

P53-Armed Oncolytic Virotherapy Promotes the Efficacy of PD1 Blockade in Murine Osteosarcoma Tumors

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Research Article

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Abstract

Background/Aim: Osteosarcoma (OS) is refractory to immune checkpoint inhibitors targeting programmed cell death 1 (PD1)/PD ligand 1 (PD-L1) due to poor immune response. We previously developed telomerase-specific, replication-competent oncolytic adenoviruses non-armed OBP-301 and P53-armed OBP-702 that exert antitumor efficacy against human OS cells. Recently, we demonstrated that P53-armed OBP-702 induces more profound immunogenic cell death and antitumor immune response against human and murine OS cells than does non-armed OBP-301. In the present study, we assessed the combined efficacy of PD1 blockade and P53-armed OBP-702 against murine OS cells. **Materials and Methods:** Three murine OS cell lines (K7M2, NHOS, NHOS-LM4) were used to assess the cytopathic effect of non-armed OBP-301 and P53-armed OBP-702 by XTT assay. Virus-induced immunogenic cell death was assessed by analyzing the levels of extracellular adenosine triphosphate and high-mobility group box protein B1. The expression of PD-L1 and PD-L2 was analyzed by flow cytometry. The malignant potential of NHOS-LM4 cells was analyzed by a migration and invasion assay. An orthotopic NHOS-LM4 tumor model was used to evaluate the antitumor efficacy of combination therapy with P53-armed OBP-702 and anti-PD1. **Results:** P53-armed OBP-702 exhibited antitumor potential for the induction of immunogenic cell death, apoptosis, autophagy, and PD-L1/2 upregulation in K7M2 and NHOS cells. NHOS-LM4 cells showed increased migratory and invasive ability compared to NHOS cells. P53-armed OBP-702 significantly suppressed the malignant potential of NHOS-LM4 cells. Combination dosing showed that P53-armed OBP-702 significantly promoted the antitumor effect of PD1 blockade against NHOS-LM4 tumors. **Conclusion:** Our

results suggest that P53-armed OBP-702 is a promising agent for improving the antitumor effect of PD1 blockade in treating invasive OS.

Osteosarcoma (OS) is the most common malignant tumor of bone in children and young adults (1, 2). The 5-year survival rate of patients with localized primary OS is increased to approximately 60% by current treatment strategies, including aggressive surgery and multi-agent chemotherapy (2). However, the prognosis of patients with unresectable, chemoresistant, and recurrent OS has remained poor over the past several decades (2, 3). As current treatment strategies against advanced OS are insufficient, novel therapeutic strategies are needed to improve the clinical outcome of patients with advanced OS.

Several immunotherapies have been developed to improve the poor prognosis of patients with advanced OS (4, 5). Among these approaches, immune checkpoint inhibitors (ICIs) that target the programmed cell death 1 (PD1)–programmed cell death ligand 1 (PD-L1) axis have been shown to improve antitumor immunity (6). ICIs including PD1 or PD-L1 antibodies have been shown to be effective in patients with certain cancer types, such as melanoma and small-cell lung cancer (7). However, several clinical trials using anti-PD1 demonstrated that patients with OS were less sensitive to PD1 blockade (8-10). Antitumor modalities, including chemotherapy and radiotherapy, have been shown to induce immunogenic cell death (ICD), which is a form of cell death in which the immune system is induced *via* the secretion of damage-associated molecular patterns (DAMPs), including adenosine triphosphate (ATP) and high-mobility group box protein 1 (HMGB1) (11). However, the combination of ICD inducers does not always promote the antitumor effect of ICIs in the clinical setting (12). Therefore, the development of novel antitumor modalities to induce ICD and promote the efficacy of ICIs is needed for the treatment of patients with OS.

