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A novel apoptotic mechanism of genetically engineered adenovirus-mediated tumour-specific p53 overexpression through E1A-dependent p21 and MDM2 suppression

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Abstract Oncolytic viruses engineered to replicate in tumour cells but not in normal cells could be used as tumour-specific vectors carrying the therapeutic genes. We previously developed a telomerase-specific oncolytic adenovirus, OBP-301, that causes cell death in human cancer cells with telomerase activities. Here, we further modified OBP-301 to express the wild-type *p53* tumour suppressor gene (OBP-702), and investigated whether OBP-702 induces stronger antitumour activity than OBP-301. The antitumour effect of OBP-702 was compared to that of OBP-301 on OBP-301-sensitive (H358 and H460) and OBP-301-resistant (T.Tn and HSC4) human cancer cells. OBP-702 suppressed the viability of both OBP-301-sensitive and OBP-301-resistant cancer cells more efficiently than OBP-301. OBP-702 caused increased apoptosis compared to OBP-301 or a replication-deficient adenovirus expressing the *p53* gene (Ad-p53) in H358 and T.Tn cells. Adenovirus E1A-mediated p21 and MDM2 downregulation was involved in the apoptosis caused by OBP-702. Moreover, OBP-702 significantly suppressed tumour growth in subcutaneous tumour xenograft models compared to monotherapy with OBP-301 or Ad-p53. Our data demonstrated that OBP-702 infection expressed adenovirus E1A and then inhibited p21 and MDM2 expression, which in turn efficiently induced apoptotic cell death. This novel apoptotic mechanism suggests that the p53-expressing OBP-702 is a promising antitumour reagent for human cancer and could improve the clinical outcome.

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1. Introduction

Replication-selective oncolytic viruses have emerged as promising antitumour reagents for induction of tumour-specific cell death.[1–4] Recent evidence from several clinical studies of oncolytic virotherapy has suggested that oncolytic viruses are well tolerated by cancer patients.[5–8] We previously developed a telomerase-specific replication-competent oncolytic adenovirus OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase (*hTERT*) promoter drives the expression of the *E1A* and *E1B* genes that are linked to an internal ribosome entry site (IRES).[9–11] A phase I clinical trial of OBP-301 in patients with advanced solid tumours has been recently completed and OBP-301 was well tolerated by these patients.[12] However, the antitumour effect of OBP-301 was limited in some of the OBP-301-injected tumours. Therefore, to efficiently eliminate tumour cells using OBP-301, and to improve the clinical outcome of cancer patients, enhancement of the OBP-301-mediated antitumour effect is required.

Genetically engineered armed oncolytic viruses that express several types of therapeutic transgenes have recently been reported that were aimed at enhancing the antitumour effect of an oncolytic virus.[6,13] Among candidate therapeutic transgenes, the tumour-suppressor *p53* gene is a potent therapeutic transgene for induction of cell cycle arrest, senescence and apoptosis.[14] Indeed, a p53-expressing replication-deficient adenovirus (Ad-p53, Advexin) has been reported to induce an antitumour effect in both *in vitro* and *in vivo* settings[15,16] as well as in various clinical studies.[17–20] Recently, p53-expressing armed replication-selective oncolytic adenoviruses have been shown to induce a stronger antitumour effect than a non-armed oncolytic adenovirus or Ad-p53.[21–23] However, the molecular mechanism of the enhanced antitumour effect of a p53-armed oncolytic adenovirus remains unclear. We recently showed that, in combination therapy, OBP-301 enhanced Ad-p53-mediated apoptosis through p53 upregulation and by suppression of the p53-downstream target p21,[24] which is not only transcriptionally activated and mainly induces cell cycle arrest, but also suppresses apoptosis.[25] These results suggest that this p53-expressing oncolytic adenovirus has a strong antitumour effect through apoptosis induction.

In the present study, we first investigated whether the p53-expressing telomerase-specific replication-competent oncolytic adenovirus OBP-702 has efficient *in vitro* antitumour activity compared with OBP-301. We next compared the induction of apoptotic cell death of human cancer cells infected with OBP-301, OBP-702 and Ad-p53. The molecular mechanism of OBP-702-mediated apoptosis induction was further addressed. Finally, the *in vivo* antitumour effect of OBP-702 was evaluated using two subcutaneous human tumour xenograft models.

2. Materials and methods

2.1. Cell lines

The human non-small cell lung cancer cell lines H1299 (p53 null), H358 (p53 null) and H460 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA, USA). The human oesophageal cancer cell line T.Tn (mutant-type p53) was purchased from the Japanese Collection Research Bioresources (JCRB, Osaka, Japan). The human oral squamous cell carcinoma cell line HSC4 (wild-type p53) was obtained from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). The human colon cancer cell lines (SW620 (mutant-type p53) and LoVo (wild-type p53)) and the human liver cancer cell line HepG2 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA, USA). The human liver cancer cell line Huh-7 (mutant-type p53) was obtained from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). H1299, H358, H460, T.Tn, SW620 and LoVo cells were maintained in RPMI 1640 medium. HSC4, HepG2 and Huh-7 cells were maintained in Dulbecco's modified Eagle's medium. All media were supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were routinely maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. 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 that are linked with an IRES, was previously constructed and characterised.[9–11] For OBP-301 induction of exogenous *p53* gene expression, a human wild-type *p53* gene expression cassette derived by the Egr-1 promoter was inserted into the E3 region of OBP-301 (Fig. 1A). The E1A-deleted adenoviral vector dl312 and the wild-type adenovirus type 5 (Ad5) were used as control vectors. Recombinant viruses were purified by ultracentrifugation using caesium chloride step gradients, their titres were determined by a plaque-forming assay using 293 cells, and viruses were stored at −80 °C.

