Original Article
Knockdown of WWP1 inhibits growth and invasion, but induces apoptosis of osteosarcoma cells

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Abstract: Recent studies have shown that WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is frequently amplified in various cancers. However, the role of WWP1 in osteosarcoma has not yet been studied. Here, we analyzed the mRNA levels of WWP1 in 25 pairs of osteosarcoma and adjacent non-tumorous samples. We found that WWP1 were higher in 88% osteosarcoma tissues as compared with their matched normal bone tissues. Knockdown of WWP1 using small interfering RNA further showed that deficiency of WWP1 blocked cell growth and cell invasion, and caused G1-phase arrest and cell apoptosis in osteosarcoma cells (MG63 and HOS). Furthermore, knocking down WWP1 affected the protein levels of apoptosis (Bcl2 and Bax) and invasion related factor (MMP2, MMP9, β-catenin and E-cadherin). These results suggest that WWP1 might be an oncogene and shed lights on targeted therapy of osteosarcoma.

Keywords: WWP1, osteosarcoma, apoptosis, invasion

Introduction

Osteosarcoma, the most common malignant bone tumor in children and adolescents, accounts for 2.4% of all malignancies in pediatric patients and around 20% of all primary bone cancers [1]. Osteosarcoma has a high tendency for metastasis. Patients who present with metastatic disease or whose tumor recurs, the survival was less than 30% and 20%, respectively [2]. Although considerable progress has been obtained in chemotherapy and surgery, the current therapy strategies for the patients with metastatic osteosarcoma have not significantly improved the clinical outcome of patients with metastatic osteosarcoma.

The ubiquitin/proteasome system is involved in fundamental cellular mechanisms like cell proliferation, differentiation and carcinogenesis. E3 ubiquitin ligase, the most essential component in ubiquitin/proteasome system, recognizes the substrate and targets it for degradation [3]. Genetic data have suggested that several E3 ubiquitin ligases could function as an oncogenic proteins [4-6]. WW domain containing E3 ubiquitin protein ligase 1 (WWP1) belongs to the C2-WW-HECT type E3 ubiquitin ligase family [7]. As an E3 ligase, WWP1 interacts with a variety of substrate proteins, such as ErbB4/HER4 [8, 9], KLF5 [10], JunB [11], p53 [12], p27 [13] and Smad4 [14], and participates in multiple physiological and pathological processes. WWP1 has been found overexpressed in human cancers, such as breast cancer [15], prostate cancer [14], hepatocellular carcinoma [16] and oral cancer [17]. WWP1 knockdown significantly suppresses cell proliferation and/or induces apoptosis of cancer cells [14-17]. However, whether WWP1 plays a role in tumorigenesis and progression of osteosarcoma have not been explored.

