Establishment and characterization of human osteosarcoma cells resistant to pyropheophorbide-α methyl ester-mediated photodynamic therapy

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Abstract. The present study was performed to establish and characterize new human osteosarcoma cell lines resistant to pyropheophorbide-α methyl ester-mediated photodynamic therapy (MPPa-PDT). MPPa-PDT-resistant cells are isolated from the human osteosarcoma MG63 and HOS cell lines and two resistant populations were finally acquired, including MG63/PDT and HOS/PDT. Cell Counting Kit-8 (CCK-8) assay was used to determine the MPPa-PDT, cisplatin (CDDP) resistance and proliferation of MG63, MG63/PDT, HOS and HOS/PDT cells. The intracellular ROS were analyzed using DCFH-DA staining. The colony formation, invasion and migration of parental and resistant cells were compared. FCM was employed to examine the cell cycle distribution, the apoptosis rate and the proportion of CD133⁺ cells. The fluorescence intensity of intracellular MPPa was observed by fluorescence microscopy and quantified using microplate reader. The protein levels were assessed by western blotting (WB). Compared with two parental cells, MG63/PDT and HOS/PDT were 1.67- and 1.61-fold resistant to MPPa-PDT, respectively, and also exhibited the resistance to CDDP. FCM assays confirmed that both MG63/PDT and HOS/PDT cells treated with MPPa-PDT displayed a significantly lower apoptosis rate in comparison with their corresponding parental cells. The expression of apoptosis-related proteins (i.e. cleaved-caspase 3 and cleaved-PARP), intracellular ROS and the antioxidant proteins (HO-1 and SOD1) in MG63/PDT and HOS/PDT cells was also lower than that in parental cells. Both MG63/PDT and HOS/PDT cells exhibited changes in proliferation, photosensitizer absorption, colony formation, invasion, migration and the cell cycle distribution as compared to MG63 and HOS cells, respectively. Compared to MG63 and HOS cells, both resistant cell lines had a higher expression of CD133, survivin, Bcl-xL, Bcl-2, MRP1, MDR1 and ABCG2, but a lower expression of Bax. The present study successfully established two resistant human osteosarcoma cell lines which are valuable to explore the resistance-related mechanisms and the approaches to overcome resistance.

Introduction

Osteosarcoma is well known as the most common primary malignant bone tumor in clinic. It is strongly invasive and has poor prognosis, and mainly occurs in children and adolescents (1-3). Photodynamic therapy (PDT), a new promising approach for tumor therapy, is featured by excellent selectivity, less side reactions, and visible light of specific wavelength to excite photosensitizer enriched in tumor tissue, which can subsequently result in reactive oxygen, mainly singlet oxygen to cause tumor cell death (4,5). In addition, PDT has already been used in the treatment of skin and esophageal cancer, and other tumors (6-8). MPPa, a second generation photosensitizer derived from chlorophyll, has many advantages including stability, single component, rapid absorption and metabolism and strong photosensitivity (9). It has been reported that MPPa-PDT can kill tumor cells such as nasopharyngeal carcinoma, prostate and breast cancer cells (10-12).

However, photodynamic therapy may lead to the resistance of tumor cells (13). Numerous mechanisms are reported to mediate the resistance of tumor cells (14). Furthermore, the surviving tumor cells play an important role in the recurrence and deterioration of tumor, and result in poor prognosis (15,16). It has been reported that the increased expression of HO1, SOD1 and other antioxidant proteins can protect tumor cells from ROS damage (17). ATP-binding cassette (ABC) transporters (e.g. ABCG2, MRP1 and MDR1) were found to mediate tumor cell resistance, and alleviate cell damage through pumping out intracellular toxins (18). Furthermore, the overexpression of anti-apoptotic protein P-survivin was responsible for

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chemoresistance and radio-resistance of tumor cells (19). The Bcl-2 protein family can regulate the permeability of mitochondrial membrane, and its expression can decrease the sensitivity of tumor cells to antitumor therapy (20). The present study aimed to establish human osteosarcoma cell lines resistant to MPPa-PDT. The resistant cell lines can also be employed to investigate the resistance mechanism of human osteosarcoma cells to MPPa-PDT and explore the approach to overcome MPPa-PDT resistance.

Materials and methods

Reagents and instruments. MPPa and 2'7'-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BCA and trypsin were purchased from Beyotime Biotech (Shanghai, China). Cell viability and cytotoxicity test kits [Cell Counting Kit-8 (CCK-8)] were obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Extracellular matrix gel was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Annexin V-propidium iodide (PI) double-staining test kit was purchased from KeyGen Biotech (Nanjing, China). LED equipment was purchased from Chongqing Jingyu Laser Technology Co. Ltd. (Chongqing, China).

Antibodies. Primary antibodies were: β-actin (1:1,000), cleaved caspase-3 (1:1,000), SOD1 (1:1,000), cleaved PARP (1:1,000), Bcl-2 (1:1,000), Bcl-xL (1:1,000), Bax (1:1,000), CD133 (1:1,000), MDR1 (1:500), MRP1 (1:500) and P-survivin (1:1,000; all from Cell Signaling Technology, Inc. Danvers, MA, USA), HO1 (1:500; Proteintech Group, Inc., Wuhan, China) and CD133-APC (1:20; BD Biosciences). Secondary antibodies were: HRP monoclonal antibody anti-IgG of mouse and HRP monoclonal antibody anti-IgG of rabbit (Cell Signaling Technology, Inc.).

Cell line and culture. MG63 and HOS cells were obtained from the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Beijing, China), 100 μg/ml penicillin and 100 μg/ml streptomycin (Beyotime Biotech) at 37˚C in a humidified atmosphere containing 5% CO_2.

Resistance induction to MPPa-PDT. MG63 and HOS cells were cultured in the dark and incubated with different MPPa concentrations (Fig. 1A and B) for 20 h, and then washed twice with phosphate-buffered saline (PBS). The culture medium was replaced, and the cells were exposed to red light (630 nm, 40 mW/cm^2) in the continuous output mode. Treatment conditions that caused survival rates of 40-60% were chosen. The dead cells were wiped off and the surviving cells were cultured in fresh complete medium continuously for 24 h. Twenty hours later, the cells were harvested and replaced. They were subjected to a new PDT with intermittently increased doses of MPPa. The final populations were obtained following a total of 10 cycles of PDT, and they were MG63/PDT and HOS/PDT.

MPPa-PDT sensitivity assay. CCK-8 was used to investigate the sensitivity of cell lines MG63, MG63/PDT, HOS and HOS/PDT to MPPa-PDT. Cells were plated in 96-well plates

at a density of 5x10^3 cells/well with 3 duplications. After a 24-h incubation, the culture medium was replaced with the fresh medium containing different concentrations of MPPa (0, 0.25, 0.5, 0.75 and 1.0 μM for MG63 and MG63/PDT; 0, 0.1, 0.15, 0.2 and 0.25 μM for HOS and HOS/PDT cells). The cells were then cultured for 20 h. The culture medium was replaced, and the cells were exposed to red light (630 nm, 40 mW/cm^2). Twenty-four hours later, the cells were incubated for 1 h with 10 μl CCK-8 in each well. A microplate reader was employed to detect the absorption values of CCK-8 at 450 nm. The cell viability was calculated according to the following formulation:

$$\text{Cell viability (\%)} = \text{Average OD in experiment group}/ \text{average OD in control group} \times 100\%$$

$$\text{Resistance indices (RIs)} = IC_{50} \text{ values for resistant cells}/ IC_{50} \text{ values for parental cells}$$

Based on the results of the cell viability test, we chose an MPPa concentration of 0.45 μM for MG63 and MG63/PDT cells and 0.15 μM for HOS and HOS/PDT cells with a light energy density of 4.8 J/cm^2 as the treatment conditions.

