Preclinical Evaluation of Differentially Targeting Dual Virotherapy for Human Solid Cancer

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Abstract
Multimodal approaches combining drugs that differentially function is the most popular regimen for treating human cancer. Understanding the molecular mechanisms underlying the synergistic, potentiative, and antagonistic effects of drug combinations could facilitate the discovery of novel efficacious combinations. We previously showed that telomerase-specific replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase promoter controls the adenoviral E1 gene expression, induces a selective antitumor effect in human cancer cells. Here, using E1-deleted replication-deficient adenovirus expressing the p53 tumor suppressor gene (Advexin, Ad-p53) and OBP-301, we investigate how these adenoviruses that kill tumor cells with different mechanisms could work in combination on human cancer. We found that E1-deficient Ad-p53 could kill cancer cells more efficiently in the presence of OBP-301 than Ad-p53 alone or OBP-301 alone, because Ad-p53 could become replication-competent by being supplied adenoviral E1 from coinfected OBP-301 in trans. Ad-p53 plus OBP-301 induced high levels of p53 protein expression without p21 induction, resulting in apoptotic cell death documented by active caspase-3 expression with a cytometric bead array and an increased subdiploid apoptotic fraction of the cell cycle. For in vivo evaluation, nude mice xenografted with human lung tumors received intratumoral injection of OBP-301 and/or Ad-p53. Analysis of the growth of implanted tumors showed an enhanced antitumor effect in combination therapy. Our data show that Ad-p53 in combination with OBP-301 induces not only oncolytic but also apoptotic cancer cell death and enhances antitumor activity in vitro and in vivo, providing potential merits as a multimodal treatment for human cancer. Mol Cancer Ther; 9(6); 1884–93. ©2010 AACR.

Introduction
Combining antineoplastic agents with different mechanisms of action has resulted in many effective regimens in cancer therapy. For example, biochemical modulation of 5-fluorouracil, one of the most active single agents presently available, with the reduced folate leucovorin has significantly improved overall response rates in the 25% to 30% range (1). To successfully achieve synergistic effects, we need to elucidate the biochemical and/or molecular mechanisms underlying the drug interaction. Recent advances in understanding the molecular mechanisms of carcinogenesis allow us to develop a number of molecularly targeted therapies. Most agents have been developed to target specific molecules which are essential for the acquisition of malignant phenotypes such as proliferation, invasion, and metastasis.

Gene- and vector-based therapies, which have long been viewed as unsuccessful, have been greatly rejuvenated by its combination with other modalities including chemotherapy and radiotherapy (2, 3). It also remains possible to increase the therapeutic benefit by combining virotherapies having different targets. Ad-p53 (Advexin, Ad5CMV-p53) consists of an E1-deleted replication-deficient type 5 adenoviral vector expressing the human wild-type p53 tumor suppressor gene under the control of a cytomegalovirus promoter. p53 is the most commonly mutated gene in human cancer (4, 5), and p53 gene therapy using Ad-p53 is currently in clinical trials as a cancer therapy (6–9); however, a number of limitations have led to the suboptimal efficacy of existing gene therapies. One reason is that replication-deficient adenoviruses infect only a small portion of the tumor and are not able to spread over the tumor entirely. To improve viral spread in tumor conferring specificity of infection, replication-competent adenoviruses have been investigated (10–12).
We previously developed a telomerase-specific, replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase (hTERT) promoter element drives the adenoviral E1 gene, and induced a selective antitumor effect in human cancer cells (13–17). Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosomal ends (18). Many studies have shown the expression of telomerase activity in more than 85% of human cancers (19), but only in few normal somatic cells (20). Telomerase activation is considered a critical step in carcinogenesis and its activity is closely correlated with hTERT expression (21). Although virotherapy using OBP-301 as a monotherapy is currently being evaluated in clinical trials (22), multimodal strategies to enhance antitumor efficacy in vivo might be essential for a successful clinical outcome.