Here, we found that WWP1 mRNA was elevated in osteosarcoma tissues compared with normal bone tissues. We then investigate the effects of WWP1 on osteosarcoma development by the knockdown its expression in two osteosarcoma cells (MG63 and HOS). WWP1 knockdown significantly inhibited cell growth and cell invasion,
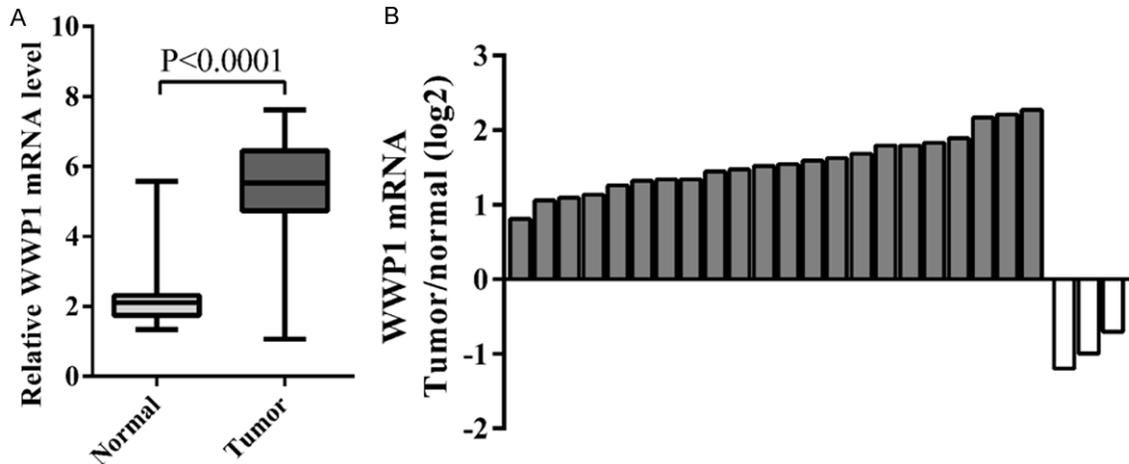
Figure 1. WWP1 was overexpressed in osteosarcoma tissues. A. WWP1 mRNA level was detected in osteosarcoma and normal tissues (n=25) by real-time PCR. B. Positive log2 (Tumor/Normal) on the y-axis indicated the increase of WWP1 expression in tumor tissue while negative log2 indicated the decrease of WWP1 expression in tumor tissue.

but induced cell apoptosis. Taken together, our findings provide the first evidence for the involvement of WWP1 in the pathophysiology of osteosarcoma.

Materials and methods

Patients and tissue samples

This study was completed with the approval of the independent ethics committee of Shanghai tenth People's Hospital, Tongji University School of Medicine (Shanghai, China). Written informed consents were obtained from all participants. From 2010 to 2012, 25 conventional osteosarcoma patients admitted to Department of Orthopedics, Shanghai tenth People's Hospital, Tongji University School of Medicine were enrolled in this study. All patients have complete clinical and pathological follow-up data. Normal bone tissues were resected within at least 5 cm of the tumor margin when the patients underwent definitive surgery.

Cell culture

All cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained at 37°C in a 5% CO_2 atmosphere. All culture media were supplemented with 10% fetal bovine serum (FBS, life technology, Grand Island, NY, USA) and 1% antibiotic (penicillin/streptomycin, life technology). MG63, HOS, Saos2 and SW1535 cells were grown in DMEM

Medium. U2OS cells were grown in RPMI 1640 medium.

Silencing of WWP1 by small interfering RNA

siRNA was designed to target the coding sequence of human WWP1 cDNA. The sequences of siRNAs were as follows: siWWP1, 5'-GGAGGCGCUUAUAUGUAAU-3'; siNC (control scrambled siRNA), 5'-UUGUACUACACAAAAGU-ACUG-3'. siRNAs duplexes were purchased from GenePharma (Shanghai, China). MG63 and HOS cells were transiently transfected with small interfering RNA (siRNA) duplexes using the lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Assays were performed 48 h after transfection.

