**ORIGINAL ARTICLE**

# Oncolytic virotherapy reverses chemoresistance in osteosarcoma by suppressing MDR1 expression

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## Abstract

**Background** Osteosarcoma (OS) is a malignant bone tumor primarily affecting children and adolescents. The prognosis of chemotherapy-refractory OS patients is poor. We developed a tumor suppressor p53–expressing oncolytic adenovirus (OBP-702) that exhibits antitumor effects against human OS cells. Here, we demonstrate the chemosensitizing effect of OBP-702 in human OS cells.

**Materials and methods** The in vitro and in vivo antitumor activities of doxorubicin (DOX) and OBP-702 were assessed using parental and DOX-resistant OS cells (U2OS, MNNG/HOS) and a DOX-resistant MNNG/HOS xenograft tumor model.

**Results** DOX-resistant OS cells exhibited high multidrug resistant 1 (MDR1) expression, which was suppressed by OBP-702 or MDR1 siRNA, resulting in enhanced DOX-induced apoptosis. Compared to monotherapy, OBP-702 and DOX combination therapy significantly suppressed tumor growth in the DOX-resistant MNNG/HOS xenograft tumor model.

**Conclusion** Our results suggest that MDR1 is an attractive therapeutic target for chemoresistant OS. Tumor-specific virotherapy is thus a promising strategy for reversing chemoresistance in OS patients via suppression of MDR1 expression.

**Keywords** Osteosarcoma · Chemoresistance · MDR1 · Oncolytic adenovirus · p53

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## Abbreviations

| | |
|---|---|
| OS | Osteosarcoma |
| DOX | Doxorubicin |
| MDR1 | Multidrug resistance 1 |
| ABCB1 | ATP-binding cassette sub-family B member 1 |
| $IC_{50}$ | 50% Inhibitory concentration |
| MOI | Multiplicity of infection |
| PFU | Plaque-forming unit |
| mAb | Monoclonal antibody |
| PARP | Poly(ADP-ribose) polymerase |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| CAR | Coxsackie and adenovirus receptor |
| JNK | C-Jun NH2-terminal kinase |

## Introduction

Osteosarcoma (OS) is the most frequent primary malignant bone tumor in children and adolescents in the United States [1]. Owing to recent advances in multi-agent chemotherapy and surgical techniques, the prognosis of OS

patients has gradually improved from 20 to 70% over the past three decades [2]. The most standard chemotherapy regimens for OS patients include doxorubicin (DOX), methotrexate, and cisplatin. However, the prognosis of OS patients who are refractory to chemotherapy remains poor due to local recurrence and distant metastasis [2]. Therefore, many clinical trials evaluating a variety of chemotherapy regimens have been conducted to improve the chemosensitivity of OS patients [3, 4]. The Children's Cancer Group/Pediatric Oncology Group trial INT-0133 demonstrated that the combination of ifosfamide and/or muramyl tripeptide–phosphatidyl ethanolamine has not improved the outcome of OS patients treated with standard chemotherapy [5]. Since efforts to develop multimodal therapies to improve chemosensitivity in OS patients have been unsuccessful [6], chemoresistance remains the primary obstacle in the treatment of OS patients.

Multidrug resistance protein 1 (MDR1), also known as ATP-binding cassette sub-family B member 1 (ABCB1) or P-glycoprotein, reduces the intracellular concentration and cytotoxicity of chemotherapeutic drugs by pumping the drugs out of cells [7]. Overexpression of MDR1 protein has been demonstrated in many types of human cancers, including OS [8]. A number of studies have examined the inhibition of MDR1 expression using small interfering RNA (siRNA) as a means of overcoming chemoresistance in human cancer cells [9–12]. In OS patients treated with DOX–based chemotherapy, MDR1 overexpression at diagnosis is an important adverse prognostic factor for clinical outcome [13]. Therefore, although MDR1 is an attractive therapeutic target for modulating chemoresistance in cancer patients, the use of MDR1-targeting inhibitors has been unsuccessful in clinical settings [8].

Oncolytic virotherapy has recently emerged as a novel anticancer therapeutic strategy [14]. Tumor-specific replication-competent oncolytic viruses are promising antitumor reagents that induce tumor-specific cell death [15]. We developed a telomerase-specific replication-competent oncolytic adenovirus, designated OBP-301 (Telomelysin), which induces tumor-specific death in a variety of human cancer cells [16, 17]. A phase I clinical trial of OBP-301 in the United States demonstrated the safety of intratumoral injection in patients with various advanced solid tumors, including sarcomas [18]. We previously evaluated the antitumor effect of OBP-301 in human OS cells [19] and determined that OBP-301 enhances the antitumor effect of chemotherapeutic drugs in human OS cells [20]. However, as some OS tumors are relatively OBP-301 resistant [19], we recently developed a p53-armed telomerase-specific oncolytic adenovirus (OBP-702) that exhibits stronger antitumor effects compared to p53-free OBP-301 due to activation of the p53-mediated cell death signaling pathway [21, 22]. Therefore, OBP-702 is expected to be

more effective than OBP-301 when combined with chemotherapy. However, the therapeutic potential of OBP-702 in combination with chemotherapy remains unclear.

In the present study, we investigated the therapeutic potential of OBP-702 as a chemosensitizing reagent against chemoresistant OS cells. The underlying mechanism of OBP-702–mediated chemosensitization was evaluated with respect to apoptosis and modulation of MDR1 protein expression. The in vivo antitumor effect of combination therapy was examined using a subcutaneous chemoresistant OS xenograft tumor model.

## Materials and methods

### Cell lines

Human U2OS cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Human MNNG/HOS cells were purchased from DS Pharma Biomedical (Osaka, Japan). Cells were cultured for no longer than 5 months following resuscitation. Authentification of cells was not performed by the authors. U2OS cells were maintained in McCoy's 5a medium. MNNG/HOS cells were maintained in Eagle's minimum essential medium containing 1% nonessential amino acids. All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were routinely maintained at 37 °C in a humidified atmosphere with 5% $CO_2$.

Chemoresistant U2OS and MNNG/HOS cells were obtained by sequential exposure to DOX over 3 months according to methods described in a previous report [23]. The concentration of DOX was determined monthly based on 50% inhibitory concentration ($IC_{50}$) values from XTT assays.