Detection of apoptosis rate by Annexin V-PI double staining and FCM. Cells were seeded in 6-well plates at a density of 1x10^5 cells/well. All cells were harvested after corresponding treatments, and assessed by FCM after Annexin V-PI double staining (KeyGen Biotech).

Cell proliferation assay by CCK-8. Cells were seeded in 96-well plates at a density of 5x10^3 cells/well. When all cells attached, the cell viability was determined using the CCK-8 assay at 0, 12, 24 and 48 h.

Cell cycle analysis by FCM. Cells were seeded in 6-well plates at a density of 1x10^5 cells/well. After incubation for ~36 h, when fusion was up to ~60-70%, all groups were collected at the same time and washed twice, and fixed by suspending in 70% ethanol at 4˚C for 24 h, and then subjected to FCM.

Assessment of intracellular ROS level by DCFH-DA staining. Cells were inoculated in 6- and 24-well plates, at a density of 1x10^5 and 5x10^4 cells/well, respectively. Following the corresponding treatments, the cells were further incubated for 2 h. Then, DCFH-DA (10 μM) was added at 37˚C for 20 min. Finally, cells in 24-well plates were observed by fluorescence microscope (FM) after being washed 3 times, and the cells in 6-well plates were determined by FCM after being trypsinized and collected.

Assessment of CD133 by FCM. Cells were seeded in 6-well plates at a density of 1x10^5 cells/well. All cells were trypsinized, centrifuged and resuspended in PBS. CD133-APC (1:20) was added to corresponding groups and incubated in the dark for 10 min at 4˚C. After being washed, the cells were resuspended in PBS and analyzed by FCM.

Measurement of intracellular MPPa. Cells were inoculated in 6- and 96-well plates at a density of 1x10^5 and 5x10^3 cells/well,
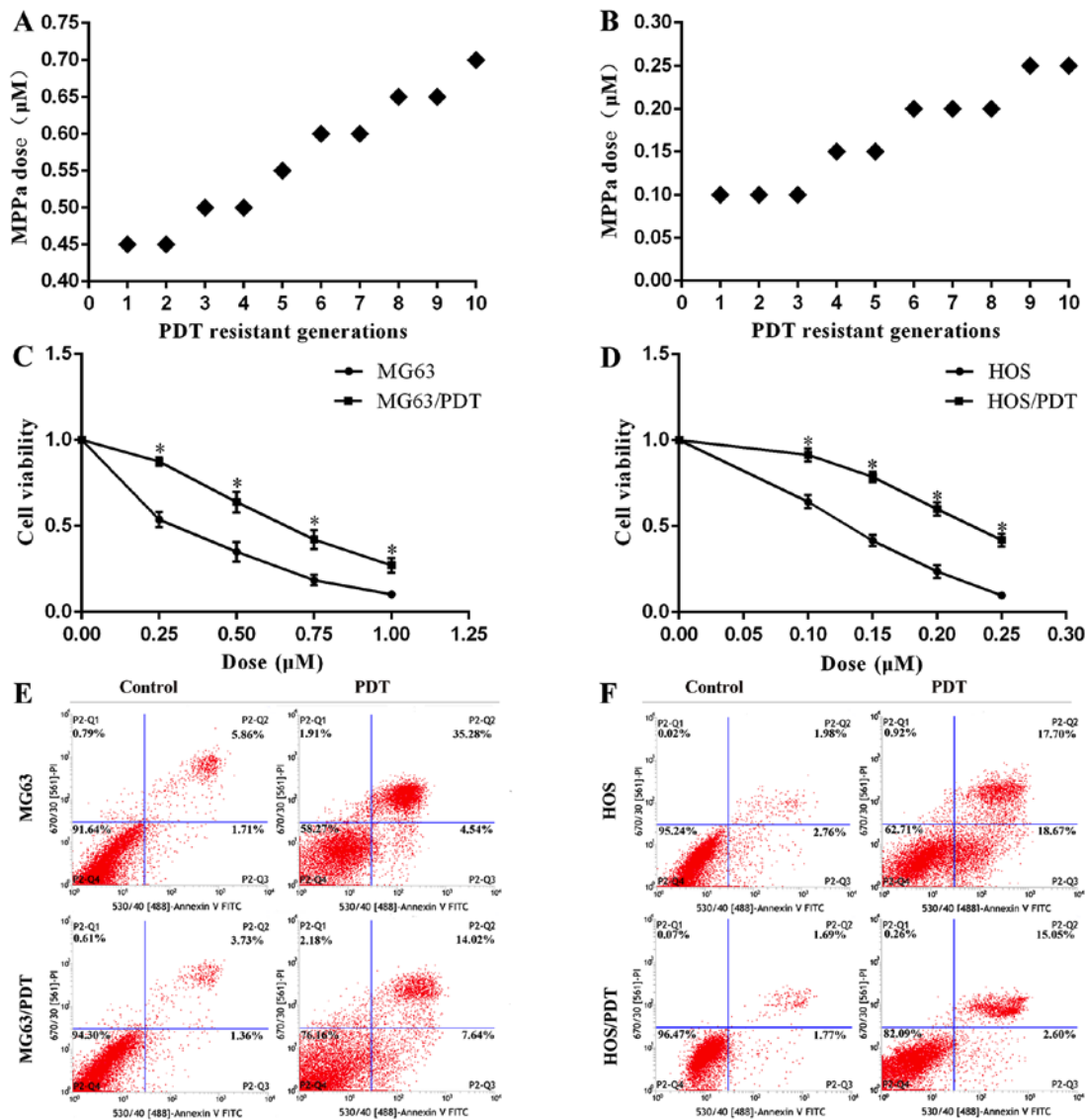
Figure 1. Establishment of the resistant cell lines. (A and B) Parental cell lines MG63 (left) and HOS (right) were subjected to 10 cycles of PDT with increasing doses of MPPa. (C and D) Cell viability was determined using CCK-8 after PDT at different concentrations of MPPa for 24 h. The cell viability of MG63/PDT and HOS/PDT were both higher than that of MG63 and HOS cells, respectively (P<0.05). (E and F) Apoptosis was assessed employing Annexin V-FITC/PI double staining followed by FCM. Apoptotic rates of MG63/PDT and HOS/PDT were markedly lower than that of their corresponding parental cells (P<0.001), after MPPa-PDT treatment for 12 h. The results indicated that the new human osteosarcoma cell lines MG63/PDT and HOS/PDT resistant to MPPa-PDT were successfully established; *P<0.05.

respectively. After attachment, the cells were incubated with different MPPa concentrations (2, 4 and 8 μM) for 20 h. Then, an inverted FM was used to observe the fluorescence of cells with 4 μM MPPa in 6-well plates, and a microplate reader was adopted to examine the fluorescence value of cells in 96-well plates (λexc 525 nm; λem 680 nm).

Colony formation assay. MG63 and MG63/PDT were seeded into 6-well plates at a density of 200 cells/well. HOS and HOS/PDT were seeded in 6-well plates at a density of 500 cells/well. The culture medium was replaced every 3 days. Two weeks later, colonies were fixed with polyformaldehyde (4%) for 20 min and visualized by crystal violet solution.

Invasion and migration assays. The ECM (1:8, 100 μl) was added to the top chamber and placed in an incubator for 2 h. When the ECM was dried, 4x10^4 MG63 and MG63/PDT or

8x10^4 HOS and HOS/PDT cells in serum-free medium were added to the top chamber. In the lower chamber, complete medium was added. After incubation at 37˚C in 5% CO$_2$ for 48 h, the cells on the upper surface of the membrane were removed using a cotton swab. The cells on the lower surface of the membrane were fixed with polyformaldehyde (4%) for 15 min and stained by crystal violet solution (0.1%). Cells were observed by inverted phase contrast microscope. Five randomized fields at a magnification of x40 were selected. For the migration assay, a protocol similar to the invasive assay was performed, but without the ECM layer in the chamber, and the cells added as well as the incubation time was half of that in invasion assay, respectively.