Real-time PCR experiments

Total RNA from tissues or cultured cells was extracted using TRIzol Reagent (Invitrogen) and reverse transcribed into cDNA using the Superscript III enzyme (Invitrogen). Real-time PCR was performed on an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Cycle parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s. The gene expression was calculated using the ΔΔ Ct method. All data represent the average of three replicates. The primers used were list as follows: WWP1 (NM_0-07013.3), 5'-GAAGGCACGAATGGAATAG-3' and 5'-GTGACAGACGCATTATCAG-3'; GAPDH (N-M_
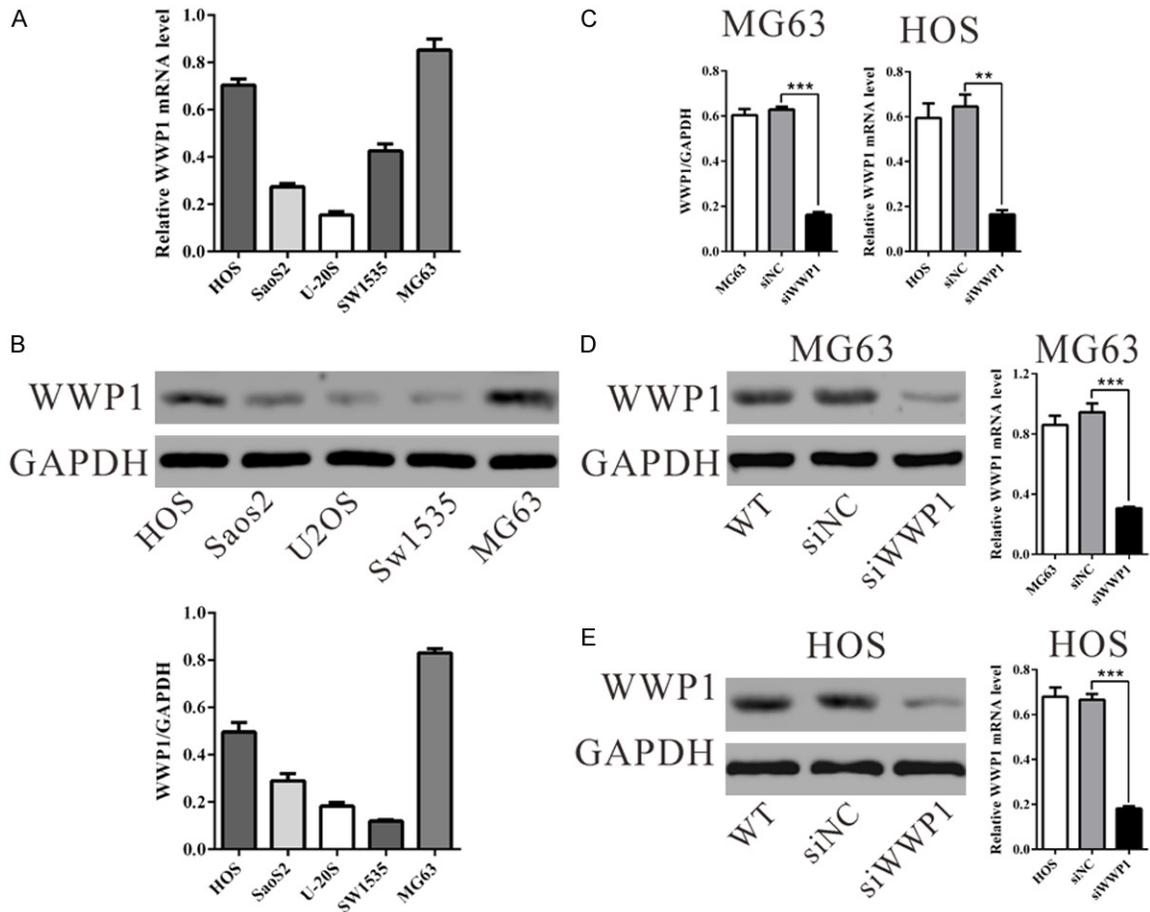
Figure 2. WWP1 gene knockdown by RNA interference. A. Real-time PCR analysis of WWP1 expression in five osteo-sarcoma cell lines. B. Western blot analysis of WWP1 and GAPDH expression in osteosarcoma cells. The representa-tive images were shown on the upper panel, and data from 3 independent experiments were expressed as the mean ± S.D. (lower panel). C. Real-time PCR analysis of MG63 and HOS cells transiently transfected with siNC or siWWP1. D and E. Western blot analysis of WWP1 and GAPDH expression in MG63 and HOS cells transiently transfected with siNC or siWWP1. The representative images were shown on the left panel, and data from 3 independent experi-ments were expressed as the mean ± S.D. (right panel, **$P<0.01$, ***$P<0.001$).

001256799.1), 5'-CACCCACTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTTGCTGTAG-3').