### Recombinant adenoviruses

The telomerase-specific replication-competent adenovirus OBP-301 (Telomelysin), in which the promoter element of the hTERT gene drives expression of the E1A and E1B genes, was previously constructed and characterized [16, 17]. For OBP-301-mediated induction of exogenous p53 gene expression, we generated OBP-702, in which a human wild-type p53 gene expression cassette was inserted into the E3 region [21]. Recombinant adenoviruses were purified using cesium chloride step gradients, and virus titer was determined using a plaque-forming assay with 293 cells. Viruses were stored at − 80 °C.

## Cell viability assay

Cells were seeded on 96-well plates at a density of $1 \times 10^3$ cells/well 24 h before administration of chemotherapeutic drugs or viral infection. For monotherapy, cells were treated with DOX at 0, 0.1, 1, 5, or 10 μg/mL or infected with OBP-702 at a multiplicity of infection (MOI) of 0, 1, 5, 10, 50, or 100 plaque-forming units (PFUs)/cell. For combination therapy involving chemotherapy and OBP-702 treatment, cells were infected with OBP-702 at the indicated MOIs. Two days after viral infection, cells were further treated with DOX for 24 h. This treatment protocol was established in our recent study [20] to induce a synergistic effect between the chemotherapeutic agent and oncolytic adenovirus. Cell viability was determined using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. The combination index was calculated using CalcuSyn software (BioSoft, Inc., Cambridge, UK).

## Cell proliferation assay

Cells were seeded on 24-well plates at a density of $1 \times 10^4$ cells/well 24 h before treatment. Twenty-four hours later, cells were infected with OBP-702 at the indicated MOIs. Two days after viral infection, cells were treated with DOX at the indicated doses for 24 h. Cells were counted using a hemocytometer.

## Western blot analysis

Cells were seeded in a 100-mm dish at a density of $2 \times 10^5$ cells/dish 24 h before treatment. The cells were then infected with OBP-702 or OBP-301 at the indicated MOIs or transfected with 10 nmol/L MDR1 siRNA or control siRNA (Applied Biosystems, Foster City, CA, USA). Two days after treatment, the cells were treated with DOX at the indicated doses for 24 h. Whole-cell lysates were prepared using lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100] containing a protease inhibitor cocktail (Complete Mini; Roche). Proteins were electrophoresed on 6–15% SDS polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). The membranes were blocked with Blocking-One (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The primary antibodies used were: mouse anti-Ad5 E1A monoclonal antibody (mAb) (BD PharMingen, Franklin Lakes, NJ, USA), rabbit anti-poly(ADP-ribose) polymerase (PARP) polyclonal antibody, rabbit anti-MDR1/ABCB1 mAb (Cell Signaling Technology, Danvers, MA, USA), and mouse anti–β-actin mAb (Sigma-Aldrich, St. Louis, MO, USA). The secondary antibodies used were: horseradish peroxidase–conjugated antibodies against rabbit IgG (GE Healthcare) and mouse IgG (GE Healthcare). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

## LC–MS analysis

Parental and DOX-resistant OS cells were lysed in lysis buffer [50 mM HEPES–NaOH (pH 8.0), 12 mM deoxycholate, 12 mM N-lauroyl sarcosinate] using low-intensity probe sonication. A total of 10 μg of protein was then reduced with 20 mM tris(2-carboxyethyl) phosphine (Sigma-Aldrich) at 100 °C for 10 min, followed by alkylation with 50 mM iodoacetamide (Sigma-Aldrich) at 25 °C in the dark for 45 min. Samples were mixed with Laemmli's sample buffer and loaded onto a polyacrylamide SDS gel. Electrophoresis was stopped when the loading dye reached 2 mm beneath the top of the separating gel. The gel was stained with Coomassie Brilliant Blue, and protein bands were excised and subjected to in-gel digestion with 100 ng of Trypsin/Lys-C Mix (Promega, Madison, WI, USA) at 37 °C for 12 h. The resulting peptides were extracted from the gel fragments and analyzed using a LTQ Orbitrap-Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to an UltiMate 3000 RSLC nano-flow HPLC system (DIONEX Corp., Sunnyvale, CA, USA). Protein identification and quantification were performed using MaxQuant software [24]. The MS/MS spectra were searched against the *Homo sapiens* protein database in SwissProt, with the false-discovery rate set to 1% for both the peptide and protein identification filters. Only "Razor + unique peptides" were used for calculation of relative protein concentration.

## Quantitative real-time reverse transcription PCR analysis

To evaluate the expression of *MDR1* mRNA, cells were seeded on 6-well plates at a density of $2 \times 10^5$ cells/well. Total RNA was extracted from the cells and tumor tissues using a miRNeasy Mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 10 ng of total RNA using a TaqMan reverse transcription kit (Applied Biosystems). *MDR1* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA expression was assessed using quantitative real-time PCR with an Applied Biosystems StepOnePlus™ real-time PCR system (Applied Biosystems). Relative expression levels of *MDR1* mRNA were calculated according to the $2^{-\Delta\Delta Ct}$ method after normalization with reference to the expression of *GAPDH* mRNA.

## Immunocytochemistry

To evaluate the subpopulation of MDR1-positive cells, cells were fixed with 4% paraformaldehyde for 15 min and were labeled with primary rabbit anti-MDR1 mAb (Cell Signaling Technology) at 4 °C overnight. Then cells were stained with secondary Alexafluor488 goat anti-rabbit IgG antibody (Invitrogen) for 30 min. DAPI was used for nuclear counterstaining. The photographs of immunostained cells were obtained under a fluorescence microscope (IX83; Olympus, Tokyo, Japan).

## In vivo subcutaneous DOX-resistant MNNG/HOS xenograft tumor model

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. DOX-resistant MNNG/HOS cells ($5 \times 10^6$ cells/site) were inoculated subcutaneously into the right flank of 6-week-old female BALB/c-*nu/nu* mice (CLEA Japan, Tokyo, Japan). Palpable tumors developed within 21 days and were permitted to grow to approximately 5 to 6 mm in diameter. At that stage, a 100-μL volume of solution containing OBP-702 ($5 \times 10^7$ PFUs) or PBS was injected into the tumors every week for 3 cycles. DOX (5 mg/kg) was injected intraperitoneally 2 days after OBP-702 injection for 3 cycles.

