, migration, and invasion in vitro and tumor growth in vivo. Therefore, DDX46 may be a potential therapeutic target for the treatment of osteosarcoma.

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