Oncolytic virotherapy has emerged as a novel antitumor strategy to enhance the antitumor efficacy of ICIs *via* ICD induction (13, 14). Previously, we developed a telomerase-specific, replication-competent, oncolytic adenovirus non-armed OBP-301 (suratadenoturev) (15), in which the human telomerase reverse transcriptase (*TERT*) gene promoter drives the expression of viral *E1A* and *E1B* genes (15, 16). The antitumor efficacy of non-armed OBP-301 against human OS cells was confirmed in monotherapy (17) and combination therapy with radiotherapy or chemotherapy (18, 19). We further reported that arginine-glycine-aspartic acid fiber-modified OBP-301 enhanced the therapeutic potential of PD1 blockade against murine OS (20). We have also generated P53-armed OBP-702 and confirmed that this construct exhibited more profound antitumor effects with apoptosis and autophagy against human OS cells than does non-armed OBP-301 (21, 22). We further demonstrated that P53-armed OBP-702-mediated P53 overexpression enhances the antitumor efficacy of ICIs *via* profound ICD induction in murine pancreatic cancer cells (23). More recently, we showed that P53-armed OBP-702 induced more profound ICD of human OS cells than did chemotherapy or non-armed OBP-301 (24). However, whether P53-armed OBP-702 promotes the antitumor effect of ICIs against murine OS tumors remains unclear. We hypothesized that P53-armed OBP-702 would enhance the antitumor efficacy of ICIs against murine OS tumors.

In the present study, we investigated the therapeutic potential of telomerase-specific oncolytic adenoviruses (non-armed OBP-301, P53-armed OBP-702) against murine OS cells (K7M2, NHOS). *In-vitro* cytopathic effect and the induction of ICD were assessed by measuring cell viability and the secretion of extracellular adenosine triphosphate (ATP) and high-mobility group box protein B1 (HMGB1). We

established invasive NHOS-LM4 cells; using an orthotopic NHOS-LM4 tumor model, we analyzed the *in-vivo* antitumor efficacy of P53-armed OBP-702 combined with PD1 antibody, including changes in the tumor immune microenvironment.

Materials and Methods

Cell lines. The murine OS cell line K7M2 (25, 26) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium. The murine OS cell line NHOS (27, 28) was obtained from RIKEN (Tokyo, Japan) and maintained in RPMI1640 medium. All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/ml streptomycin. Cells were not cultured for more than 5 months following resuscitation. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Recombinant adenoviruses. The construction and characterization of recombinant telomerase-specific replication-competent adenovirus non-armed OBP-301 (suratadenoturev), in which the promoter element of the *hTERT* gene drives the expression of the *E1A* and *E1B* genes, has been described previously (15, 16). To induce exogenous P53 expression by OBP-301, we generated P53-armed OBP-702, in which a human wild-type *P53* gene expression cassette was inserted into the *E3* region of the viral vector; the construction and characterization of this adenovirus is described elsewhere (29). Recombinant adenoviruses were purified using cesium chloride step gradients; titers were determined by a plaque-forming assay using 293 cells (Sumitomo Pharma Co., Ltd, Osaka, Japan); and viral stocks were stored at -80°C.

Immune checkpoint inhibitor. Anti-mouse PD1 antibody (Clone 4H2) was obtained from Ono Pharmaceutical Co., Ltd. (Osaka, Japan).

Cell viability assay. Cells were seeded in 96-well plates at a density of 1×10^3 cells/well. After 24 h, cells were infected with non-armed OBP-301 or P53-armed OBP-702 at multiplicities of infection (MOI) of 0, 10, 50, 100, 500, or 1000 plaque-forming units (PFU)/cell for 24 or 72 h (n=5). Cell viability was then determined using Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol.

Western blot analysis. Cells were seeded in a 100-mm dish at a density of 2×10^5 cells/dish. After 24 h, cells were infected with P53-armed OBP-702 at MOIs of 0, 10, 50, 100, and 500 PFU/cell for 72 h. Whole-cell lysates were then prepared in a lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100] containing a protease inhibitor cocktail (Complete Mini; Roche Molecular Biochemicals). Proteins were electrophoresed on 6-15% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Hybond-P; GE HealthCare, Amersham, UK). Blots were blocked with Blocking-One (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. Primary antibodies used were as follows: mouse monoclonal antibody to Ad5 E1A (554155; BD PharMingen, Franklin Lakes, NJ, USA), rabbit monoclonal antibody to P53 (2527; Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal antibody to poly(ADP-ribose) polymerase (PARP) (9542; Cell Signaling Technology), rabbit polyclonal antibody to P62 (5114; Cell Signaling Technology), and mouse monoclonal antibody to β -actin (A5441; Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies were as follows:

horseradish peroxidase-conjugated antibodies against rabbit immunoglobulin G (IgG) (NA934; GE Healthcare) or mouse IgG (NA931; GE Healthcare).

Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Prime; GE Healthcare). The expression of each protein was calculated relative to that of mock-infected cells, which was set at 1.0, using ImageJ software (1.54g; National Institutes of Health, Bethesda, MA, USA).