As a replication-deficient adenovirus could replicate in cancer cells and enhance the anticancer effect when co-transfected with a replication-competent adenovirus that could produce E1 proteins, we reasoned that combined treatment with Ad-p53 and OBP-301 might offer a way to more efficiently kill human tumor cells. In the present study, we investigated the synergistic effects of Ad-p53 combined with OBP-301 both in vitro and in vivo.

Materials and Methods

Cell lines and cell cultures
The human non–small cell lung cancer cell line H1299, which has a homozygously deleted p53, and the human colorectal carcinoma cell line SW620, which contains mutated p53, were propagated in monolayer culture in RPMI 1640 supplemented with 10% FCS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

Recombinant adenoviruses
The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the hTERT promoter element drives the expression of E1A and E1B genes linked with an IRES, was constructed and characterized previously (16). Replication-deficient adenoviral vectors containing human wild-type p53 cDNA (Ad-p53) and β-galactosidase cDNA (Ad-lacZ) were also used (23). These viruses were purified by CsCl2 step gradient ultracentrifugation followed by CsCl2 linear density gradient ultracentrifugation.
gradient ultracentrifugation, and their titers were determined by plaque assay in the 293 cells.

**Cell proliferation assay**

Cells were seeded at 1,000 cells/well in 96-well plate and infected with OBP-301, Ad-p53, or OBP-301 and Ad-p53 simultaneously at the indicated multiplicities of infection (MOI) 18 to 20 hours later. Cell viability was assessed at the indicated times after adenoviral infection using sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazoli-um]bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay with the Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

**Western blot analysis**

The primary antibodies against p53 (Ab-2; Calbiochem), p21 (EA10; Oncogene Science), β-actin (AC-15; Sigma Chemical, Co.), and peroxidase-linked secondary antibody (Amersham) were used. Cells were washed twice in cold PBS and collected then lysed in lysis buffer [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L EDTA, 10% glycerol, and 0.5% NP40] containing proteinase inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride and 0.5 mmol/L Na3VO4). After 20 minutes on ice, the lysates were spun at 14,000 rpm in a microcentrifuge at 4°C for 5 minutes. The supernatants were used as whole cell extracts. Protein concentration was determined using the Bio-Rad protein determination method (Bio-Rad). Equal amounts of proteins were boiled for 5 minutes and electrophoresed under reducing conditions in 4% to 12% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to Hybond polyvinylidene difluoride transfer membranes (Amerham Life Science) and incubated with the primary antibody followed by peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent western system (Amerham) was used to detect secondary probes.

**Human apoptosis cytometric bead array for active caspase-3**

Caspase-3 activation was quantitated using the Human Apoptosis Cytometric Bead Array kit (Becton Dickinson) according to the instructions of the manufacturer. Briefly, cell lysates were incubated with cytometric caspase-3 capture beads coated with antibody specific for caspase-3 and a secondary antibody specific for the cleaved site of active caspase-3 conjugated to phycoerythrin. Cytometric bead/caspase-3 conjugates were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). Flow cytometry measures the amount of active caspase-3 attached to beads. Becton Dickinson Cytometric Bead Array software was used to analyze beads and transform data from samples. Sample data

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Expression of p53 in combination therapy of Ad-p53 and OBP-301. H1299 and SW620 cells were infected with the indicated viruses and harvested at the indicated time. Cell lysates were subjected to immunoblot analysis for p53 and β-actin. H1299 cells infected with 50 MOI of Ad-p53 and SW620 cells infected with 500 MOI of Ad-p53 were used as a positive control for apoptosis. A, cells were harvested in the early phase (24 and 48 h after infection), p53 expression level was quantified by densitometric scanning using NIH Image software and normalization by dividing the β-actin signal. The background was subtracted and corrected so that the control became 0, because H1299 cells were p53-null. B, cells were harvested in the late phase (6 d after infection).
was normalized with specific protein standards to provide quantification of the proteins of interest.

