

ORIGINAL ARTICLE

Expression of p53 synergistically augments caspases-mediated apoptosis induced by replication-competent adenoviruses in pancreatic carcinoma cells

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We examined cytotoxicity of replication-competent type 5 adenoviruses (Ad5) in human pancreatic carcinoma cells with a p53-defective genotype. The replication-competent Ad5 of which *E1A* gene was activated by exogenous transcriptional regulatory sequences, derived from the *midkine* and *survivin* genes, achieved cytotoxicity to the pancreatic carcinoma. These cells were susceptible to replication-incompetent Ad5 expressing the wild-type p53 gene. We also produced the replication-competent Ad5 bearing the same exogenous regulatory sequences and the type 35 Ad-derived fiber-knob region, and showed that the cytotoxicity was comparable to that of the replication-competent Ad5 prototype. We then investigated possible combinatory effects of the fiber-modified replication-competent Ad and Ad5 expressing the wild-type p53 gene, both of which did not interfere respective infections. The combination produced synergistic cytotoxic effects with enhanced cleavages of caspase-3 and PARP molecules, and with increased sub-G1 fractions and annexin V-positive populations although the viral production of the replication-competent Ad was rather suppressed by expressed p53. Pancreatic cells infected with both Ad showed increase of p53 and decrease of MDM2 and p21 levels, compared with those infected with Ad expressing the p53 gene. These data collectively indicated that replication-competent Ad augmented susceptibility of pancreatic cells to apoptosis through upregulated p53 expression.

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INTRODUCTION

Pancreatic carcinoma remains one of the intractable diseases partly because of the invasive property and lack in symptomatic signs until the advanced stage.¹ Recent chemotherapeutic agents such as gemcitabine and 5-fluorouracil derivatives prolonged survival of the advanced or the recurrent cases², and a novel treatment modality can further contribute to improvements in the prognosis and quality of the patient's life. A gene medicine that can activate cell death pathways is one of such candidates. Replication-competent adenoviruses (Ad) achieve cytotoxic effects on the infected cells through the viral replications, and augment the cytotoxicity by spreading the viral progenies into tumors in the vicinity.

A regulatory region of the genes that are preferentially expressed in tumor cells but scarcely in normal tissues can be a tool to activate a therapeutic gene in a tumor-specific manner. We demonstrated that the regulatory region of the *survivin* (*Sur*) gene and the *midkine* (*MK*) gene transactivated an exogenous gene preferentially in tumors and produced anti-tumor effects in a suicide gene system.^{3,4} Replication-competent Ad of which the *E1A* gene is controlled with such a regulatory region in fact induced preferential tumor cell death.⁵ Type 5 Ad (Ad5), commonly used for gene transduction, uses the coxsackie adenovirus receptor (CAR) molecules as the major

receptor for infection.⁶ Expression of the CAR molecules is often downregulated in human tumors and consequently transduction efficacy of Ad5 was low in some of the tumors. In contrast, type 35 Ad uses CD46 molecules as a cellular receptor and the expression level of CD46 is not suppressed in human tumors.^{6,7} Replacement of the Ad fiber-knob region, which is responsible for the binding to CAR and CD46 molecules, can thereby convert the Ad-infectious tropism between type 5 and type 35. Ad5 bearing type 35-derived fiber-knob region (AdF) can augment the infectivity in particular to tumor cells expressing CAR at a low level.^{8,9} The chimeric AdF have another advantage in terms of gene transfer. AdF in combination with Ad5 vectors can transduce target cells with two different genes encoded by the respective vectors. A simultaneous infection with the same Ad5 vectors decreases the transduction efficacy because Ad5 infection downregulates the CAR expression to inhibit further Ad5-mediated gene transfer. A combinatory use of Ad5 and AdF, however, did not influence expression levels of respective receptors and enabled dual gene transfer.¹⁰

A majority of pancreatic carcinoma has defective or mutated p53 gene and transduction of such tumors with the p53 gene achieved cytotoxic effects.¹¹ Nevertheless, activation of the p53 pathways can limit the viral proliferation and a possible role of the activated p53 pathways in viral replication-induced cell death

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remain uncertain. In this study, we examined cytotoxicity of the promoter-mediated replication-competent Ad5 and AdF on pancreatic carcinoma and then investigated the possible combinatory effects of the replication-competent AdF and Ad5 bearing the wild-type *p53* gene (Ad5-*p53*).

MATERIALS AND METHODS

Cells and anti-cancer reagents

Human pancreatic carcinoma cells, PANC-1 (*p53* mutated), AsPC-1 (*p53* defective), MIA-PaCa-2 (*p53* mutated) and BxPC-3 (*p53* mutated), and A549 human lung carcinoma cells were from Cell Resource Center for Biomedical Research (Sendai, Japan). HEK293 cells were from ATCC (Manassas, VA, USA), and all the cells were cultured with RPMI 1640 supplemented with 10% fetal calf serum.

Ad preparation

Replication-incompetent Ad5-*p53* and Ad expressing the β -galactosidase gene (accession number: NM066611) (Ad5-LacZ) were prepared with an Adeno-X expression system (Takara, Shiga, Japan) and HEK293 cells. These Ad5 vectors used the same cytomegalovirus (CMV) promoter (BK000394) to activate the respective genes. Replication-competent Ad5 in which the *E1* gene was activated by an exogenous regulatory element, Ad5-Sur, Ad5-MK and Ad5-CMV, were prepared by replacing authentic *E1* promoter region with regulatory sequences of the 0.5 kb *Sur*³ (U75285) or the 0.6 kb *MK*¹² (D10604) gene, or with the CMV promoter. Replication-competent AdF-MK and AdF-Sur, and -incompetent AdF-LacZ were produced by replacing the fiber-knob region of Ad5 (Clonetech, Mountain View, CA, USA) with that of type 35 Ad (Avior Therapeutic, Seattle, WA, USA) (AY271307 at 30827-33609). Consequently, difference between Ad5-MK/Ad5-Sur/Ad5-LacZ and AdF-MK/AdF-Sur/AdF-LacZ was the fiber-knob region, derived from either type 5 or type 35. Ad were purified with an Adeno-X virus purification kit (BD Biosciences, San Jose, CA, USA) and the numbers of virus particles (vp) per milliliter was estimated with the formula, absorbance at 260 nm of purified Ad in the presence of 0.1% sodium dodecyl sulfate $\times 1.1 \times 10^{12}$.