Western blot analysis. Cells were rinsed with PBS and lysed by RIPA buffer containing a phosphatase and protease inhibitor cocktail. Protein concentration was assessed by
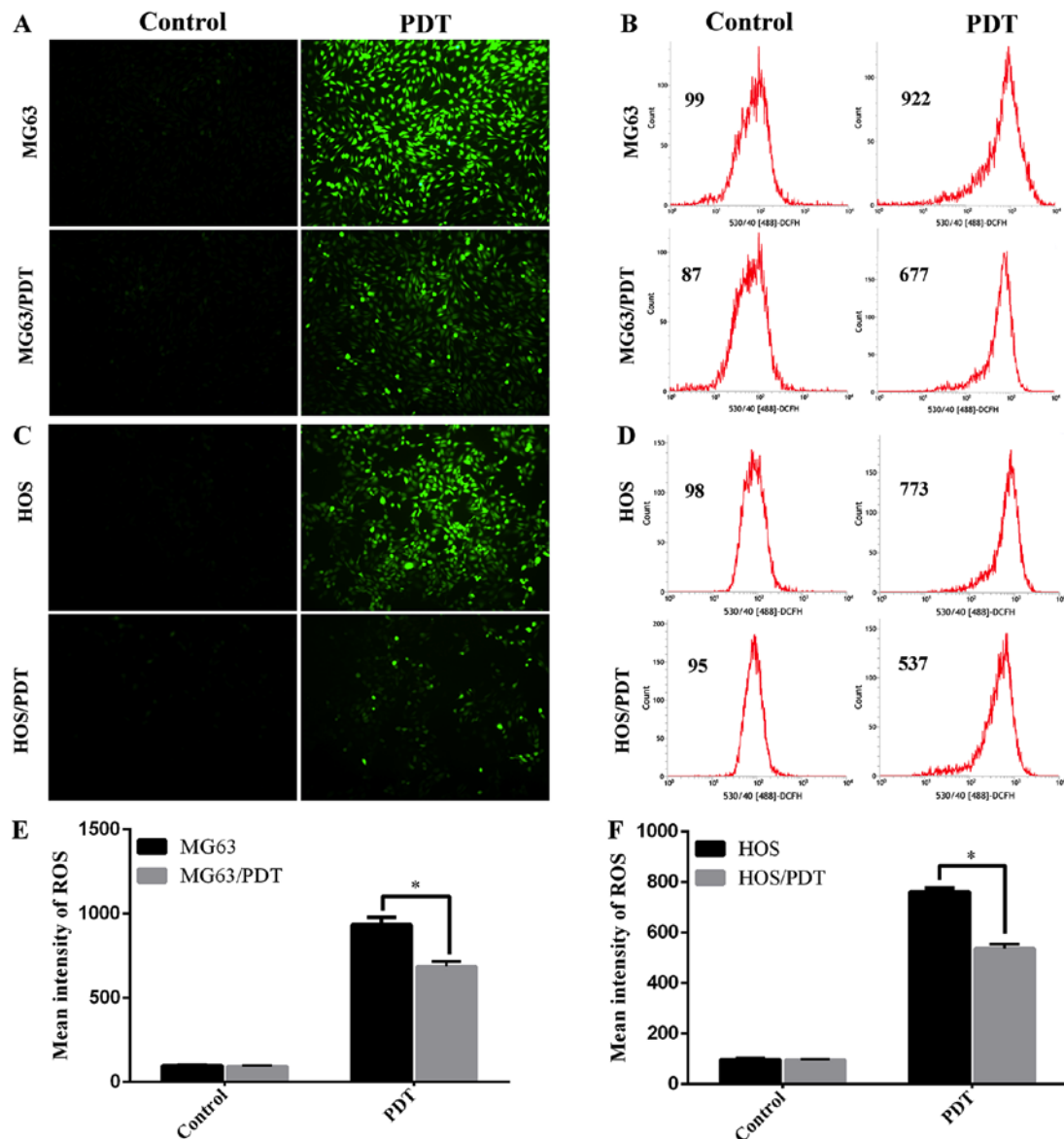
Figure 2. Cellular ROS in different cell types was examined using DCFH-DA (10 μM) followed by FCM and inverted FM. (A and B) MG63 and MG63/PDT cells were treated with MPPa (0.45 μM) for 20 h, and then irradiated with light (4.8 J/cm^2). At 2 h after irradiation, the ROS level was observed by inverted FM and detected by FCM. (C and D) The ROS levels of HOS and HOS/PDT cells, undergoing the same treatment of MG63 and MG63/PDT cells except for MPPa (0.15 μM), were assessed by the same methods as those for MG63 and MG63/PDT cells (A and C, magnification, x100). (E) The FCM status for MG63 and MG63/PDT cells from 3 independent experiments displayed in bar format. (F) The FCM data for HOS and HOS/PDT from 3 independent experiments displayed in bar format. The results revealed that the ROS level of MG63/PDT and HOS/PDT was significantly lower than that of parental MG63 and HOS cells, induced by MPPa-PDT; *P<0.05.

BCA. Protein samples (40 μg) were electrophoresed and blotted on polyvinylidene fluoride membranes, which were blocked in Tris-buffered saline containing Tween-20 and 5% non-fat milk for 1 h at room temperature, and incubated with the corresponding primary antibodies overnight at 4˚C. After being rinsed, the membranes were subjected to the horseradish peroxidase-conjugated secondary antibody for 1 h and developed by electrochemiluminescence. Quantity One software was used to detect the gray values of some western bands. The relative expression of target proteins was displayed using the ratio of target protein/β-actin. Three independent experiments were performed.

Statistical analysis. Data are expressed as the mean ± SD and analyzed by SPSS (SPSS, Inc., Chicago, IL, USA). Differences between groups were determined using the one-way or two-way ANOVA test for intergroup and independent-sample t-test for two groups. At P<0.05, the difference was considered significant.

Results

Establishment of the resistant cell lines. Parental cells, MG63 and HOS cells, were treated by PDT with increased concentration of MPPa for 10 cycles (Fig. 1A and B). The 10th generation of resistant cells obtained were named MG63/PDT and HOS/PDT, respectively. We employed MG63, HOS and MG63/PDT, HOS/PDT as experimental objects for the following studies. In order to verify the tolerance of resistant cells compared with their corresponding parental cells, the
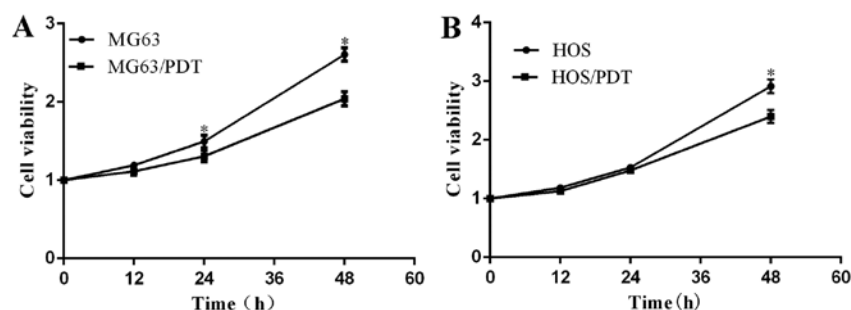
Figure 3. Cell proliferation of MG63, MG63/PDT, HOS and HOS/PDT cells. Cell viability was evaluated by CCK-8 at 0, 12, 24 and 48 h, respectively; *P<0.05.
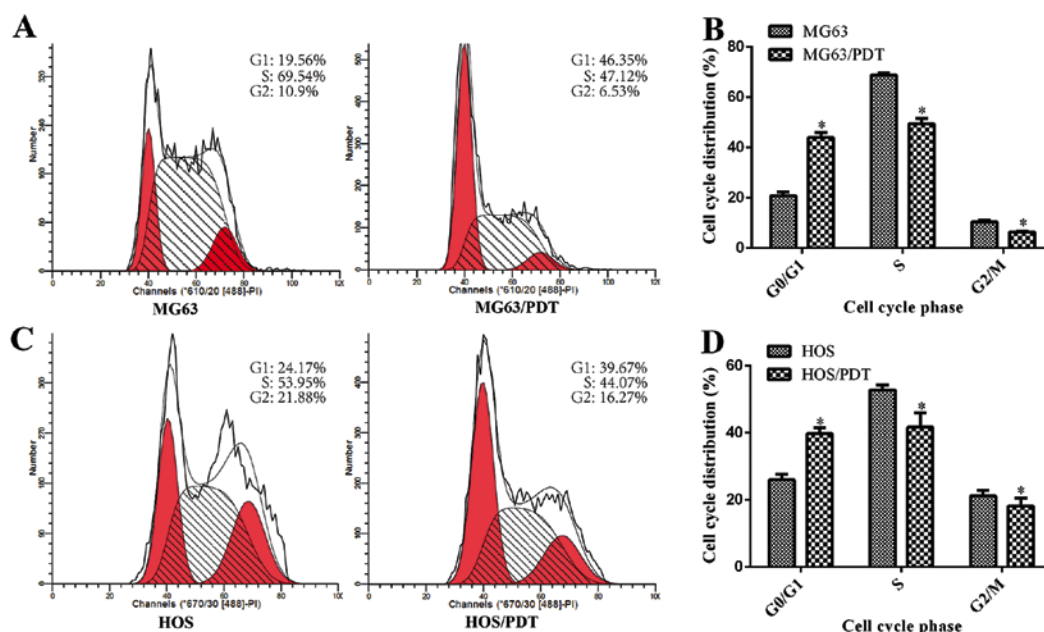