**In vitro replication assay**

H1299 cells were infected with OBP-301 (1 MOI), Ad-p53 (5 MOI), OBP-301 (1 MOI) + Ad-p53 (5 MOI), or OBP-301 (1 MOI) + Ad-lacZ (5 MOI) for 24 hours after seeding for 2 hours. Following the removal of virus inocula, the cells were further incubated at 37°C, trypsinized, and harvested at 2, 24, 48, and 72 hours after infection. DNA purification was done using QIAamp DNA mini kit (Qiagen, Inc.). The E1A and p53 DNA copy numbers were determined by quantitative real-time PCR using a LightCycler instrument and LightCycler-DNA Master SYBR Green I (Roche Diagnostics).

**Cell cycle analysis**

Cells treated with various concentrations of OBP-301, Ad-p53, or a combination of both were harvested in 0.125% trypsin, washed twice in PBS with 2% FCS. After centrifugation, $1 \times 10^6$ cells were resuspended in 70% ethanol at 4°C overnight. Cells were washed twice in PBS. After centrifugation, the pellet was resuspended in 0.25 μg/mL of RNase A for 30 minutes at 37°C. The samples were then stained with 50 μg/mL of propidium iodide and incubated at 4°C for 30 minutes. Cell cycle analysis was determined by FACSCalibur flow cytometer using FlowJo software (TreeStar, Inc.).

**In vivo human tumor model**

H1299 cells ($7.5 \times 10^6$ cells/mouse) were injected s.c. into the flank of 5-week-old female BALB/c nu/nu mice and permitted to grow to 4 to 10 mm in diameter. At that time, the mice were randomly assigned into six groups, and a 50 μL solution containing Ad-p53 [$1 \times 10^9$ plaque-forming units (pfu)], OBP-301 [$1 \times 10^7$ pfu], OBP-301 (1 × 10^7 pfu) + Ad-p53 (1 × 10^8 pfu), or Ad-lacZ (1 × 10^5 pfu), Ad-p53 (1 × 10^7 pfu) + Ad-lacZ (1 × 10^7 pfu), or PBS was injected into the tumor for three cycles every 2 days. The perpendicular diameter of each tumor was measured every 3 or 4 days, and tumor volume was calculated using the following formula:

$$V = \frac{1}{2} \times (l \times w)$$

where $V$ is the tumor volume (mm$^3$), $l$ is the length (mm), and $w$ is the width (mm).
tumor volume (mm$^3$) = $a \times b^2 \times 0.5$, where $a$ is the longest diameter, $b$ is the shortest diameter and 0.5 is a constant to calculate the volume of an ellipsoid. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine.

**Statistical analysis**

The statistical significance of the differences in the in vitro and in vivo antitumor effects of viruses was determined by using the (two-tailed) Student’s $t$ test.

**Results**

**Enhanced antitumor effect of OBP-301 plus Ad-p53 in human cancer cell lines in vitro**

To achieve optimum antitumor effect, Ad-p53 at the MOI of 50 and 500 was needed in H1299 and SW620 cells, respectively (Fig. 1A). We have previously confirmed that these cells expressed detectable hTERT mRNA (13, 14). To examine the potential interaction of Ad-p53 with OBP-301, we did a dose-response analysis of the cell-killing effect at various doses in H1299 and SW620 cells. Ad-p53 at 5 or 50 MOI could have little antitumor effect in H1299 and SW620 cells, respectively. Combination therapy with 1 MOI of OBP-301 plus Ad-p53 resulted in more cell killing than OBP-301 alone or Ad-p53 alone in both cells (Fig. 1B). In contrast, OBP-301 plus Ad-lacZ did not increase the antitumor effect (data not shown). We next examined a time course analysis of the cell-killing effect. The antitumor effect of Ad-p53 plus OBP-301 was augmented after 4 days of infection (Fig. 1C). To determine whether the timing of administration of the adenoviruses affected the combined cytotoxic effect, H1299 and SW620 cells were treated with Ad-p53 24 hours after infection or synchronously with OBP-301. The results showed no apparent differences in cytotoxic activity by the treatment schedules (data not shown).