DAMP analysis. Cells were seeded on 6-well plates at a density of 2×10^5 cells/well. After 24 h, cells were infected with non-armed OBP-301 or P53-armed OBP-702 at MOIs of 0 and 100 PFU/cell for 24 h (n=3). Spent medium was then collected and analyzed using ENLITEN ATP assay (Promega, Madison, WI, USA) and HMGB1 ELISA Kit II (Shino-Test, Kanagawa, Japan) according to the respective manufacturer's protocol.

Flow-cytometric analysis. Cells were seeded on 6-well plates at a density of 2×10^5 cells/well. After 24 h, cells were infected with P53-armed OBP-702 at MOIs of 0 and 100 PFU/cell for 24 h (n=3). To analyze the expression of PD-L1 or PD-L2 on murine OS cells, cells were incubated for 30 min at 4°C with primary antibodies consisting of allophycocyanin-conjugated mouse monoclonal antibody to PD-L1 (124311; Biolegend, San Diego, CA, USA) or rat monoclonal antibody to PD-L2 (ab21107; Abcam, Cambridge, UK). Cells then were further incubated with a secondary antibody consisting of fluorescein isothiocyanate-conjugated donkey anti-rat IgG (ab102181; Abcam) for 15 min at 4°C. Allophycocyanin-conjugated isotype IgG or fluorescein isothiocyanate-conjugated isotype IgG was used as a control IgG. Cells were analyzed using a FACS Lyric (BD Biosciences, San Jose, CA, USA) flow

cytometer. The mean fluorescence intensity for each cell line was determined by calculating the difference between the mean fluorescence intensity in antibody-incubated and isotype control IgG-incubated cells from three independent experiments.

Migration and invasion assay. To analyze the malignant potential of murine OS cells, migration and invasion assays were performed using 24-well Boyden chambers equipped with 8- μ m pore size filter membranes, both uncoated and coated with Matrigel matrix (BD Biosciences). Cells (5×10^4 cells/well) were seeded in a 500- μ L of serum-free medium in the upper chambers and infected with P53-armed OBP-702 at MOIs of 0 and 100 PFU/cell. After 24 h, migrating or invading cells on the lower surface of the membrane were stained with crystal violet (Sigma-Aldrich); cells were counted in five different randomly selected fields under a microscope.

In-vivo orthotopic NHOS-LM4 tumor model. Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine (Approval No. OKU-2020172). NHOS-LM4 cells (2×10^6 cells per site) were orthotopically inoculated into the proximal tibia of 6-week-old female BALB/c mice (n=5 in each group) (CLEA Japan, Tokyo, Japan). When palpable tumors developed (at 4 days after implantation), a 20- μ L volume of solution containing P53-armed OBP-702 at a dose of 5×10^7 PFU (or phosphate-buffered saline) was injected into the tumors; dosing was repeated once per week for a total of three cycles. Mice also were dosed once weekly for a total of three cycles by intraperitoneal injection with 100- μ L of solution containing anti-PD1 (1st cycle: 4 mg/kg; 2nd and 3rd cycles: 2 mg/kg) or phosphate-buffered saline. Throughout the

study (following the first viral injection), tumor size was monitored twice per week by measuring tumor length (L) and width (W) using calipers. Tumor volume was calculated using the formula for the volume of an ellipsoid sphere: tumor volume (mm^3) = $L \times W^2 \times 0.5$. At 21 days after the first dose, mice were euthanized and tumors were harvested; tumors were weighed and then fixed in neutral buffered formalin at room temperature.

Immunohistochemistry. Fixed tumor tissues were processed and embedded in paraffin using standard methods. Paraffin-embedded tissues were sectioned at 4- μm thicknesses, and the sections then were deparaffinized in xylene and rehydrated in a graded ethanol series. After blocking endogenous peroxidases by incubation with 3% H_2O_2 for 10 min, the sections were subjected to antigen retrieval by boiling in citrate buffer or ethylenediaminetetraacetic acid buffer for 14 min in a microwave oven. The sections were incubated (for 1 h at room temperature or overnight at 4°C) with primary antibodies against CD8 (eBioscience, San Diego, CA, USA) and CD4 (eBioscience), and then for 30 min at room temperature with horseradish peroxidase-conjugated secondary antibody. Following signal generation by staining with 3,3-diaminobenzidine staining and counterstaining with Mayer's hematoxylin, samples were dehydrated and mounted onto coverslips. The numbers of cells expressing CD8 and CD4 (markers of cytotoxic T-lymphocytes and helper T-lymphocytes, respectively) were calculated from five different randomly selected fields. All sections were analyzed by light microscopy.

