Downregulation of Ubiquitin-Specific Protease 22 Inhibits Proliferation, Invasion, and Epithelial–Mesenchymal Transition in Osteosarcoma Cells

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

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

Ubiquitin-specific protease 22 (USP22), a novel deubiquitinating enzyme, belongs to an extended family of proteins that have ubiquitin hydrolase activity. Recently, USP22 has attracted widespread attention because of its implication in carcinogenesis. However, there have been no studies, to our knowledge, investigating the expression of USP22 in osteosarcoma (OS) and its association with OS progression. In this study, we explored the role of USP22 in OS. We demonstrated that USP22 was highly expressed in OS tissue and cell lines. Downregulation of USP22 inhibited OS cell proliferation, invasion, and epithelial–mesenchymal transition (EMT) in vitro. In addition, downregulation of USP22 suppressed OS tumor growth and metastasis in vivo. We also found that the PI3K/Akt signaling pathway was involved in the tumor-promoting effect of USP22 on OS progression. Taken together, we suggest USP22 as a novel therapeutic target for OS.

Key words: Ubiquitin-specific protease 22 (USP22); Proliferation; Invasion; Epithelial–mesenchymal transition (EMT); Osteosarcoma (OS)

INTRODUCTION

Osteosarcoma (OS) is a common type of bone tumor and accounts for a not insignificant proportion of malignant tumors in children1–3. With the origin of primitive bone-forming mesenchymal stem cells, OS frequently starts from the metaphysis of long bones4,5. In the past, surgical resection dominated OS therapies, leading to poor prognosis of OS patients6. OS therapies have recently developed to a great degree and include neoadjuvant chemotherapies that are performed with cisplatin, ifosfamide, doxorubicin, and methotrexate7,8. The application of chemotherapies has increased the 5-year survival rate of OS patients to about 80% but has produced adverse effects such as renal and cardiac toxicity, suppression of bone marrow, and gastrointestinal problems1,7,9. Therefore, the development of a novel therapy for OS is urgently needed.

Ubiquitin-specific protease 22 (USP22), a novel deubiquitinating enzyme, belongs to an extended family of proteins having ubiquitin hydrolase activity10. First identified in a microarray-based study by Glinsky et al., USP22 is considered to be important in many physiological and pathological processes such as cell cycle, cell proliferation, and tumor invasion10–14. It has been found that USP22 is frequently overexpressed in different types of cancers15–17. Furthermore, the elevated expression of USP22 has proven to be associated with tumor recurrence, distant metastasis, poor prognosis, and therapeutic failure in various cancer patients18–20. Because of its significant role in cancer, USP22 has been given a lot of attention and has been extensively investigated. However, the expression pattern and biological significance of USP22 in OS remain largely unknown.

In this study, we investigated the role of USP22 in OS. We reported that USP22 was upregulated in OS tissues and cell lines. USP22 downregulation inhibited OS cell proliferation, invasion, and epithelial–mesenchymal transition (EMT) in vitro. It also suppressed OS tumor growth and metastasis in vivo. In addition, we demonstrated that USP22 functioned as a tumor promoter in OS by suppressing the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Patients and Tissue Samples

Twenty-eight pairs of OS tissues and matched non-cancerous bone tissues were obtained from OS patients who were admitted to the Huaihe Hospital of Henan University (Kaifeng, P.R. China). Each patient taking part in the study provided written consent. All tissue samples
were frozen in liquid nitrogen and stored at −80°C before use. The study was approved by the ethics committee of the Huaihe Hospital of Henan University.

**Cell Lines and Cell Culture**

Human OS cell lines (U2OS and MG-63) and osteoblastic cell line hFOB were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco).

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

Total RNA was extracted from tissues or cells with the TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA) and then reversely transcribed into cDNA using the Primer Script Kit (TaKaRa, Dalian, P.R. China). RT-PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 30 s, 35 cycles of 95°C for 15 s and 60°C for 30 s. The PCR primers were as follows: USP22, 5’-CCATTGATCTGATGTACGGAGG-3’ (forward) and 5’-TCCTTGGCGATTATTTCCATGTC-3’. The mRNA and protein expression levels of USP22 were marked-
dly increased in OS tissues compared with the corresponding normal tissues. The expression of USP22 was much higher in the OS cell lines U2OS and MG-63 than in the osteoblastic cell line hFOB at both mRNA and protein levels. *p<0.05.

![Figure 1](image-url). USP22 was upregulated in OS tissues and cell lines. (A, B) The mRNA and protein expression levels of USP22 were markedly increased in OS tissues compared with the corresponding normal tissues. (C, D) The expression of USP22 was much higher in the OS cell lines U2OS and MG-63 than in the osteoblastic cell line hFOB at both mRNA and protein levels. *p<0.05.
(reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GAGTCACCGATTGCTGGT-3' (forward) and 5'-GACAAGCTTCCGTTCTCAG-3' (reverse). GAPDH was used as an internal control. Data analysis was performed through the 2-ΔΔCT method21.

