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P-Glycoprotein Overexpression Is Associated With Cisplatin Resistance in Human Osteosarcoma

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Abstract. *Background/Aim: Osteosarcoma (OS) is a diagnosed primary cancer of the bone. Despite the great advances that have been made during the past decades in OS therapy, drug resistance and tumor recurrence are still major problems. It is urgent to find novel strategies to overcome drug resistance in order to prolong the survival time of OS patients. Materials and Methods: Cell viability was investigated by the cell count kit-8 (CCK-8) and colony formation assays. P-Glycoprotein (P-gp) expression was analyzed by RT-qPCR and western blot. A xenograft mouse model was used to identify the synergistic efficacy of a P-gp inhibitor with cisplatin. Student's t-test was used to determine statistically significant differences. Results: P-gp expression levels were associated with cisplatin efficacy in OS patients. OS cells with higher P-gp expression were more resistant to cisplatin. Knockdown or inhibition of P-gp sensitized OS cells to cisplatin. Conclusion: Down-regulating the expression of P-gp in OS maybe a promising strategy to overcome cisplatin resistance.*

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Osteosarcoma (OS) is a rare primary cancer of the bone that mainly affects adolescents (1). The main treatment of OS is surgery and adjuvant chemotherapy. First-line chemotherapy usually includes a combination of methotrexate, doxorubicin, cisplatin, and ifosfamide. Unfortunately, chemotherapy often fails due to the appearance of multidrug resistance (MDR) (2). The prognosis of OS remains unchanged for decades. The survival time in recurrent OS patients with drug resistance is approximately one year (3-5). Hence, it is critical to overcome MDR to prolong the survival time of OS patients (2, 6-8). The up-regulation of the drug efflux pump P-glycoprotein (P-gp) appears critical for MDR (8-10).

P-gp is coded by the gene *ABCB1* (or *MDR1*) and belongs to the ATP-binding cassette (ABC) membrane transport superfamily (11). P-gp has 12 transmembrane regions and two ATP-binding sites. This structure enables efflux of broad-spectrum drugs including positively-charged hydrophobic drugs (12). The substrates of P-gp include taxanes, vinca alkaloids, podophyllotoxins and anthracyclines (13). P-gp was found up-regulated in many cancers, such as breast cancer, neuroblastoma, acute lymphocytic leukemia (ALL), acute nonlymphocytic leukemia (ANLL), neuroblastoma and pheochromocytoma (14). Overexpressed P-gp will accelerate the excretion of drugs and attenuate chemotherapy efficacy against these tumors.

In our study, expression of P-gp was found to be higher in OS patient specimens resistant to cisplatin compared to those sensitive to cisplatin. Also, knockdown or inhibition of P-gp sensitized OS to cisplatin *in vitro* and *in vivo*. The results suggested that P-gp is a promising target for cisplatin -resistance in OS.

Materials and Methods

Tissue samples collection. The patients involved in this study had histologically confirmed OS and they provided informed consents. The study was performed following the regulations of the ethics

committee of the Shanghai Ninth People's Hospital. A total of 12 patients (4 cisplatin sensitive and 8 cisplatin resistant OS patients) were included.

Immunohistochemistry (IHC). IHC was produced as described (15). Briefly, after deparaffinization, rehydration, antigen retrieval and endogenous peroxidase blocking, sections were incubated with the primary antibody: anti-P-gp (Cell Signaling Technology, Danvers, MA USA) at 4˚C overnight. After rinsing, the secondary antibody was used for 20 min at room temperature and the sections were counterstained with hematoxylin (Sigma, St. Louis, MO, USA).

Cells. Human OS cell lines, HOS, U-2OS, Saos-2, and MG-63 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China. Cells were grown in DMEM (HyClone, Waltham, MA, USA) supplemented with 10% FBS (Gibco, Waltham, MA, USA) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and 5% CO_2 at 37˚C humidified atmosphere.

Cell viability assay. The assay was performed as described (16). In brief, cells were seeded in 96-well plates. After incubation for 72 h with different concentrations of cisplatin, with or without dofequidar fumarate (5 μM) (MedChemExpress, Shanghai, PR China) - an inhibitor of P-gp (17-20), we added 10 μl CCK-8 to each well and incubated at 37˚C for 2 h.