## Histopathologic analysis

Tumors were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. Sections were stained with hematoxylin/eosin to assess the tumor region. Proliferation and MDR1 expression of tumor cells within tumor tissues was assessed by immunostaining with rabbit anti-Ki67 mAb (Abcam, Cambridge, MA, USA) and rabbit anti-MDR1 mAb (Cell Signaling Technology), respectively, using standard techniques. All sections were analyzed under a light microscope. The number of immunoreactive cells for Ki67 or percentage of immunoreactive area for MDR1 were calculated in three randomly selected fields in each tumor using ImageJ software.

## Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The significance of differences was assessed using the Student's *t* test. Differences between groups in animal experiments were assessed using one-way analysis of variance followed by Tukey's multiple-group comparison test. Statistical significance was defined as a *P* value of $< 0.05$.

## Results

### Establishment of DOX-resistant OS cells

To analyze the therapeutic potential of OBP-702 against chemoresistant OS cells, we first established two types of DOX-resistant human OS cells (U2OS and MNNG/HOS) by sequential exposure to the standard chemotherapeutic agent DOX over 3 months. After establishment of chemoresistant OS cells, we compared the sensitivity of 4 human OS cell lines (parental U2OS, DOX-resistant U2OS, parental MNNG/HOS, and DOX-resistant MNNG/HOS) to DOX treatment. As expected, DOX-resistant U2OS and MNNG/HOS cells were significantly more resistant to DOX compared to parental cells (Fig. 1A). The DOX $IC_{50}$ values for DOX-resistant U2OS and MNNG/HOS cells were approximately 20-fold higher than those of parental cells (Fig. 1B). Western blot analyses showed that DOX treatment induced apoptosis in a dose-dependent manner, which was confirmed by the increased expression of cleaved PARP, in parental U2OS and MNNG/HOS cells (Fig. 1C). By contrast, the expression of cleaved PARP was lower in DOX-resistant U2OS and MNNG/HOS cells than in parental cells with DOX treatment (Fig. 1C). The rate of DOX-resistant U2OS and MNNG/HOS cell proliferation was also lower than that of the parental cells (Supplementary Fig. S1); however, the morphology of DOX-resistant U2OS and MNNG/HOS cells was similar to that of the parental cells (Supplementary Fig. S2). These results indicate that DOX-resistant OS cells are less sensitive to DOX-induced apoptosis compared to parental cells.

### Identification of chemoresistance-related proteins in DOX-resistant OS cells

To identify the molecular targets associated with chemoresistance in OS cells, we employed comprehensive and quantitative label-free proteomic analysis which finally provided relative expression levels of 2897 proteins in parental and DOX-resistant OS cells. A Venn diagram showed that 270 and 191 proteins were expressed at levels more than twofold higher in DOX-resistant U2OS and MNNG/HOS cells, respectively, and 97 proteins were commonly expressed at levels more than twofold higher in both DOX-resistant U2OS and MNNG/HOS cells when compared to parental cells (Fig. 2A and Supplementary Table S1). Among the 97 proteins that were expressed at levels more than twofold higher in DOX-resistant OS cells than parental cells, the top 5 included MDR1, PTHB1, A-kinase anchor protein
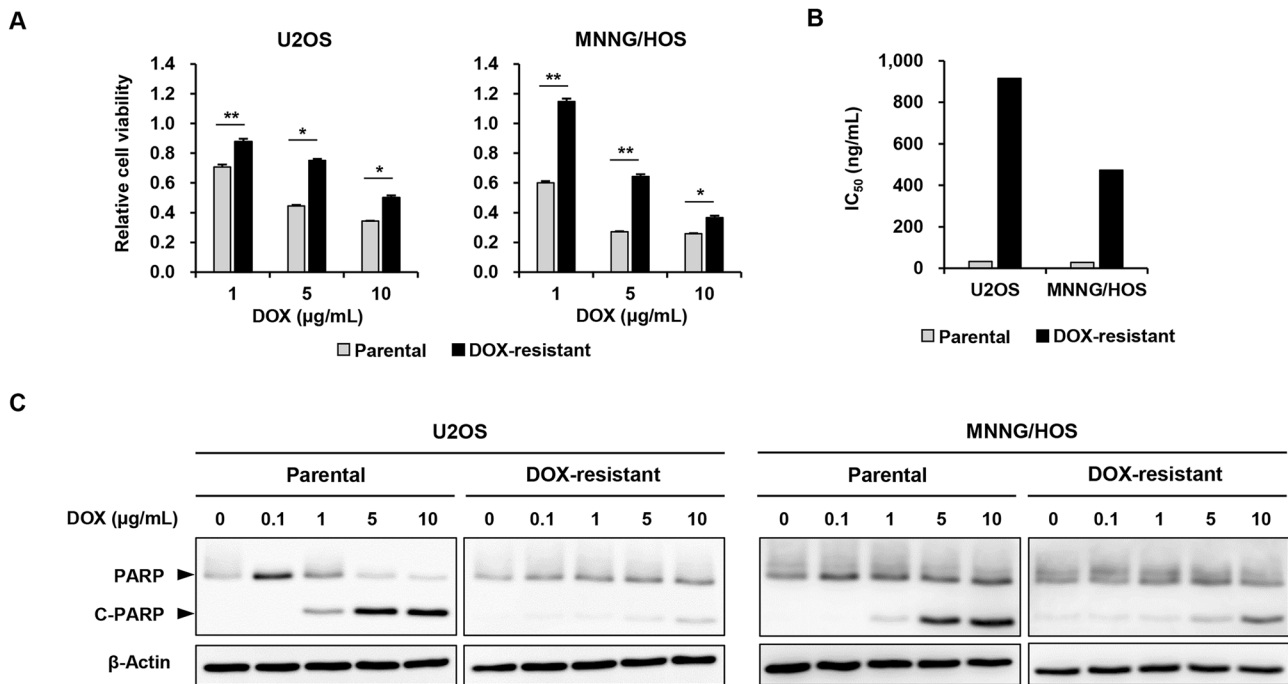
**Fig. 1** Establishment of DOX-resistant human OS cells. **A** Four human OS cell lines (U2OS, DOX-resistant U2OS, MNNG/HOS, DOX-resistant MNNG/HOS) were treated with DOX at the indicated doses for 24 h. Cell viability was quantified using the XTT assay and calculated relative to that of parental cells, which was set at 1.0. Cell viability data are expressed as mean $\pm$ SD ($n=5$; $*P<0.05$, $**P<0.01$). **B** Relative $IC_{50}$ values of DOX for parental and DOX-resistant OS cells. $IC_{50}$ values were calculated using cell viability data obtained on day 4 after DOX treatment. **C** Expression of PARP and cleaved PARP (C-PARP) proteins in human OS cells treated with DOX at the indicated doses for 24 h. β-Actin was used as a loading control