In vitro cytotoxicity

Cells (5×10^3 per well) were seeded in 96-well plates and were cultured for 5 days with different amounts of Ad (vp per cell). Cell viability was determined with a cell-counting WST kit (Wako, Osaka, Japan). The amount of formazan produced was determined with the absorbance at 450 nm and the relative viability was calculated based on the absorbance without any treatments. Combinatory effects were examined with CalcuSyn software (Biosoft, Cambridge, UK). Combination index (CI) values at respective fractions affected (Fa) points, which showed relative levels of suppressed cell viability, were calculated based on the WST assay. $CI < 1$, $CI = 1$ and $CI > 1$ indicate synergistic, additive and antagonistic actions, respectively.

Cell cycle analysis

Cells were treated with Ad were fixed in ice-cold 70% ethanol, incubated with RNase ($50 \mu\text{g ml}^{-1}$) and stained with propidium iodide ($50 \mu\text{g ml}^{-1}$). The staining profiles were analyzed with FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences).

Production of viral progeny

AsPC-1 cells were uninfected or infected with Ad5-*p53* or Ad5-LacZ (5000 vp per cell) and were treated with or without AdF-Sur (5000 vp per cell) for 72 or 96 h. Lysate of respective cell samples was tested for the virus amounts with the 50% tissue culture infectious dose (TCID₅₀) assay using A549 cells that were defective of Ad *E1* region. The virus production was expressed as vp per milliliter.

Detection of apoptosis

AsPC-1 cells were uninfected or infected with Ad5-*p53* or AdF-Sur (5000 vp per cell), or were treated with combination of Ad5-*p53* and AdF-Sur (5000 vp per cell for respective Ad) for 3 days. The cells were stained with annexin V Alexa Fluor 488 and then analyzed with an image-based cytometer (Invitrogen, Carlsbad, CA, USA).

Western blot analysis

Lysate of Ad-infected AsPC-1 cells was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was transferred to a nylon filter and was hybridized with antibody against phosphorylated *p53* at serine (Ser) residues 15 (catalog number: #9284), cleaved caspase-3 (#9664), poly (ADP-ribose) polymerase (PARP) (that detects cleaved PARP molecules as well) (#9542), LC3A/B (#4108), Atg5 (#2630), Beclin-1 (#3495), p21 (#2947) (Cell Signaling, Danvers, MA, USA), MDM2 (SC-965), E1A (SC-25) (Santa Cruz Biotech, Dallas, TX, USA), MAD2 (ab70385), Hexon E11 (ab2596), NBS1 (ab23996) (Abcam, Cambridge, UK), phosphorylated H2AX at Ser 139 (613401) (BioLegend, San Diego, CA, USA), *p53* (MAB1976) and tubulin- α (MS-581-P1) (Thermo Fisher Scientific, Fremont, CA, USA) as a control. The membranes were developed with the ECL system (GE Healthcare, Buckinghamshire, UK).

RESULTS

Cytotoxicity of replication-competent Ad to pancreatic carcinoma cells

We examined cytotoxicity of Ad5-Sur, Ad5-MK, Ad5-CMV and Ad5-*p53* in four kinds of pancreatic cell lines, PANC-1, AsPC-1, BxPC-1 and MIA-PaCa-2 (Figure 1). The cytotoxicity was different among the cells tested, but all the cells were susceptible to the replication-competent Ad5 and Ad5-*p53*. In contrast, Ad5-LacZ minimally suppressed the viability even at high virus doses. The cytotoxicity of the regulatory region-mediated Ad5 was not greatly different from that of Ad5-CMV, suggesting that transcriptional activities of the regulatory regions were comparable to the CMV promoter. These data also implied that Ad5-*p53* activated the *p53* downstream pathways in these pancreatic carcinoma cells and achieved cytotoxic effects.

Combinatory effects of replication-competent AdF and Ad5-*p53*

We investigated differential cytotoxicity between Ad5-Sur and AdF-Sur or between Ad5-MK and AdF-MK to know the possible benefits of receptor exchange (Figure 2). AdF-Sur produced greater cytotoxicity than Ad5-Sur in both PANC-1 and AsPC-1 cells, whereas cytotoxicity of AdF-MK and Ad5-MK was not different in both cells. AdF-LacZ achieved minimal cytotoxicity as found in Ad5-LacZ. These data showed that replication-competent AdF achieved cytotoxicity to pancreatic carcinoma cells at least at a similar level as did replication-competent Ad5. The reason for lack of differential cytotoxicity between Ad5-MK and AdF-MK remains unknown. It could be attributable to a limited amount of transcriptional factors necessary for MK transcripts activation. The limited factors can subsequently restrict the *E1A* activation and the cytotoxicity becomes independent of infected viral amounts.

We then examined combinatory cytotoxicity induced by Ad5-*p53* and either AdF-Sur or AdF-MK. We infected PANC-1 or AsPC-1 cells with AdF35-Sur at various doses and Ad5-*p53* or Ad5-LacZ (Figure 3a). AdF-Sur-mediated cytotoxicity was enhanced by simultaneous transduction with Ad5-*p53* but not with Ad5-LacZ in both cells. We calculated CI values at several fraction-affected points that corresponded to the percentages of live cells tested with the WST assay. The CI values in combination of AdF-Sur and Ad5-*p53* were less than 1 in the majority of the Fa points tested, demonstrating synergistic effects produced by the combination. We also examined the combination of AdF-MK at various doses and Ad5-*p53* (Figure 3b). Additional Ad5-*p53* enhanced the AdF-MK-mediated cytotoxicity in PANC-1 and AsPC-1 cells. The CI values of the combinatory use were less than 1 in all the Fa points tested and indicated synergistic cytotoxicity in the combination. These data collectively suggested that transduction with Ad5-*p53* enhanced cytotoxicity of replication-competent AdF in a synergistic manner in pancreatic carcinoma cells.