2.3. Western blot analysis

Cells were seeded in a 100-mm dish at a density of 1×10^5 cells/dish 12 h before infection and were infected with OBP-301, OBP-702 or Ad-p53 at the indicated multiplicity of infection (MOI). 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) at the indicated time points. Proteins were electrophoresed on 6–15% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). Blots were blocked with 5% non-fat dry milk in TBS-T (Tris-buffered saline and 0.1% Tween-20, pH 7.4) at room temperature for 30 min. The primary antibodies used were: mouse anti-p53 monoclonal antibody (mAb) (Calbiochem, Darmstadt, Germany), mouse anti-$p21^{WAF1}$ mAb (Calbiochem), mouse anti-MDM2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-BAX polyclonal antibody (pAb) (Santa Cruz Biotechnology), rabbit anti-poly (ADP-ribose) polymerase (PARP) pAb (Cell Signaling Technology, Beverly, MA, USA), mouse anti-Ad5 E1A mAb (BD PharMingen, Franklin Lakes, NJ, USA) and mouse anti-β-actin mAb (Sigma–Aldrich, St. Louis, MO, USA). The secondary antibodies used were: horseradish peroxidase-conjugated antibodies against rabbit IgG (GE Healthcare) or mouse IgG (GE Healthcare). Immunoreactive bands on the blots were visualised using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

2.4. Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 12 h before infection and were infected with OBP-301 or OBP-702 at MOIs of 0, 0.1, 1, 10 or 100 plaque-forming units (PFU)/cell. Cell viability was determined on days 2, 3 and 5 after virus infection using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA), which is based on an XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulphonic acid hydrate assay, according to the manufacturer's protocol. The 50% inhibiting dose (ID_{50}) value of OBP-301 and OBP-702 for each cell line was calculated using cell viability data obtained on day 5 after virus infection.

2.5. Flow cytometric analysis of active caspase-3 expression

Cells were incubated for 20 min on ice in Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, USA), were labelled with phycoerythrin-conjugated rabbit anti-active caspase-3 mAb (BD Biosciences) for