SDS-PAGE and Western blotting

Cell lysates were prepared with radioimmuno-precipitation assay buffer. Aliquots of the cell lysates (30 μg) were separated by SDS-PAGE and blotted onto nitrocellulose membrane (Millipore, Bredford, USA). The blots were blocked with 5% nonfat dry milk in PBS over-night and incubated with primary antibodies for 1 hour at room temperature, followed by appro-priate secondary antibodies for 1 hour at room temperature. The labeled bands were revealed by chemiluminescence using enhanced chemi-luminescence detection reagents (ECL, Milli-

pore) and exposed to Kodak X-ray film. Density of each band was quantitated with NIH Image software. Antibodies against E-cadherin, β-catenin and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against WWP1, MMP2 and MMP9 were from Abcam (Cambridge, MA, USA). Antibodies against Bcl2 and Bax were from Santa Cruz (Santa Cruz, CA, USA).

Cell proliferation assay

Cell proliferation was detected by using the Cell Count Kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan). Briefly, cells were treated with various siRNA and 48 h later, cells were seeded to 96-well plates in 0.2 ml of complete medium
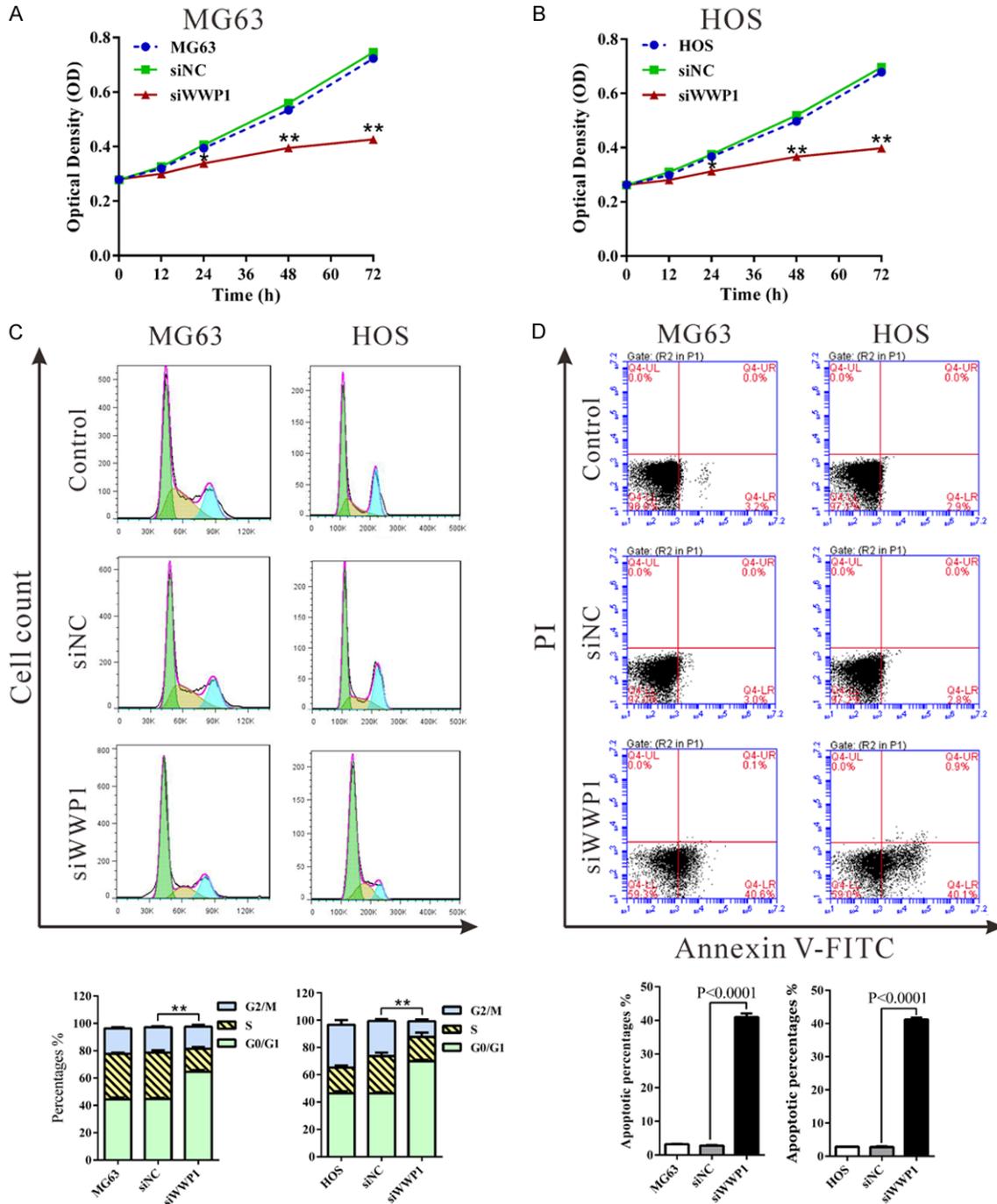
Figure 3. WWP1 knockdown suppressed osteosarcoma cells proliferation. A and B. WWP1 knockdown in MG63 and HOS cells significantly reduced their proliferative capacities, as determined by CCK-8 assay. C and D. Silencing of WWP1 in MG63 and HOS cells resulted in cell arrest in G1 phase of cell cycle and a dramatic increase of apoptosis. Representative images were shown on the upper panel, and data from 3 independent experiments were expressed as the mean ± S.D. (lower panel, *P<0.05, **P<0.01).