SPHKAP, myosin regulatory light polypeptide 9, and epidermal growth factor receptor kinase substrate 8–like protein 2 (Fig. 2B). As MDR1 is a member of the ATP-binding cassette transporter family and associated with chemoresistance, we further confirmed that the expression level of MDR1 mRNA and protein was upregulated in DOX-resistant OS cells using quantitative real-time PCR and Western blot analyses, respectively (Fig. 2C, D). Immunocytochemistry for MDR1 demonstrated that the subpopulations of MDR1-positive cells were small and large in parental and DOX-resistant OS cells, respectively. These results suggest that MDR1-positive OS cells are selectively enriched under DOX treatment and targeting MDR1 activation holds therapeutic potential for overcoming chemoresistance in OS cells.

## Chemosensitizing effect of OBP-702 in DOX-resistant OS cells

We recently confirmed the chemosensitizing effect of OBP-301 against various types of human malignant tumor cells [25], including OS cells [20]. To investigate the chemosensitizing effect of OBP-702 against human OS cells, we treated parental and DOX-resistant OS cells with DOX and OBP-702 as combination therapy. The number and viability of cells treated with the combination of OBP-702 and DOX

decreased in a dose-dependent manner (Fig. 3A, B). Calculation of the combination index demonstrated a synergistic antitumor effect of combination therapy in parental and DOX-resistant OS cells (Fig. 3C). These results suggest that OBP-702 chemosensitizes both parental and DOX-resistant OS cells.

As parental OS cells were more sensitive to OBP-702 infection than DOX-resistant cells, we analyzed the expression level of coxsackie and adenovirus receptor (CAR) in parental and DOX-resistant OS cells by flow cytometric analysis. The expression of CAR protein was significantly higher in parental OS cells than DOX-treated cells (Supplementary Fig. S4). These results suggest that chemoresistance is associated with low CAR expression.

## OBP-702–mediated suppression of MDR1 expression plays a critical role in enhancing DOX-induced apoptosis

To elucidate the underlying mechanism of OBP-702–mediated enhancement of chemosensitivity in DOX-resistant OS cells, we examined whether OBP-702 enhances DOX-mediated apoptosis. DOX-resistant U2OS and MNNG/HOS cells were treated with DOX and/or OBP-702, and then apoptosis status was assessed by Western blot analysis. In
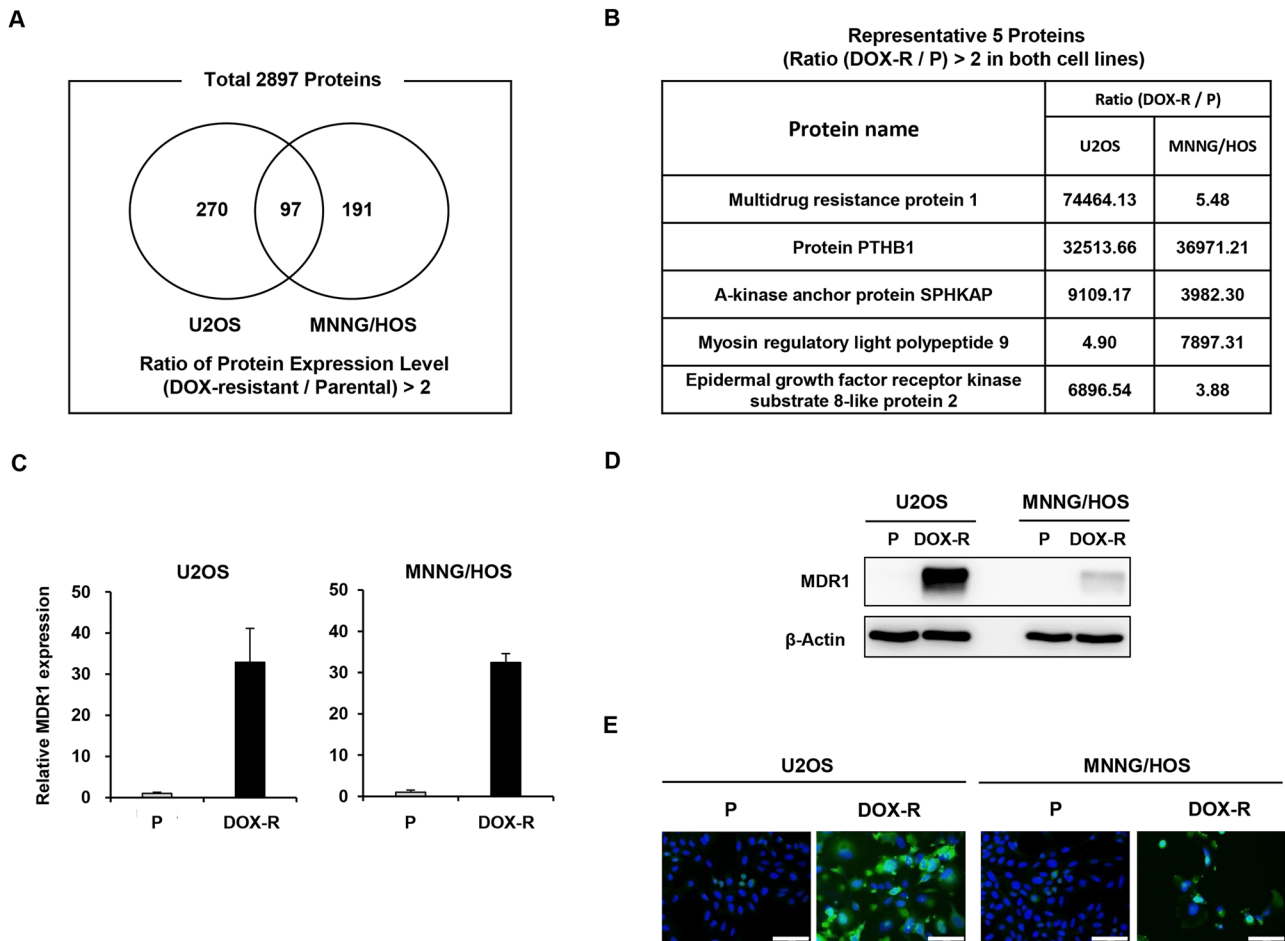
**A**



