Original Article

Recombinant adenovirus-p53 (Gendicine) sensitizes a pancreatic carcinoma cell line to radiation

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Objective: In this study, we examine the effects of recombinant adenovirus-p53 (rAd-p53) on the pancreatic carcinoma cell line SW1990. Specifically, we determine if expression of rAd-p53 sensitizes these cells to radiation.

Methods: Following transfection of SW1990 cells with rAd-p53, we measured expression of P53, P21 and Bax by immunocytochemistry. Both transfected and control cell lines were irradiated with a range of doses, and the survival fractions (SF) were calculated. Dose survival curves were constructed and modeled for comparison.

Results: Transfection of SW1990 cells with rAd-p53 resulted in increased expression of P53, P21 and Bax in a time-dependent manner. At 96 h after transfection, 89.92% of cells expressed P53, 56.8% expressed P21, and 76.50% expressed Bax. The SF following radiation was lower in the rAd-p53 transfected cells compared to the control cells, suggesting that rAd-p53 sensitizes SW1990 cells to radiation (D_0 for the experimental and control groups was 2.199 and 2.462, respectively).

Conclusions: Use of the adenoviral vector is an effective means of transfecting SW1990 cells with wild-type P53, and this sensitizes the cell line to irradiation. This work suggests that combining rAd-p53 with radiation therapy in pancreatic cancer may be therapeutically beneficial.

Keywords: Pancreatic carcinoma; recombinant adenovirus-p53 (rAd-p53); transfection; radiosensitization

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Introduction

In 2012, approximately 44,000 new cases of pancreatic cancer were diagnosed in the United States, making it the tenth most common cancer in both men and women (1). Pancreatic cancer is a lethal disease, with a 5-year relative survival rate for all stages of only 6%. While surgery offers the best chance at prolonged survival, only 10-20% of patients present with resectable disease at the time of diagnosis (2). Even still, locoregional failure remains common in resected cases, and radiation therapy has been used in the neoadjuvant and adjuvant settings to improve local control (3). While the recently published Eastern Cooperative Oncology Group trial showed a survival benefit with the addition of radiation therapy to gemcitabine for locally advanced pancreatic cancer (4), local control at conventional doses remains poor (5). Gemcitabine and fluoropyrimidine-based radiosensitizers are currently in clinical use, but both are associated with moderate to severe toxicities in most patients (4,6). This highlights the urgent need for novel radiosensitizers with less toxicity.

P53, a key mediator of DNA damage and apoptotic responses, is mutated in approximately 60% of pancreatic

Table 1 The design of transfection

Group	4 (96 h)		3 (72 h)		2 (48 h)		1 (24 h)	
	A	B	A	B	A	B	A	B
N	1×10^6	1×10^6	1.5×10^6	1.5×10^6	3×10^6	3×10^6	4×10^6	4×10^6
rAd-p53	1×10^8	/	1.5×10^8	/	3×10^8	/	4×10^8	/

N, cell number; rAd-p53, vector particles.

cancers. Mohiuddin *et al*. (7) have shown that pancreatic cancer cells with wild-type P53 are significantly more radiosensitive than P53 mutant cells. Another study showed that adenovirus-mediated transfer of wild-type P16 and P53 to pancreatic cancer cells results in multiple effects, including G1 cell cycle arrest and increased apoptosis (8). Recombinant adenovirus-p53 (rAd-p53) (Gendicine; China Shenzhen SiBiono GeneTech Co., Ltd., Shenzhen, China) is a newly developed medicine of gene therapy that relies on the function of wild-type P53; it was recently licenced for clinical use in China for head and neck malignancies. A randomized trial in nasopharyngeal carcinoma has demonstrated improved locoregional control with weekly intratumoral injections of rAd-p53 combined with radiation therapy compared to radiation therapy alone (9). In pancreatic cancer, a pilot study has also demonstrated improved disease control when rAd-p53 is added to chemoradiation therapy (10); however, the mechanism of this combined action is unclear. In the present study, we tested the hypothesis that transfection of pancreatic carcinoma cell line SW1990 (harboring mutant P53) with rAd-p53 can alter expression of target genes, including *Bax* and *p21*, and increase sensitivity to radiation. This would provide a scientific foundation for further clinical trials of rAd-p53 as a novel radiosensitizer of pancreatic cancer.