(3×10^3 cells per well). At indicated time point, CCK-8 solution was added to each well and incubated for 1 h. The optical density (OD) of each well at 450 nm was recorded on a micro-plate reader (Bio-Rad, Richmond, CA, USA). Three wells were measured for cell viability in each treatment group, and all independent treatments were performed in triplicate.
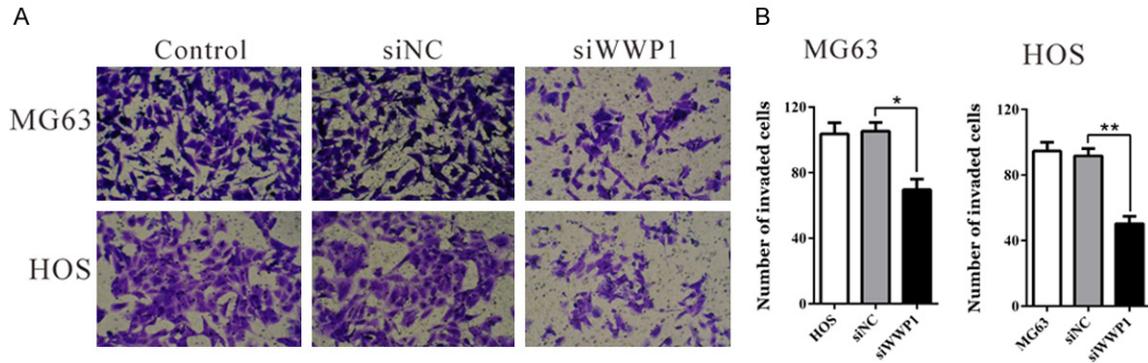
Figure 4. Suppressing WWP1 expression inhibited cell invasion in osteosarcoma cells. A. Invasion assay was performed in Matrigel-coated transwell chambers. Cells that migrated from the upper well into the lower well were stained, photographed and counted. Representative images of cell invasion assay were shown. B. Data from 3 independent experiments were expressed as the mean ± S.D. (*$P<0.05$, **$P<0.01$).

Flow cytometric assay of cell cycle and apoptosis

Cells were harvested and fixed with cold 70% ethanol stored overnight at -20°C, stained with in PI staining buffer (1 mg/ml RNase A, 0.1% Triton X-100, 50 mg/ml PI in PBS) and analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Experiments were performed in triplicate. G1, S, and G2/M fractions were quantified with the FlowJo software (Tree Star).

