Research Article

# Telomerase-specific oncolytic immunotherapy for promoting efficacy of PD-1 blockade in osteosarcoma

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**Running title**: Combination immunotherapy for osteosarcoma

**Key words**: oncolytic adenovirus, hTERT, immunogenic cell death, ATP, CD8

**Footnotes:**

**Abbreviations:** PD-1, programmed cell death 1; OS, osteosarcoma; CAR, coxsackie and adenovirus receptor; PD-L1; programmed cell death ligand 1; HMGB1, high mobility group box 1; ICI, immune checkpoint inhibitor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; hTERT, human telomerase reverse transcriptase; ICD, immunogenic cell death; DAMPs, damage-associated molecular patterns; ATP, adenosine triphosphate; MOI, multiplicity of infection; PFU, plaque-forming units; PARP, poly (ADP-ribose) polymerase; LC3, microtubule-associated protein 1 light chain 3; APC, allophycocyanin; MFI, mean fluorescence intensity; PBS, phosphate buffered saline.

**Disclosure of potential conflict of interest:** Y. Urata is President & CEO of Oncolys BioPharma, Inc. H. Tazawa and T. Fujiwara are consultants of Oncolys BioPharma, Inc. The other authors disclosed no potential conflicts of interest.

**Grant support:** This study was supported in part by grants from the Ministry of Education, Science, and Culture, Japan (to T. Fujiwara, Nos. 16H05416 and 19H03731; T. Ozaki, No. 25293323; T. Kunisada, No. 16K10862; K. Sugiu, No. 15K10446; T. Komatsubara, No. 18K15242; H. Tazawa, No. 16K10596; Y. Mochizuki, No. 19K16835).

**Word counts:** 4576 words (excluding references)

**Abstract**

Immune checkpoint inhibitors including anti-programmed cell death 1 (PD-1) antibody have recently improved clinical outcome in certain cancer patients; however, osteosarcoma (OS) patients are refractory to PD-1 blockade. Oncolytic virotherapy has emerged as novel immunogenic therapy to augment antitumor immune response. We developed a telomerase-specific replication-competent oncolytic adenovirus OBP-502 that induces lytic cell death via binding to integrins. In this study, we assessed the combined effect of PD-1 blockade and OBP-502 in OS cells. The expression of coxsackie and adenovirus receptor (CAR), integrins αvβ3 and αvβ5, and programmed cell death ligand 1 (PD-L1) was analyzed in two murine OS cells (K7M2, NHOS). The cytopathic activity of OBP-502 in both cells was analyzed by using the XTT assay. OBP-502-induced immunogenic cell death was assessed by analyzing the level of extracellular ATP and high-mobility group box protein B1 (HMGB1). Subcutaneous tumor models for K7M2 and NHOS cells were used to evaluate the antitumor effect and number of tumor-infiltrating CD8+ cells in combination therapy. K7M2 and NHOS cells showed high expression of integrins αvβ3 and αvβ5, but not CAR. OBP-502 significantly suppressed the viability of both cells, in which PD-L1 expression and the release of ATP and HMGB1 were significantly increased. Intratumoral injection of OBP-502 significantly augmented the efficacy of PD-1 blockade on subcutaneous K2M2 and NHOS tumor models via enhancement of tumor-infiltrating CD8+ T cells. Our results suggest that telomerase-specific oncolytic virotherapy is a promising antitumor strategy to promote the efficacy of PD-1 blockade in OS.

## Introduction

Osteosarcoma (OS) is one of the most common malignant tumors in children and young adults. Over the past few decades, current treatment strategies, including multi-agent chemotherapy and aggressive surgery, have improved the prognosis of OS patients with a 5-year survival rate from 60% to 80% (1-4). However, OS patients with unresectable advanced tumors, distant metastases or refractory to chemotherapy still show poor prognosis (1, 2, 5). In OS patients with metastatic tumors at diagnosis, the 5-year survival rate has reached only 20%, even after multi-modal chemotherapy and surgical resection of primary and metastatic tumors (4, 6, 7). Therefore, the development of novel therapeutic strategies is required to improve the clinical outcome of OS patients.