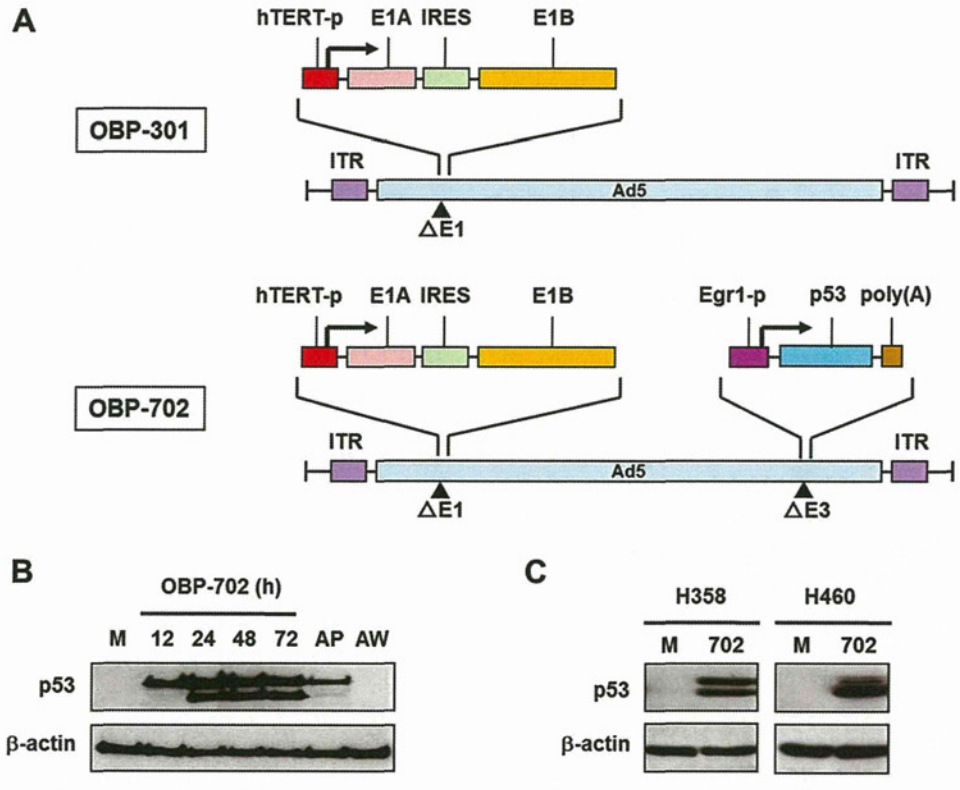

Fig. 1. p53 upregulation in human cancer cells infected with OBP-702. (A) Schematic diagrams of OBP-301 and OBP-702 structures. OBP-301 is a telomerase-specific replication-competent adenovirus, in which the *hTERT* promoter drives the expression of *E1A* and *E1B* genes that are linked with an IRES. OBP-702 is a p53-armed OBP-301, in which the *Egr-1* promoter drives expression of the *p53* gene that is inserted into the E3 region. (B) Expression of the p53 protein in p53-null H1299 cells infected with OBP-702 (10 MOI) at the indicated time points. A replication-deficient p53-expressing adenovirus Ad-p53 (AP) and a wild-type adenovirus Ad5 (AW) were also infected at an MOI of 10 for 24 h as a positive and negative control, respectively. Cell lysates were subjected to Western blot analysis with an anti-p53 antibody. β-Actin was assayed as a loading control. (C) Expression of the p53 protein in H358 and H460 cells infected with OBP-702 (702) at an MOI of 10 for 24 h. Mock-infected cells (M) were used as controls.

30 min, and were then analysed using FACS array (BD Biosciences).

2.6. In vivo subcutaneous H358 and T.Tn xenograft tumour models

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. The H358 and T.Tn cells (5×10^6 cells per site) were inoculated into the flanks of 5-week-old female athymic nude mice (Charles River Laboratories, Wilmington, MA, USA). When tumours reached approximately 5–6 mm in diameter, a 50 μl volume of solution containing OBP-301, OBP-702 or Ad-p53 at a dose of 1×10^8 PFU or phosphate buffered saline (PBS) was injected into the tumours for three cycles every 2 days. Tumour size was monitored by measuring tumour length and width using calipers. Each tumour volume was calculated using the following formula: tumour volume (mm^3) = $L \times W^2 \times 0.5$, where L is the length and W is the width. The survival rate of mice with H358 tumours or T.Tn tumours was assessed until 90 or 180 days, respectively, after first treatment.

2.7. Statistical analysis

Data are expressed as means ± standard deviation (SD). Student's t test was used to compare differences between groups. Log-rank test was also used to compare differences between groups in the survival rate of mice. Statistical significance was defined as a P value less than 0.05.

3. Results

3.1. p53 induction in human cancer cells infected with OBP-702

To examine the level of p53 expression induced by OBP-702 in human cancer cells, we first evaluated p53 expression of p53-null human lung cancer H1299 cells after OBP-702 infection using Western blot analysis. The p53 expression level was increased within 24 h after OBP-702 infection, and a high expression level was maintained for up to 72 h (Fig. 1B). OBP-702-induced p53 expression was higher than Ad-p53-induced p53 expression 24 h after infection. Detectable 40 kDa protein expression in OBP-702-infected H1299 cells may be due to higher p53 expression. In contrast, no p53 expression was induced by OBP-301 infection (data not shown). OBP-702 further induced p53 expression in other human lung cancer cells (H358 (p53-null) and H460 (wild-type p53)) and in human colon cancer cells (SW620 (mutant p53), LoVo cells (wild-type p53)) and human liver cancer cells (HepG2 (wild-type p53) and Huh7 (mutant p53)) (Fig. 1C and Supplementary Fig. 1A). These results indicate that OBP-702 efficiently induces exogenous p53 expression in human cancer cells independent of the status of endogenous p53.

3.2. OBP-702 has enhanced antitumour activity against human cancer cells compared to OBP-301

To compare the *in vitro* antitumour activity of OBP-702 and OBP-301, we used the two OBP-301-sensitive human cancer cells (H358 and H460) and the two OBP-301-resistant human cancer cells (T.Tn and HSC4) that were previously reported.[11] OBP-301-resistant cells showed lower the coxsackie and adenovirus receptor (CAR) expression compared to OBP-301-sensitive cells (data not shown). The cell viability of each cell line was assessed over 5 days after infection using the XTT assay. OBP-702 suppressed the viability of OBP-301-sensitive and OBP-301-resistant cells more efficiently than OBP-301, although at least 48 h are required for the sufficient viral replication (Fig. 2A). Furthermore, OBP-702 also showed increased antitumour activity against human colon and liver cancer cells compared to OBP-301 (Supplementary Fig. 1B). Calculation of the ID_{50} values indicated that all cell lines were more sensitive to OBP-702 than to OBP-301 (Supplementary Table S1). These results suggest that OBP-702 is more cytopathic for human cancer cells than OBP-301.

3.3. Increased induction of apoptosis by OBP-702 compared to OBP-301 or Ad-p53

We next investigated whether OBP-702 has a greater apoptotic effect than OBP-301 or Ad-p53. OBP-301-sensitive H358 cells and OBP-301-resistant T.Tn cells were each infected with OBP-702, OBP-301 or Ad-p53 at MOIs of 10 and 100 for 48 h, and apoptosis was analysed. Western blot analysis showed that OBP-702, but not OBP-301 or Ad-p53, induced the cleavage of PARP at 48 and 72 h after infection (Fig. 3A). Furthermore, flow cytometric analysis demonstrated that OBP-702 infection significantly increased the percentage of apoptotic H358 and T.Tn cells that expressed active caspase-3 compared to Ad-p53 infection (Fig. 3B and C). However, no apoptosis was induced after OBP-301 infection. These results suggest that OBP-702 has a stronger effect on apoptosis than Ad-p53 or OBP-301.