Statistical analysis. All data are expressed as the mean $\pm$ standard deviation. Inferential statistical analysis was performed using SPSS Statistics v.26 (SPSS,

Chicago, IL, USA). Two-tailed non-paired Student's *t*-tests were used to compare mean differences between two groups. Two-tailed one-way ANOVA with *post hoc* Tukey's tests were used to compare mean differences among multiple groups in animal experiments. Statistical significance was defined as a *p*-value less than 0.05.

Results

Non-armed OBP-301 and P53-armed OBP-702 exhibited in-vitro cytopathic effects against K7M2 and NHOS cells. To evaluate the therapeutic potential of non-armed OBP-301 and P53-armed OBP-702 against murine OS cells, the viabilities of K7M2 and NHOS cells were assessed (using XTT assay) over 3 days following viral infection. The viability of K7M2 and NHOS cells was not reduced at 24 h after infection with non-armed OBP-301, whereas non-armed OBP-301 at high dose (>500 MOI) significantly suppressed the viability of K7M2 and NHOS cells at 72 h after infection (**Figure 1A**). In contrast, the viability of NHOS cells was reduced even at 24 h after infection with P53-armed OBP-702 at high dose (>500 MOI) (**Figure 1B**). P53-armed OBP-702 at high dose (>500 MOI) significantly suppressed the viability of K7M2 and NHOS cells at 72 h after infection (**Figure 1B**). Moreover, NHOS cells showed high sensitivity to P53-armed OBP-702 even at low doses (<100 MOI) at 72 h after infection (**Figure 1B**). These results suggest that non-armed OBP-301 and P53-armed OBP-702 have therapeutic potential against murine OS cells and that NHOS cells are more sensitive to non-armed OBP-301 and P53-armed OBP-702 than are K7M2 cells.

In previous work, we showed that P53-armed OBP-702 induces apoptosis- and autophagy-related cell death of human OS cells (21). To investigate whether P53-armed OBP-702 induces apoptosis and autophagy in K7M2 and NHOS cells, cells

were infected with P53-armed OBP-702 for 72 h. Notably, we were unable to obtain a cell lysate from NHOS cells infected with P53-armed OBP-702 at 500 MOI because of the strong induction of cell death. Instead, we analyzed the protein levels of P53, adenoviral E1A, PARP, cleaved PARP (an apoptosis marker), and P62 (an autophagy marker) by western blot analysis. We observed that P53-armed OBP-702 induced the expression of E1A and P53 proteins in K7M2 and NHOS cells in a dose-dependent manner (**Figure 1C**). Consistent with the accumulation of E1A and P53 proteins, the level of C-PARP increased and that of P62 decreased in P53-armed OBP-702-infected K7M2 and NHOS cells (**Figure 1C**). These results suggested that P53-armed OBP-702 induces apoptosis and autophagy of murine OS cells.

P53-armed OBP-702 induced the secretion of ATP and HMGB1 in K7M2 and NHOS cells. Oncolytic viruses have been shown to induce ICD and to promote an antitumor immune response by inducing the secretion of DAMPs, including ATP and HMGB1 (30). To investigate the ICD-inducing capabilities of non-armed OBP-301 and P53-armed OBP-702 in murine OS cells, the levels of extracellular ATP and HMGB1 were analyzed using the conditioned medium from K7M2 and NHOS cells infected with non-armed OBP-301 and P53-armed OBP-702 at 100 MOI for 24 h, a condition in which non-armed OBP-301 and P53-armed OBP-702 did not suppress the viability of murine OS cells (**Figure 1**). At this MOI and time point, P53-armed OBP-702 significantly increased the secretion of extracellular ATP in NHOS cells, but not in K7M2 cells (**Figure 2A**). In contrast, the amount of extracellular HMGB1 was increased in K7M2 cells, but not in NHOS cells, after infection with P53-armed OBP-702 under these conditions (**Figure 2B**). These results suggest that P53-armed OBP-702 induces ICD with secretion of DAMPs in murine OS cells.