Total 2897 Proteins

270 | 97 | 191

U2OS | MNNG/HOS

Ratio of Protein Expression Level
(DOX-resistant / Parental) > 2

**B**

Representative 5 Proteins
(Ratio (DOX-R / P) > 2 in both cell lines)

| Protein name | Ratio (DOX-R / P) | |
| --- | --- | --- |
| | U2OS | MNNG/HOS |
| Multidrug resistance protein 1 | 74464.13 | 5.48 |
| Protein PTHB1 | 32513.66 | 36971.21 |
| A-kinase anchor protein SPHKAP | 9109.17 | 3982.30 |
| Myosin regulatory light polypeptide 9 | 4.90 | 7897.31 |
| Epidermal growth factor receptor kinase substrate 8-like protein 2 | 6896.54 | 3.88 |

**C**



**D**



**E**



**Fig. 2** Expression of MDR1 protein is upregulated in DOX-resistant OS cells. **A** parental and DOX-resistant OS cells were subjected to proteomics analysis. The Venn diagram shows the number of proteins for which expression was greater than twofold higher in DOX-resistant OS cells than parental cells. **B** Five proteins were commonly upregulated in DOX-resistant U2OS and MNNG/HOS cells. **C** Expression level of MDR1 mRNA in DOX-resistant OS cells was determined by quantitative RT-PCR. **D** MDR1 protein expression in DOX-resistant OS cells was assessed by Western blot analysis. β-Actin was used as a loading control. **E** MDR1 protein expression in parental and DOX-resistant OS cells were assessed by immunocytochemistry. Scale bars: 100 um

DOX-resistant U2OS and MNNG/HOS cells treated with OBP-702 alone, cleaved PARP expression increased and MDR1 expression decreased in a dose-dependent manner (Fig. 4A). To explore the mechanism of OBP-702-mediated MDR1 suppression, we analyzed whether OBP-301 without p53 induction downregulates MDR1 expression in DOX-resistant OS cells. Western blot analysis demonstrated that OBP-301 suppressed MDR1 expression in a dose-dependent manner as similar with OBP-702 (Fig. 4B). These results suggest that OBP-702-mediated MDR1 suppression is mainly due to adenoviral infection rather than p53 induction.

To investigate the role of OBP-702-mediated MDR1 suppression in DOX-resistant OS cells, we examined whether OBP-702 enhances DOX-induced apoptosis. In cells treated with DOX and OBP-702 in combination, MDR1 expression was suppressed, leading to enhanced DOX-induced apoptosis of DOX-resistant OS cells (Fig. 4C). To further confirm the role of MDR1 suppression in enhanced DOX-induced apoptosis, we assessed the effect of MDR1 knockdown by RNA interference in combination with DOX treatment. When MDR1 siRNA was combined with DOX, DOX-induced apoptosis was enhanced in DOX-resistant OS cells, similar to treatment with OBP-702 (Fig. 4D). These results suggest that suppression of MDR1 expression plays a crucial role in OBP-702–mediated enhancement of DOX-induced apoptosis.

## In vivo antitumor effect of combination therapy with DOX and OBP-702

To assess the in vivo antitumor effect of combination therapy with DOX and OBP-702, we used a subcutaneous DOX-resistant MNNG/HOS xenograft tumor model. We injected OBP-702 or PBS into tumors weekly, and DOX was injected
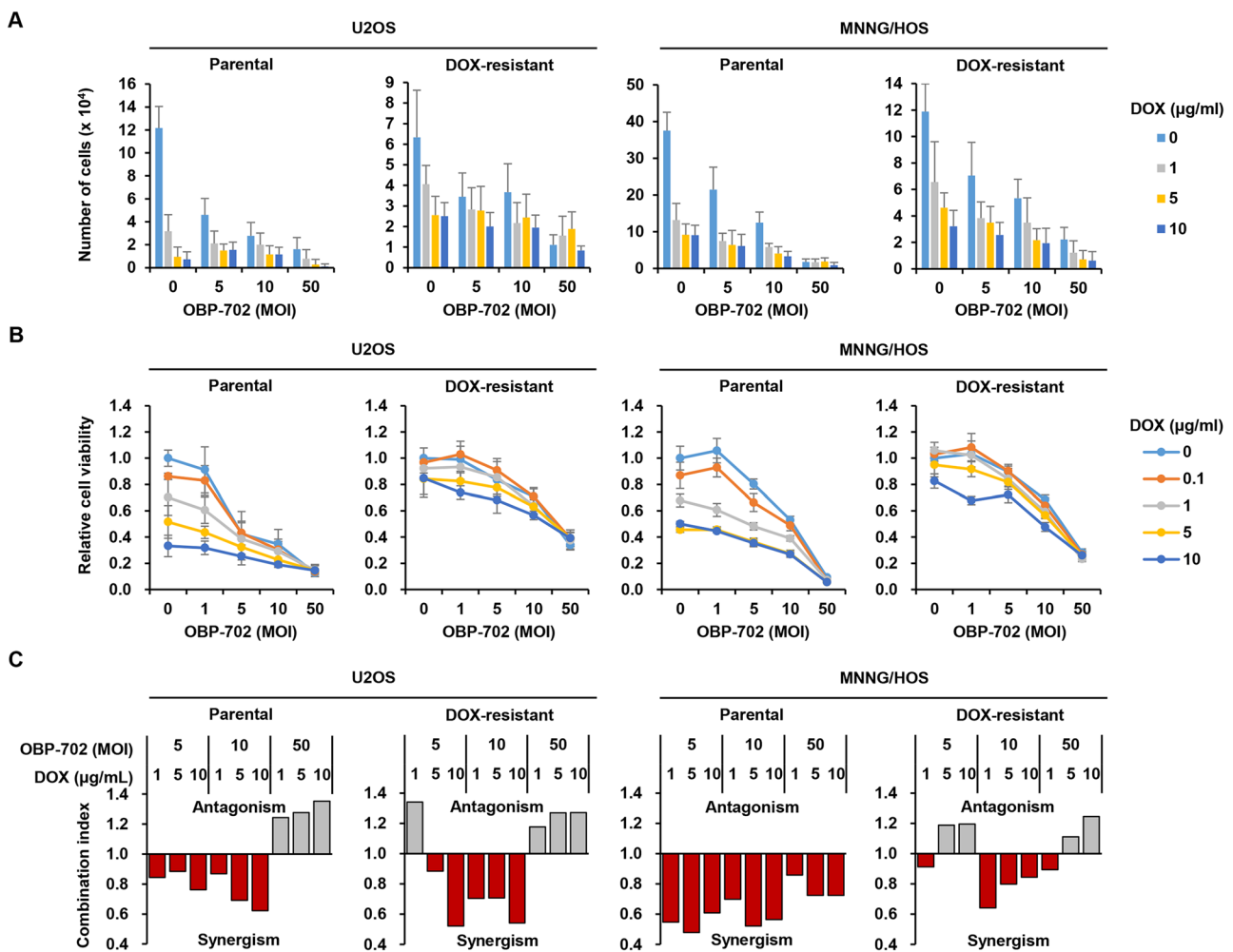
**Fig. 3** Combination treatment of DOX-resistant OS cells with OBP-702 and DOX. Four human osteosarcoma cell lines (U2OS, DOX-resistant U2OS, MNNG/HOS, DOX-resistant MNNG/HOS) were infected with OBP-702 at the indicated MOIs. Two days after viral infection, cells were further treated with DOX at the indicated doses for 24 h. **A** The number of viable cells was counted using a hemocytometer. Cell number data are expressed as mean ± SD ($n = 3$). **B** Cell viability was quantified using the XTT assay and calculated relative to that of parental cells, which was set at 1.0. Cell viability data are expressed as mean ± SD ($n = 5$). **C** the combination index was calculated using CalcuSyn software (BioSoft, Inc.). Synergism and antagonism were defined as interaction indices of < 1 or > 1, respectively