Colony formation assay. Cells were added in 6-well plates at 5×10^3 cells per well. After cell attachment, different concentrations of cisplatin (0 μM, 0.4 μM, 0.8 μM) with or without dofequidar fumarate (5 μM) were added. After 7 days, we fixed colonies and stained them with 0.1% crystal violet.

RNA extraction and RT-qPCR. The experiments were conducted as previously described (21), RNA was extracted by TRIzol Reagent (Takara, Shanghai, PR China). RT-qPCR was performed according to the protocols. Data were calculated as $2^{-\Delta\Delta Ct}$. The primer sequences are listed in Table I.

Western blot. An equal amount of protein was subjected to 10% dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to 0.22 μm polyvinylidene fluoride (PVDF) membranes. Then blocking the membranes in 10% non-fat milk at room temperature for 1 h, next incubating them with the primary antibodies, including anti-P-gp, anti-GAPDH overnight at 4˚C. The membranes were incubated with secondary antibody for 2 h at room temperature after 3 washes with Tris-buffered saline-Tween (TBST). The signal was visualized with an enhanced ECL detection kit (Beyotime, Shanghai, China).

shRNA. P-gp shRNAs were synthesized by Sangon Biotech (Shanghai, PR China) and cloned onto the pLVX plasmid. The sequences are shown in Table II. Non-silencing lentiviral shRNA vector was used as a negative control. To obtain stable P-gp knockdown cells, lentivirus supernatant was added to cells, which were then treated with 2 μg/ml puromycin for two weeks.

Mice. All mice were maintained in a specific pathogen-free (SPF) facility, and all animal experiments were conducted following the protocols approved by the ethics committee of the Shanghai Ninth People's Hospital. 2×10^6 HOS cells were suspended in sterile 100 μl PBS and injected subcutaneously into the flanks of 6-8-week-old male nude mice. After 7 days, the mice were divided into four groups randomly (n=5): Control; Dof. (dofequidar fumarate 200 mg/kg, p.o. q3d×6); Cis (cisplatin 8 mg/kg, i.p., q3d×6); Cis+Dof. (dofequidar fumarate 200 mg/kg, p.o. and cisplatin 8 mg/kg, i.p., q3d×6). Administration of dofequidar fumarate was 30 min before the i.p. administration of cisplatin. Tumor sizes were measured as 0.5 × length × width^2 every 7 days.

Table I. *Sequences of the RT-qPCR primers*.

P-gp-F	AATGCGACAGGAGATAGG
P-gp-R	TGTTGCCATTGACTGAAA
GAPDH-F	TCTGATTTGGTCGTATTGGG
GAPDH-R	GGAAGATGGTGATGGGATT

Table II. *shRNA Sequences Targeting P-gp*.

sh P-gp -1F	TTCCACTGTAATAATAGGC
sh P-gp -1R	GCCTATTATTACAGTGGAA
sh P-gp -2F	GCAGGAAATGAAGTTGAAT
sh P-gp -2R	ATTCAACTTCATTTCCTGC

Statistical analysis. All experiments were conducted three times. The mean, standard error of mean (SEM) and *p*-values were analyzed by Excel (Microsoft) using the 2-tailed student's *t*-test. Differences were considered significant at $p<0.05$ ($^*p<0.05$, $^{**}p<0.01$).

Results

P-gp was associated with cisplatin efficacy against OS. First, the relevance between P-gp expression in patient specimens and cisplatin treatment efficacy was analyzed. According to the cisplatin sensitivity rate, the patients were divided into two groups: cisplatin sensitive group (n=4) and cisplatin resistant group (n=8). Approximately 70.51% of the OS cells responded to cisplatin treatment in the sensitive group while 6.85% responded in the resistant group. The expression profile of P-gp analyzed by IHC indicated that P-gp expression was significantly higher in cisplatin -resistant specimens than in cisplatin sensitive specimens (Figure 1A and B). Altogether, P-gp was overexpressed in cisplatin resistant specimens compared to cisplatin sensitive ones.