P53-armed OBP-702 increased the expression of PD-L1 and PD-L2 on K7M2

and NHOS cells. We previously showed that murine K7M2 and NHOS cells express PD-L1 protein, and that infection with non-armed OBP-301 increased the expression of PD-L1 (20). Therefore, using flow cytometry, we next investigated whether P53-armed OBP-702 also potentiated the expression of PD-L1 and PD-L2 proteins in murine OS cells. Infection by P53-armed OBP-702 resulted in significant accumulation of PD-L1 and PD-L2 proteins on the surface of K7M2 and NHOS cells (**Figure 3**). These results suggest that P53-armed OBP-702 has therapeutic potential to enhance the expression of PD-L1 and PD-L2 proteins on murine OS tumors.

Establishment of NHOS-LM4 cells with enhanced invasive potential. To obtain invasive murine OS cells, we established NHOS-LM4 cells by repeated orthotopic inoculation of NHOS cells and culturing of metastatic NHOS cells in the lung for a total of four rounds. To evaluate their malignant potential, we compared the migratory and invasive capabilities of NHOS and NHOS-LM4 cells. NHOS-LM4 cells showed significantly higher migratory and invasive capabilities compared to parental cells (**Figure 4**). These results suggest that NHOS-LM4 cells have greater malignant potential compared to parental NHOS cells.

P53-armed OBP-702 reduced NHOS-LM4 cell viability, migration and invasion, and induced the secretion of DAMPs. To investigate the therapeutic potential of P53-armed OBP-702 against NHOS-LM4 cells, the viability of NHOS-LM4 cells was assessed over 3 days after infection. At a high dose of P53-armed OBP-702 (1000

MOI), the viability of NHOS-LM4 cells was reduced at 24 h, whereas even moderate doses (>50 MOI) reduced the viability of NHOS-LM4 cells at 72 h after infection (**Figure 5A**). In contrast, when infected with P53-armed OBP-702 at a moderate dose (<100 MOI), the viabilities of K7M2 and NHOS cells were not reduced at 24 h (**Figure 5A**). Therefore, infection with P53-armed OBP-702 at an MOI of 100 PFU/cell for 24 h was used to analyze the levels of extracellular ATP and HMGB1 and migration and invasion in K7M2 and NHOS cells. P53-armed OBP-702 infection significantly increased the secretion of ATP and HMGB1 in NHOS-LM4 cells (**Figure 5B**). P53-armed OBP-702 infection also significantly suppressed the numbers of migrating and invading OS cells (**Figure 5C**). These results suggest that P53-armed OBP-702 has potential for inducing cytopathic effects and ICD, and for suppressing migration and invasion of NHOS-LM4 cells.

Combination dosing with P53-armed OBP-702 and anti-PD1 potentiated the antitumor effect of the antibody in an orthotopic NHOS-LM4 tumor model. To assess the therapeutic potential of combination therapy with P53-armed OBP-702 and anti-PD1 against murine OS tumors, we used a syngeneic mouse model with NHOS-LM4 tumors. NHOS-LM4 cells were orthotopically inoculated into the proximal tibia of female BALB/c mice (day 0) (**Figure 6A**). P53-armed OBP-702 was intratumorally injected once per week for three cycles (on days 4, 11, and 18), and anti-PD1 was intraperitoneally injected once per week for three cycles (on days 7, 14, and 21) (**Figure 6A**). Monotherapy with the anti-PD1 or P53-armed OBP-702 alone did not suppress tumor growth compared to the control group (**Figure 6B** and **C**). In contrast, combination therapy with anti-PD1 and P53-armed OBP-702 significantly suppressed the growth and necropsy weight of orthotopic NHOS-LM4

tumors compared to the control group (**Figure 6B and C**). Immunohistochemical analysis demonstrated that combination therapy-treated tumors contained significantly increased levels of CD8⁺ and CD4⁺ T-cells compared to monotherapy-treated tumors (**Figure 6D**). These results suggested that P53-armed OBP-702 has potential for promoting the antitumor effect of PD1 blockade against NHOS-LM4 tumors *via* activation of antitumor immunity.