**Increased expression of p53 by cotransduction with OBP-301 and Ad-p53**

To explore the effect of the combination of Ad-p53 and OBP-301, the expression levels of p53 protein were compared by immunoblotting analyses at 24 and 48 hours after infection. By cotransduction with Ad-p53 and OBP-301, the expression of p53 was 33.6-fold higher in H1299 cells at 48 hours and 4.34-fold higher in SW620...
The augmented expression of p53 was maintained in cotransduced cells with both adenoviruses on day 5 (Fig. 2B). The prolonged upregulation of p53 suggests that Ad-p53 replicates in cancer cells by E1 protein supplied by cotransduced OBP-301, although the pattern of increased p53 expression was different between H1299 and SW620 cells. These cells expressed equivalent levels of coxsackievirus and adenovirus receptor (15); p53-null H1299 cells were, however, more sensitive to Ad-p53 compared with SW620 cells containing mutated p53, as shown in Fig. 1A, suggesting that Ad-p53 induced rapid apoptosis within 48 hours in infected H1299 cells and, therefore, p53 expression declined at 48 hours after infection. In contrast, as Ad-p53 could replicate and infect more H1299 cells in the presence of OBP-301, the level of p53 expression increased after coinfection of Ad-p53 and OBP-301.

**Combined treatment of OBP-301 plus Ad-p53 more efficiently induced apoptosis**

Overexpression of p53 is known to induce apoptosis in most cancer cells. We hypothesized that the enhanced antitumor effect of Ad-p53 plus OBP-301 would result in elevated apoptotic cell death. Two days after infection, combined treatment of Ad-p53 plus OBP-301 induced significantly elevated levels of active caspase-3, whereas a low dose of Ad-p53 alone did not (Fig. 3). Moreover, interestingly, the expression level of p21, which is a
downstream target of p53 in combination therapy with OBP-301 and Ad-p53, was lower than in low doses of Ad-p53 alone (Fig. 4A).

**p53 DNA copy number is significantly amplified in combination therapy with OBP-301 and Ad-p53, whereas p53 does not impair the replication of OBP-301**

Then we examined the p53 DNA levels in H1299 cells. The amplification of p53 DNA in the cells treated with Ad-p53 plus OBP-301 by one to two orders of magnitude was observed (Fig. 4B). These results suggest that Ad-p53 actually replicates by using the E1 protein supplied by OBP-301, which is consistent with increased expression of p53 protein (Fig. 2A). In this combination treatment, proapoptotic p53 expression might impair the replication of oncolytic virus because of premature early cell death provoked by p53. Therefore, we examined the effect of p53 on the replication of OBP-301 by quantitative real-time PCR analysis. H1299 cells were infected with OBP-301 and/or Ad-p53. Cells were harvested 2, 24, and 48 hours after infection, and extracted DNA were subjected to the assay. As shown in Fig. 4C, the presence of Ad-p53 did not impair the replication of OBP-301.

**Augmentation of apoptosis in human tumor cells after coinfection with Ad-p53 and OBP-301**

We next did a cell cycle analysis in H1299 and SW620 cells on days 2 and 5 after infection. Neither group showed any differences between treatment 2 days after infection; however, a sub-G1 population (an apoptotic cell population) was markedly increased in H1299 and SW620 cells infected with Ad-p53 plus OBP-301 after 5 days of infection, whereas OBP-301 or Ad-p53 alone did not increase apoptosis (Fig. 5; Table 1). These results were compatible with those of the time course analyses (Fig. 1C), suggesting that the augmented antitumor effect of combined treatment of Ad-p53 and OBP-301 was due to enhanced apoptotic cancer cell death.

**Antitumor effect of OBP-301 plus Ad-p53 in human tumor xenografts**

Based on the *in vitro* combination effect of Ad-p53 and OBP-301, the *in vivo* therapeutic efficacy of H1299 tumors was further assessed. Mice bearing H1299-xenografted tumors measuring 4 to 10 mm in diameter received intratumoral injection of Ad-p53 at a dose of $1 \times 10^9$ pfu and OBP-301 at a dose of $1 \times 10^7$ pfu, singly or in combination every 2 days for three cycles starting at day 0. As shown in Fig. 6, Ad-p53 alone and Ad-p53 + Ad-lacZ had no apparent antitumor effect, but OBP-301 alone resulted in significant tumor suppression compared with controls ($P < 0.05$). OBP-301 plus Ad-lacZ did not improve the tumor suppression compared with OBP-301 alone ($P > 0.05$). Nevertheless, the combination of OBP-301 plus Ad-p53 produced a more profound and significant inhibition of tumor growth compared with OBP-301 alone or OBP-301 plus Ad-lacZ ($P < 0.05$).