Figure 4. Cell cycle distribution of MG63, MG63/PDT, HOS and HOS/PDT cells tested by FCM. (A) MG63 and MG63/PDT, (C) HOS and HOS/PDT, as well as (B) FCM data for MG63 and MG63/PDT are shown in bar format. (D) FCM data for HOS and HOS/PDT exhibited in bar format. The proportion of MG63/PDT and HOS/PDT cells in the G2/M and S phases were significantly increased compared with the EC109 cells with a concomitant decrease in the G0/G1 phase. Similar results were observed between HOS and HOS/PDT cells; *P<0.05.

cell viability and apoptosis rate were assessed after PDT. A CCK-8 assay demonstrated that the concentration at 50% inhibition (IC_{50}) for MG63/PDT (0.704±0.016 μM) was 1.67-fold more resistant than that for MG63 (0.421±0.028 μM; P<0.001), and the concentration at 50% inhibition (IC_{50}) for HOS/PDT (0.226±0.008 μM) was 1.61-fold higher as compared to that for HOS (0.140±0.004 μM) (P<0.001; Fig. 1C and D). FCM indicated that the apoptosis rate of MG63/PDT (20.04±2.16%) was markedly lower than that of MG63 (40.58±2.34%) (P<0.001; Fig. 1E), when they were subjected to PDT with 0.45 μM MPPa. The apoptosis rate of HOS/PDT (17.80±1.26%) subjected to PDT with 0.15 μM MPPa was also significantly lower than that of HOS (37.22±0.81%) (P<0.001; Fig. 1F).

Measurement of intracellular ROS by FCM and FM. FCM and FM demonstrated that there was no significant difference of intracellular ROS levels between MG63 and MG63/PDT cells without PDT treatment (P=0.267). However, the ROS level in MG63 cells was markedly higher than that in MG63/PDT after PDT treatment (P=0.001) (Fig. 2A, B and E). Similar results were observed between HOS and HOS/PDT cells (Fig. 2C, D and F).

Cell proliferation test. After 12 h of culture, the MG63/PDT cells began to grow slower compared with the MG63 cells and the difference of growth speed was significant after 24 h (P=0.037; Fig. 3A). However, HOS/PDT began to grow slower than the parental HOS cells after 24 h, and the difference of growth speed was significant after 48 h (P=0.005; Fig. 3B). The proliferation curves of two types of resistant cells were more flat compared with that of the relatively primitive cells.

Examination of cell cycle distribution by FCM. FCM revealed that the proportion of cells in the G0/G1, S and G2/M phase were 19.56, 69.54 and 10.9% for MG63; 46.35, 47.12 and 6.53% for MG63/PDT (Fig. 4A); 24.17, 53.95 and 21.88% for HOS; and 39.67, 44.07 and 16.27% for HOS/PDT (Fig. 4C), respectively. The MG63/PDT and HOS/PDT cells exhibited shorter S and G2/M phases with a concomitantly longer G0/G1 phase in cycle distribution than their comparative cells (P<0.05; Fig. 4B and D).

Invasion assay. Transwell assays with a layer of Matrigel on the top inserts were employed to examine the invasive capability.
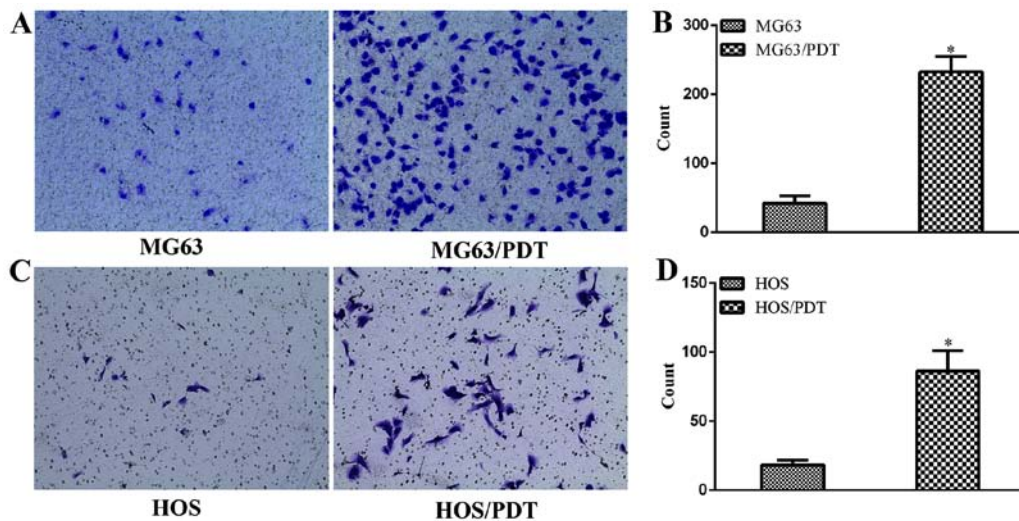
Figure 5. Invasive capability of (A) MG63, MG63/PDT and (C) HOS, HOS/PDT cells. (B and D) MG63/PDT and HOS/PDT cells were more invasive compared to parental MG63 and HOS cells; *P<0.05. (A and C, magnification, x40).
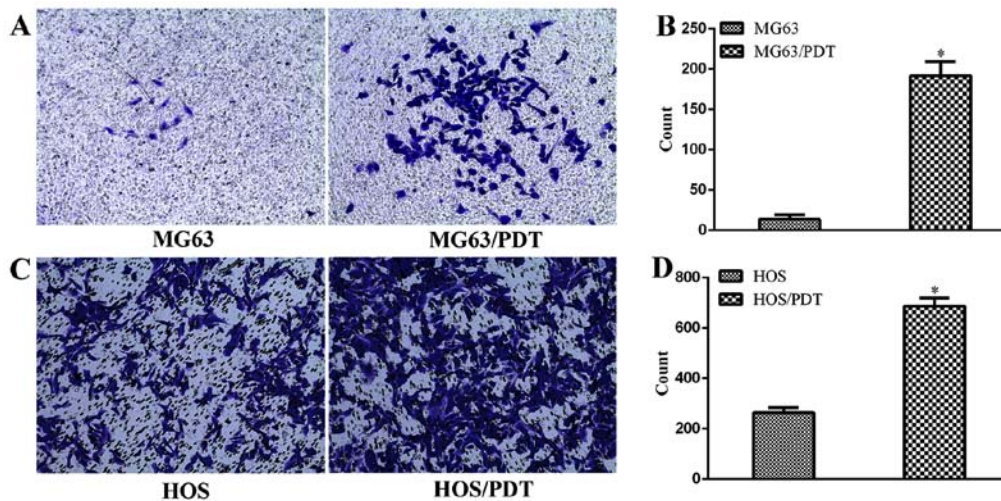

Figure 6. Migration capability of (A) MG63, MG63/PDT and (C) HOS, HOS/PDT cells. (B and D) The migration capability of the MG63/PDT and HOS/PDT cells was increased compared to parental MG63 and HOS cells, respectively; *P<0.05. (A and C, magnification, x40).