The immune checkpoint pathway is a biological mechanism that modulates excessive immune reactions in normal tissues. However, this pathway is often used to help cancer cells escape from antitumor immune system in tumor tissues. Immune checkpoint inhibitors (ICIs) have therefore been developed to improve the cancer immunity cycle (8). There are various types of ICIs to target programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on normal and tumor cells. Clinical trials have shown that anti-PD-1 antibody treatment significantly increased the response rate and prolonged the overall survival in metastatic melanoma patients (9). However, the therapeutic efficacy of anti-PD-1 antibody is limited to certain cancer types. Clinical trials using anti-PD-1 antibody demonstrated that patients with bone and soft-tissue sarcomas were refractory to immunotherapy, as progressive disease is approximately 70% and partial response rate is only 5% (10). Thus, the development of combination immunotherapy to enhance the therapeutic efficacy of anti-PD-1 antibody is needed for

the treatment of OS patients.

Tumor-specific replication-competent oncolytic viruses are being developed as novel antitumor reagent, in which the promoters of cancer-related genes are used to regulate virus replication. We have previously developed a telomerase-specific replication-competent oncolytic adenovirus OBP-301 (Telomelysin) (11), in which the *hTERT* (*human telomerase reverse transcriptase*) gene promoter drives the expression of the *E1A* and *E1B* genes (11, 12). In monotherapy (11-13) or combination therapy with radiotherapy or chemotherapy (14, 15), we confirmed the antitumor efficacy of OBP-301 on both epithelial and mesenchymal types of malignant tumor cells. A phase I clinical trial of OBP-301, which was conducted in the United States on patients with advanced solid tumors including sarcoma, indicated that OBP-301 was well tolerated by patients (16). However, whether telomerase-specific oncolytic adenovirus enhances the efficacy of anti-PD-1 antibody against OS cells remains unclear.

Immunogenic cell death (ICD) represents a type of cell death that releases damage associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1), leading to the induction of antitumor immune response (17). Chemotherapeutic agents and ionizing radiation have been shown to induce ICD in tumor cells (18). Oncolytic adenoviruses have also been shown to induce ICD through the secretion of ATP, HMGB1, and uric acid (19). Recent accumulating evidences have suggested the promoting effect of oncolytic virotherapy in the antitumor immune responses (20, 21), particularly when combined with ICIs (22, 23). However, the therapeutic potential of combination therapy with oncolytic adenovirus and PD-1 blockade against OS cells remains unclear. Therefore, we hypothesize that telomerase-specific oncolytic adenovirus enhances the therapeutic efficacy of anti-PD-1

antibody against OS cells.

In the present study, we analyzed the efficacy of combination therapy with PD-1 blockade and telomerase-specific oncolytic virus in murine OS cells *in vitro* and *in vivo*. The expression of coxsackie and adenovirus receptor (CAR), integrins, and PD-L1 on murine OS cells were analyzed by flow cytometry. *In vitro* cytopathic effects and induction of ICD were assessed in the cell viability and secretion of extracellular ATP and HMGB1. *In vivo* antitumor efficacy and infiltration of CD8+ T cells were analyzed using subcutaneous murine OS tumor models.

## Materials and methods

### Cell lines

The murine osteosarcoma cell lines K7M2 (24, 25) and NHOS (26, 27) were obtained from the American Type Culture Collection (Manassas, VA, USA) and the RIKEN (Tokyo, Japan), respectively. K7M2 and NHOS cells were maintained in Dulbecco's modified Eagle's medium and RPMI1640 medium, respectively. All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were not cultures for more than 5 months following resuscitation. The cells were maintained at 37°C in a humidified atmosphere with 5% $CO_2$.

### Recombinant adenoviruses

The recombinant telomerase-specific replication-competent adenovirus OBP-301 (Telomelysin), in which the promoter element of the *hTERT* gene drives the expression of *E1A* and *E1B* genes, was previously constructed and characterized (11, 12). To target integrin-positive tumor cells, OBP-502 was generated by inserting the RGD peptide in

the fiber knob of OBP-301 (Supplementary Fig. S1). Recombinant viruses were purified by ultracentrifugation using cesium chloride step gradients, their titers were determined by a plaque-forming assay using 293 cells, and they were stored at -80°C.

**Immune checkpoint inhibitor**

Anti-mouse PD-1 antibody (clone 4H2) was obtained from Ono Pharmaceutical CO.LTD.

**Cell viability assay**

Cells were seeded on 96-well plates at a density of $1 \times 10^3$ cells/well 24 h before viral infection. Cells were then infected with OBP-502 at multiplicity of infections (MOIs) of 0, 10, 50, 100, 500 or 1000 plaque forming units (PFU)/cell. Cell viability was determined at 3 days after virus infection using Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA).