Western Blot Analysis

Lysis buffer was used to extract total protein from tissues and cells. After centrifugation of lysates at 13,200 rpm for 10 min, the supernatants were collected for Western blot analysis. The protein was resolved by 12% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Subsequent to blocking with nonfat milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against USP22, E-cadherin, N-cadherin, vimentin, p-Pi3K, Pi3K, p-Akt, Akt, or GAPDH. After washing with TBST three times, the membranes were incubated with HRP-conjugated secondary antibody. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Protein bands were visualized by enhanced chemiluminescence. The protein expression was analyzed using an Odyssey infrared laser imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Small Interfering RNA (siRNA) and Transfection

The siRNA method was used for USP22 down-regulation in U2OS and MG-63 cells. USP22 siRNA was purchased from RiboBio (Guangzhou, P.R. China), and the sequence was 5'-GGAGAAGAGUACCCUGAA dTdT-3'. Ncontrol_05815 (NCsi; RiboBio) was used as a negative control. U2OS and MG-63 cells were transfected with USP22si or NCsi using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The transfection efficiency was determined via Western blot analysis.

MTT Assay

Cell proliferation was tested using an MTT assay. Transfected cells were seeded into 96-well plates at a density of 5×10⁴ cells/well, followed by incubation for 24, 48, 72, and 96 h, respectively. After 25 μl of MTT (5 mg/ml; Sigma-Aldrich) was added to each well, cells were further incubated for 4 h. Subsequently, supernatants were removed, and 150 μl of DMSO (Sigma-Aldrich) was added to each well. The absorbance value (OD) was measured at a wavelength of 450 nm.

Transwell Assay

Transwell chambers (8-μm pore size; Costar, Cambridge, MA, USA) were used for the cell invasion assay. Briefly, 5×10⁴ transfected cells were suspended in RPMI medium and seeded into the Matrigel-coated upper chamber. RPMI medium (500 μl) containing 10% FBS was added to the lower chamber. After incubation for 24 h with 5% CO₂ at 37°C, cells invading the lower surface of the filters were fixed with cold methanol and stained with 0.1% crystal violet. The number of invading cells was counted in four randomly selected fields under a microscope (400×).

In Vivo Xenograft Tumor Assay

Male BALB/c nude mice (4 to 6 weeks old) were purchased from the Experimental Animal Center of Henan University and maintained under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of Henan University. U2OS cells (1×10⁵) transfected with USP22si or NCsi were suspended in 200 μl of PBS and subcutaneously injected into the left flank of nude mice (n=6 per group). Tumor volume was measured every 7 days and calculated by the following formula: tumor volume=(length×width²)/2. After 35 days, mice were euthanatized, and the tumors were weighed.

To test tumor metastasis in vivo, 1×10⁵ transfected U2OS cells were suspended in 100 μl of PBS and injected into the lateral tail vein of nude mice (n=6 per group). Thirty-five days later, mice were sacrificed to evaluate lung metastasis. The number of lung nodules was counted under a dissecting microscope.

Statistical Analysis

Data were shown as means±standard deviation (SD). Differences were analyzed by the Student’s t-tests. Statistical analysis was performed using SPSS 19.0 software (Chicago, IL, USA). A value of p<0.05 was considered statistically significant.

RESULTS

USP22 Was Upregulated in OS Tissues and Cell Lines

To investigate the role of USP22 in OS, we first examined the expression levels of USP22 in OS tissues via RT-PCR and Western blot analysis. The results indicated that USP22 was significantly upregulated in OS tissues compared with their corresponding normal tissues (Fig. 1A and B). In addition, we detected the expression of USP22 in the OS cell lines U2OS and MG-63. The expression levels of USP22 were remarkably increased in OS cell lines compared with the osteoblastic cell line hFOB (Fig. 1C and D). These results showed that USP22 was upregulated in both OS tissues and cell lines.

Downregulation of USP22 Inhibited OS Cell Proliferation and Invasion In Vitro

To explore the effect of USP22 on OS cell proliferation and invasion, we decreased the expression of
USP22 depletion suppressed cell survival and proliferation in vitro. We performed in vivo experiments to verify the in vitro results. As expected, downregulation of USP22 suppressed OS tumor growth and metastasis in vivo. The findings in our study agreed with the fact that a growing body of evidence indicated the suppressive effect of USP22 knockdown on malignant behavior of cancer cells. For instance, Ding et al. demonstrated that USP22 silencing inhibited in vitro proliferation and in vivo tumor growth of non-small cell lung cancer cells. Similarly, Zhao et al. reported that USP22 depletion suppressed cell survival and proliferation as well as tumor growth and lung metastasis of anaplastic thyroid carcinoma cells. These observations support the notion that USP22 acts as an oncogene in cancer development.