3.4. Induction of apoptosis by OBP-702 through p53-dependent BAX upregulation and E1A-dependent p21 and MDM2 downregulation

Overexpression of p53 is well known to induce apoptosis through induction of p53-downstream target genes.[14] To investigate the molecular mechanism of OBP-702-induced apoptotic cell death, the expression

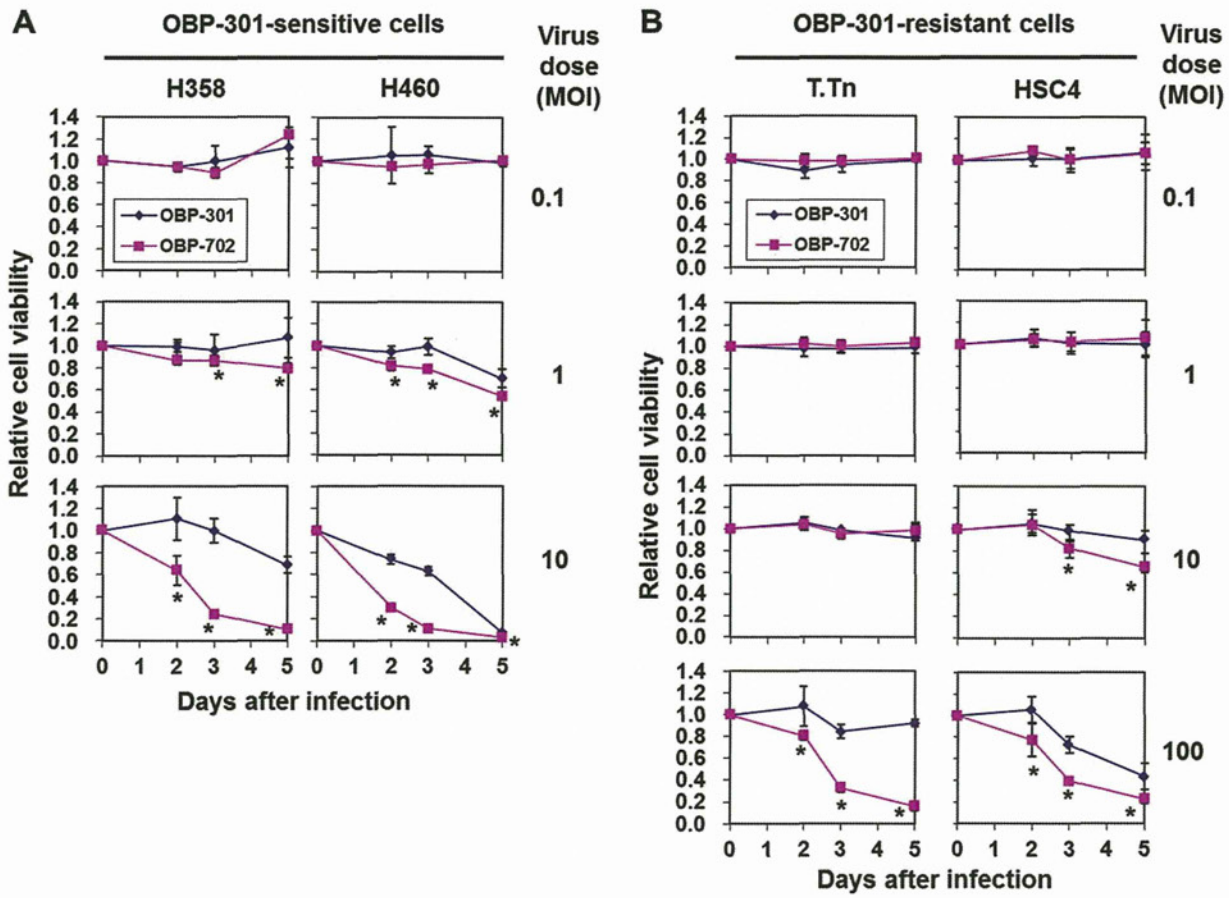

Fig. 2. OBP-702 has enhanced antitumour activity against human cancer cells compared to OBP-301. OBP-301-sensitive cells (H358 and H460) (A) and OBP-301-resistant cells (T.Tn and HSC4) (B) were infected with OBP-301 or OBP-702 at the indicated doses and cell viability was measured using the XTT assay on days 2, 3 and 5 after infection. Cell viability was calculated relative to that of the mock-treated group on each day, which was set at 1.0. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance was determined using Student's t test. $^{*}P < 0.05$. The data are representative of three separate experiments.

level of p53, and p53-downstream target proteins such as p21, BAX and MDM2, was evaluated by Western blot analysis. OBP-702 infection induced higher p53 expression than that induced by Ad-p53 between 24 and 72 h after infection (Fig. 4A). Ad-p53 infection upregulated the expression of p21, MDM2 and BAX proteins. In contrast, OBP-702 infection upregulated the BAX protein as well as Ad-p53, but expression of p21 and MDM2 was low despite strong p53 activation. PARP cleavage was observed 48 and 72 h after OBP-702 infection, consistent with suppression of p21 and MDM2 expression. Overexpression of the adenoviral E1A protein was observed in OBP-702-infected cells. These results suggest that OBP-702 upregulates p53 expression and subsequent BAX expression, but downregulates p21 and MDM2 expression, resulting in the induction of apoptosis.