**DAMPs analysis**

Cells, seeded on 6-well plates at a density of $2 \times 10^5$ cells/well, were infected with OBP-502 at MOIs of 0, 500, and 1000 PFU/cell for 24 and 48 h (n=3). Supernatant was collected and analyzed with ENLITEN ATP assay (Promega, Madison, WI, USA) and HMGB1 ELISA kit II (Shino-Test, Kanagawa, Japan).

**Western blot analysis**

K7M2 and NHOS cells, seeded in a 100-mm dish at a density of $2 \times 10^5$ cells/dish, were infected with OBP-502 at MOIs of 0, 10, 50, 100, 500, and 1000 PFU/cell for 72 h.

Whole-cell lysates were 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, Indianapolis, IN, USA). Proteins were electrophoresed on 6% to 15% sodium dodecyl sulfate polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). Blots were blocked with Blocking-One (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. 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 (pAb) (Cell Signaling Technology, Beverly, MA, USA), mouse anti-p62 mAb (MBL, Nagoya, japan), rabbit anti- PD-L1 pAb (Abcam, Cambridge, MA, USA), and mouse anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies used were: horseradish peroxidase-conjugated antibodies against rabbit IgG (GE Healthcare) or mouse IgG (GE Healthcare). Immunoreactive bands on the blots were visualized using enhanced chemoluminescence substrates (ECL Plus; GE Healthcare).

**Flow cytometric analysis**

To analyze the expression of CAR, integrin αvβ3, and integrin αvβ5, cells were incubated with antibody to CAR (Upstate Biotechnology, Lake Placid, NY, USA), integrin αvβ3 (Bioss Antibodies, Woburn, MA, USA), and integrin αvβ5 (Bioss Antibodies) or isotype control IgG for 60 min on ice, then were labeled with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG secondary antibody (Invitrogen, Carlsbad, CA, USA) or Alexa Fluor 647-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) for 30 min, and were then analyzed using FACS Array (BD

Biosciences). To analyze the PD-L1 expression, cells were incubated with allophycocyanin (APC)-conjugated mouse anti PD-L1 mAb (Biolegned, San Diego, CA, USA) or APC-conjugated isotype control IgG for 30 min at 4°C and were analyzed using flow cytometry (FACS Array; BD Bioscience). The mean fluorescence intensity (MFI) for each cell line was determined by calculating the difference between the MFI in antibody-treated and isotype control IgG-treated cells from 3 independent experiments.

***In vivo* subcutaneous tumor model**

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. K7M2 and NHOS cells ($5 \times 10^6$ cells per site) were inoculated into the flank of female BALB/c mice aged 6 weeks (CLEA Japan, Tokyo, Japan). Palpable tumors developed within 14 days and were permitted to grow to approximately 5 to 6 mm in diameter. At that stage, a 50-μl volume of solution containing OBP-502 at a dose of $1 \times 10^9$ PFU or phosphate buffered saline (PBS) was injected into the tumors once a week for three cycles. A-100μl volume of solution containing anti-PD-1 antibody (1st: 4 mg/kg, 2nd/3rd: 2 mg/kg) or PBS was injected into the peritoneal cavities once a week for three cycles. Tumor volume was monitored twice a week after virus infection. Mice were suffocated after infection days 7, 14 and 28 respectively.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue samples cut at 4 μm 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 samples were boiled in citrate buffer or EDTA buffer for 14 min in a microwave oven for antigen retrieval. Samples were incubated with primary antibodies for 1 h at room temperature or overnight at 4°C and then with peroxidase-linked secondary antibody for 30 min at room temperature. After 3,3-diaminobenzidine staining for signal generation and counterstaining with Mayer's hematoxylin, samples were dehydrated and mounted onto coverslips. Antibodies against CD8, CD4, and Foxp3 (eBioscience, San Diego, CA, USA) were used as primary antibodies. The number of cells with expression of CD8, CD4, and Foxp3, which indicate immune cells of cytotoxic T lymphocytes, helper T lymphocytes and regulatory T lymphocytes, respectively, was calculated from five different randomly selected fields. All sections were analyzed under a light microscope.

**Statistical analysis**

Data are expressed as means ± SD. One-way ANOVA test or Student's *t* test was used to compare differences between groups. Statistical significance was defined as a *P* value less than 0.05.