OS cells with higher P-gp expression were more resistant to cisplatin. In order to verify the relationship between P-gp expression and cisplatin sensitivity in OS, the expression of P-gp in four OS cell lines was analyzed. HOS and U-2OS expressed higher levels of P-gp than MG-63 and Saos-2 did as assessed by RT-qPCR (Figure 2A) and western blot (Figure

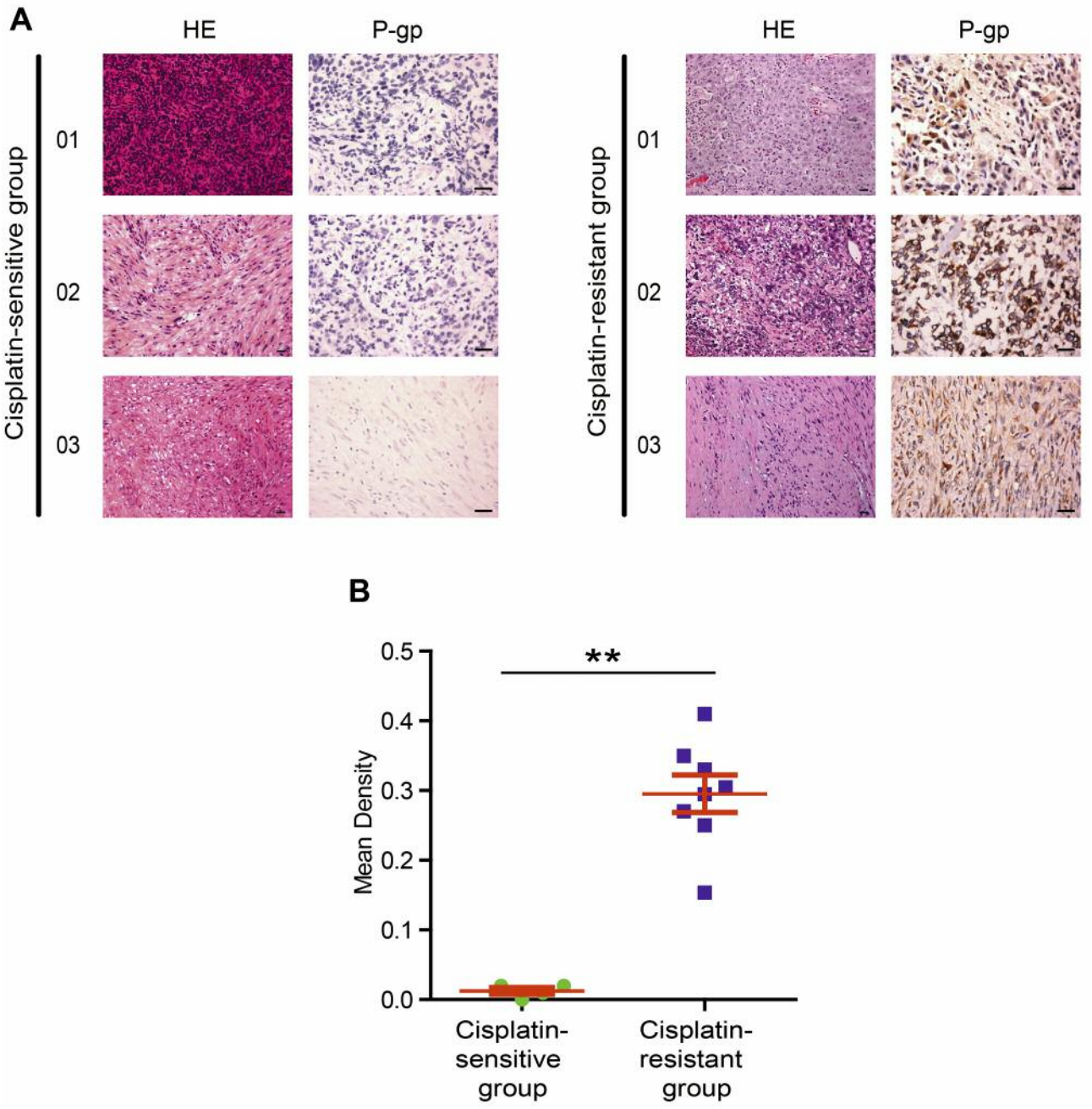

Figure 1. *Expression of P-gp in OS patients. (A) Expression of P-gp was assessed in cisplatin-sensitive and -resistant OS patient specimens by immunohistochemical staining (IHC). (B) Quantification of the results from (A). **p<0.01. Scale bar=100 μm.*