We recently reported that OBP-301 enhances Ad-p53-induced apoptosis through p53 overexpression and p21 suppression.[24] Furthermore, adenovirus-mediated E2F1 overexpression also enhanced Ad-p53-induced apoptosis through MDM2 downregulation.[26] Since adenoviral E1A is known to activate E2F1 expression,[27] we hypothesised that OBP-702-mediated E1A expression may enhance Ad-p53-induced apoptosis through suppression of p21 and MDM2 expression. To address this hypothesis, H358 cells were coinfected with E1A-deficient dl312 or E1A-expressing wild-type Ad5 after Ad-p53 infection. Ad-p53-induced p53 overexpression was enhanced in the Ad5-coinfected H358 cells, but not in the dl312-coinfected H358 cells (Fig. 4B). Consistent with p53 overexpression, BAX expression was also upregulated. However, despite the enhanced p53 expression, the expression of p21 and MDM2 proteins was lower in Ad5-coinfected cells than in dl312-coinfected cells. Furthermore, PARP cleavage was only detected in H358 cells 72 h after coinfection of Ad-p53 with Ad5. As expected, OBP-301 infection had no apparent effect of the expression of p53, and p53-downstream target proteins (Supplementary Fig. 2). These results suggest that adenoviral E1A suppresses the expression of p21 and MDM2 thereby enhancing apoptosis through p53-dependent BAX upregulation (Fig. 4C, Supplementary Fig. 3).

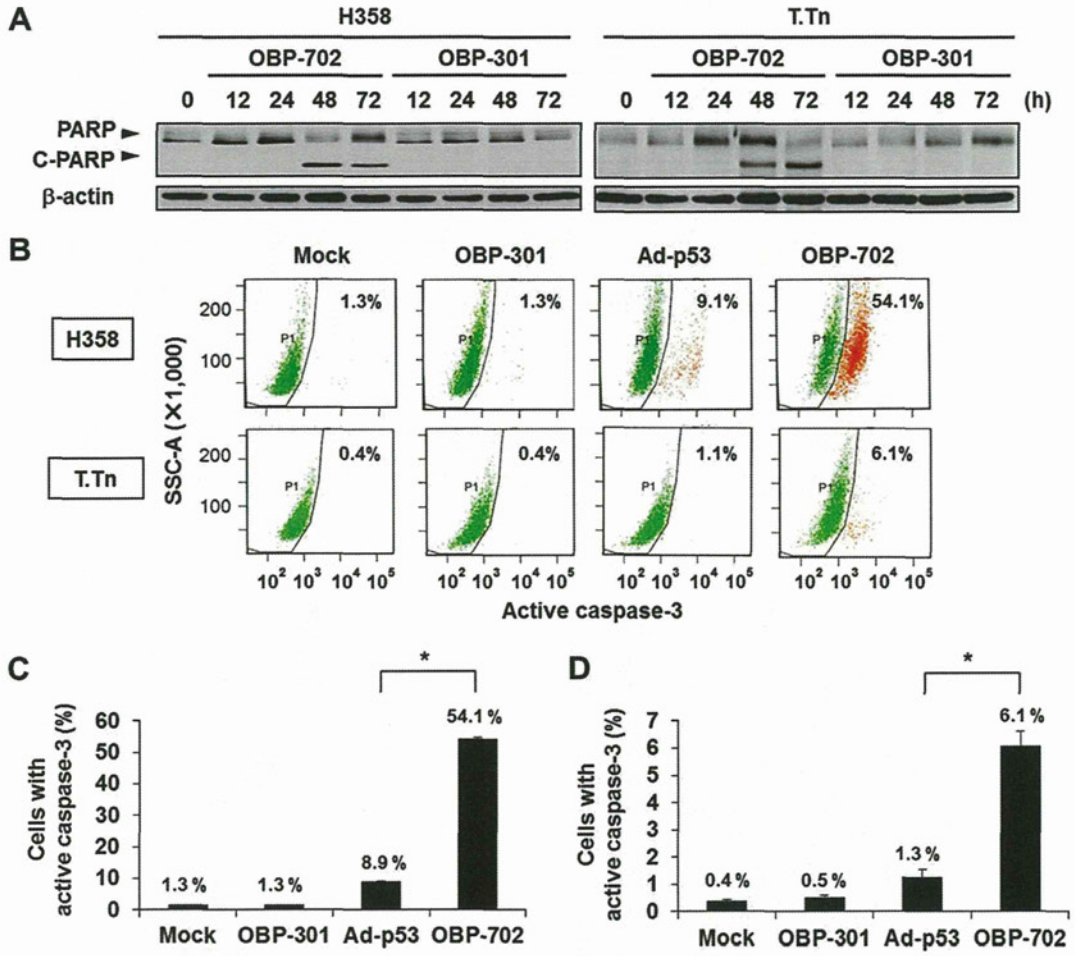

Fig. 3. OBP-702 induces increased apoptosis compared to OBP-301 or Ad-p53. (A) OBP-301-sensitive H358 cells and OBP-301-resistant T.Tn cells were infected with OBP-301 or OBP-702 at an MOI of 10 and 100, respectively, for 48 h. The level of cleaved PARP (C-PARP) and intact PARP in cell lysates was analysed using Western blotting. β-Actin was assayed as a loading control. (B–D), H358 and T.Tn cells were infected with OBP-702, OBP-301 or Ad-p53 at an MOI of 10 and 100, respectively, for 48 h. Mock-infected cells were used as controls. Caspase-3 activation was quantified using flow cytometric analysis. Representative flow cytometric data are shown (B). The mean percentage of H358 cells (C) and T.Tn cells (D) that express active caspase-3 was calculated based on three-independent experiments. Bars, SD. Statistical significance was determined using Student's *t* test. $^{*}P < 0.05$.