**Results**

**Expression of CAR and integrins on murine K7M2 and NHOS cells**

To evaluate the combined effects of PD-1 blockade and telomerase-specific oncolytic adenovirus in OS tumors, we used two murine OS cell lines, K7M2 and NHOS, that have been reported to have the tumorigenic potential in the immunocompetent mice (24-27). As adenovirus serotype 5 (Ad5), a basal virus of telomerase-specific oncolytic adenovirus, enters the target cells via binding to CAR and integrins (28, 29), we first

analyzed the expression of CAR and integrins αvβ3 and αvβ5 on the surface of K7M2 and NHOS cells by flow cytometric analysis. Although the expression of CAR protein was quite low in K7M2 and NHOS cells, both cells strongly expressed integrins αvβ3 and αvβ5 (Fig. 1A), which were more abundant in NHOS cells than in K7M2 cells (Fig. 1B). These results indicate that K7M2 and NHOS cells are available to evaluate the effects of oncolytic adenoviruses with the RGD-modified fiber via binding to integrins.

***In vitro* cytopathic effect of OBP-502 in murine K7M2 and NHOS cells**

To evaluate the therapeutic potential of oncolytic adenovirus in integrin-expressing K7M2 and NHOS cells, we used a fiber-modified OBP-301 (OBP-502), which is generated by inserting the RGD-modified fiber to bind to integrins on target cells more strongly compared to OBP-301 (Supplementary Fig. S1). Cell viability was assessed over 3 days after infection using the XTT assay. OBP-502 significantly suppressed the viability of K7M2 and NHOS cells in a dose-dependent manner (Fig. 2A). The viabilities of K7M2 and NHOS cells was significantly decreased when treated with more than 500 MOIs of OBP-502 (Fig. 2A). As oncolytic adenoviruses have the therapeutic potential to induce apoptosis- and autophagy-related cell death in human OS cells (30, 31), we next investigated whether OBP-502 induces apoptosis and autophagy in K7M2 and NHOS cells. Western blot analysis demonstrated that OBP-502 increased the expression of cleaved PARP, which is an apoptosis-related marker, in K7M2 and NHOS cells when treated with more than 500 MOIs of OBP-502 (Fig. 2B). Moreover, the downregulation of p62, which is an autophagy-related marker, was also induced in K7M2 and NHOS cells when treated with more than 100 MOIs of OBP-502 (Fig. 2B). These results suggest that OBP-502 has the therapeutic potential to induce apoptosis

and autophagy-related cell death in K7M2 and NHOS cells.

**OBP-502 induces immunogenic cell death in murine K7M2 and NHOS cells**

Recent evidences have suggested that oncolytic viruses induce ICD to activate the antitumor immune response via the secretion of DAMPs, such as ATP and HMGB1 (32-34). We next investigated whether OBP-502 induces ICD in K7M2 and NHOS cells. The level of extracellular ATP and HMGB1 was analyzed using conditioned medium from K7M2 and NHOS cells at 24 and 48 hours after infection with OBP-502. OBP-502 significantly increased the secretion of extracellular ATP from K7M2 and NHOS cells at 24 hours after infection (Fig. 3A). The amount of extracellular ATP was approximately 100-fold higher in NHOS cells when compared to K7M2 cells (Fig. 3A). In contrast, the release of HMGB1 was significantly increased in only K7M2 cells (Fig. 3B). These results suggest that OBP-502 has the potential to induce ICD in K7M2 and NHOS cells.

**OBP-502 increases PD-L1 expression on murine K7M2 and NHOS cells**

The expression of PD-L1 on tumor cells has been shown to be associated with sensitivity to immunotherapy with PD-1 blockade (35). We next investigated whether K7M2 and NHOS cells express PD-L1 and OBP-502 upregulates the PD-L1 expression. Flow cytometric analysis demonstrated that K7M2 and NHOS cells show expression of PD-L1 protein (Fig. 4). OBP-502 significantly increased the expression of PD-L1 protein on the surface of K7M2 and NHOS cells (Fig. 4). These results suggest that OBP-502 has the potential to upregulate the PD-L1 expression in K7M2 and NHOS cells.