Molecular mechanisms of enhanced cytotoxicity in combination

We investigated a possible mechanism of cytotoxicity induced by the combination of Ad5-*p53* and AdF-Sur (Figure 4). AsPC-1 cells

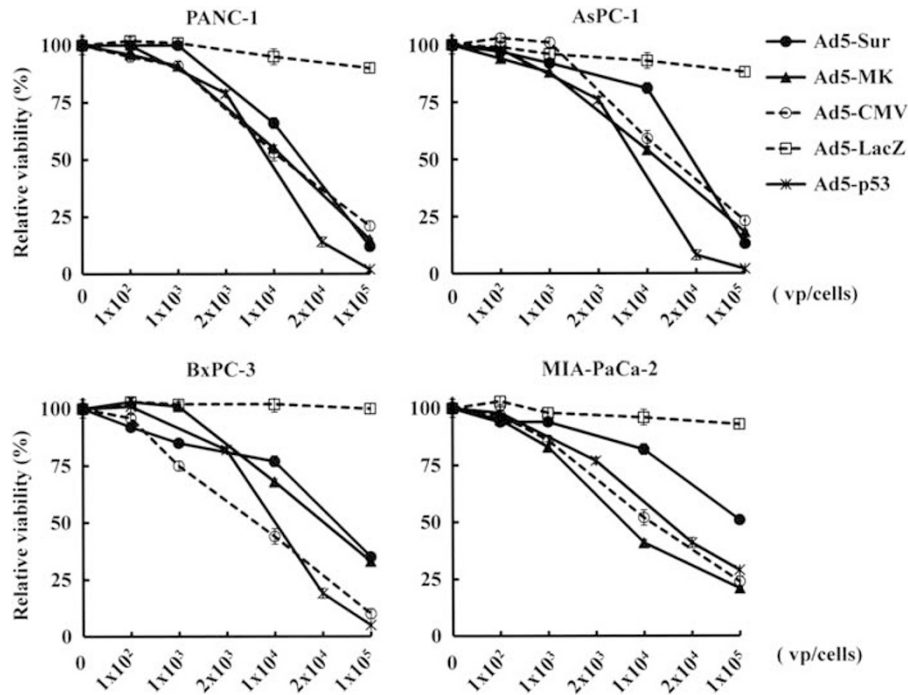


Figure 1. Cytotoxicity of replication-competent and -incompetent Ad to pancreatic carcinoma cells. Cells were infected with Ad5-Sur, Ad5-MK, Ad5-CMV, Ad5-p53 or Ad5-LacZ as a control at various virus particles and relative viability was calculated based on uninfected cells. Averages and s.e. bars are shown ($n=3$).

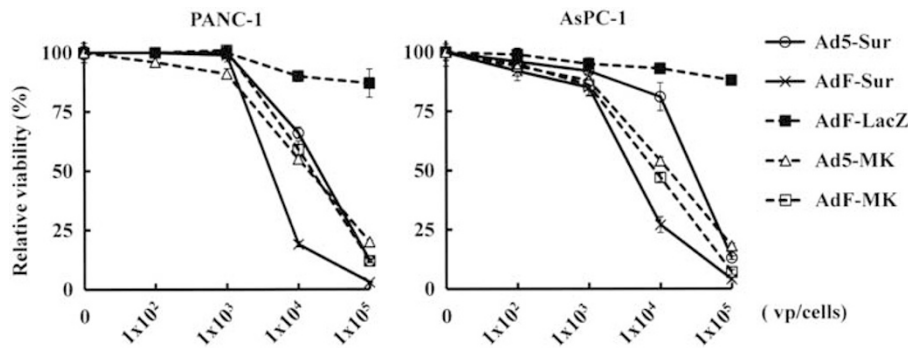


Figure 2. Cytotoxicity of replication-competent Ad5 and AdF to pancreatic carcinoma. PANC-1 and AsPC-1 cells were treated with Ad5-Sur, Ad5-MK, AdF-Sur, AdF-MK or AdF-LacZ as a control at various virus particles. Relative viability was calculated based on uninfected cells. Averages and s.e. bars are shown ($n=3$).

infected with Ad5-p53 induced p53 expression and the phosphorylation of p53. The endogenous p53 gene in AsPC-1 cells was deleted in both alleles and expressed p53 was thereby produced by transduction with Ad5-p53. Expression of p21 and MDM2 was also induced with Ad5-p53, whereas Ad5-LacZ did not activate the respective endogenous genes. Cleavages of caspase-3 and PARP were detected with Ad5-p53, but expression of Atg5 and Beclin-1 remained unchanged. Transition of LC3A/B I to LC3A/B II was minimally detected in Ad5-p53-transduced cells only after 96 h, but the same transition was also observed in Ad5-LacZ-infected cells at 120 h. AdF-Sur infection did not induce p21 or MDM2 expression but activated cleavages of caspase-3 and PARP. Transition of LC3A/B I to LC3A/B II was minimally detected as found in Ad5-p53-infected cells.

A combinatory use of Ad5-p53 and AdF-Sur augmented Ad5-p53-induced p53 levels until 72 h but rather decreased the

p53 levels after 96 h. The p53 phosphorylation levels were not different between cells infected with Ad5-p53 and those with Ad5-p53 and AdF-Sur. Nevertheless, the combination facilitated cleavages of caspase-3 and PART compared with infections with Ad5-p53 or AdF-Sur. The combination decreased expression levels of Ad5-p53-induced p21 and MDM2 in particular after 96 h, whereas the dual infection did not influence the expression levels of Atg5 and Beclin-1, or transition of LC3A/B I to LC3A/B II. These data collectively suggested that the dual infection with Ad5-p53 and AdF-Sur augmented p53-mediated apoptosis by inhibiting p21 and MDM2. We found that E1A and hexon expression levels in cells infected both with Ad5-p53 and AdF-Sur were lower than those in AdF-Sur-infected cells after 48 h. We also investigated the expression of p53, E1A and hexon in an early phase of infection (Supplementary Figure 1). Expression of p53 and E1A but not of hexon was detected as early as 12 h after the infection, and the combinatory