Cells were stained with Annexin V-FITC (BD Biosciences) in Annexin V staining buffer for 15 min at 4°C and counterstained with 50 mg/ml PI (Sigma) and analyzed with a flow cytometer. The experiments were performed in triplicate.

In vitro invasion assay

The in vitro invasion assay was performed using Transwell cell culture chambers (Corning Costar, Corning, NY, USA) separated by 8.0-μm pore filter precoated with Matrigel (BD Biosciences) on the upper surface. The bottom chamber was filled with DMEM supplemented with 10% FBS, and cell suspensions in serum-free medium (1×10^5 cells per well) were added onto the upper chamber. After 24 hours of incubation at 37°C, unmigrated cells were removed from the upper surface by a cotton swab, and migrated cells on the lower membrane surface was fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Viable cells were counted under a microscope. Each assay was done in triplicate.

Statistical analysis

The unpaired Student's t test was used to evaluate the statistical significance of the difference between two groups. The results were presented as the mean value ± S.D. Data were considered significant for *P*-value <0.05.

Results

Increased expression of WWP1 in human osteosarcoma tissues

Compared with normal bone tissues, osteosarcoma tissues exhibited significantly increased mRNA level of WWP1 (5.38±0.32 versus 2.23±0.18, n=25, $P<0.0001$, **Figure 1A**). The log2 ratio of tumor versus normal for WWP1 was then calculated. As shown in **Figure 1B**, positive log2 (Tumor/Normal), indicating the increased expression of WWP1, was observed in 88% (22/25) of tested osteosarcoma tissues.