**Combination of PD-1 blockade and OBP-502 induces massive infiltration of CD8+ T cells in K7M2 tumors**

To assess the immune-modulating effect of OBP-502 in combination with PD-1 blockade, we used a subcutaneous K7M2 tumor model. OBP-502 was injected into the tumor once a week for one or two cycles. Three days after OBP-502 injection, anti-PD-1 antibody was injected into the peritoneal cavity once a week for one or two cycles (Fig. 5A). Intratumoral injection of OBP-502 significantly increased the number of CD8+ T cells in tumor tissues when combined with PD-1 blockade (Fig. 5B). To further evaluate the immune modulating effects of OBP-502 in combination with PD-1 blockade, OBP-502 was intratumorally injected every 2 days for 3 cycles and anti-PD-1 antibody was intraperitoneally injected once a week. Intratumoral injection of OBP-502 significantly increased the number of CD8+ T cells in tumor tissues when combined with PD-1 blockade (Supplementary Fig. S2). These results indicate that combination of PD-1 blockade and OBP-502 induces the infiltration of CD8+ T cells into K7M2 tumor tissues.

**Combination of PD-1 blockade and OBP-502 suppresses growth of K7M2 and NHOS tumors associated enhancement of tumor-infiltrating CD8+ T cells**

Finally, to assess the *in vivo* antitumor effects of combination therapy with PD-1 blockade and OBP-502, we used subcutaneous tumor models for K7M2 and NHOS cells. OBP-502 was intratumorally injected once a week for three cycles, whereas anti-PD-1 antibody was intraperitoneally injected once a week for three cycles. Monotherapy of OBP-502 significantly suppressed the growth of NHOS tumors, whereas the growth of K7M2 tumors was not suppressed by treatment with OBP-502

alone (Fig. 6A). Combination of PD-1 blockade and OBP-502 significantly suppressed tumor growth when compared to control group or monotherapy with PD-1 blockade in K7M2 and NHOS tumors (Fig. 6A). Immunohistochemistry revealed that the number of CD8+ T cells was significantly higher in tumors treated with combination therapy when compared to control tumors or tumors treated with PD-1 blockade (Fig. 6B). However, there were no significant differences in the number of CD4+ cells and Foxp3+ cells (Supplementary Fig. S3). These results suggest that OBP-502 treatment has the therapeutic potential to enhance the antitumor effects of PD-1 blockade in OS tumors by inducing tumor-infiltrating CD8+ T cells.

**Discussion**

Immune checkpoint inhibitors including anti PD-1 antibody have been shown to improve clinical outcome in certain cancer patients (36-38); however, clinical trials using anti PD-1 antibody demonstrated that bone and soft-tissue sarcomas were less sensitive to PD-1 blockade (10). Therefore, the development of combination immunotherapy with PD-1 blockade and immunogenic therapy is needed in patients with bone and soft-tissue sarcomas. In this study, we demonstrated that telomerase-specific oncolytic adenovirus OBP-502 has the therapeutic potential to induce ICD to promote the efficacy of PD-1 blockade in murine OS cells. A combination of anti-PD-1 antibody and OBP-502 significantly suppressed the growth of K7M2 and NHOS tumors through enhancement of tumor-infiltrating CD8+ T cells. Thus, combination immunotherapy with PD-1 blockade and oncolytic adenovirus OBP-502 is a promising antitumor strategy for eliminating OS cells via enhancement of antitumor immune response.

OBP-502 showed the therapeutic potential for inducing apoptosis and autophagy in K7M2 and NHOS cells, in which NHOS cells were more sensitive to OBP-502 than K7M2 cells, probably due to higher expression of integrins. Moreover, OBP-502 treatment significantly induced more abundant secretion of ATP in NHOS cells than in K7M2 cells. On the underlying mechanism of ICD-related ATP secretion from dying cells, autophagy has been shown to play a critical role in chemotherapy-induced ICD of human OS cells (39). Although autophagy is known to be associated with both cell survival and cell death in OS cells (40), autophagy inducers, including mTOR inhibitors, are currently expected to induce autophagy-related cell death in OS tumors in some clinical trials (41). Autophagy induction has also been shown to be involved in the antitumor efficacy of various oncolytic adenoviruses (42). Our collaborators demonstrated that combination of mTOR inhibitor rapamycin synergistically enhances the antitumor effects of telomerase-specific oncolytic adenovirus in human cancer cells via enhancement of autophagy induction (43). Thus, exploration of the role of autophagy in the oncolytic adenovirus-induced ATP secretion may provide the novel insights to enhance the antitumor efficacy in OS tumors.