intraperitoneally 2 days after OBP-702 injection for 3 cycles (Fig. 5A). Combined treatment with DOX and OBP-702 significantly suppressed tumor growth when compared with mock treatment, whereas there was no significant difference between mock treatment and DOX or OBP-702 monotherapy (Fig. 5A, B). In the tumor growth curves of individual mice bearing DOX-resistant MNNG/HOS tumors (Supplementary Fig. S4), combination therapy completely suppressed tumor growth in one mouse. Histopathologic analysis for Ki67 and MDR1 revealed large necrotic areas lacking Ki67 and MDR1 expression in tumors treated with combination therapy (Fig. 5C). Combination treatment with OBP-702 significantly decreased the number of Ki67-positive cells and percentage of MDR1 expression area in DOX-treated OS tumors (Fig. 5D). These results suggest that OBP-702

enhances DOX-associated antitumor effects against DOX-resistant OS tumors via suppression of MDR1 expression.

## Discussion

The prognosis of chemotherapy-refractory OS patients is typically poor, despite the recent advances in multi-agent chemotherapy and surgical resection therapies. Chemoresistance is thus the primary obstacle to improving clinical outcomes in OS patients. In this study, we demonstrated that combination therapy with DOX and OBP-702 induces a synergistic antitumor effect in parental and DOX-resistant OS cells. DOX-resistant OS cells exhibited higher MDR1 expression than parental OS cells. OBP-702 efficiently
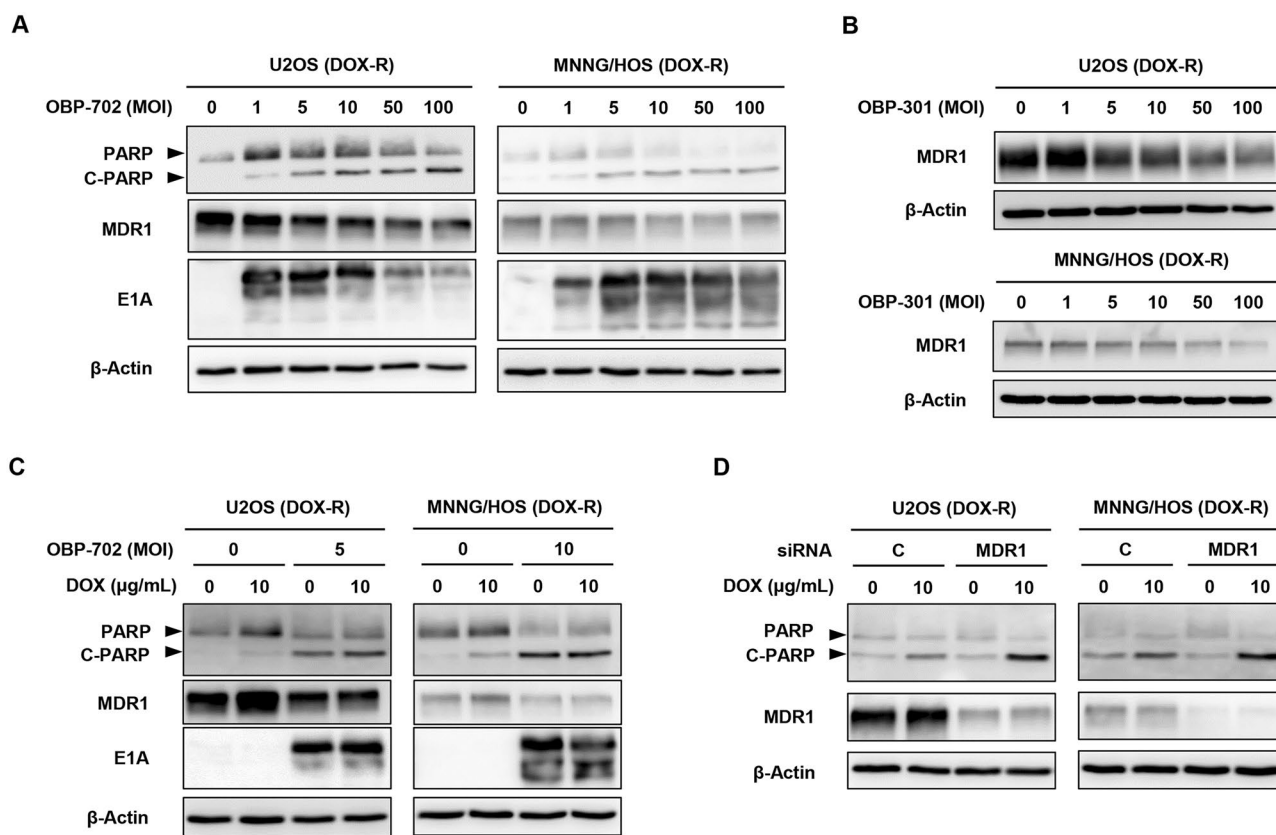
**Fig. 4** OBP-702–mediated suppression of MDR1 expression enhances DOX-induced apoptosis. **A** expression of PARP, C-PARP, MDR1, and adenoviral E1A proteins in DOX-resistant OS cells infected with OBP-702 at the indicated MOIs for 72 h. **B** Expression of MDR1 in DOX-resistant OS cells infected with OBP-301 at the indicated MOIs for 72 h. **C** Expression of PARP, C-PARP, MDR1, and adenoviral E1A proteins in DOX-resistant OS cells treated with OBP-702 and DOX. Two days after OBP-702 infection, cells were treated with DOX at 10 μg/mL for 24 h. **D** expression of PARP, C-PARP, and MDR1 proteins in DOX-resistant OS cells treated with DOX and MDR1 siRNA or control siRNA. Cells were treated with 10 nmol/L MDR1 siRNA or control siRNA. Two days after siRNA treatment, cells were treated with DOX at 10 μg/mL for 24 h. β-Actin was used as a loading control