### Table 1. Cell cycle analysis following infection with OBP-301, Ad-p53, or Ad-p53 plus OBP-301 in H1299 and SW620 cells

<table>
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<th>Cell lines</th>
<th>Days</th>
<th>Treatments</th>
<th>Sub–G0-G1</th>
<th>G0-G1</th>
<th>S</th>
<th>G2-M</th>
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<td>H1299</td>
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<td></td>
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<tr>
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NOTE: H1299 cells were infected with 1 MOI of OBP-301, 5 MOI of Ad-p53, or a combination of both and harvested on days 2 and 5. SW620 cells were infected with 1 MOI of OBP-301, 50 MOI of Ad-p53, or a combination of both and harvested on days 2 and 5. Cell cycle analysis was determined by the fluorescence-activated cell sorter (FACSCalibur) flow cytometer.
Resistance to apoptosis is a major cause of treatment failure in human cancers. Many combination regimens with clinically available agents are currently being used; however, there is a need for a better understanding of the molecular interaction of drugs to efficiently induce apoptosis in human cancer cells. In the present study, our goal was to determine whether dual virotherapy, which mediates telomerase-specific enhancement of exogenous wild-type p53 gene expression, was effective for inducing apoptosis. We found that Ad-p53 and OBP-301, with different mechanisms of action, could be more effective in the growth of human cancer cells than Ad-p53 or OBP-301 alone in vitro as well as in vivo. Moreover, our data suggest that p53-induced p21 induction was inhibited in the presence of OBP-301 infection, which might in turn sensitize tumor cells to apoptosis by blocking cell cycle arrest.

Virotherapy for p53 gene transfer by Ad-p53 (Advexin) is currently in clinical trials as a cancer therapeutic (24). Overexpression of p53 gene caused preferential cancer cell–killing, although this was less toxic to normal cells, which means that p53 gene therapy itself is a cancer-targeting therapy; however, the low transduction rate and the narrow spread of Ad-p53 limits the anticancer effect in vivo. The major problem for p53 therapy with replication-defective adenovirus vector is incomplete transduction of target cancer cells. Therefore, cancer cells beyond transduced cells will escape the antitumor effect. To overcome the low transduction rate of replication-defective adenoviral vectors, an increment in the dose would be the simple resolution; however, a higher dose of Ad-p53 might not always contribute to the improvement of transduction efficacy and could potentially cause side effects. In this study, even the low dose of Ad-p53, administration of which solely was not enough to induce apoptosis, enabled an increase anticancer effect in combination with OBP-301 by replicating selectively in tumor cells. OBP-301 was genetically designed to replicate specifically in tumor cells, causing specific "oncolysis" (13, 15, 16), thereby increasing the effective treatment radius in tumors. This combination might contribute to the mitigation of unfavorable effects. OBP-301 is currently being evaluated in phase I trials for clinical safety as of the writing of this article.

Virotherapy with adenovirus Onyx-015, the first oncolytic virus, has been evaluated in clinical trials, and showed that administration of oncolytic adenovirus via intratumor, intraperitoneum, or intravenous methods was well tolerated (25); however, the efficacy of Onyx-015 as a single agent is also limited, possibly because of inefficient cell lysis and release of their progeny, which may be defective in cancer cells. Our strategy is aiming not only to enhance the antitumor activity of Ad-p53 by OBP-301, but also to augment the oncolytic activity of OBP-301 in combination with Ad-p53. Oncolysis is due to cytotoxic effects intrinsic to the adenovirus. Adenovirus must be able to induce cell death in infected cells for the virus to generate a cytotoxic effect and be released from the cell. Hall et al. showed that wild-type p53 enhances the ability of adenoviruses to induce cell death, whereas the loss of functional p53 in cancer cells resulted in a defect in adenovirus-induced cytotoxic effects (26). Thus, functional p53 is required for a productive adenovirus infection. Consistent with their report, our strategy to compensate or overexpress functional wild-type p53 must be conducive to efficient adenovirus replication, which leads to effective oncolysis by OBP-301.