Previous report has shown that the replication of human Ad5 is suppressed in murine cells (44). However, OBP-502 induced the expression of E1A and subsequent cell death in murine OS cells. As the replication of OBP-502 is regulated by hTERT promoter, hTERT promoter may be activated in murine OS cells. Ritz *et al.* generated the transgenic mice with hTERT promoter-driven lacZ reporter system to analyze the activity of hTERT promoter in murine tumor tissues (45). They showed the activation of hTERT promoter in murine tumor tissues. Moreover, Jia *et al.* demonstrated that hTERT is activated in murine tumor tissues of transgenic mice with bacterial artificial

chromosome containing the hTERT locus (46). These reports suggest that hTERT promoter can be activated to support the replication of OBP-502 in murine tumor cells.

Although K7M2 and NHOS cells showed high levels of PD-L1 expression, K7M2 and NHOS tumors were resistant to PD-1 blockade. As K7M2 and NHOS tumors showed cold tumors with low number of tumor-infiltrating CD8+ T cells, the interaction between CD8+ T cells and OS cells via PD-1/PD-L1 binding may be important to improve the resistance to PD-1 blockade in OS tumors. To promote this interaction, there may be at least two strategies. One is to increase the number of tumor-infiltrating CD8+ T cells. Various types of oncolytic viruses including adenovirus have been shown to induce ICD and promote the antitumor immune response with increased CD8+ T cells (32). Similar with these viruses, OBP-502 induced ICD and increased the number of tumor-infiltrating CD8+ T cells. This effect may be commonly caused by ICD-inducing oncolytic viruses. The other is to increase the binding of PD-1/PD-L1 between CD8+ T cells and OS cells. Infection with many kinds of viruses including adenovirus has been shown to increase the PD-L1 expression in normal cells (47). Similar with these viruses, OBP-502 increased the expression of PD-L1 in OS cells. Therefore, OBP-502 may promote the interaction between CD8+ T cells and OS cells to improve the resistance to PD-1 blockade in OS tumors.

OBP-502 treatment upregulated the expression of PD-L1 on K7M2 and NHOS cells. However, the underlying mechanism of OBP-502-mediated PD-L1 upregulation in OS cells remains unclear. Adenovirus infection has been shown to increase the PD-L1 expression in hepatocytes via induction of antiviral cytokines, type I and II interferons (48). Adenoviral E1A induces the stabilization of oncoprotein Myc (49), which can upregulate the PD-L1 expression in murine tumor cells (50). Recent reports

have suggested the complex network to regulate the PD-L1 expression on tumor cells at the transcriptional, posttranscriptional, and protein level (51). In contrast, tumor microenvironment also has the potential to induce the PD-L1 upregulation in tumor cells. Hypoxic condition increases the PD-L1 expression in tumor cells via induction of hypoxia-inducible factors (52). These reports suggest that intrinsic and extrinsic factors cooperatively induce the PD-L1 upregulation in tumor cells. Therefore, further *in vitro* and *in vivo* experiments should be warranted to explore the underlying mechanism of OBP-502-induced PD-L1 upregulation in OS cells.

A Phase 2 clinical trial of anti-PD-1 antibody demonstrated that only 5% (1 out of 22) of OS patients had an objective response (10), suggesting the requirement of combination therapy for improving the efficacy of PD-1 blockade therapy. Several antitumor therapies have recently been investigated in combination therapy with PD-1 blockade (53). A Phase 2 clinical trial of combination therapy with PD-1 blockade and anti-CTLA-4 antibody demonstrated no objective responses in patients with bone sarcomas (54). Combination with radiation has been shown to improve the efficacy of PD-1 blockade in mice models with various types of cancers, including OS (55). However, the therapeutic potential of combination immunotherapy with PD-1 blockade and radiotherapy remains to be elucidated. In our study, combination of telomerase-specific oncolytic adenovirus OBP-502 significantly suppressed tumor growth when compared to monotherapy of PD-1 blockade in K7M2 and NHOS tumors via increase in the number of tumor-infiltrating CD8+ T cells. Thus, further clinical studies are warranted to investigate the safety, tolerability, and efficacy of combination immunotherapy with PD-1 blockade and telomerase-specific oncolytic virotherapy in OS patients.