promoted the DOX-induced apoptosis of DOX-resistant OS cells by suppressing the expression of MDR1 as effectively as MDR1 siRNA. Moreover, combination therapy with OBP-702 and DOX significantly suppressed tumor growth in a subcutaneous DOX-resistant OS xenograft tumor model. Thus, OBP-702 is a promising antitumor reagent for modulating chemoresistance in OS cells via suppression of MDR1 expression.

Our proteomics analysis demonstrated that MDR1 protein was one of 97 proteins expressed at greater than twofold higher levels in DOX-resistant U2OS and MNNG/HOS cells than parental cells. The expression of MDR1 mRNA was also upregulated in DOX-resistant OS cells and tumors. Consistent with our findings, several reports have demonstrated the importance of MDR1 activation in the chemoresistance of OS cells [23] and OS tissues [26, 27]. Regarding the underlying mechanism of chemotherapy-associated MDR1 activation, interplay between genetic and epigenetic effects is thought to induce MDR1 expression in chemoresistant

OS cells following chemotherapy [28]. Activation of the *MDR1* gene is associated with the selection of spontaneous mutations and acetylation of histone H3 within the *MDR1* upstream promoter. Liu et al. [29] suggested that disruption of the *MDR1* gene via genome editing using the clustered regulatory interspaced short palindrome repeats–associated Cas9 system is associated with reversal of chemoresistance in OS cells. Thus, inhibition of MDR1 expression appears to be a promising therapeutic strategy for modulating chemoresistance in OS cells.

Various MDR1-targeting inhibitors have been developed to overcome chemoresistance in human cancers [8, 30]. However, the effect of oncolytic virotherapy on MDR1 expression in chemoresistant OS cells remains unclear. In this study, we demonstrated that OBP-702 suppresses MDR1 expression and subsequent enhancement of DOX-induced apoptosis. As OBP-301 without p53 induction also suppressed MDR1 expression, at least two possible mechanisms induced by adenoviral infection could govern
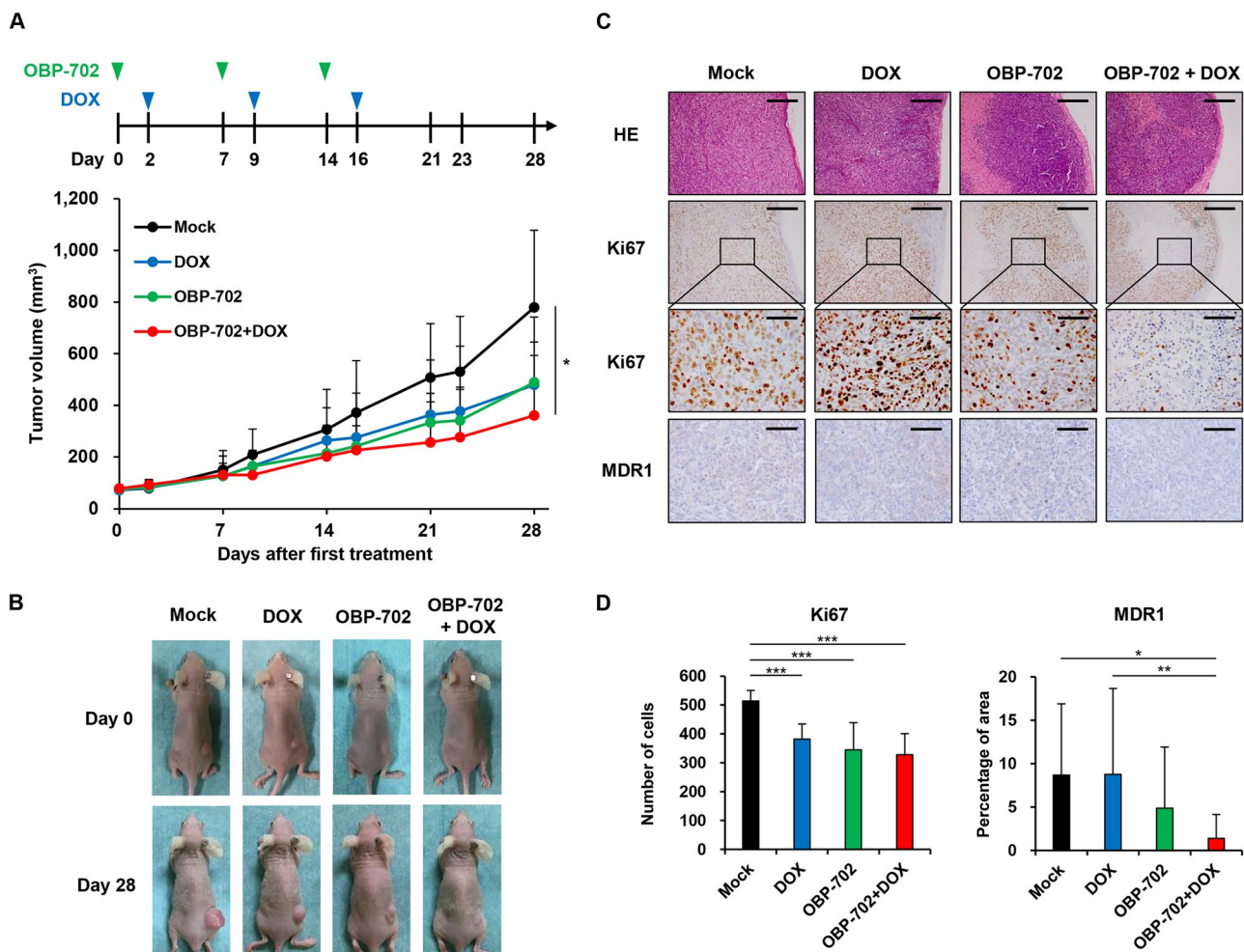
**Fig. 5** Enhancement of the DOX-mediated antitumor effect in combination treatment with OBP-702 in a subcutaneous DOX-resistant MNNG/HOS xenograft tumor model. Athymic nude mice were inoculated subcutaneously with DOX-resistant MNNG/HOS cells ($5 \times 10^6$ cells/site). OBP-702 ($5 \times 10^7$ PFU) was injected into the tumor at 3, 4, and 5 weeks after tumor inoculation. DOX (5 mg/kg body weight) was injected intraperitoneally 2 days after OBP-702 injection. **A** Tumor growth expressed as mean ± SD ($n = 7$ or 8 in each group; $*P < 0.05$). **B** Representative photographs of tumor-bearing mice treated with PBS (mock), DOX, OBP-702, or OBP-702 and DOX. **C** Histologic analysis of DOX-resistant MNNG/HOS tumors. Tumor tissues were obtained at 7 weeks after tumor inoculation.