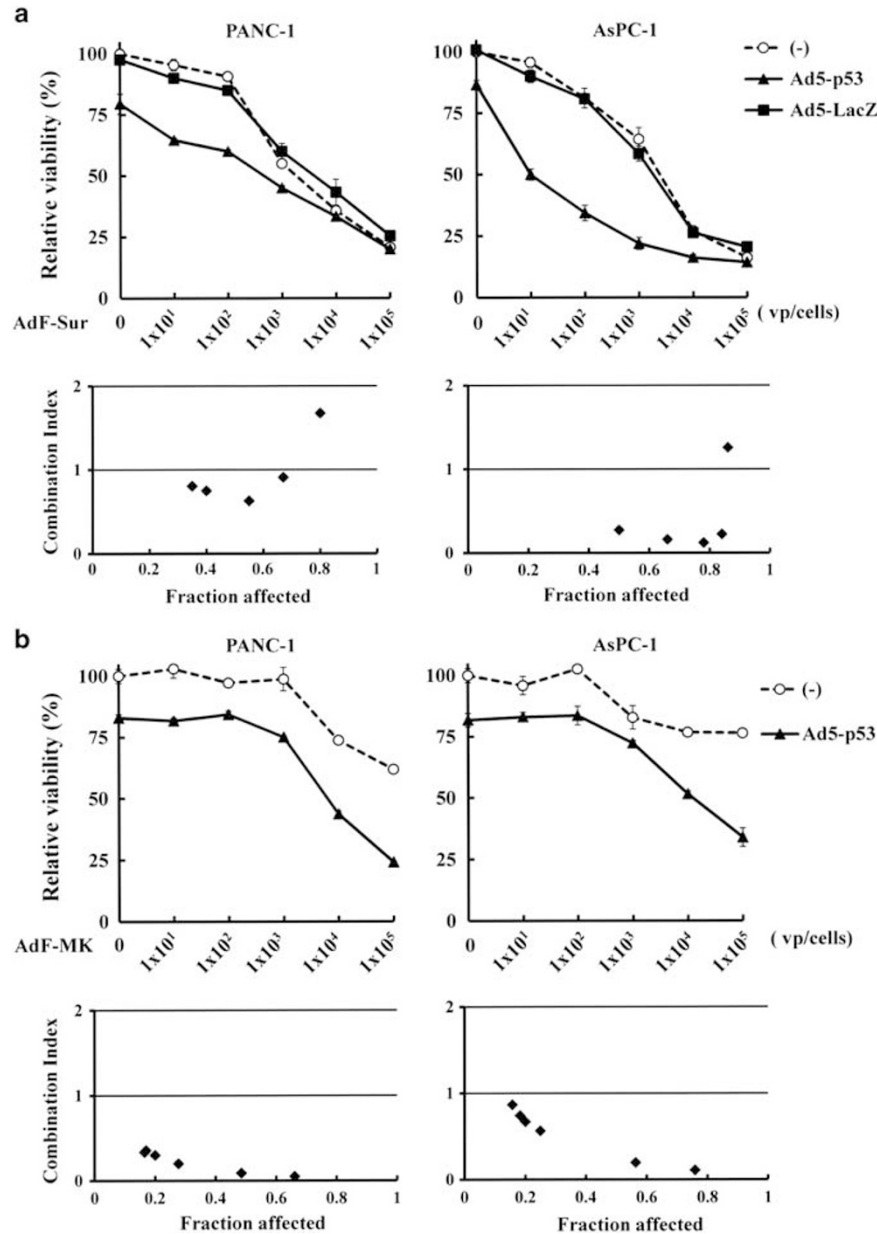


Figure 3. Cytotoxicity in combination of replication-competent AdF and Ad5-p53. PANC-1 (**a**) or AsPC-1 (**b**) cells were infected with various doses of (**a**) AdF-Sur or (**b**) AdF-MK together with either Ad5-p53 (4000 vp per cell) or Ad5-LacZ (4000 vp per cell). The relative viability was calculated based on uninfected cells. Averages and s.e. bars are shown ($n=3$). Combinatory effects were assessed with combination index values at different fractions affected (Fa) points.

infection at 24 h showed upregulated p53 expression compared with Ad-p53 alone, and downregulated E1A expression compared with AdF-Sur alone. These data suggested that induced p53 expression was correlated with suppressed viral replications of Ad.

Cell cycle changes induced by replication-competent AdF and Ad5-p53

We also examined cell cycle changes of AsPC-1 cells infected with Ad5-p53, AdF-Sur, AdF-MK or both Ad5-p53 and AdF-Sur or AdF-MK (Figure 5, Table 1). Ad5-p53-infected cells showed increased sub-G1 fractions, whereas Ad5-LacZ did not influence cell cycle progression. Cells infected with AdF-Sur and less significantly with AdF-MK also increased sub-G1 populations in a time-dependent manner. Interestingly, AdF-Sur- but not AdF-MK-infected cells showed

hyperploid populations with more than a 4N fraction. We also detected small percentages of the hyperploidy in untransduced and Ad5-LacZ-transduced cells but the population remained unchanged thereafter. We further investigated a possible molecular mechanism that could be involved in hyperploidy by comparing AdF-Sur- and AdF-MK-infected cells from the standpoint of DNA damages and the M-phase checkpoint (Supplementary Figure 2). Both AdF-Sur and AdF-MK augmented phosphorylated H2AX and downregulated NBS1 expression, whereas MAD2 expression remained unchanged. The augmentation of phosphorylated H2AX expression was greater in AdF-MK-infected cells than AdF-Sur-infected cells. These data thereby suggested that the hyperploidy was not linked with DNA damages or the spindle checkpoint at M-phase.

Combination of Ad5-p53 and AdF35-Sur or AdF35-MK increased sub-G1 populations greater than infection with Ad5-p53, AdF-Sur