Materials and methods

rAd-p53 and cell culture

rAd-p53 is a recombinant replication-incompetent human serotype 5 adenovirus, in which the E1 region is replaced by a human wild-type *p53* expression cassette. rAd-p53 was stored at –20 ℃ in a concentration of 1×10^{12} virus particles/mL. The human pancreatic carcinoma cell line SW1990 (mutant *p53*) was obtained from the Chinese Academy Of Medical Sciences & Peking Union Medical College (CAMS & PUMC, Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen Corporation, Australia), 50 unit/mL penicillin, and 50 μg/mL streptomycin (Invitrogen Corporation, USA) at 37 ℃ in an atmosphere of 5% CO_2 (300/3,000 Incubator, Revco Scientific, USA).

Immunocytochemistry (ICC)

Glass slides were sterilized by dipping them in 90% ethanol and carefully drying them over a flame for a few seconds. Each slide was placed in a sterile 100-mm diameter tissue culture dish, and a specific number of cells were seeded (*Table 1*). Dishes were divided into an experimental group (group A) and a control group (group B). Cells in group A were infected with rAd-p53 solution at a viral multiplicity of infection (MOI) of 100 (1:100 MOI) for various lengths of time (*Table 1*). Cells were fixed by incubating them in 4% (V/V) paraformaldehyde in PBS for 20 min at room temperature. Immunocytochemical staining for P53, P21 and Bax was performed using anti-P53 (DO-1; 1/100 dilution; Santa Cruz Biotechnology, USA), anti-P21 (F-5; 1/100 dilution; Santa Cruz Biotechnology, USA), and anti-Bax (B-9; 1/100 dilution; Santa Cruz Biotechnology, USA) antibodies, respectively. ICC-positive cells were stained brown, and ICC-negative cells were stained blue. Slides were visualized using an inverted microscope (Olympus CKX31, Japan). The experiment was repeated three times to minimize random error.

Cell irradiation

A set number of logarithmic phase cells were grown in 25 cm^2 culture flasks, as shown in *Table 2*. Cells in group A were infected with rAd-p53 solution at a viral MOI of 100. Group B served as the control group. Cultures were terminated by Giemsa staining at different times. The experiment was repeated three times.

Cells were irradiated with 6 MV X-ray (Varian 600CD linear accelerator, Varian Medical Systems, Inc. USA) at room temperature at a central dose rate of 300 MU/min. The output factor was 1.041, with a 20 cm × 20 cm field.

Table 2 Radiation design for the cell survival curve

Group	1 (0 Gy)		2 (2 Gy)		3 (4 Gy)		4 (6 Gy)		5 (8 Gy)	
	A	B	A	B	A	B	A	B	A	B
N	50	50	200	200	500	500	1×10^3	1×10^3	1×10^4	1×10^4
rAd -p53	5×10^3	/	2×10^4	/	5×10^4	/	1×10^5	/	1×10^6	/

N, cell number; rAd-p53, vector particles.

Table 3 Pearson chi-square test of ICC results between experimental and control groups

Group	1 (4×10^6 cells)			2 (3×10^6 cells)			3 (1.5×10^6 cells)			4 (1×10^6 cells)		
	Positive rate (%)	χ^2	P	Positive rate (%)	χ^2	P	Positive rate (%)	χ^2	P	Positive rate (%)	χ^2	P
P53												
A	28.50	13.36	0.000	30.48	17.47	0.000	51.28	69.84	0.000	89.92	555.04	0.000
B	18.66			19.08			25.28			15.34		
P21												
A	38.04	135.58	0.000	39.16	93.80	0.000	40.20	113.75	0.000	56.80	187.78	0.000
B	7.16			12.40			10.72			15.12		
Bax												
A	54.10	77.88	0.000	64.10	196.76	0.000	70.30	64.02	0.000	76.50	232.82	0.000
B	26.71			20.20			45.25			28.30		

Five clinically relevant doses were chosen: 0 Gy (group 1), 2 Gy (group 2), 4 Gy (group 3), 6 Gy (group 4), and 8 Gy (group 5) (*Table 2*). Cells were cultured in the CO_2 incubator for two weeks and then colony-forming units (≥50 cells) were calculated.

Curve fitting models

Plating efficacy (PE) was defined as colonies observed/number of cells plated. Survival fraction (SF) was the ratio of PE for the irradiation group compared to PE for the control group. Curve fitting analyses were made according to the single-hit multitarget (SHMT) [$SF=1-(1-e^{-D/D_0})^N$, $D_q=D_0\times\ln(N)$], the modified single-hit multitarget (mSHMT) {$S=e^{-D/D_1}[1-(1-e^{-D/D_2})]^N$}, and the linear-quadratic (L-Q) ($S=e^{-\alpha D-\beta D^2}$) models, which are the most commonly used models in radiation oncology. The mean values were recorded as data.