The efficacy of combination therapy with conventional gene therapy using replication-deficient adenovirus plus virotherapy has been reported previously (27, 28). Combined with tumor-specific replication-selective adenovirus, replication-deficient adenoviruses were able to replicate and spread to surrounding tumor cells. Antitumor activity was increased compared with gene therapy alone or virotherapy alone in agreement with our study. In our previous study, co-infection with a replication-deficient adenovirus expressing the green fluorescent protein (GFP) gene and OBP-301 was able to specifically visualize human lung cancer cells in an orthotopic murine model (14). Although the study showed the diagnostic application of combinative use of replication deficient adenoviral vector and OBP-301, the specific replication of coinfected adenovirus in tumor was visually evidenced. The current study applied this principle to therapeutic purpose. When Ad-p53 was used together with OBP-301, the total amount of the viruses might be higher than that of OBP-301 alone that shows the equivalent efficacy; the dose of replicative OBP-301, however, should be reduced as much as possible from the viewpoint of safety.

Figure 6. H1299 cells (7.5 x 10⁶ cells/mouse) were injected s.c. into the flank of BALB/c nu/nu mice and permitted to grow to 4 to 10 mm in diameter. A 50 μL solution containing Ad-p53 (1 x 10⁷ pfu), OBP-301 (1 x 10⁷ pfu), OBP-301 (1 x 10⁷ pfu) + Ad-p53 (1 x 10⁷ pfu), or Ad-lacZ (1 x 10⁷ pfu), Ad-p53 (1 x 10⁸ pfu) + Ad-lacZ (1 x 10⁷ pfu), or PBS was injected into the tumor for three cycles every 2 d. The perpendicular diameter of each tumor was measured every 3 or 4 d. Six or seven mice were used for each group. Points, mean tumor growth volume; bars, SE. *, P < 0.05, statistical significance (Student’s t test). Arrows, days of treatment.
Indeed, the pharmacodynamic data in a phase I clinical trial of OBP-301 showed the transient systemic dissemination of OBP-301 following intratumoral injection (29).

Another interesting finding in the current study is that OBP-301 infection apparently induced endogenous p53-mediated expression of p21, which is a key player in arresting cells in the G1 phase. This suggests that OBP-301 infection might be an important requirement for rendering tumor cells sensitive to apoptosis rather than cell cycle arrest. Indeed, it has been reported that the adenovirus E1A protein could enhance the sensitivity of tumor cells to chemotherapeutic agents by promoting apoptosis (30). This is primarily because of the ability of E1A to interact with p21 and thereby inactivate it, which in turn, leads to apoptosis. Further mechanisms of this interaction are now under investigation.

Our strategies aim to enhance the efficiency and specificity of viral agents through incorporation of tumor-targeting mechanisms via therapeutic gene and transcripational regulation. Although this study examined the combinative use of two viral agents, oncolytic adenoviruses carrying therapeutic genes in the E1 or E3 region have also been reported (31, 32). Gene therapy and oncolytic virotherapy, however, do not always work effectively in combination (33). In case the expression level of therapeutic genes needs to be titrated, therapeutic genes might work better when separated from oncolytic virus. In addition, both p53 therapy and OBP-301 oncolytic therapy are currently being tested clinically. Once they are approved for clinical use, this study would provide realistic therapeutic applications as a preclinical study in the future.

In conclusion, our data showed that combination therapy of Ad-p53 and OBP-301 efficiently inhibited human cancer cell growth in vitro and in vivo, and that this approach has important implications for the treatment of human cancers.

Disclosure of Potential Conflicts of Interest

Y. Urata: employee of Oncolys BioPharma, Inc. T. Fujinara: consultant to Oncolys BioPharma, Inc. No other potential conflicts of interest were disclosed.

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