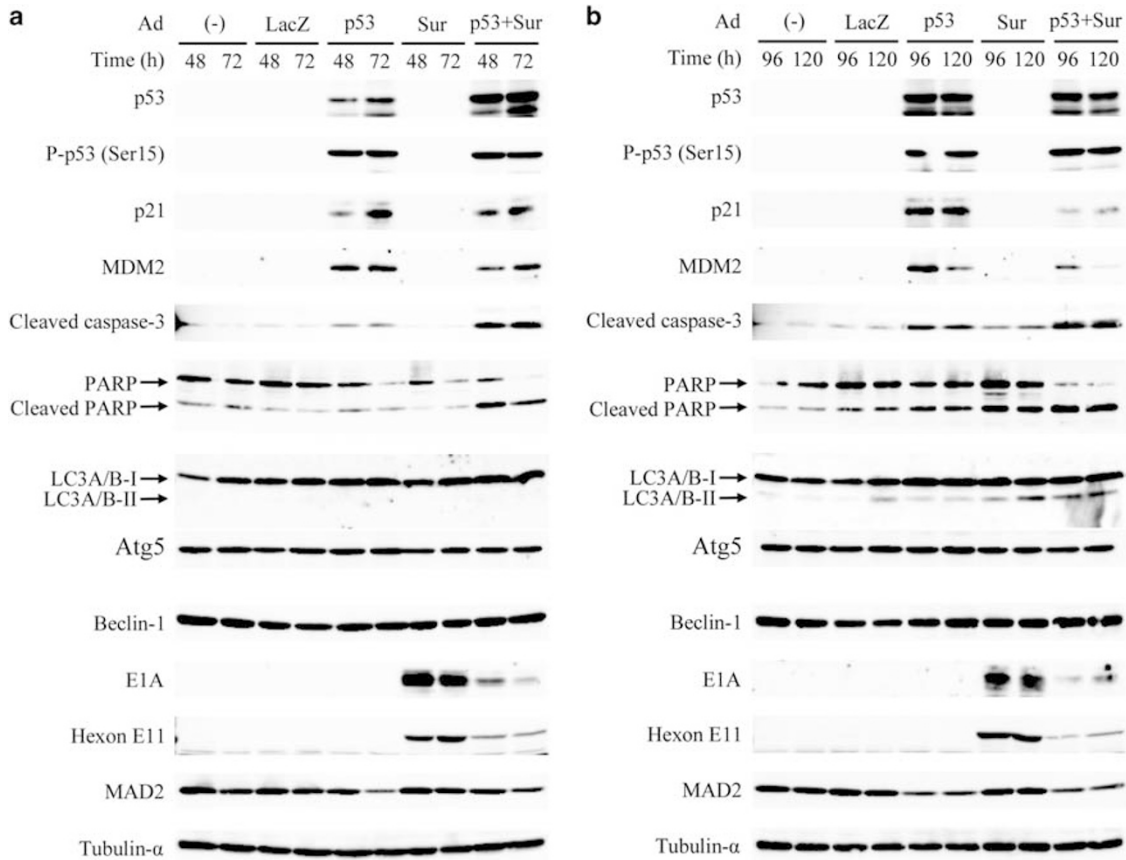


Figure 4. Expression of molecules linked with apoptosis and autophagy. AsPC-1 cells, which were uninfected or infected with Ad5-LacZ (LacZ) (10 000 vp per cell), Ad5-p53 (p53) (5000 vp per cell), AdF-Sur (Sur) (5000 vp per cell) or in combination with Ad5-p53 and AdF-Sur (5000 vp per cell for respective Ad), were cultured for (a) 48 and 72 h, (b) 96 and 120 h. The cell lysate were subjected to western blot analysis with antibody as indicated. Note that exposed periods of (a) and (b) filters were not the same and consequently it is difficult to compare the expression levels of respective molecules between (a) and (b) filters. Expression of tubulin- α was used for a loading control.

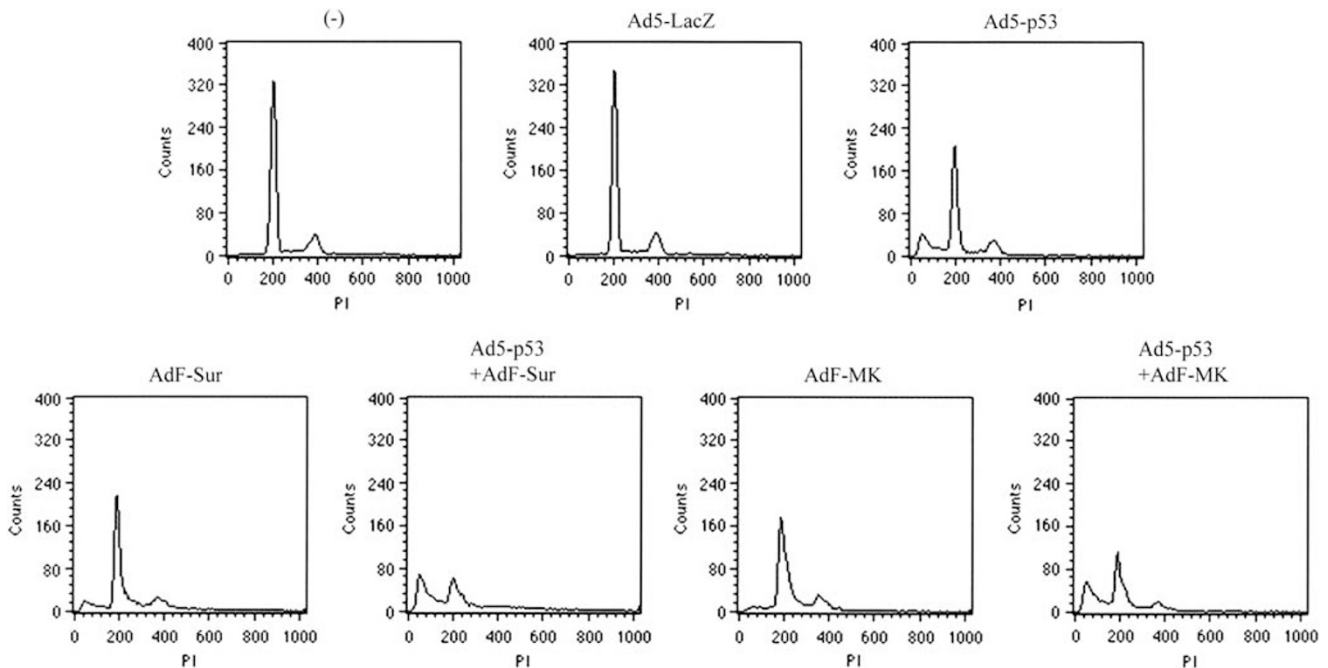


Figure 5. Representative data of cell cycle analysis. AsPC-1 cells were uninfected or infected with Ad5-LacZ, Ad5-p53, AdF-Sur, AdF-MK or Ad5-p53 in combination with AdF-Sur or AdF-MK. These cells were subjected to cell cycle analysis on day 8 with flow cytometry.

