Knockdown of DDX46 Inhibits the Invasion and Tumorigenesis in Osteosarcoma Cells

Feng Jiang,1 Dengfeng Zhang,1 Guojun Li, and Xiao Wang

Department of Orthopedics, Huaihe Hospital of Henan University, Kaifeng, Henan Province, P.R. China

INTRODUCTION

Osteosarcoma is the most prevalent primary malignant bone tumor and mainly affects children and young adults1. Despite very aggressive treatments including surgery, multiagent chemotherapy, and radiotherapy2,3, the 5-year survival rate of osteosarcoma patients remains poor, and most of them die of pulmonary metastases eventually4,5. 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 transcription6. 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 assembly7,8. It was reported that DDX46 is required for the development of digestive organs and the brain, mainly via regulating pre-mRNA splicing9. In addition, DDX46 is involved in the development of several tumors10–12. 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 proliferation10. 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% CO₂ 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'-TTCTCCGAACGTCAGCTTCT-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.

**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 1×10⁴ 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, 5×10⁴ cells/well were transfected with sh-DDX46 or sh-NC and 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 (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 (1×10⁶ 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: \( V=1/2 \times \text{length} \times \text{width} \times \text{length} \). 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-PCR.
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](image_url)

**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 for 24 h. (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.
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...
Cancer (CRC) tissues compared to adjacent tissues, and downregulation of DDX46 markedly suppressed CRC cell proliferation. 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.

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. 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. Compelling evidence suggests that DDX46, through its relationship with EMT-related molecules, might serve as a potential therapeutic target for osteosarcoma.

**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.
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. Akt, a member of the AGC serine–threonine kinase family, is a major signaling molecule downstream of PI3K. The activation of Akt further phosphorylates multiple proteins that regulate various cellular responses, including cell proliferation, metastasis, and the EMT process. So inhibition of the PI3K/Akt signaling pathway represents an attractive potential therapeutic approach for osteosarcoma. 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. 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.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