It is well known that the PI3K/Akt signaling pathway is a player in the regulation of cell proliferation, migration, and invasion of diverse cancers. PI3K is activated by oncoproteins, and activation of PI3K contributes to cancer cell growth and survival. Akt, a key molecule in the PI3K pathway, is frequently found to...
Figure 2. Downregulation of USP22 inhibited OS cell proliferation and invasion in vitro. (A, B) USP22 downregulation in U2OS and MG-63 cells was confirmed by Western blot analysis. (C, D) USP22 downregulation significantly inhibited the proliferative ability of U2OS and MG-63 cells. (E, F) USP22 downregulation obviously reduced the number of invading U2OS and MG-63 cells. *p<0.05.
Figure 3. Downregulation of USP22 inhibited OS tumor growth and metastasis in vivo. (A, B) USP22 downregulation significantly decreased the tumor volume and weight of the U2OS/USP22si group compared with the U2OS/NCsi group. (C) The number of lung nodules was obviously reduced in the U2OS/USP22si group compared with the U2OS/NCsi group. *p<0.05.
be activated in cancers, impacting various downstream targets. Thus, the PI3K/Akt signaling pathway is considered to be a potential target for cancer therapies. More importantly, the PI3K/Akt signaling pathway is found to play a significant role in OS progression. Therefore, we inferred that USP22 downregulation exerted the suppressive effect on OS cells via inactivating the PI3K/Akt signaling pathway. To prove our hypothesis, we detected the protein expression levels of p-PI3K, PI3K, p-Akt, and Akt after USP22 downregulation. The results showed that USP22 downregulation remarkably decreased the protein expression of p-PI3K and p-Akt without change in the total protein levels of PI3K and Akt. In addition, we tested the effect of an Akt inhibitor (MK-2206) on USP22si-mediated OS cell invasion, finding that MK-2206 significantly potentiated USP22si-inhibited OS cell invasion. These results suggest that USP22 downregulation inhibits OS cells by suppressing the PI3K/Akt pathway. Considering the complex relationship affecting cancer development, the mechanisms behind the oncogenic role of USP22 in OS need to be studied further.

In conclusion, we demonstrated that USP22 was highly expressed in OS tissues and cells lines. Downregulation of USP22 inhibited OS cell proliferation, invasion, and EMT in vitro. In addition, downregulation of USP22

Figure 4. Downregulation of USP22 inhibited the EMT process in OS cells. (A) The protein expression levels of EMT-related markers in U2OS cells were detected by Western blot. (B) The protein expression of EMT-related markers in U2OS cells was quantified by an Odyssey infrared laser imaging system. *p<0.05.
suppressed OS tumor growth and metastasis in vivo. We also found that the PI3K/Akt signaling pathway was involved in the tumor-promoting effect of USP22 on OS progression.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