2B). Furthermore, all cell lines showed a dose-dependent response to cisplatin treatment for 72 h (Figure 2C, D and E). HOS and U-2OS cells were more resistant to cisplatin than MG-63 and Saos-2 cells as measured by the CCK-8 assay (Figure 2C) and colony formation assay (Figure 2D and E). Together, our result indicated that OS cells with higher P-gp expression were more resistant to cisplatin.

Knockdown of P-gp could sensitize OS cells to cisplatin. To further test whether P-gp was involved in cisplatin resistance in OS, two shRNAs were designed to knockdown P-gp. The knockdown efficacy was verified by RT-qPCR (Figure 3A) and western blot (Figure 3B). P-gp-knockdown could significantly enhance cisplatin efficacy in both HOS and U-2OS cells as assessed by the CCK-8 assay (Figure 3C) and

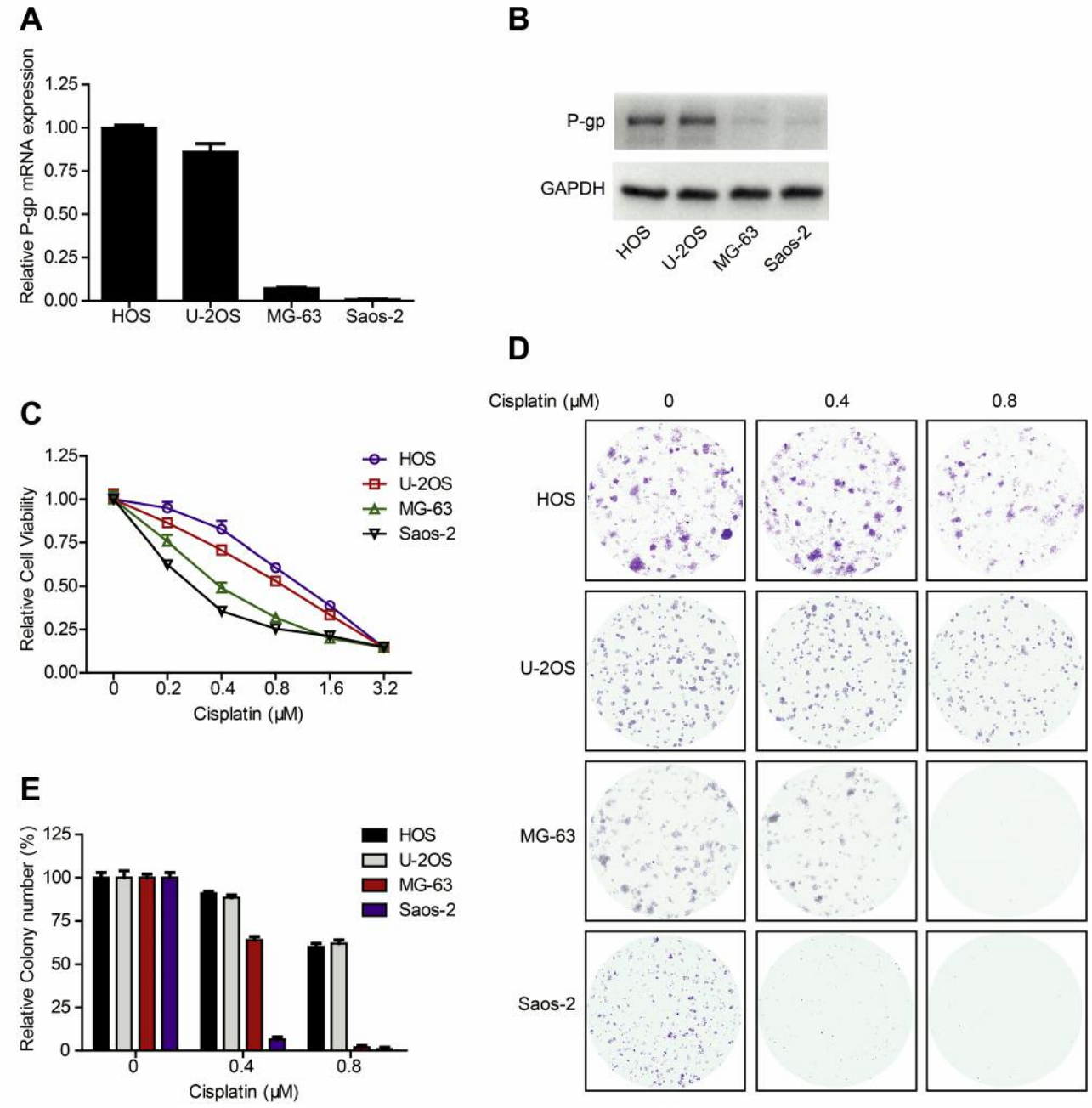

Figure 2. *OS cells with higher P-gp were more resistant to cisplatin. P-gp expression in OS cells were analyzed by RT-qPCR (A) and western blot (B). Viability of OS cells exposed to increasing concentrations of cisplatin measured by CCK- 8 assay (C) and colony formation assay (D and E).*

colony formation assay (Figure 3D). Collectively, knockdown of P-gp sensitized OS cells to cisplatin.

Dofequidar fumarate enhanced cisplatin efficacy on OS in vitro and in vivo. Additionally, dofequidar fumarate (Dof.) (17-20), an inhibitor of P-gp, was also used to further confirm the role of P-gp in OS chemotherapy resistance. Dofequidar fumarate sensitized HOS and U-2OS to cisplatin, as determined by the CCK-8 (Figure 4A) and colony formation assays (Figure 4B). Moreover, dofequidar fumarate synergized with cisplatin *in vivo*. As shown, tumor weight and volume were much smaller in the Cis+Dof. group than in any of the other three groups (Figure 4C, D and E). Overall, these data indicated that inhibition of P-gp enhanced cisplatin efficacy *in vitro* and *in vivo*.