Suppression of WWP1 expression in osteosarcoma cells by siRNA

We then assessed the expression of WWP1 in five osteosarcoma cell lines by real-time PCR (**Figure 2A**) and western blot (**Figure 2B**). To investigate the function of WWP1 in osteosarcoma, two cell lines, MG63 and HOS, which showed higher WWP1 mRNA and protein level, were transiently transfected with siRNA against WWP1 (siWWP1) or control scrambled siRNA (siNC). As shown in **Figure 2C-E**, siWWP1 markedly decreased WWP1 expression in both osteosarcoma cells, whereas WWP1 expression remained unaffected in siNC-transfected cells.
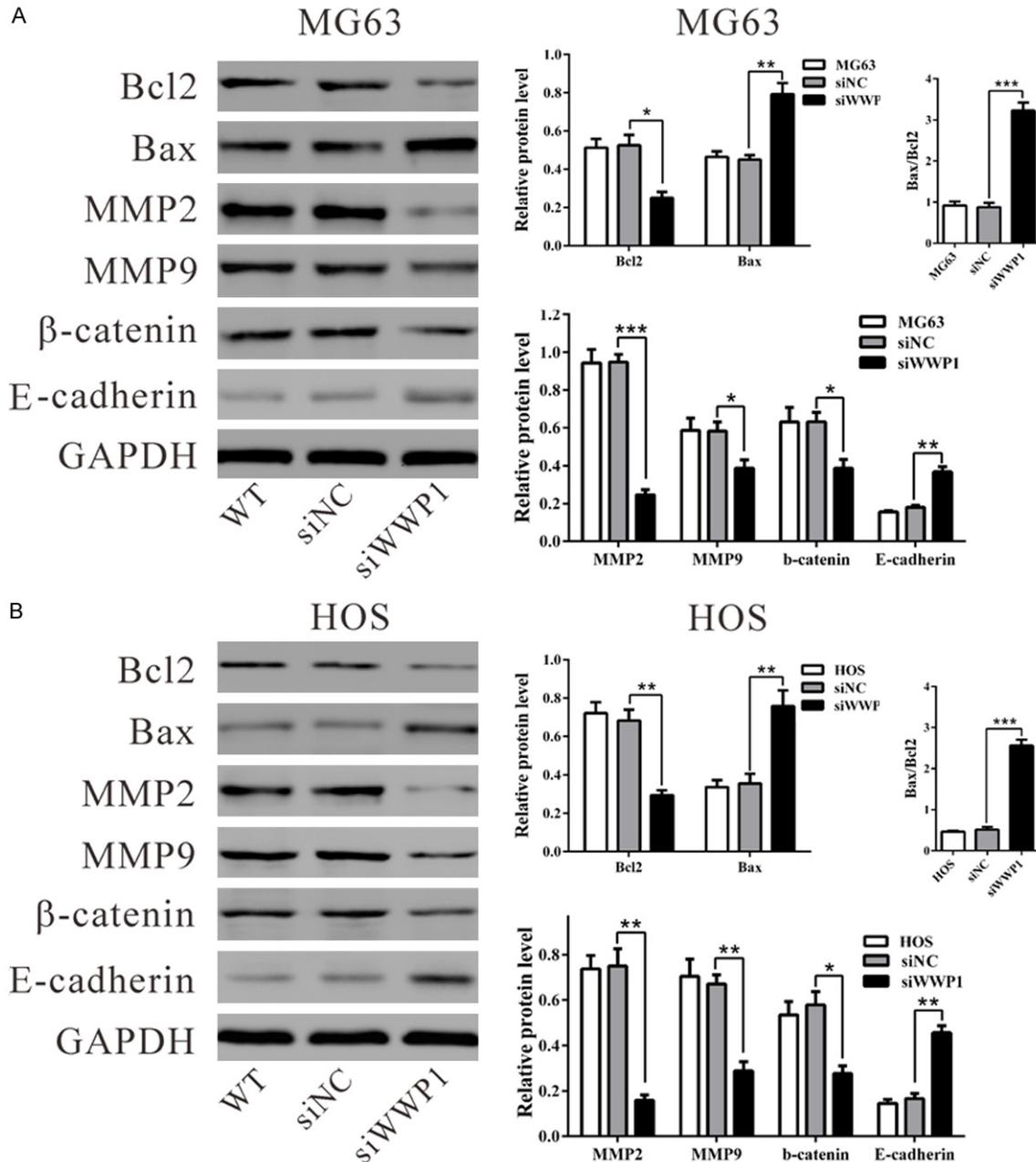
Figure 5. Effects of WWP1 knockdown on the expression of apoptosis and invasion-related factors. Western blot analysis was performed in MG63 (A) and HOS cells (B). Left panel, representative results of western blot; right panel, protein levels relative to GAPDH. Data were presented as the mean value from 3 independent experiments ± S.D. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

WWP1 silencing inhibited the rate of proliferation of osteosarcoma cells

To explore the effects of WWP1 silencing in cell proliferation, a fundamental process in tumor development, CCK8 assay was performed (**Figure 3A** and **3B**). The rate of proliferation was not significantly different between cells transfected with siNC and control cells, while

proliferation was remarkably reduced in siW-WP1-transfected cells at 24, 48 and 72 hours.

Silencing of WWP1 induced G1-phase arrest and cell apoptosis in osteosarcoma cells

We then determined the possible inhibitory effect of WWP1 knockdown on cell cycle progression. As shown in **Figure 3C**, FACS cell cycle

analyses of MG63 and HOS cells revealed that siWWP1 treatment increased the percentage of G1 phase of the cell cycle.

To examine the effects of WWP1 on cell apoptosis, Annexin V-FITC/propidium iodide (PI) staining of osteosarcoma cells (MG63 and HOS) was performed (**Figure 3B**). The results indicated that there were very few PI-positive and Annexin-V-positive cells in the control cells (3.2%) and siNC-transfected cells (2.8%). In comparison, approximately 41% of WWP1 siR-NA-treated MG63 cells were positive for Annexin-V labeling. Annexin V-FITC/PI staining was also completed for the HOS cells with 2.9% and 2.8% positive cells observed for control cells and siNC-transfected cells, respectively, while greater than 41% positive cells were noted for HOS cells transfected with WWP1 siRNA.