REFERENCES
1. Damron TA, Ward WG, Stewart A. Osteosarcoma, chondrosarcoma, and Ewing’s sarcoma: National Cancer Data Base Report. Clin Orthop Relat Res. 2007;459:40–7.
2. Heare T, Hensley MA, Dell’Orfano S. Bone tumors: Osteosarcoma and Ewing’s sarcoma. Curr Opin Pediatr. 2009;21:365–72.
3. Picci P. Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis. 2007;2:1–4.
4. Benayahu D, Shur I, Marom R, Mellor I, Issakov J. Cellular and molecular properties associated with osteosarcoma cells. J Cell Biochem. 2001;84:108–14.
5. Klein MJ, Siegal GP. Osteosarcoma: Anatomic and histologic variants. Am J Clin Pathol. 2006;125:555–81.
6. Zhao H, Li M, Li L, Yang X, Lan G, Zhang Y. MiR-133b is downregulated in human osteosarcoma and inhibits osteosarcoma cells proliferation, migration and invasion, and promotes apoptosis. PLoS One 2013;8:e83571.
7. Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. Cancer Treat Res. 2009;152:3–13.
8. Tan ML, Choong PF, Dass CR. Osteosarcoma: Conventional treatment vs. gene therapy. Cancer Biol Ther. 2009;8:106–17.
9. Wenzong Z, Qingjun L, Zhida C, Xinyu W, Yuanfu Z, Jia W. Silencing of HEG1 gene inhibits proliferation and invasion, and induces apoptosis in human osteosarcoma cells by targeting the NF-kB pathway. J Cancer 2016;7:746–57.
10. Lee HJ, Kim MS, Shin JM, Park TJ, Chung HM, Baek KH. The expression patterns of deubiquitinating enzymes, USP22 and Usp22. Gene Expr Patterns 2006;6:277–84.
11. Glinsky GV, Berezovskaya O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. J Clin Invest. 2005;115:1503–21.
12. Glinsky GV. Genomic models of metastatic cancer: Functional analysis of death-from-cancer signature genes reveals aneuploid, aneikis-resistant, metastasis-enabling phenotype with altered cell cycle control and activated Polycomb group (PcG) protein chromatin silencing. Cell Cycle 2006;5:1208–16.
13. Zhao Y, Lang G, Ito S, Bonnet J, Metzger E, Sawatsubashi S, Suzuki E, Le Guezennec X, Stunnenberg HG, Krasnov A, Georgiev SG, Schüle R, Takeyama K, Kato S, Tora L, Devys DA. TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. Mol Cell 2008;29:92–101.
14. Zhang XY, Varthi M, Sykes SM, Phillips C, Warzecha C, Zhu W, Wyce A, Thorne AW, Berger SL, McMahon SB. The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. Mol Cell 2008;29:102–11.
15. Yan LL, Yan MY, Hui X, Xin SD. Increased expression of ubiquitin-specific protease 22 can promote cancer progression and predict therapy failure in human colorectal cancer. J Gastroenterol Hepatol. 2010;25:1800–5.
16. Hu J, Liu YL, Piao SL, Yang DD, Yang YM, Cai L. Expression patterns of USP22 and potential targets BMI-1, PTEN, p-AKT in non-small-cell lung cancer. Lung Cancer 2012;77:593–9.
17. Ning J, Zhang J, Liu W, Lang Y, Xue Y, Xu S. Overexpression of ubiquitin-specific protease 22 predicts poor survival in patients with early-stage non-small cell lung cancer. Eur J Histochem. 2012;56:289–94.
18. Liu Y, Yang Y, Xu H, Dong X. Implication of USP22 in the regulation of BMI-1, c-Myc, p16INK4a, p14ARF, and cyclin D2 expression in primary colorectal carcinomas. Diagn Mol Pathol. 2010;19:194–200.
19. Zhang Y, Yao L, Zhang X, Ji H, Wang L, Sun S, Pang D. Elevated expression of USP22 in correlation with poor prognosis in patients with invasive breast cancer. J Cancer Res Clin Oncol. 2011;137:1245–53.
20. Yang DD, Cui BB, Sun LY, Zheng HQ, Huang Q, Tong JX, Zhang QF. The co-expression of USP22 and BMI-1 may promote cancer progression and predict therapy failure in gastric carcinoma. Cell Biochem Biophys. 2011;61:703–10.
21. Brand TM, Iida M, Luthar N, Starr MM, Huppert EJ, Wheeler DL. Nuclear EGFR as a molecular target in cancer. Radiother Oncol. 2013;108:370–7.
22. Shih MC, Chen JY, Wu YC, Yan YH, Yang BM, Lu PJ, Cheng HC, Huang MS, Yang CJ, Hsiao M. TOPK/PBK promotes cell migration via modulation of the PI3K/PTEN/AKT pathway and is associated with poor prognosis in lung cancer. Oncogene 2012;31:2389–400.
23. Liu YL, Yang YM, Hui XM, Dong XS. Aberrant expression of USP22 is associated with liver metastasis and poor prognosis of colorectal cancer. J Surg Oncol. 2011;103:283–9.
24. Ding F, Bao C, Tian Y, Xiao H, Wang M, Xie X, Hu F, Mei J. USP22 promotes NSCLC tumorigenesis via MDM2 upregulation and subsequent p53 inhibition. Int J Mol Sci. 2015;16:307–20.
25. Zhao HD, Tang HL, Liu NN, Zhao YL, Liu QQ, Zhu XS, Jia LT, Gao CF, Yang AG, Li JT. Targeting ubiquitin-specific protease 22 suppresses growth and metastasis of anaplastic thyroid carcinoma. Oncotarget 2016;7:31191–203.
26. Qiao M, Chang X, Jing F, Rojanasakul Y, Jiang BH. Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion and proliferation through the p70S6K1 pathway. Cell Signal 2006;18:2262–71.
27. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase pathway. Cell Signal 2006;18:2262–71.
28. Wheeler DL. Nuclear EGFR as a molecular target in cancer. Radiother Oncol. 2013;108:370–7.
29. Benayahu D, Shur I, Marom R, Mellor I, Issakov J. Cellular and molecular properties associated with osteosarcoma cells. J Cell Biochem. 2001;84:108–14.
30. Klein MJ, Siegal GP. Osteosarcoma: Anatomic and histologic variants. Am J Clin Pathol. 2006;125:555–81.