3.5. Enhanced antitumour effect of OBP-702 in tumour xenograft animal models

Finally, to assess the *in vivo* antitumour effect of OBP-702, we used subcutaneous H358 and T.Tn tumour xenograft models. OBP-702, OBP-301, Ad-p53 or PBS was intratumourally injected for three cycles every 2 days. OBP-702 administration significantly suppressed tumour growth compared to OBP-301, Ad-p53 or PBS in H358 and T.Tn tumour xenograft models (Fig. 5A). Furthermore, H358 tumour-bearing mice treated with OBP-702 significantly survived longer than those treated with OBP-301 or Ad-p53 (Fig. 5B). Although there was no significant difference in the survival rates between OBP-702-treated and OBP-301-treated mice with T.Tn tumours, OBP-702 treatment significantly increased the survival rate of T.Tn tumour-bearing mice compared to Ad-p53. These results suggest that OBP-702 eliminates tumour tissues more efficiently than OBP-301 or Ad-p53.

4. Discussion

Genetically engineered transgene-expressing armed oncolytic adenoviruses are expected to be a third-generation oncolytic virus for induction of a strong antitumour effect through induction of oncolytic and transgene-induced cell death.[6,13] Although the tumour suppressor *p53* gene is a potent therapeutic transgene for enhancement of an oncolytic adenovirus-mediated antitumour effect,[21–23] the molecular mechanisms by which p53 mediates enhancement of the antitumour effect remain unclear. In this study, we showed that the p53-expressing telomerase-specific oncolytic adenovirus OBP-702 exerted stronger *in vitro* and *in vivo* antitumour effects than OBP-301 or Ad-p53 (Figs. 2 and 5). This enhanced antitumour effect was due to p53-induced apoptosis, and adenoviral E1A enhanced this apoptosis via suppression of the expression of anti-apoptotic p21 and p53-inhibitory MDM2 (Figs. 3 and 4). Although