Knockdown of WWP1 inhibited invasion of osteosarcoma cells in vitro

The effect of siWWP1 on osteosarcoma cell invasion was evaluated using Matrigel-coated Transwell invasion assays. siWWP1 treatment decreased cell invasion by 55.4% and 33.3% in MG63 and HOS cells, respectively (**Figure 4**). These data suggested a role of WWP1 in the promotion of osteosarcoma metastasis.

Knockdown of WWP1 affected the expression of apoptosis and invasion-related factors

The expression levels of important factors to regulate cell apoptosis and invasion were then estimated by Western blot (**Figure 5**). WWP1 knockdown in both cells led to a significant decrease in the expression of the anti-apoptotic protein (Bcl2 [18]) and a notable increase in the expression of proapoptotic protein (Bax [18]) compared with the control cells, which may contribute to the induction of cell apoptosis.

The protein levels of three important invasion regulators (MMP2, MMP9 [19] and β-catenin [20]) were significantly down-regulated, while the anti-invasion protein (E-cadherin [21]) was remarkably increased in WWP1 knockdown cells.

Discussion

WWP1 expression is reported upregulated in various cancer tissues [14-17]. Consistent with the previous studies, we found that WWP1 mRNA expression was higher in 88% osteosarcoma tissues than their adjacent non-tumor tissues (**Figure 1**), suggesting the WWP1 might play an oncogenic role in osteosarcoma.

In order to investigate the function of WWP1 in osteosarcoma, WWP1 expression was suppressed in two osteosarcoma cells (MG63 and HOS cells) (**Figure 2**) and then subjected to cell proliferation analysis by CCK-8 assay (**Figure 3A** and **3B**). WWP1 knockdown significantly decreased cell proliferation from 24 to 72 hours, which was consistent with previous studies [14-17]. We then evaluated the cell cycle distribution and cell apoptosis of WWP1 knockdown cells by flow cytometry (**Figure 3C** and **3D**). Our results suggested that knockdown of WWP1 significantly induced G1-phase arrest and cell apoptosis, which was causes of the anti-proliferation effects of WWP1 siRNA and consistent with the findings in other type of cancer cell lines [15-17]. Further, the increase ratio of Bax/Bcl-2 was observed in WWP1 knockdown cells, indicating that WWP1 exerted an anti-apoptosis role in osteosarcoma cells (**Figure 5**).

Metastasis is a multi-step process, and local tumor cell invasion is one of the most essential step [22]. Since metastases are the leading cause of mortality in patients with osteosarcoma [2], we then try to explore whether WWP1 knockdown influence cell invasion. As shown in **Figure 4**, WWP1 siRNA treatment notably impaired the invasive ability of osteosarcoma cells. Matrix metalloproteinases (MMPs), especially MMP2 and MMP9, have been identified as important enzymes in metastasis [19]. Consistent with the decrease invasive ability of WWP1 knockdown cells, the expression of MMP2 and MMP9 were significantly decreased (**Figure 5**). Activation of epithelial-mesenchymal transition (EMT) is crucial for the invasion of cancer cells [23]. We then detected the expression of β-catenin [20] and E-cadherin [21], two important regulators in the induction of EMT during tumorigenesis. As shown in **Figure 5**, WWP1 knockdown resulted in a notably increase in E-cadherin expression and a remarkably decrease in β-catenin expression, which was in accordance with our data of *in vitro* invasion assay. These results suggested that WWP1 may promote cancer cell invasion via decreasing the expression of MMPs and inducing EMT. Further investigations are re-

quired to understand how WWP1 regulates MMPs and EMT.

In conclusion, we demonstrated the elevation of WWP1 expression in osteosarcoma tissues. Our in vitro functional assay suggests that WWP1 may act as a functional oncogene in osteosarcoma cell line. We provide mechanistic evidence that cell invasion inhibited by WWP1 siRNA was mediated through MMPs and EMT, which may provide useful information for targeted therapy.

Disclosure of conflict of interest

None.

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