Discussion

Several clinical trials have shown that OS tumors are less sensitive to immunotherapy with PD1 blockade than are tumors of other types (8-10). Therefore, the development of immunotherapy combining PD1 blockade with immunological agents is needed to improve the clinical outcomes of patients with OS. In the present study, we demonstrated that the P53-armed, telomerase-specific oncolytic adenovirus OBP-702 has the therapeutic potential to induce cytopathic effects, ICD, and the accumulation of PD-L1 and PD-L2 proteins while suppressing migration and invasion by murine OS cells. To evaluate the therapeutic potential of P53-armed OBP-702 against more malignant murine OS cells, we established NHOS-LM4 cells with enhanced migration and invasion capabilities. The combination of P53-armed OBP-702 and PD1 blockade significantly suppressed the growth of orthotopic NHOS-LM4 tumors. Furthermore, the numbers of tumor-infiltrating CD8⁺ T-cells and CD4⁺ T-cells were significantly increased in the combination therapy-treated tumors compared to tumors from the control and monotherapy groups. Therefore, we conjecture that P53-armed OBP-702 would be a promising antitumor agent for potentiating PD1 blockade against OS, reflecting the ability of the viral construct to induce an antitumor immune response.

P53-armed OBP-702 was superior to non-armed OBP-301 in suppressing the viability of murine OS cells. Consistent with the suppression of cell viability, P53-armed OBP-702 increased the expression of apoptosis- and autophagy-related proteins in K7M2 and NHOS cells. These findings suggest that P53-armed OBP-702 has the therapeutic potential to induce apoptosis- and autophagy-related cell death in murine OS cells *via* activation of human P53 protein. Many kinds of animal models with OS tumors have been developed in cancer research (31). Regarding the relationship between P53 function and OS development, we note that genetically engineered mouse models lacking P53 function are known to be prone to developing OS tumors, mimicking the features of human OS tumors (32, 33). These findings suggest that P53 function plays a central role in suppressing the development of murine OS tumors. Although the P53 gene-regulatory network is not same between human and mouse, a recent report has shown that a core set of 86 genes shared between humans and mice that are direct targets of P53 are particularly enriched for functioning in apoptosis in both species (34). Therefore, P53-armed OBP-702-mediated human P53 activation may play a crucial role in the induction of apoptosis-related cell death in murine OS cells.

P53-armed OBP-702 increased the secretion of ATP in virus-sensitive NHOS cells and the secretion of HMGB1 in virus-resistant K7M2 cells. These findings suggest that murine OS cells show distinct responses in P53-armed OBP-702-mediated ICD induction. We recently demonstrated that P53-armed OBP-702 induced the secretion of ATP more strongly than that of HMGB1 in human OS cells (24). Oncolytic viruses have been shown to induce autophagy-related cell death in tumor cells (35). Autophagy has been suggested to be involved in the mechanism that sustains high ATP levels in cells undergoing ICD (36). As recent review

suggested that ATF6 plays a crucial role in maintaining autophagy-related degradation of mutant P53 in response to endoplasmic reticulum (37), P53-armed OBP-702-mediated induction of autophagy may be effective to reduce mutant P53 in P53-mutant OS cells.

In contrast, we recently showed that chemotherapeutic agents, including cisplatin and doxorubicin, induce the secretion of HMGB1 more strongly than that of ATP in human OS cells (24). Tang *et al.* also recently demonstrated that chemotherapy enhanced autophagy to induce the release of HMGB1 in human tumor cells (37). Huang *et al.* demonstrated that chemotherapy resulted in the accumulation of HMGB1, along with inducing chemoresistance and autophagy in human OS cells (38). Although the underlying mechanism in the secretion of ATP and HMGB1 in P53-armed OBP-702-infected OS cells remains unclear, P53-armed OBP-702-mediated induction of autophagy may be involved in promoting the secretion of ATP and HMGB1 by murine OS cells.

P53-armed OBP-702 increased the expression of PD-L1 and PD-L2 on the surface of K7M2 and NHOS cells at 24 h after infection. Many viruses have been shown to increase the expression of PD-L1 and PD-L2 on hematopoietic and non-hematopoietic cells (39). The expression of PD-L1 and PD-L2 is regulated by both pro- and anti-inflammatory signals (39). Type I interferons, including interferon- α and interferon- β , play a central role as antiviral cytokines that suppress the activation of virus-specific CD8⁺ T-cells in the early phase of acute infection. Therefore, P53-armed OBP-702-mediated upregulation of PD-L1 and PD-L2 on murine OS cells may suppress the activation of virus-specific CD8⁺ T-cells that would otherwise eliminate P53-armed OBP-702-infected OS cells. In contrast, PD1 antibody would activate virus-specific CD8⁺ T-cells that serve to eliminate P53-armed OBP-702-

infected OS cells and promote the release of tumor-associated antigens. Therefore, combination of P53-armed OBP-702 and PD1 blockade may be an effective strategy for activating both antiviral and antitumor immunities.