The results revealed that the number of cells penetrated both the Matrigel and membrane was 232±22.7 for MG63/PDT cells, 42±11 for MG63 cells (Fig. 5A and B), 86.33±14.64 for HOS/PDT cells and 18.33±3.51 for HOS cells (Fig. 5C and D), demonstrating that MG63/PDT and HOS/PDT had better ability of invasiveness compared with their corresponding parental cells (P<0.05).

Migration assay. Transwell assay was performed to detect migration ability. The results indicated that the number of cells penetrating the membrane was 195±12.12 for MG63/PDT cells, 13.67±5.68 for MG63 cells (Fig. 6A and B), 687.33±31.72 for HOS/PDT cells and 263.67±20.84 for HOS cells (Fig. 6C and D), revealing that the migration ability of MG63/PDT and HOS/PDT cells were increased compared with parental MG63 and HOS cells, respectively (P<0.05).

Colony formation assay. Both MG63/PDT and HOS/PDT cells had more potential in forming colonies. The number of

MG63/PDT cell colonies (36.3±5.0) was significantly higher compared to MG63 cells (21.3±5.7, P=0.027; Fig. 7A and B). The number of HOS/PDT cell colonies (22.3±4.5) was also markedly higher than HOS cells (6.3±1.5, P=0.004; Fig. 7C and D).

Determination of osteosarcoma stem marker CD133 by FCM. FCM revealed that the proportion of CD133+ cells in MG63/PDT, MG63, HOS/PDT and HOS cells was 11.49±0.71, 0.43±0.09, 5.76±0.52 and 0.50±0.06%, respectively. MG63/PDT and HOS/PDT exhibited more CD133+ cells than their corresponding original cells (Fig. 8; P<0.05).

Measurement of intracellular MPPa. The fluorescence intensity of MPPa cells was observed by FM. The results revealed that the fluorescence intensity of MPPa in MG63 cells was stronger than that of MG63/PDT (Fig. 9A), and the fluorescence intensity of MPPa in HOS cells was stronger than that of HOS/PDT cells (Fig. 9C). Microplate reader was
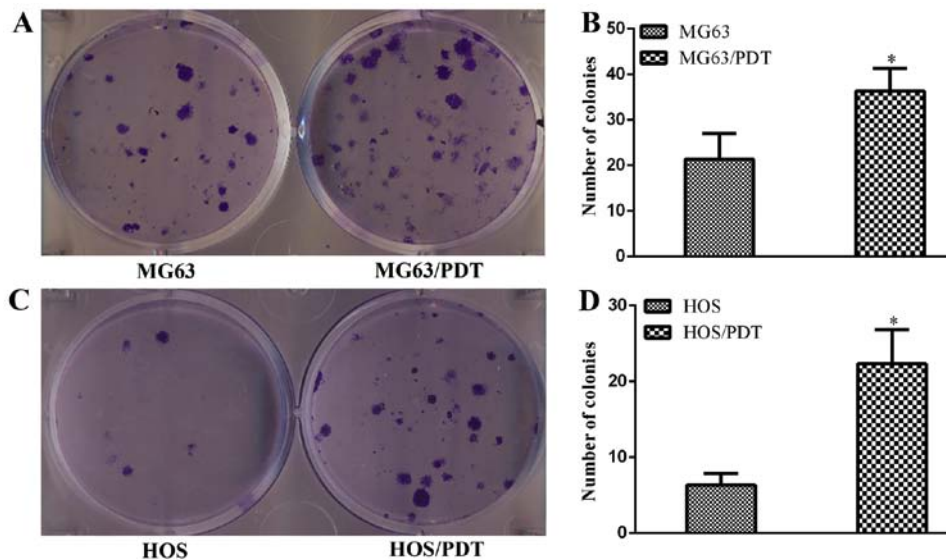
Figure 7. The colony formation of MG63, MG63/PDT, HOS and HOS/PDT cells. (A and C) MG63/PDT and HOS/PDT cells resulted in markedly more colonies than their corresponding parental cells. (B and D) The data are displayed in bar format. The MG63/PDT and HOS/PDT cells formed significantly more colonies compared to parental MG63 and HOS cells, respectively; $^{*}P<0.05$.
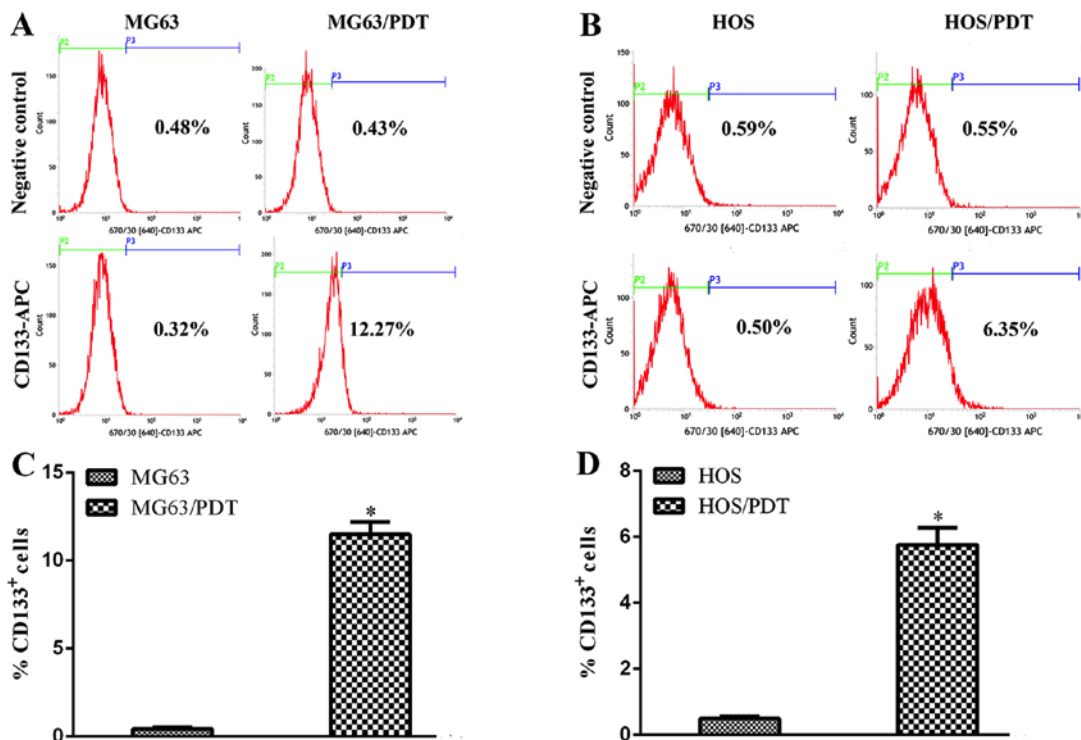

Figure 8. The proportion of cells expressing CD133. (A and B) The CD133+ proportion in all groups was determined by FCM, revealing that both MG63/PDT and HOS/PDT cell lines had more CD133+ cells than their parental MG63 and HOS cells, respectively. (C and D) FCM data for each group from 3 independent experiments exhibited in bar format. The results revealed that the proportion of CD133+ cells in MG63/PDT and HOS/PDT were significantly higher than that in their corresponding parental cells; $^{*}P<0.05$.

employed to quantify fluorescence intensity of MPPa in the cells, revealing that MPPa content in MG63 and HOS cells was significantly higher than that of MG63/PDT and HOS/PDT cells, respectively (P<0.05; Fig. 9B and D).