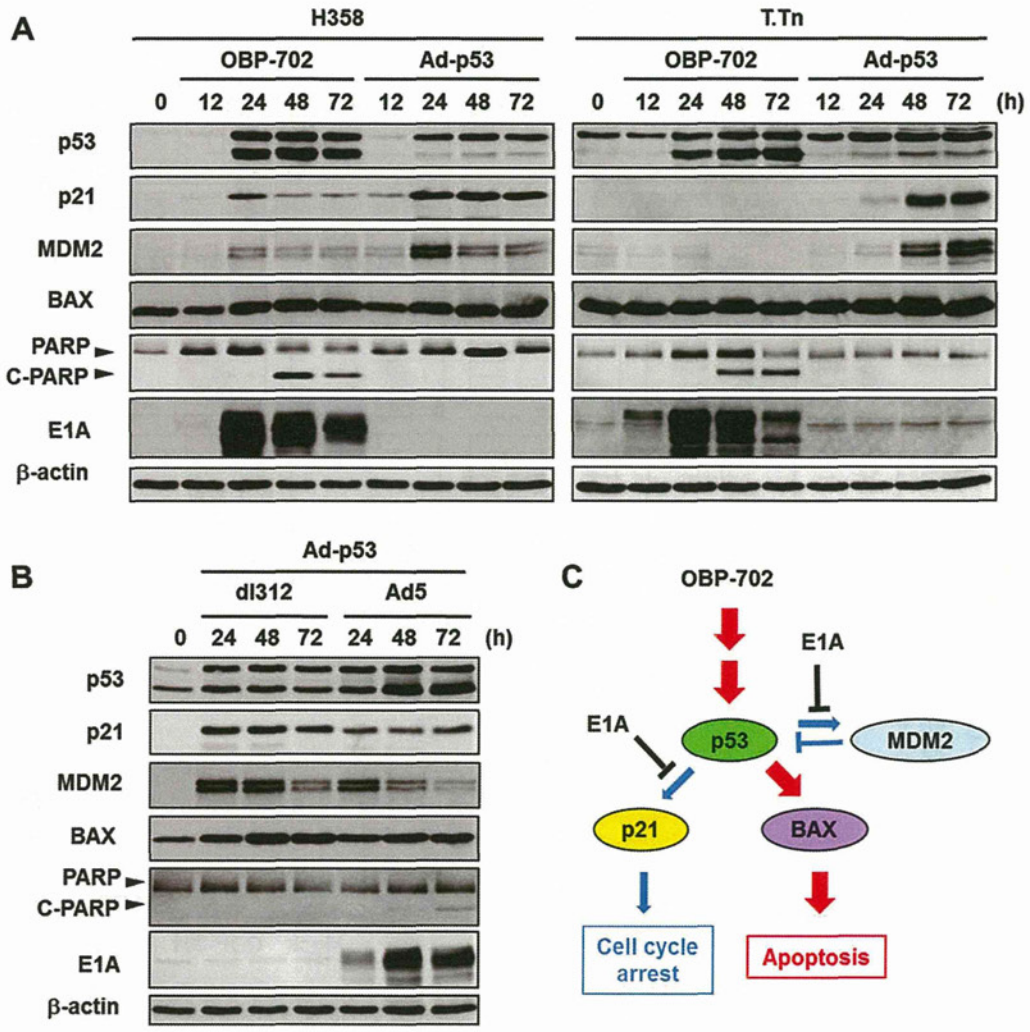

Fig. 4. OBP-702-mediated activation of p53, p53-target proteins and PARP in an E1A-dependent manner. (A) H358 and T.Tn cells were infected with OBP-702 or Ad-p53 at an MOI of 10 and 100, respectively, and infected cells were harvested at the indicated time points. The level of p53, p21, MDM2, BAX, PARP, cleaved PARP (C-PARP) and E1A proteins in cell lysates was analysed by Western blotting. β-Actin was assayed as a loading control. (B) H358 cells were infected with Ad-p53, following which they were coinfected with the E1A-deficient adenovirus (dl312) or an E1A-expressing wild-type adenovirus (Ad5) at the indicated time points. (C) Outline of OBP-702-mediated apoptosis induction through p53-dependent BAX upregulation and E1A-dependent downregulation of p21 and MDM2.

replication-competent adenovirus-mediated *p53* gene transduction has been suggested to exert an increased antitumour effect compared to replication-deficient Ad-p53 through replication-mediated p53 overexpression,[22] adenoviral E1A also enhanced p53-mediated apoptosis through suppression of expression of the p53-downstream targets p21 and MDM2 (Fig. 4). The adenoviral E1A protein has been previously shown to suppress p53-induced p21 and MDM2 expression.[28,29] E1A-mediated p21 and MDM2 suppression has also been shown to induce apoptosis in DNA-damaged cells that overexpress p53.[30,31] These reports support our findings that adenoviral E1A protein enhances p53-induced apoptosis through p21 and MDM2 suppression. It has recently been further shown that replication-deficient Ad-p53 enhances apoptosis through p21 suppression in combination with artificial microRNAs[32] or with OBP-301.[24] Thus, replication-competent oncolytic adenovirus-mediated *p53* gene transfer would strongly induce apoptosis not only through replication-dependent p53 overexpression, but also through E1A-dependent enhancement of p53-mediated apoptosis.

The molecular mechanism by which E1A suppresses p53-mediated upregulation of p21 and MDM2 remains unclear. Since adenoviral E1A has been shown to repress the expression of many target genes through activation of p300/CBP [cyclic adenosine monophosphate response element-binding protein (CREB)-binding protein] histone acetyltransferases that cause global histone modification,[33,34] p300/CBP activation may be involved in E1A-mediated p21 and MDM2 suppression. Indeed, E1A-mediated p21 and MDM2 suppression has been shown to be regulated in a p300/CBP dependent manner.[29,31] A recent report also suggested that an E1B-defective adenovirus activates p53 expression, but