NHOS-LM4 cells showed more malignant potential in migration and invasion than did parental NHOS cells. Orthotopic NHOS-LM4 tumor models demonstrated that the combination of P53-armed OBP-702 and anti-PD1 significantly suppressed the growth of NHOS-LM4 tumors *via* induction of tumor-infiltrating CD8⁺ and CD4⁺ T-cells. These findings suggest that the combination of P53-armed OBP-702 and PD1 blockade would be an effective strategy for treating advanced OS tumors. We previously demonstrated that P53-armed OBP-702 suppresses tumor growth and bone destruction in orthotopic mouse tumor models employing human MNNG/HOS cells (21). Martinez-Velez *et al.* showed that oncolytic adenovirus delta-24-ACT suppressed tumor growth and prolonged the survival of tumor-bearing mice by activating the massive accumulation of CD8⁺ T-cells in orthotopic K7M2 tumor mouse models (40). Recently, Christie *et al.* demonstrated that combination of ICIs and loaded oncolytic myxoma virus expressing human tumor necrosis factor in peripheral blood mononuclear cells suppressed lung metastasis of K7M2 cells (41). Sakuda *et al.* showed that intratumoral injection of vesicular stomatitis virus incorporating tumor-suppressor *miRNA143* reduced primary OS tumor (43). However, whether intratumoral injection of oncolytic viruses in combination with ICIs is able to suppress lung metastasis in orthotopic OS tumor models remains to be elucidated. Therefore, further experiments are warranted to evaluate the therapeutic potential of combination therapy with P53-armed OBP-702 and PD1 antibody against lung metastasis of murine OS.

In conclusion, we demonstrated that the P53-armed telomerase-specific oncolytic adenovirus OBP-702 has potential for promoting the antitumor effect of PD1 blockade against murine OS. Taken together, our results indicate that the combination of P53-armed, telomerase-specific oncolytic adenovirus with ICI immunotherapy may provide a novel therapeutic option for treating invasive OS.

Conflicts of Interest

Y. Urata. is the President and CEO of Oncolys BioPharma, Inc. H. Tazawa and Toshiyoshi Fujiwara are consultants of Oncolys BioPharma, Inc. The other Authors disclosed no potential conflicts of interest.

Authors' Contributions

Conception and design: H. Tazawa, T. Ozaki and Toshiyoshi Fujiwara. Development of methodology: M. Kure, K. Demiya, H. Kondo and Y. Mochizuki. Acquisition of data: M. Kure, K. Demiya, H. Kondo, Y. Mochizuki, T. Komatsubara and A. Yoshida. Analysis and interpretation of data: M. Kure, K. Demiya, H. Kondo, Y. Mochizuki, T. Komatsubara and A. Yoshida. Writing, review, and/or revision of the manuscript: M. Kure, H. Tazawa and Toshiyoshi Fujiwara. Resources: Y. Urata. Study supervision: H. Tazawa, K. Uotani, J. Hasei, Tomohiro Fujiwara, T. Kunisada, S. Kagawa, T. Ozaki and Toshiyoshi Fujiwara.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this manuscript.

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Figure legends

Figure 1. *In-vitro* cytopathic effect of non-armed OBP-301 and P53-armed OBP-702 against murine osteosarcoma cells. K7M2 and NHOS cells were infected with non-armed OBP-301 (**A**) or P53-armed OBP-702 (**B**) at the indicated multiplicity of infection (MOI), and cell viability was quantified (using XTT assay) at 24 and 72 h after infection. Cell viability was calculated relative to that of uninfected cells, which was set at 1.0. Cell viability data are expressed as the mean $\pm$ standard deviation (n=5). (**C**) K7M2 and NHOS cells were infected with P53-armed OBP-702 at the indicated MOIs for 72 h. Cell lysates were subjected to western blot analysis for E1A, P53, poly (ADP-ribose) polymerase (PARP), cleaved PARP (C-PARP), and P62; β -actin was assayed as a loading control. The expression of each protein was calculated relative to that of mock-infected cells, which was set at 1.0. Statistical significance was determined using two-tailed non-paired Student's *t*-test. Significantly different from 0 MOI at: * $p < 0.05$, ** $p < 0.01$.