REFERENCES
1. Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004;9:422–41.
2. Ferrari S, Palmerini E. Adjuvant and neoadjuvant combination chemotherapy for osteogenic sarcoma. Curr Opin Oncol. 2007;19:341–6.
3. Isakoff MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: Current treatment and a collaborative pathway to success. J Clin Oncol. 2015;33:3029–35.
4. Mankin HJ, Hornicek FJ, Rosenberg AE, Harmon DC, Gebhardt MC. Survival data for 648 patients with osteosarcoma treated at one institution. Clin Orthop Relat Res. 2005;429:286–91.
5. Jaffe N. Osteosarcoma: Review of the past, impact on the future. The American experience. Cancer Treat Res. 2009;152:239–62.
6. Rocak S, Linder F. DEAD-box proteins: The driving forces behind RNA metabolism. Nat Rev Mol Cell Biol. 2004;5:232–41.
7. Will CL, Henning U, Tilmann A, Marc G, Matthias W, Reinhard L. Characterization of novel SF3b and 17S U snRNP proteins, including a human Prp5p homologue and an SF3b DEAD-box protein. EMBO J. 2002;21:4978–88.
8. Hirabayashi R, Hozumi S, Higashijima S, Kikuchi Y. DDX46 is required for multi-lineage differentiation of hematopoietic stem cells in zebrafish. Stem Cells Dev. 2013;22:2532–42.
9. Hozumi S, Hirabayashi R, Yoshizawa A, Ogata M, Ishitani T, Tsutsumi M, Kuroiwa A, Itoh M, Kikuchi Y. DEAD-box protein Ddx46 is required for the development of the digestive organs and brain in zebrafish. PLoS One 2012;7:e33675.
10. Li B, Li YM, He WT, Chen H, Zhu HW, Liu T, Zhang JH, Song TN, Zhou YL. Knockdown of DDX46 inhibits proliferation and induces apoptosis in esophageal squamous cell carcinoma cells. Oncol Rep. 2016;36:223–30.
11. Admoni-Elisha L, Nakdimon I, Shteinfer A, Prezma T, Arif T, Arbel N, Melkov A, Zelichov O, Levi L, Shoshan-Barmatz V. Novel biomarker proteins in chronic lymphocytic leukemia: Impact on diagnosis, prognosis and treatment. PLoS One 2016;11:e0148500.
12. Li M, Ma Y, Huang P, Du A, Yang X, Zhang S, Xing C, Liu F, Cao J. Lentiviral DDX46 knockdown inhibits growth and induces apoptosis in human colorectal cancer cells. Gene 2015;560:237–44.
13. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: New insights in signaling, development, and disease. J Cell Biol. 2006;172:973–81.
14. Yang G, Yuan J, Li K. EMT transcription factors: Implication in osteosarcoma. Med Oncol. 2013;30:697–701.
15. Ishikawa T, Shimizu T, Ueki A, Yamaguchi SI, Onishi N, Sugithara E, Kuninaka S, Miyamoto T, Morioha K, Nakayama R. TWIST2 functions as a tumor suppressor in murine osteosarcoma cells. Cancer Sci. 2013;104:880–8.
16. Machado I, López-Guerrero JA, Navarro S, Alberghini M, Scotlandi K, Picci P, Llombart-Bosch A. Epithelial cell adhesion molecules and epithelial mesenchymal transition (EMT) markers in Ewing’s sarcoma family of tumors (ESFTs). Do they offer any prognostic significance? Virchows Arch. 2012;461:337–7.
17. Brader S, Eccles SA. Phosphoinositide 3-kinase signaling pathways in tumor progression, invasion and angiogenesis. Tumori 2004;90:2–8.
18. Wong KK, Engelman JA, Cantley LC. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev. 2010;20:87–90.
19. Zhang J, Yu XH, Yan YM, Wang C, Wang WJ. PI3K/Akt signaling in osteosarcoma. Clin Chim Acta 2015;444:182–92.
20. Yang L, Shu T, Liang Y, Gu W, Wang C, Song X, Fan C, Wang W. GDC-0152 attenuates the malignant progression of osteosarcoma promoted by ANGPTL2 via PI3K/AKT but not p38MAPK signaling pathway. Int J Oncol. 2015;46:1651–8.
21. Cohen-Solal KA, Boregowda RK, Lasfar A. RUNX2 and the PI3K/AKT axis reciprocal activation as a driving force for tumor progression. Mol Cancer 2015;14:1–10.
22. Manning BD, Cantley LC. AKT/PI3K signaling: Navigating downstream. Cell 2007;129:1261–74.
23. Bellacosa A, Kumar CC, Cristofano AD, Testa JR. Activation of Akt kinases in cancer: Implications for therapeutic targeting. Adv Cancer Res. 2005;94:29–86.
24. Li YJ, Dong BK, Fan M, Jiang WX. BTG2 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt pathway. Int J Clin Exp Pathol. 2015;8:12410–8.
25. Zhang A, He S, Sun X, Ding L, Bao X, Wang N. Wnt5a promotes migration of human osteosarcoma cells by triggering a phosphatidylinositol-3 kinase/Akt signals. Cancer Cell Int. 2014;14:612–3.
26. Dong Y, Liang G, Yuan B, Yang C, Gao R, Zhou X. MALAT1 promotes the proliferation and metastasis of osteosarcoma cells by activating the PI3K/Akt pathway. Tumour Biol. 2015;36:1477–86.
27. Zhou Y, Zhu LB, Peng AF, Wang TF, Long XH, Gao S, Zhou RP, Liu ZL. LY294002 inhibits the malignant phenotype of osteosarcoma cells by modulating the phosphatidylinositol 3-kinase/Akt/fatty acid synthase signaling pathway in vitro. Mol Med Rep. 2014;11:1352–7.