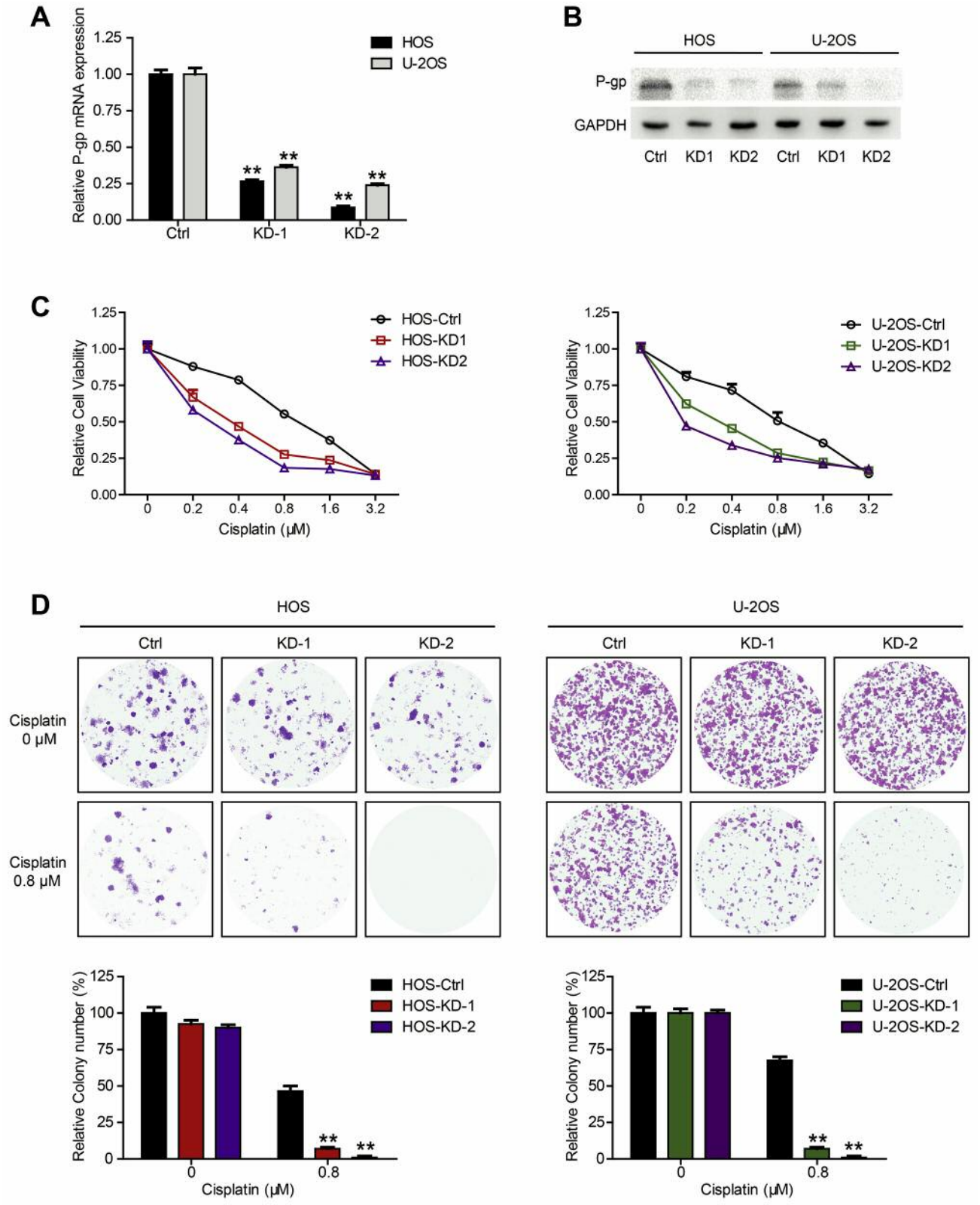

Figure 3. *Knockdown of P-gp could sensitize OS cell to cisplatin. P-gp expression in HOS and U-2OS parental cells and P-gp knockdown cells (KD-1 and KD-2) determined by RT-qPCR (A) and western blot (B). Viability of HOS and U-2OS parental cells and P-gp knockdown cells with the indicated treatment determined by the CCK-8 assay (C) and colony formation assay (D). **p<0.01 compared with control.*