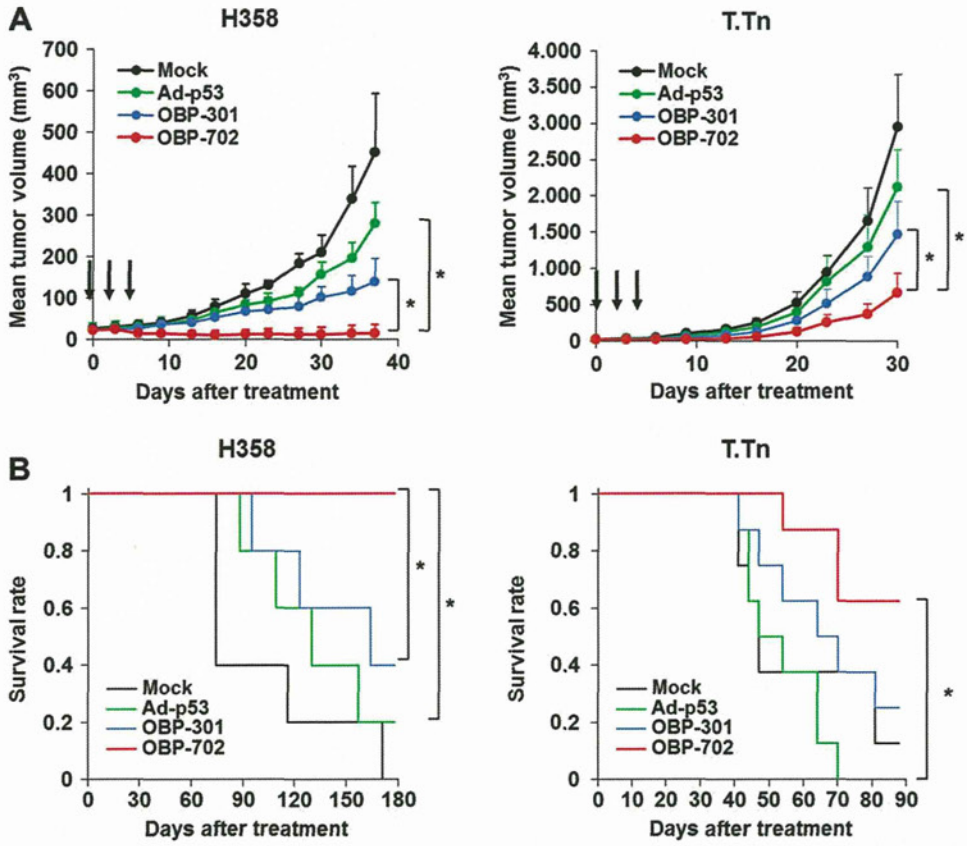

Fig. 5. Strong antitumour effect of OBP-702 on subcutaneous human tumours in xenograft models. (A) H358 or T.Tn cells (5×10^6 cells per site) were inoculated into the flank of 5-week-old female BALB/c *nu/nu* mice. When the tumours reached 3–5 mm in diameter, OBP-702 (10^8 PFU/tumour), OBP-301 (10^8 PFU/tumour), Ad-p53 (10^8 PFU/tumour) or PBS (Mock) was intratumourally injected on days 0, 2 and 4 (Black arrows). Tumour growth is expressed as the mean tumour volume ± SD in each group of H358 tumours ($n = 5$) or T.Tn tumours ($n = 8$). Statistical significance was determined using Student's t test. $^*P < 0.05$. The data are representative of three separate experiments. (B) Survival rate in each group of H358 tumours-bearing mice ($n = 5$) or T.Tn tumours-bearing mice ($n = 8$) was shown using the Kaplan–Meier method. Statistical significance was determined using log-rank test. $^*P < 0.05$.

suppresses p21 and MDM2 expression, through the binding of E1A with p300/CBP.[35] However, p300 disruption has also been shown to both increase p53 stability through MDM2 suppression, and to suppress p21 expression, resulting in apoptosis in UV-irradiated human cancer cells.[36] Therefore, the role of p300/CBP in adenoviral E1A-mediated p21 and MDM2 suppression may be cell type-specific.

It has recently been shown that siRNA-mediated p21 suppression enhances the antitumour effect of an oncolytic adenovirus,[37,38] suggesting that p21 suppression further induces oncolytic cell death. Oncolytic adenovirus-mediated cell death has been shown to be associated with autophagy-related cell death, which is distinct from apoptosis.[39,40] Autophagy has been shown to be positively regulated by p53,[14] but negatively regulated by p21.[41] These results suggest that p53 upregulation without p21 activation enhances autophagic cell death. Thus, oncolytic adenovirus-mediated p21 suppression may enhance not only p53-mediated apoptosis, but also autophagic cell death during the OBP-702-mediated antitumour effect.

Telomerase-specific replication-competent OBP-301 that possesses the *hTERT* gene promoter replicates, and induces an antitumour effect in, human cancer cells in a telomerase-dependent manner.[9–11] Previous reports have shown that Ad-p53-mediated p53 overexpression suppresses *hTERT* mRNA expression,[42,43] suggesting possible suppression of OBP-301 and OBP-702 replication by p53 overexpression. However, we previously reported that Ad-p53-mediated p53 overexpression did not suppress OBP-301 replication during combination therapy.[24] Shats et al. previously reported that knockdown of p21 eliminated the p53-dependent repression of *hTERT* mRNA expression.[44] Since OBP-702, or combination therapy of OBP-301 with Ad-p53, induces p53 overexpression together with E1A-mediated p21 downregulation, p53 overexpression may not suppress hTERT expression. Furthermore, we recently demonstrated that OBP-301 infection itself induces a 1.1- to 50-fold increase in *hTERT* mRNA expression in an E1A-dependent manner.[45] Thus, OBP-702-mediated p53 overexpression would induce apoptosis without affecting hTERT expression.

An anti-tumour effect of Ad-p53-mediated gene therapy has been shown in various clinical studies.[17–20] We previously reported that Ad-p53 induces sensitivity to chemotherapeutic drugs, resulting in enhancement of the antitumour effect.[46,47] Since OBP-702-mediated p53 gene transfer has a stronger antitumour effect than Ad-p53 (Fig. 5), combination therapy of OBP-702 with chemotherapeutic agents may be a more effective antitumour therapy than monotherapy of OBP-702. The adenoviral E1A protein has been shown to enhance chemotherapy-induced apoptosis.[48,49] In particular, p21 suppression has been suggested to be involved in E1A-mediated chemosensitisation.[30] Indeed, artificial miRNA-mediated p21 suppression on Ad-p53-induced p53 overexpression enhanced tumour sensitivity to chemotherapeutic agents.[32] Thus, combination therapy of a p53-armed oncolytic adenovirus with chemotherapy may be a more efficient antitumour strategy for eradication of tumour cells through p53 and E1A-mediated chemosensitisation than monotherapy.

In conclusion, we have clearly demonstrated that the p53-expressing oncolytic adenovirus OBP-702 has a much stronger antitumour effect than OBP-301 or Ad-p53 through p53-mediated apoptosis that is enhanced by E1A-dependent p21 and MDM2 suppression. Oncolytic adenovirus-mediated *p53* gene transduction should therefore be a promising antitumour therapy for efficient elimination of tumour cells.

Conflict of interest statement

Yasuo Urata is an employee of Oncolys BioPharma Inc., the manufacturer of OBP-301 (Telomelysin). Other authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.12.020.

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