Cytotoxicity by CDDP. CCK-8 was adopted to assess the cytotoxicity of CDDP on PDT resistant and parental cells. Survival rates of MG63/PDT and HOS/PDT were significantly higher

than those of the corresponding original MG63 and HOS cells (P<0.05; Fig. 10).

Western blot assays. WB was used to detect the expression of different proteins. The results demonstrated that there was no significant difference of the expression of antioxidant-related protein HO-1, SOD1 and pro-apoptotic proteins cleaved-caspase 3, cleaved-PARP between MG63 and
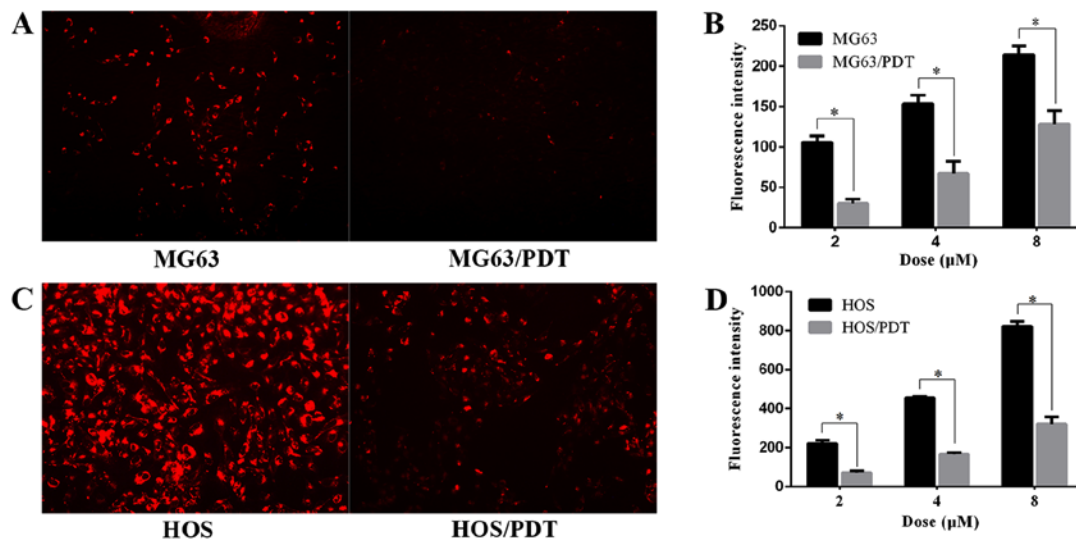
Figure 9. Intracellular MPPa content was analyzed by inverted FM and microplate reader. (A and C) Cells were treated with MPPa (4 μM) for 20 h, and then washed using PBS 3 times. The fluorescence intensity representing MPPa content in MG63 and HOS cells was stronger than that in MG63/PDT and HOS/PDT cells. (B and D) Cells were treated with different concentrations of MPPa, 2, 4 and 8 μM for 20 h, and subjected to microplate reader (λexc 525 nm; λem 680 nm). The results indicated that MPPa content in MG63/PDT and HOS/PDT cells was more than that in MG63 and HOS cells, respectively; *P<0.05.
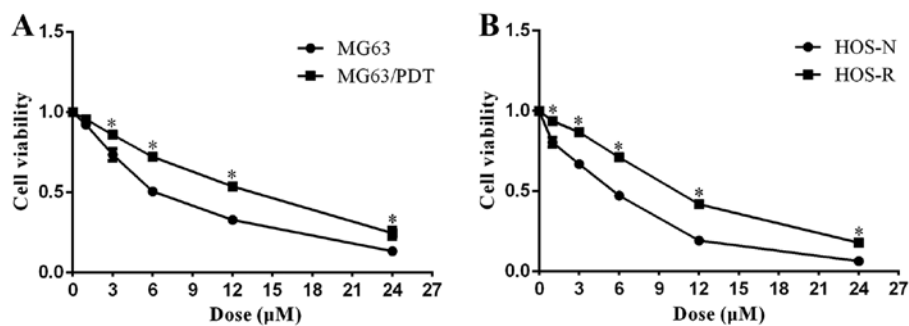

Figure 10. Cell resistance to CDDP. (A) MG63 and MG63/PDT cells were treated with different concentrations of CDDP for 24 h, and then subjected to CCK-8 assay. (B) HOS and HOS/PDT underwent the same treatment. The results indicated that MG63/PDT and HOS-PDT were more resistant to CDDP compared to parental cells MG63 and HOS, respectively; *P<0.05.

MG63/PDT cells (P>0.05). After treatment with MPPa-PDT for 12 h, the expression of HO-1, SOD1, cleaved-caspase 3 and cleaved-PARP in MG63 and MG63/PDT cells was increased, but their expression in MG63/PDT cells was lower than those in MG63 cells (P<0.05; Fig. 11A and B). The expression of HO-1, SOD1, cleaved-caspase 3 and cleaved-PARP in HOS and HOS/PDT cells displayed similar results (Fig. 11C and D).

To further study the characteristics of the resistance and the mechanisms, the expression of drug resistance-related proteins (e.g. ABCG2, MRP1, MDR, apoptosis-related proteins, Bcl-2, Bcl-xL, P-survivin and Bax) and cancer stemness markers CD133 in MG63, MG63/PDT, HOS and HOS/PDT were detected. The results indicated that the levels of ABCG2, MRP1, MDR and the anti-apoptotic proteins Bcl-2, Bcl-xL, P-survivin in MG63/PDT and HOS/PDT cells were all more highly expressed than those in MG63 and HOS cells (P<0.05; Fig. 12). However, the pro-apoptotic protein Bax in MG63/PDT and HOS/PDT cells was significantly downregulated compared with that in their parental cells (P<0.05; Fig. 12). Furthermore, the expression for the cancer stem marker CD133 in MG63/PDT cells and HOS/PDT cells was markedly increased relative to MG63 and HOS cells (P<0.05; Fig. 12).

Discussion

The 5-year survival rate of osteosarcoma patients has reached 60-70% with the continuous improvement of treatment, but the treatment is still not effective for the patients with recurrence, metastasis and chemoresistance. PDT has emerged as an important approach to treat tumors, with some advantages including strong targeting, non-invasive and repeatable treatment (4). Our previous study found that MPPa-mediated photodynamic therapy could significantly kill osteosarcoma MG63 cells, suggesting that it may be used in the clinical treatment of osteosarcoma patients (3). It is well known that resistance is very common in chemotherapy, radiotherapy and other cancer therapy (21,22). This may be related to the existence of heterogeneous cells resistant to the therapy or the induction of resistance in tumor cells (23). Some surviving cells after the treatment become more aggravated, more prone to invasion and metastasis (13,16). However, PDT still encountered the problem of resistance due to the expression of resistance-related proteins.