Table 1. Cell cycle progressions of AsPC-1 cells treated with replication-competent AdF and Ad5-p53

Treatment	Days	Cell cycle distribution (%)				
		Sub-G1	G0/G1	S	G2/M	4N <
(-)	2	0.59 ± 0.03	57.04 ± 0.26	19.26 ± 0.22	20.52 ± 0.38	3.04 ± 0.10
	4	3.35 ± 0.13	73.13 ± 0.24	5.19 ± 0.06	15.57 ± 0.25	2.96 ± 0.09
	6	3.18 ± 0.13	70.88 ± 0.65	7.57 ± 0.15	13.92 ± 0.17	4.71 ± 0.08
	8	1.16 ± 0.05	72.68 ± 0.65	6.44 ± 0.21	15.16 ± 0.52	4.81 ± 0.03
Ad5-LacZ	2	0.67 ± 0.02	54.94 ± 0.82	20.10 ± 0.38	21.65 ± 0.98	3.21 ± 0.27
	4	3.23 ± 0.14	72.93 ± 0.19	6.04 ± 0.09	15.71 ± 0.20	2.31 ± 0.01
	6	2.81 ± 0.05	68.78 ± 0.21	8.42 ± 0.08	15.73 ± 0.49	4.79 ± 0.04
	8	1.11 ± 0.05	71.55 ± 0.12	6.11 ± 0.13	16.26 ± 0.10	5.19 ± 0.10
Ad5-p53	2	0.77 ± 0.03	65.76 ± 1.98	11.62 ± 0.06	19.56 ± 0.93	1.73 ± 0.27
	4	6.06 ± 0.19	70.81 ± 0.43	6.33 ± 0.30	15.60 ± 0.57	1.46 ± 0.14
	6	14.26 ± 0.14	63.55 ± 1.21	5.37 ± 0.09	14.22 ± 0.13	1.86 ± 0.02
	8	25.07 ± 0.28	55.24 ± 0.18	6.35 ± 0.19	11.75 ± 0.02	1.91 ± 0.04
AdF-Sur	2	0.82 ± 0.06	57.28 ± 0.16	19.20 ± 0.12	18.81 ± 0.11	4.39 ± 0.06
	4	2.51 ± 0.08	64.50 ± 0.18	7.55 ± 0.15	15.04 ± 0.18	10.68 ± 0.24
	6	6.01 ± 0.06	63.44 ± 0.24	9.25 ± 0.08	12.75 ± 0.23	8.99 ± 0.17
	8	11.18 ± 0.21	57.52 ± 0.38	11.61 ± 0.08	11.89 ± 0.22	8.44 ± 0.12
Ad5-p53+ AdF-Sur	2	0.85 ± 0.05	63.21 ± 1.71	11.93 ± 0.14	17.07 ± 1.35	7.41 ± 0.56
	4	14.76 ± 0.14	50.82 ± 0.20	10.25 ± 0.22	9.95 ± 0.20	14.75 ± 0.35
	6	32.51 ± 0.08	35.87 ± 0.32	10.82 ± 0.17	6.36 ± 0.06	14.92 ± 0.60
	8	40.76 ± 0.78	29.40 ± 0.56	11.90 ± 0.33	5.10 ± 0.24	13.38 ± 0.23
AdF-MK	2	0.63 ± 0.04	57.85 ± 0.06	20.48 ± 0.17	18.73 ± 0.08	2.82 ± 0.13
	4	3.43 ± 0.02	72.21 ± 0.26	5.93 ± 0.14	15.51 ± 0.13	3.13 ± 0.19
	6	6.53 ± 0.15	66.52 ± 0.08	11.49 ± 0.32	12.29 ± 0.17	3.76 ± 0.20
	8	7.34 ± 0.11	63.99 ± 0.43	13.09 ± 0.27	11.89 ± 0.25	4.31 ± 0.16
Ad5-p53+ AdF-MK	2	0.63 ± 0.07	65.30 ± 0.20	11.08 ± 0.42	20.59 ± 0.48	2.76 ± 0.13
	4	8.76 ± 0.14	65.51 ± 0.06	7.24 ± 0.49	14.78 ± 0.23	4.03 ± 0.39
	6	21.79 ± 0.29	53.95 ± 0.16	9.54 ± 0.20	11.65 ± 0.20	3.59 ± 0.10
	8	37.41 ± 0.41	42.10 ± 0.32	9.03 ± 0.13	8.42 ± 0.22	3.46 ± 0.11

AsPC-1 cells were uninfected or infected with Ad5-LacZ, Ad5-p53, AdF-Sur, AdF-MK, (3000 vp per cell) or in combination with Ad5-p53 and AdF-Sur or AdF-MK as indicated (3000 vp per cell for respective Ad) and the cell cycle progressions were analyzed with flow cytometry. Average and s.e. are shown ($n = 3$).

or AdF-MK alone. The dual infection with Ad5-p53 and AdF-Sur also upregulated hyperplod populations, and the sequential cell cycle changes suggested that increase of the hyperplod fractions was followed by that of sub-G1 populations. We found that MAD2 expression was downregulated by Ad5-p53, probably due to p53-mediated inhibition of cell cycle progression, and that the expression level was not influenced by combination with AdF-Sur (Figure 4). In contrast, combination of Ad5-p53 and AdF-MK increased sub-G1 populations without inducing the hyperplod. It is thereby uncertain how hyperplod contributes to the AdF-mediated cytotoxicity.

We also investigated apoptotic cell death induced by the Ad combination with an annexin V staining (Figure 6). Infection with Ad5-p53 or AdF-Sur increased annexin V-positive cells compared with untreated cells ($P < 0.01$) and the combination achieved greater cytotoxicity than infection with Ad5-p53 or AdF-Sur ($P < 0.01$). These data collectively indicated that a combinatory use of Ad5-p53 and replication-competent AdF enhanced apoptotic cell death.

Viral production influenced by Ad5-p53

We investigated effects of Ad5-p53 transduction on production of viral progenies. AsPC-1 cells were infected with AdF-Sur or in combination with AdF-Sur and Ad5-p53 or Ad5-LacZ, and the cell lysate was tested for the viral amounts with E1-negative A549 cells (Figure 7). Lysate of cells infected with either Ad5-LacZ or Ad5-p53 did not produce any cytopathic effects in A549 cells (data not shown), indicating that recombination of the replication-incompetent Ad was not induced in AsPC-1 cells. In contrast, lysate of cells infected with AdF-Sur showed cytopathic effects in A549

cells and the viral amounts produced in AsPC-1 were similar in samples harvested at 72 and 96 h after the infection. Transduction with Ad5-p53 downregulated viral production of AdF-Sur, whereas coinfection with Ad5-LacZ did not suppress the production.