Figure 2. P53-armed OBP-702-mediated induction of immunogenic cell death of murine osteosarcoma cells. K7M2 and NHOS cells were infected with non-armed OBP-301 or P53-armed OBP-702 at a multiplicity of infection (MOI) of 100 plaque-forming units/cell for 24 h. Spent medium was analyzed using an ENLITEN ATP assay (**A**) and a high-mobility group box protein B1 (HMGB1) enzyme-linked immunosorbent assay kit (**B**). Data are expressed as the mean $\pm$ standard deviation (n=3). Statistical significance was determined using two-tailed one-way analysis of variance followed by Tukey's comparison tests. Significantly different at: * $p < 0.05$, ** $p < 0.01$.

Figure 3. P53-armed OBP-702-mediated upregulation of programmed cell death ligand 1 (PD-L1) and PD-L2 on murine osteosarcoma cells. Expression of PD-L1 (**A**) and PD-L2 (**B**) in K7M2 and NHOS cells infected with P53-armed OBP-702 at the indicated multiplicity of infection (MOI) for 24 h was assessed using flow cytometry. Mean fluorescence intensity (MFI) of PD-L1 and PD-L2 expression was determined by calculating the difference between MFI in antibody-incubated and isotype control IgG-incubated cells. Data are expressed as mean $\pm$ standard deviation (n=3). Statistical significance was determined using two-tailed non-paired Student's *t*-test. Significantly different from 0 MOI at: * $p < 0.05$, ** $p < 0.01$.

Figure 4. Comparison of the migration and invasion of NHOS cells and NHOS-LM4 cells with enhanced invasive potential. The number of migrating (**A**) and invading (**B**) cells in five randomly selected fields was determined under a light microscope. Representative photographs of migrating cells stained with crystal violet are shown in the left panels. Scale bars: 100 μ m. Data are expressed as the mean $\pm$ standard deviation (n=5). Statistical significance was determined using two-tailed non-paired Student's *t*-test. **Significantly different from NHOS cells at $p < 0.01$.

Figure 5. P53-armed OBP-702-mediated suppression of malignant potential in NHOS-LM4 cells. Highly invasive NHOS-LM4 cells were infected with non-armed OBP-301 (**A**) or P53-armed OBP-702 (**B**) at the indicated multiplicity of infection (MOI), and cell viability was quantified using XTT assay at 24 and 72 h after infection. Cell viability was calculated relative to that of uninfected cells, which was set at 1.0. Cell viability data are expressed as mean $\pm$ SD (n=5). NHOS-LM4 cells were infected with P53-armed OBP-702 at an MOI of 100 plaque-forming units/cell

for 24 h. Spent medium was analyzed using ENLITEN ATP assay (**C**) and a high-mobility group box protein B1 (HMGB1) enzyme-linked immunosorbent assay kit (**D**). Data are expressed as mean $\pm$ SD (n=3). The numbers of migrating (**E**) and invading (**F**) cells in five randomly selected fields were determined under a light microscope. Data are expressed as the mean $\pm$ standard deviation (n=5). Statistical significance was determined using two-tailed non-paired Student's *t*-test.

Significantly different from 0 MOI at: **p*<0.05, ***p*<0.01.

Figure 6. *In-vivo* antitumor effect of combination therapy with P53-armed OBP-702 and anti-programmed cell death protein 1 (PD1) in orthotopic NHOS-LM4 tumor models. (**A**) NHOS-LM4 cells (2×10^6 cells per site) were orthotopically inoculated into the proximal tibia of BALB/c mice on DAY 0. P53-armed OBP-702 was intratumorally injected at 5×10^7 plaque-forming units/cell once per week for three cycles (green arrowheads). Phosphate-buffered saline was used as a control. Anti-PD1 (PD1 Ab) was intraperitoneally administered once per week for three cycles (blue arrowheads). (**B**) Tumor growth in mice expressed as the mean tumor volume $\pm$ standard deviation (n=5). (**C**) Necropsy weight of control, PD1 Ab-treated, P53-armed OBP-702-treated, and combination-treated tumors. Representative photographs are shown of immunohistochemical staining for CD8⁺ (**D**) and CD4⁺ (**E**) T-cells in tumors from each group. Scale bars, 100 μ m. The numbers of CD8⁺ and CD4⁺ cells were calculated from five different randomly selected fields. Data are expressed the mean $\pm$ standard deviation (n=5). Statistical significance was determined using two-tailed one-way analysis of variance followed by Tukey's comparison tests. **Significantly different at *p*<0.01.