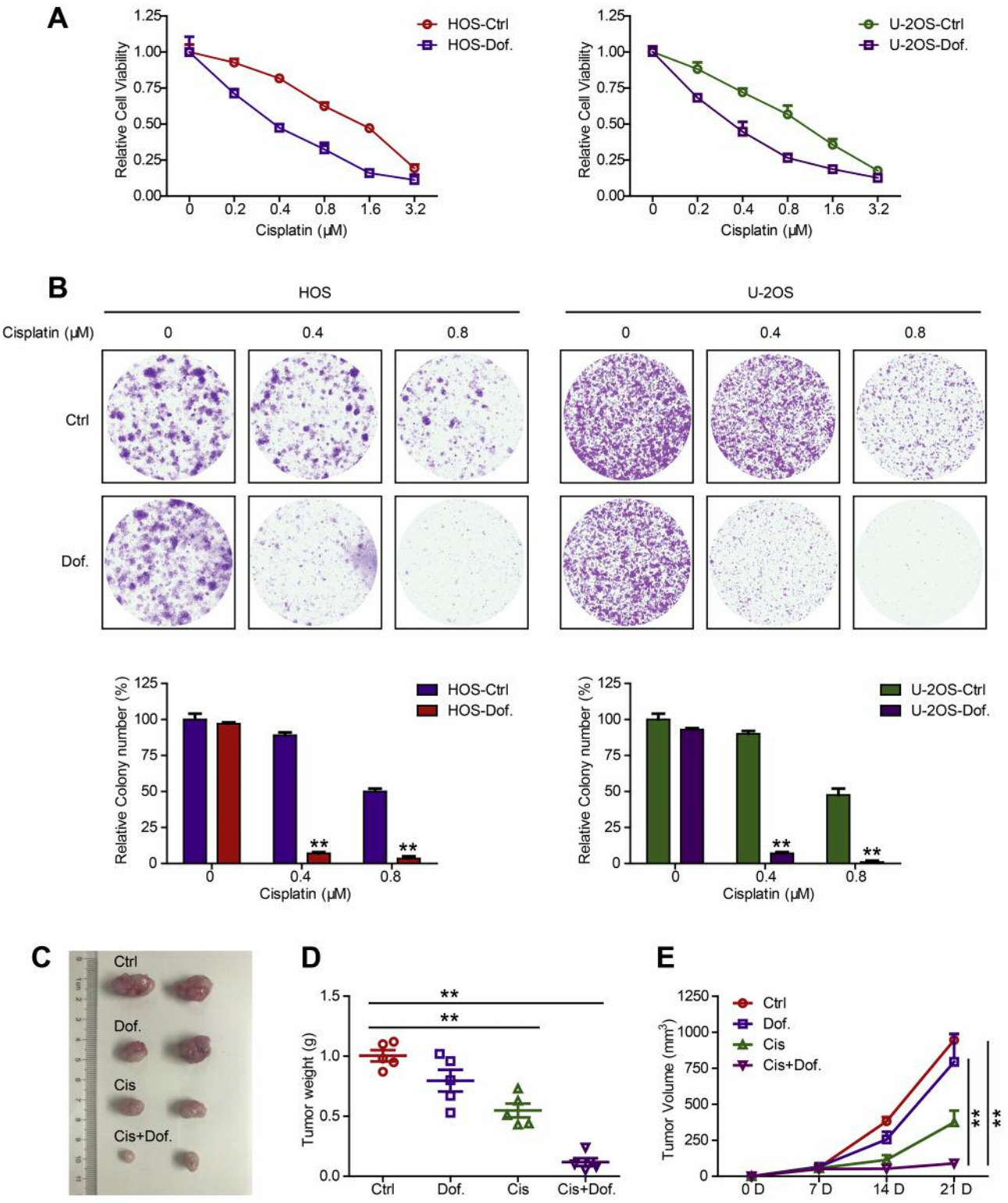

Figure 4. *Dofequidar fumarate enhanced cisplatin efficacy against OS in vitro and in vivo. Viability of HOS and U-2OS cells exposed to increasing concentrations of cisplatin with or without dofequidar fumarate measured by CCK-8 assay (A) and colony formation assay (B). Representative images of tumors removed from mice (C). Tumor weight (D) and volume (E) at the indicated times are shown in the diagram. **p<0.01 compared with control.*

Discussion

OS is the most commonly primary malignancy of the bone diagnosed among children and adolescents (22-24). Despite the great advances in chemotherapy and surgery, the outcome of therapy for OS still remains unsatisfactory (25). Cisplatin is the most commonly used anti osteosarcoma drug due to its unique therapeutic advantages. However, cisplatin resistance is frequently reported, meaning that the enhancement of cisplatin sensitivity is imperative (26).

Several studies have reported that inhibition of P-gp reverses drug resistance in OS (27-30). In the current study, P-gp was found to be overexpressed in cisplatin resistant OS specimens compared to cisplatin sensitive ones. OS cells with higher P-gp expression were more resistant to cisplatin. In addition, knockdown or inhibition of P-gp sensitized OS to cisplatin *in vitro* and *in vivo*.

Several drug resistance-related molecular mechanisms have been found in association with cisplatin resistance in OS. Kim *et al*. (31) revealed that GDNF receptor alpha 1 could overcome cisplatin resistance in OS by inhibiting AMP activated protein activated kinase-dependent autophagy. Zheng *et al*. (32) demonstrated that MAX dimerization protein 1 mediated hypoxia-induced cisplatin resistance in OS cells by suppressing the expression of phosphatase and tensin homolog. MicroRNAs have also been identified as novel modulators that regulate the effect of cisplatin in OS (33). Our study revealed an association of P-gp with cisplatin resistance in OS.

In conclusion, P-gp mediated cisplatin resistance in OS, suggesting that P-gp can be used as a potential therapeutic target for overcoming cisplatin resistance in OS.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Yongqiang Hao and Lei Wang designed the experiments, Chao He, Zhenyu Sun and Yuhang Jiang performed the experiments. Yuhang Jiang drafted the manuscript. Robert M. Hoffman and Zhijian Yang revised the manuscript. All Authors approved the final manuscript.

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