Milla *et al* successfully isolated squamous carcinoma cells (SCCs) resistant to PDT by repeated methyl d-aminolevulinic
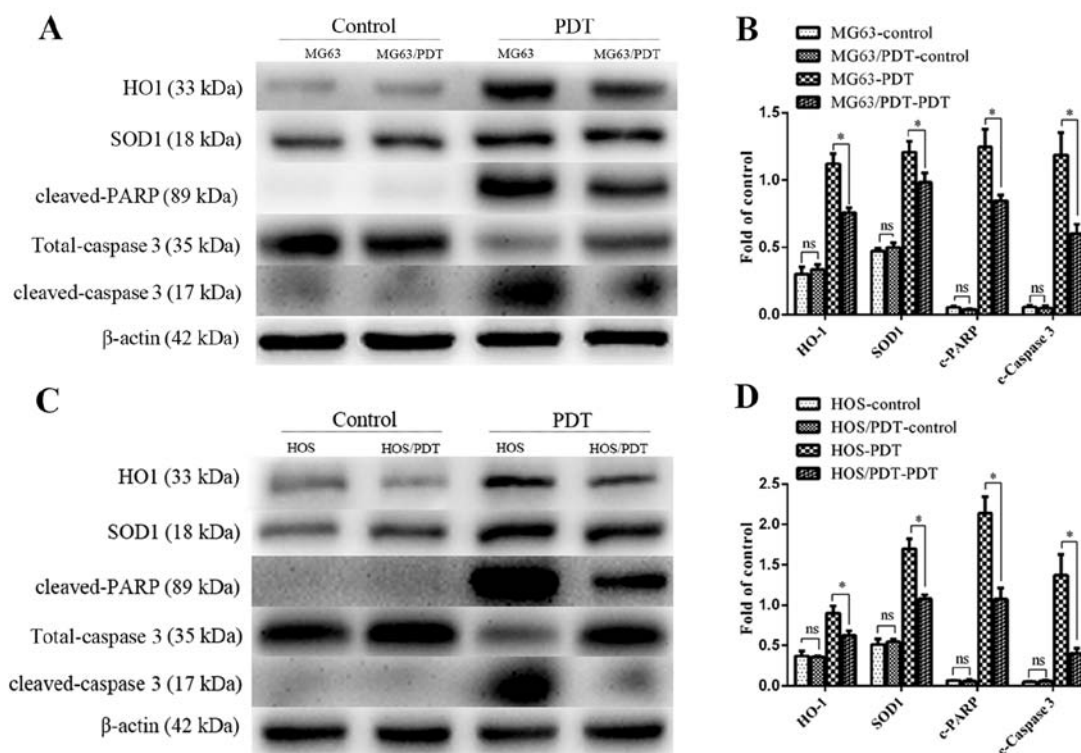
Figure 11. Changes in the expression of antioxidant proteins HO-1, SOD1 and apoptosis proteins cleaved-PARP, total-caspase 3, cleaved-caspase 3 following MPPa-PDT were analyzed by western blotting as depicted in the Materials and methods section. (A and C) WB images of HO-1, SOD1, cleaved-PARP, total-caspase 3, cleaved-caspase 3 in cells. (B and D) The relative protein expression levels of HO-1, SOD1, cleaved-PARP, cleaved-caspase 3 to β-actin; *P<0.05; ns >0.05.
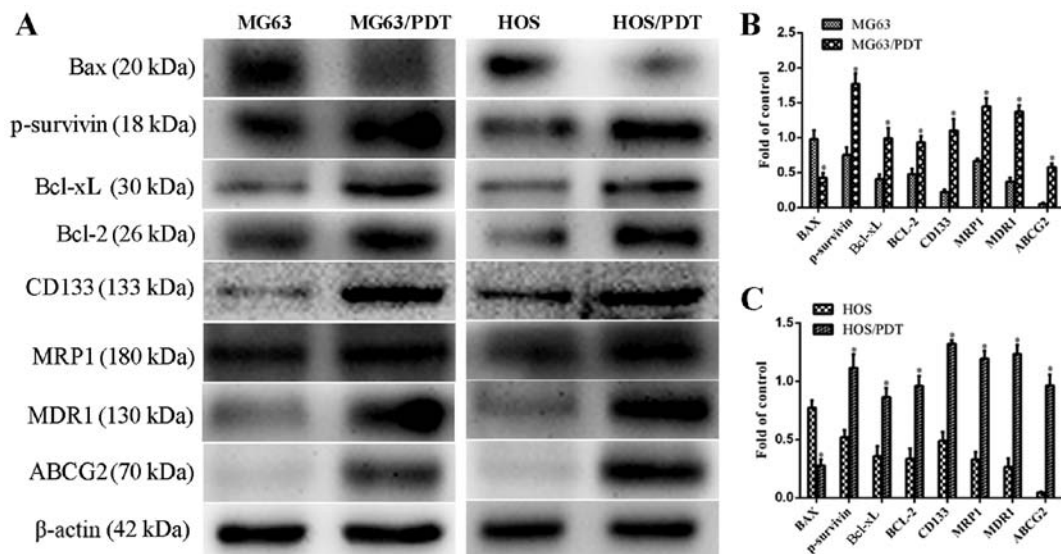

Figure 12. Different expression of Bax, P-survivin, Bcl-xL, Bcl-2, CD133, MRP1, MDR1, ABCG2 between MG63 and MG63/PDT cells and between HOS and HOS/PDT cells. (A) WB images of Bax, P-survivin, Bcl-xL, Bcl-2, CD133, MRP1, MDR1, ABCG2 and β-actin in MG63, MG63/PDT cells, HOS and HOS/PDT cells. (B and C) Protein expression levels of Bax, P-survivin, Bcl-xL, Bcl-2, CD133, MRP1, MDR1 and ABCG2 after normalization relative to β-actin; *P<0.05.

acid (Me-ALA-PDT) treatment of LD90 doses for tumor cells (24). The present study selected LD90 doses of MPPa-PDT for human osteosarcoma cell lines MG63 and HOS to establish new human osteosarcoma cell lines. However, after 3 days of treatment, all the cells died and failed to form resistance. This may be related to mismatch speed of resistance-related molecule expression. Thus, we chose a relatively mild treatment

condition of IC40-IC60. The MG63 and HOS cells were subjected to 10 cycles of PDT by gradually increasing the dose of MPPa, and finally MPPa-PDT-resistant cells were obtained, named MG63/PDT and HOS/PDT, respectively. In order to verify the resistance of newly constructed osteosarcoma cell lines MG63/PDT and HOS/PDT to MPPa-PDT, we examined the expression of cleaved-caspase 3 and cleaved-PARP,

apoptosis, cell viability in MG63, MG63/PDT, HOS and HOS/PDT cells after MPPa-PDT treatment. The results revealed that MG63/PDT and HOS/PDT cells were more resistant to MPPa-PDT compared to their corresponding parental cells. There may be some mechanisms that protected them from the damage of MPPa-PDT in osteosarcoma cells.

ROS is the main mechanism by which PDT kills osteosarcoma cells (3,25). In the present study, ROS in resistant cells MG63/PDT and HOS/PDT and parental cells MG63 and HOS, was analyzed by FCM and FM. The results demonstrated that there was no difference in the ROS level between resistant and parental cells in the absence of treatment. However, after treatment with PDT, the amount of ROS in resistant cells was significantly lower than that in parental cells, suggesting that the resistant cells changed some signal molecules to decrease the production of ROS. The amount of ROS induced by PDT depends on the type and the dose of the photosensitizer, irradiation time and the ability of cells to antioxidative stress. HO-1 not only degrades heme, but also promotes antioxidation, anti-inflammation and anti-apoptosis (26,27). Ciesla *et al* found that upregulation of HO-1 expression in rhabdomyosarcoma could reduce intracellular ROS content and promote cell survival (28). Lv *et al* reported that inhibition of HO-1 could increase the sensitivity of laryngeal carcinoma to CDDP. Early studies also found that HO-1 expression could decrease the damage of photodynamic therapy to tumors (29). SOD1 is an important antioxidant enzyme in cells, and is capable of decomposing superoxide, and free cells of ROS damage. Soares *et al* reported that inhibition of SOD1 increased the sensitivity of tumor cells to photodynamic therapy (30,31). In the present study, HO-1 and SOD1 expression were examined after MPPa-PDT treatment by same MPPa and light dose. However, the results were contrary to our expectation. The expression of HO-1 and SOD1 in resistant cells was significantly lower than those in parental cells, though both of them were induced by MPPa-PDT. In addition, there was no significant difference in the expression of HO-1 and SOD1 between resistant and parental cells without MPPa-PDT treatment. The results indicated that there may be another pathway in resistant cells that induces the resistance to MPPa-PDT. Higher expression of antioxidant machinery of cells definitely should result in low ROS levels in response to a particular treatment. Primitively, we hypothesized that MPPa-PDT-resistant osteosarcoma cells may produce more antioxidant proteins than original cells in order to clean out ROS. However, the results demonstrated that MPPa-PDT-resistant osteosarcoma cells had less antioxidant proteins than original osteosarcoma cells. Tian *et al* found that the inhibition of antioxidants may increase ROS and the damage of MPPa-PDT on tumor cells (32). There may be another reason for the high expression of antioxidants in parental cells. In one treatment, the expression of antioxidant proteins was adjusted in osteosarcoma cells according to the amount of ROS and formed tolerance to treatment, however this warrants further exploration. Concomitantly, the expression of other anti-oxidative stress kinases in resistant cells was also worthy of further study.