DISCUSSION

We examined cytotoxicity of replication-competent AdF and Ad5-p53 in p53-null or p53-mutated pancreatic carcinoma and demonstrated that the combination of both Ad types produced synergistic effects through augmented apoptotic cell death. We used two kinds of Ad with different fiber-knob structures to conduct dual infection as type 5 and type 35 Ad do not share the receptor molecules or interfere respective infections.

The present study revealed synergism of Ad5-p53 and AdF-Sur or AdF-MK in the anti-tumor effects, which were associated with apoptosis but not autophagy. Furthermore, the western blot analyses suggested that the synergistic cytotoxicity was due to enhanced activation of the p53 pathways by replication-competent Ad. We presumed that Ad5-p53 might enhance viral replication-mediated cell death, but transduction of Ad5-p53 was rather inhibitory to production of replication-competent Ad. Furthermore, activation of the p53 pathways induced cell cycle arrest, and the suppressed cell proliferation reduced viral replication.¹³ We therefore think that enhancement of the p53 pathways contributed to the synergism. Previous studies analyzed the role of p53 in replication-competent Ad, which harbored the p53 gene in the same Ad construct and consequently induced p53 expression in coordination with the viral replications.¹⁴⁻¹⁶ In contrast to the present study, these reports demonstrated that expression of p53 augmented viral replications or release of viral

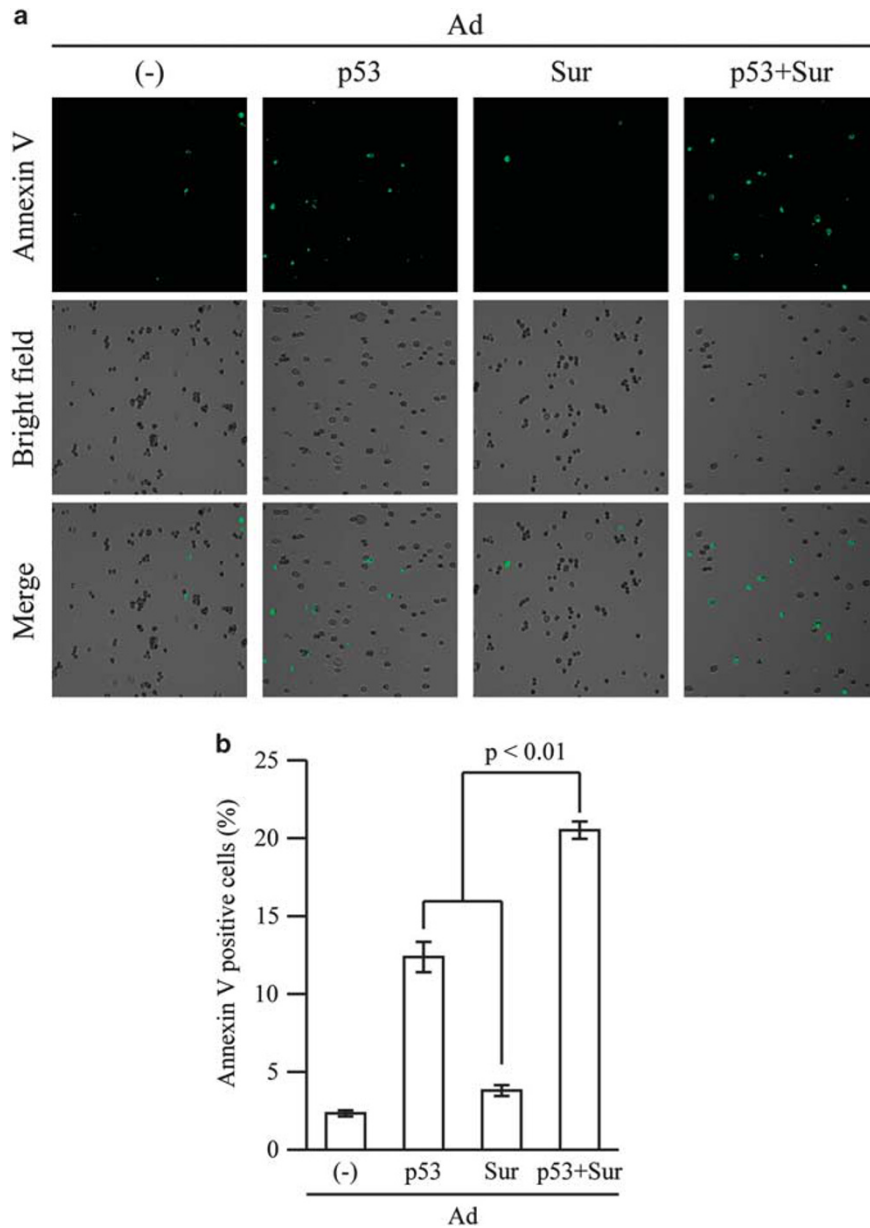


Figure 6. Apoptosis induced by combination of Ad5-p53 and AdF-Sur. **(a)** AsPC-1 cells, which were uninfected or infected with Ad5-p53 (p53) (5000 vp per cell), AdF-Sur (Sur) (5000 vp per cell) or in combination with Ad5-p53 and AdF-Sur (5000 vp per cell for respective Ad), were stained with annexin V. Apoptotic cells were stained green and representative figures are shown. **(b)** Percentage of the annexin V-positive cells. Averages and s.e. bars are shown ($n = 20$). Analysis of variance is used for statistical analysis.

progenies,¹⁴ and increased autophagy as well as apoptosis.^{15,16} These studies did not analyze whether the combinatory effects were additive or synergistic in the cytotoxicity. The experimental systems of the previous studies were not the same as the present study in terms of p53 transduction. Expression of p53 was induced in an initial phase of viral replications in the present study, whereas in the previous studies, expression of p53 was coordinated with the viral replication.

The enhanced cytotoxicity despite decreased viral replications suggested that the synergistic cell death was associated with cross talks between Ad5-p53-mediated and AdF-Sur- or AdF-MK-mediated signaling. Selection of cell death pathways, either apoptosis or autophagy, is also influenced by types of activated signal pathways. We therefore analyzed the pathways that were associated with p53 activation. The present study showed that transduction of p53-null

AsPC-1 cells with Ad5-p53 induced the p53 phosphorylation and the phosphorylation could involve two possible mechanisms. One is increased amounts of p53, which trigger the phosphorylation and the other is Ad-mediated DNA damages, which subsequently activate the p53 phosphorylation. Our previous studies showed that Ad5-LacZ infection in wild-type p53-bearing tumors did not phosphorylate endogenous p53¹⁷ and transfection of plasmid DNA encoding the wild-type p53 gene induced phosphorylation of the exogenous p53.¹⁸ These data indicated that increased amounts of p53 itself activated the phosphorylation.