Paraffin-embedded sections of DOX-resistant MNNG/HOS tumors were stained with hematoxylin and eosin or anti-Ki67 antibody. Upper and middle panels show low-magnification images of tumor tissues stained with hematoxylin and eosin and Ki67, respectively. Lower panels are high-magnification images of the areas outlined by black squares in the images of anti-Ki67 antibody staining. Upper scale bars, 200 μm; lower scale bars, 50 μm. **D** The number of Ki67-positive cells and percentage of MDR1 expression area in tumor tissues were calculated using ImageJ software. Data are expressed as mean ± SD ($n = 7$ or 8 in each group; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$)

OBP-702–mediated MDR1 suppression. One mechanism involves adenoviral E1A–dependent suppression of MDR1 expression. E1A protein has demonstrated therapeutic potential for enhancing the chemosensitivity of various human tumor cells [31]. Mantwill et al. [32] reported that E1A plays a critical role in adenovirus-mediated suppression of MDR1 expression. The second potential mechanism involves c-Jun NH2-terminal kinase (JNK)-dependent suppression of MDR1 expression. Adenoviral infection has been shown to induce JNK activation in the infected cells [33]. Zhou et al. [34] demonstrated that JNK represses transcription of the

*MDR1* gene. Taken together, these data suggest that OBP-702–mediated activation of E1A and JNK protein cooperatively downregulates MDR1 expression in DOX-resistant OS cells.

MDR1 protein is expressed in normal tissues as well as tumor tissues [8], suggesting that the use of MDR1-targeting inhibitors could have adverse effects [30]. MDR1 protein is expressed in hematopoietic stem cells [35] and various immune cells [36], including dendritic cells, NK cells, and CD8+ T cells. Indeed, MDR1-targeting inhibitors have been shown to attenuate the maturation of dendritic cells [37] and

the cytotoxic function of NK cells [38] and CD8+ T cells [39]. Therefore, systemic administration of MDR1-targeting inhibitors could adversely affect antitumor immunity, although such inhibitors could enhance chemosensitivity. By contrast, oncolytic virotherapy is a promising antitumor strategy for specifically targeting tumor cells without affecting normal cells [14, 15]. Recent evidence suggests that oncolytic virotherapy enhances antitumor immunity by inducing the release of damage-associated molecular pattern molecules and tumor-associated antigens [40, 41]. Therefore, to overcome chemoresistance without adversely affecting antitumor immunity, OBP-702–mediated tumor-specific suppression of MDR1 expression may be more beneficial than MDR1-targeting inhibitors.

It is worth noting that DOX-resistant OS cells show the low proliferative ability, which is thought to be one of the characteristics for cancer stem-like cells. DOX treatment mainly targets proliferating cancer cells by inducing the intercalation into DNA base pairs, nucleosome destabilization, breakage of DNA strands, and inhibition of both DNA and RNA synthesis [42]. Dormant cancer stem-like cells would resist the DOX-mediated cytotoxic effect. In contrast, DOX-resistant OS cells showed the low CAR expression. CAR expression has been shown to be associated with cell cycle status, in which CAR expression is higher in the M phase than in the G1 and G2 phases [43]. Dormant cancer stem-like cells may be less sensitive to adenovirus infection than cycling cancer cells. Previous report has shown that DOX treatment induces chemoresistance with stem-like phenotype in U2OS and MNNG/HOS cells [44]. As we previously demonstrated that OBP-301 efficiently eliminates gastric cancer stem-like cells with chemoradioresistant phenotype by suppressing their dormancy [45], further experiments would be warranted to evaluate whether OBP-702 shows the therapeutic potential against dormant OS stem-like cells.

In conclusion, we demonstrated that the oncolytic adenovirus OBP-702 enhances the DOX-induced apoptosis of human OS cells via suppression of MDR1 expression, which is a critical factor in the chemoresistance of OS cells. Thus, combination therapy with DOX and OBP-702 is a novel and promising strategy for overcoming chemoresistance in OS patients. To investigate the tolerability and efficacy of combination therapy with OBP-702 and chemotherapy, further clinical studies are warranted in chemotherapy-refractory OS patients.

## Declarations

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