Our previous study revealed that the cytotoxicity of MPPa-PDT on osteosarcoma cells was in a dose-dependent manner (3). Milla *et al* found that the amount of Me-ALA in resistant SCC was less than that in parental SCC cells after only Me-ALA treatment for 4 h (24). We hypothesized that certain intracellular content after MPPa treatment resulted in the difference of ROS between resistant and parental cells. By detecting the fluorescence intensity of intracellular MPPa, we found that the content of MPPa in the resistant cells was significantly less than that in parental cells after the identical MPPa treatment, suggesting that the resistant cells could decrease the production of ROS induced by PDT owing to the less content of intracellular photosensitizer compared with that in parental cells. The ABC transporter family proteins with ATP enzyme activity (i.e. ABCG2, MDR1 and MRP1) can diminish intracellular drug concentration by accelerating drug efflux. Ishikawa *et al* found that inhibition of ABCG2 increased the uptake of photosensitizer PpIX by tumor cells (33). Liu *et al* reported that inhibition of MDR1 or MRP1 could increase the sensitivity of osteosarcoma cells to chemotherapy (34,35). In the present study, WB results implied that the expression of ABCG2, MDR1 and MRP1 in the resistant cells was significantly unregulated compared to parental cells. The decrease of MPPa content in the tolerant cells may be linked to the high expression of ABC transporter protein. However, the definite relationship between their high expression and the resistance to MPPa-PDT of MG63/PDT and HOS/PDT cells warrants further studies.

In addition to the upregulation of the ABC transporter, the anti-apoptotic and pro-apoptotic proteins may also be involved in the formation of cell resistance. The BCL-2 protein family is widely involved in the process of cell apoptosis. The pro-apoptotic protein Bax can increase the permeability of the mitochondrial membrane to facilitate the release of cytochrome *c* from the mitochondria into the cytoplasm. On the contrary, anti-apoptotic protein Bcl-2 and Bax-xL can stabilize the mitochondrial membrane by inhibiting Bax (20). Previous studies have found that MPPa-PDT can induce apoptosis of osteosarcoma MG63 cells by downregulation of Bcl-2 and the promotion of the expression of Bax (3). Survivin is a member of the inhibitor of apoptosis protein, which can bind caspase 3, an apoptosis executor, thereby inhibiting its activity (19). Ferrario *et al* found that a reduction of survivin can increase the cytotoxicity of PDT on breast cancer cells (36). The expression of P-survivin is unregulated in PDT-resistant squamous carcinoma cells (24). Our results revealed that the expression of anti-apoptotic protein Bcl-2, Bcl-xL and P-survivin was significantly increased in the resistant cells MG63/PDT and HOS/PDT compared with the parental cells MG63 and HOS, and the expression of pro-apoptotic protein Bax was notably decreased. The results demonstrated that upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins may be one of the mechanisms responsible for PDT-resistance of MG63/PDT and HOS/PDT cells.

Under normal circumstances, the resistant cells may accelerate proliferation to overcome the cytotoxicity of drugs (37). In our experiments, we found that the proliferation ability of the resistant cells MG63/PDT and HOS/PDT was weaker than that of their primary MG63 and HOS cells, respectively. The results are similar to those found in the CDDP resistant osteosarcoma cells SOSP-9607/CDDP (38). The slow proliferation of the resistant cells may be caused by the increased quiescent cells (16). In line with this, our results also demonstrated that there were more cells in the G0/G1 phase in resistant cells MG63/PDT and HOS/PDT compared with their

corresponding parental cells. PDT can cause DNA damage, which can activate or inhibit some signal molecules to block the cell cycle in the G1 phase and promote DNA repair (39,40). The results revealed that more cells in the G0/G1 phase of the resistant cells MG63/PDT and HOS/PDT could enhance their DNA repair ability, which also may be one of the mechanisms responsible for the PDT-resistance.

Casas *et al* found that the ability of invasion and migration in PDT-resistant cells was significantly weaker than that of their primary cells (41). However, Milla *et al* found that the migration ability of PDT-resistant SCC cells was stronger than that of their original cells (24). Han *et al* found that the invasion ability of CDDP-resistant osteosarcoma cells was also significantly enhanced compared to their primary cells (37). Our results revealed that the invasion, migration and clone formation capacity of resistant cells MG63/PDT and HOS/PDT were also significantly stronger than those of parental cells. The invasion, migration and clone formation may vary depending on the treatment methods, and the type of cells and photosensitizers. The results revealed that the recurrence of osteosarcoma after PDT may become more troublesome.

Cancer stem cells (CSCs) have the capacity of self-renewal and differentiation, and are accountable for proliferation, metastasis, drug resistance and recurrence of tumors (42). CD133 is currently recognized as a tumor stem cell marker, and many researchers have chosen CD133 as a marker for osteosarcoma stem cells to screen osteosarcoma stem cells (43-47). ABCG2 is not only associated with drug resistance, but also as a surface marker of CSC (48,49). It was reported that osteosarcoma-cancer-stem cells exhibited higher expression of ABCG2 and CD133 (50). In the present study, ABCG2 was upregulated in resistant cells. The present study also revealed that MG63/PDT and HOS/PDT cells presented higher expression of CD133 and possessed more CD133$^+$ cells compared to their parental cells, demonstrating that the stemness of osteosarcoma cells was enhanced during resistant cell construction by MPPa-PDT, and osteosarcoma stem cells may be involved in PDT-resistance of MG63/PDT and HOS/PDT cells. However, the proportion of CD44$^+$ cells, another cancer stem cell maker (51), between parental and resistant cells did not exhibit any significant difference and the slow proliferation and less cells in the G2/M phase of MG63/PDT and HOS/PDT cells were inconsistent with the appearance of cancer stem cells. It has been reported that PDT can conquer the resistance of tumor cells to chemotherapeutic drugs (52). CCK-8 assay was used to detect the viability of the cells treated with different doses of CDDP. The results revealed that the PDT-resistant osteosarcoma cells also appeared to be more resistant to CDDP, suggesting that MG63/PDT and HOS/PDT cells were not only resistant to PDT, but also to chemotherapy.

In conclusion, we successfully constructed two new PDT-resistant osteosarcoma cell lines MG63/PDT and HOS/PDT which featured by strong invasiveness, migration and stemness, and high expression of anti-apoptotic proteins as well as the ABC transporter family proteins, and low expression of pro-apoptotic protein and CDDP tolerance. The newly constructed PDT-resistant cell lines will be beneficial in the exploration of the biological characteristics of recurrent osteosarcoma, the methods of conquering PDT-resistance and to clarify possibly related mechanisms.

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