Transduction of the p53 gene increased p21 expression and induced a cleavage of caspase-3 and PARP molecules, showing that the p53 downstream pathways were intact. Combination of Ad5-p53 and AdF-Sur induced p53 expression levels greater than Ad5-p53 alone at 48 and 72 h after the infection, suggesting that

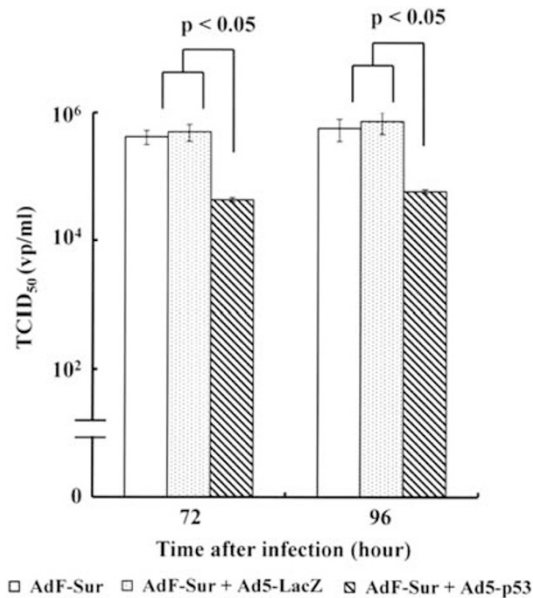


Figure 7. Viral replications influenced by Ad5-p53. AsPC-1 cells were infected with AdF-Sur, AdF-Sur plus Ad5-LacZ or AdF-Sur plus Ad5-p53 (5000 vp per cell for respective Ad) for 72 and 96 h. Cell lysates were assessed for production of AdF-Sur progenies with the TCID₅₀ assay using A549 cells. Averages and s.e. bars are shown ($n=3$). Analysis of variance is used for statistical analysis.

replication of AdF-Sur augmented the p53 expression. AdF-Sur infection upregulated phosphorylation of H2AX and downregulated NBS1 expressions, which implied that viral replication induced DNA damages and suppressed DNA repair systems. These damage responses consequently activate p53 phosphorylation. Furthermore, E1A suppressed expression levels of MDM2, which promoted p53 degradation through the ubiquitination processes and contributed to upregulation of p53 expression.¹⁵ The present study showed that AdF-Sur-mediated E1A and p53-mediated MDM2 levels were downregulated by the combination. The E1A downregulation could be partly due to p53-mediated inhibition of cell cycle progression and cell growth. We thereby presume that induction of E1A suppressed MDM2 and augmented p53 levels, but the upregulated p53 reversely inhibited viral replication and subsequently suppressed E1A expression. Nevertheless, combination of Ad5-p53 and AdF-Sur induced cleavages of caspase-3 and PARP greater than infection by Ad5-p53 or AdF-Sur alone. The enhanced cytotoxicity in the combination could be attributable to increased p53 levels and apoptosis subsequently induced, which was also evidenced by the cell cycle analysis and staining with annexin V. The p21 expression level was rather suppressed by the combinatory infection at 96 and 120 h although p21 was a target of p53. In fact, p21 was rather inhibitory to apoptosis^{19,20} and E1A suppressed p21 expression.¹⁵ The downregulation of p21 in the combination was therefore favorable to apoptotic cell death. These data suggested that E1A expression contributed to cytotoxicity by Ad5-p53 and previous studies in fact showed that transduction of tumors with the *E1A* gene sensitized the tumors to DNA-damaging agents.^{21,22} On the other hand, autophagy was minimally induced by Ad5-p53 and AdF35-Sur, and did not contribute to the combinatory cytotoxicity in the present study. Previous studies, however, demonstrated that p53 positively regulated autophagy and p21 was rather inhibitory to autophagy.^{23,24} The current study indicated that cytotoxicity in the combination was attributable to apoptosis rather than autophagy despite increased p53 and decreased p21, suggesting that balance between apoptotic and autophagic cell death can be independent of p53 levels. E1A has

multiple functions and regulates a number of genes including molecules involved in the p53 pathways.¹⁶ A period of p53 expression during viral proliferation processes can thereby have a certain role in the replication-induced cell death.²⁵ The combinatory cytotoxicity can also be derived from p53-mediated augmentation of replication-induced cell death as expressed p53 may lower a threshold of the viral replications-induced cell death.

The present study showed hyperploidy that was differentially detected between AdF-Sur- and AdF-MK-infected cells. MAD2 expression was not associated with the hyperploidy although a previous study suggested that hyperploidy induced by replication-competent Ad was linked with upregulated MAD2, which has a role in spindle checkpoint activation and induces retarded mitosis.²⁶ The upregulation of phosphorylated H2AX was greater in AdF-MK- than AdF-Sur-infected cells, but downregulation of NBS1 was not different between AdF-MK and AdF-Sur. These data collectively suggest that AdF-Sur-induced hyperploidy is independent of DNA damages and sensing of DNA damages for DNA repair. The discrepant results regarding hyperploidy may be linked with differential expression of Sur and MK in the context of cell cycle progression. The *Sur* gene is expressed only in G2/M phase but the *MK* gene expression does not have such cell cycle specificity. Consequently, the transcriptional regulatory region from the *Sur* gene is active in G2/M phase, but E1, which promotes cell cycle progression into S phase, was regulated by the *Sur* regulatory region in the present Ad construct. The discordant driving activity in cell cycle may be responsible for induction of hyperploidy fraction.

In conclusions, we demonstrated synergistic cytotoxicity of Ad5-p53 and AdF replication in p53-null and p53-mutated pancreatic carcinoma cells and showed that apoptotic cell death was induced by augmented activation of the p53 pathways. The present study suggested a combinatory use of a gene medicine expressing the *p53* or the *E1A* gene and a small molecule inhibiting p53 degradation.²⁷ The strategy can be a possible therapeutic option for pancreatic carcinoma and requires further evaluation in an animal experimental model.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (<http://www.nature.com/cgt>)