In conclusion, we demonstrated that telomerase-specific oncolytic adenovirus OBP-502 improves the efficacy of PD-1 blockade in OS tumors by inducing autophagy, ICD, and massive infiltration of CD8+ T cells. Combination immunotherapy with PD-1 blockade and oncolytic adenovirus is a promising antitumor strategy for promoting the therapeutic potential of PD-1 blockade against OS tumors. Taken together, the combination of telomerase-specific oncolytic virotherapy would provide a novel therapeutic option for the treatment of immunotherapy-refractory OS tumors.

**Acknowledgements**

We would like to thank Takeshi Ieda, Tomoko Sueishi, Yuko Hoshijima, and Tae Yamanishi for their excellent technical support.

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**Figure legends**

**Fig. 1. Expression of integrin and CAR proteins on the surface of murine OS cells.** Expression levels of CAR ex, integrin α5β3 and Integrin α5β5 on K7M2 and NHOS cells were assessed by flow cytometric analysis. The mean fluorescence intensity (MFI) for each cell line was determined by calculating the differences between the mean fluorescence intensity (MFI). Data are expressed as mean values ± SD (n = 3 in each group; *, $P < 0.05$).

**Fig. 2. *In vitro* cytopathic effects of OBP-502 in murine OS cells. A**, K7M2 and NHOS cells were infected with OBP-502 at the indicated multiplicity of infection (MOI), and cell viability was quantified over 5 days using the XTT assay. Cell viability was calculated relative to that of the mock-infected group on each day, which was set at 1.0. Cell viability data are expressed as mean values ± SD (n = 5). **B**, K7M2 and NHOS cells were infected with OBP-502 at the indicated MOIs for 72 h. Cell lysates were subjected to Western blot analysis for the cleaved PARP (C-PARP), PARP and p62. β-actin was assayed as a loading control.

**Fig. 3. OBP-502-mediated induction of immunogenic cell death with secretion of ATP and HMGB1 in murine OS cells.** Cells were treated with OBP-502 (0, 500 and 1000 MOI) for 24 and 48 hours (n=5). Supernatant was analyzed with ENLITEN ATP assay (Promega, Madison, WI, USA) and HMGB1 ELISA kit (Shino-Test, Kanagawa, Japan), respectively. Data are expressed as mean values ± SD (n = 5 in each group; *, $P < 0.05$ (vs 0 MOI)).

**Fig. 4. OBP-502-mediated PD-L1 upregulation in murine OS cells. A,** Expression of PD-L1 in K7M2 and NHOS cells infected with OBP-502 at the indicated MOIs for 72 h was assessed using flow cytometric analysis. **B**, Mean fluorescence intensity (MFI) of PD-L1 expression was assessed by flow cytometric analysis. Data are expressed as mean values ± SD (n = 3 in each group; ** $P < 0.01$).

**Fig. 5. Number of CD8+ T cells in K7M2 tumors treated with PD-1 blockade and OBP-502. A**, K7M2 cells ($5 \times 10^6$ cells/site) were inoculated into the flanks of BALB/c mice. OBP-502 was intratumorally injected with $1 \times 10^9$ PFUs once a week for one or two cycles (n = 3 to 4; green arrow heads). PBS was used as a control. Anti-PD-1 Ab was intraperitoneally administered once a week for one or two cycles (blue arrow heads). **B**, Number of CD8+ T cells was calculated from five different randomly selected fields. Data are expressed as mean values ± SD (n = 3 to 4 in each group; *, $P < 0.05$). Representative photographs of immunohistochemical staining for CD8-positive TILs in each group. Scale bar, 100 μm.

**Fig. 6. *In vivo* antitumor effects of OBP-502 and anti PD-L1 antibody in K7M2 and NHOS tumor models. A**, K7M2 or NHOS cells ($5 \times 10^6$ cells/site) were inoculated into the flanks of BALB/c mice. OBP-502 was intratumorally injected with $1 \times 10^9$ PFUs once a week for three cycles (green arrow heads). PBS was used as a control. Anti-PD-1 Ab was intraperitoneally administered once a week for three cycles (blue arrow heads). Tumor growth was expressed as mean tumor volume ± SD (n = 7 to 9 in each group; *, $P < 0.05$; **, $P < 0.01$). **B**, The number of CD8+ T cells was calculated from five different randomly selected fields. Data are expressed as mean values ± SD (n = 7 to 9

in each group; *, $P < 0.05$; **, $P < 0.01$).