Statistical analysis

Statistical comparisons were performed using the Pearson chi-square test. A two-tailed $P<0.05$ was considered statistically significant. SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for the calculation of SF prediction, and Origin 8.6 (OriginLab, USA) was used for cell survival curve fitting.

Results

Here, we investigated whether transfection of the pancreatic carcinoma cell line SW1990 with rAd-p53 sensitizes the cells to radiation. Results from ICC staining (*Table 3*) showed that expressions of P53, P21 and Bax were all significantly increased ($P<0.05$) after transfection with rAd-p53. Additionally, this increased expression was dependent on time, with the group at 96 h achieving the highest level of expression (*Figure 1*). Thus, we were able to successfully transfect SW1990 cells with rAd-p53, and this subsequently influenced expression of downstream targets. Average numbers of counted colonies and plating efficiencies are shown in *Table 4*.

We calculated SF of SW1990 cells in both group A and group B at different radiation doses (0, 2, 4, 6 and 8 Gy), and cell survival curves based on the three different models (SHMT, mSHMT, L-Q) were generated (*Table 5*).

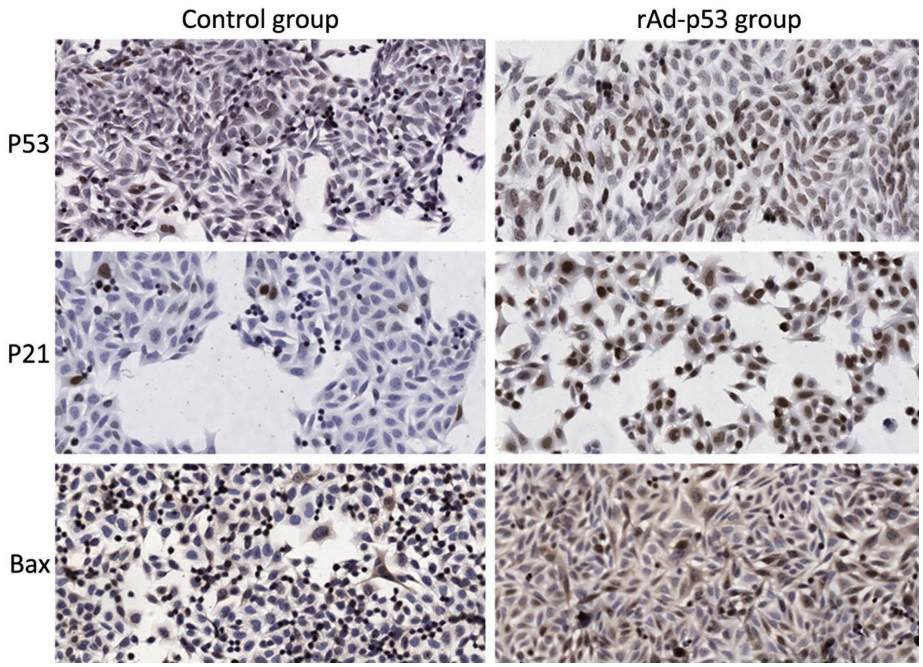

Figure 1 ICC staining (×200) for P53, P21 and Bax for SW1990 after 96 h of transfection. ICC-positive cells are stained brown.

Table 4 Cell irradiation experiment

Group	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
Colonies	39	41	83	92	90	160	61	81	77	101
PE	0.780	0.820	0.415	0.460	0.180	0.320	0.061	0.081	0.008	0.010
SF ($\bar{x}\pm s$)	1.000±0.000	1.000±0.000	0.534±0.094	0.572±0.084	0.231±0.117	0.393±0.055	0.078±0.031	0.100±0.011	0.010±0.001	0.012±0.001

Colonies, the average number of counted colonies; PE, the average plating efficiency. The experiment was reapeated three times.

Table 5 The fitting parameters of three different models

Model	SER	Group	Parameter 1	Parameter 2	Parameter 3	GOF
SHMT			D_0	D_q	N	R^2
	1.215	A	2.199	0.754	1.409	0.998
		B	2.462	1.239	1.654	0.981
mSHMT			D_1	D_2	N	R^2
	1.219	A	3.423	8.269	0.578	0.991
		B	3.734	9.202	0.267	0.969
L-Q			α	β		R^2
	1.228	A	0.264	0.027	–	1.000
		B	0.170	0.028	–	0.985

SER, sensitizing enhancement ratio; GOF, goodness of fit.

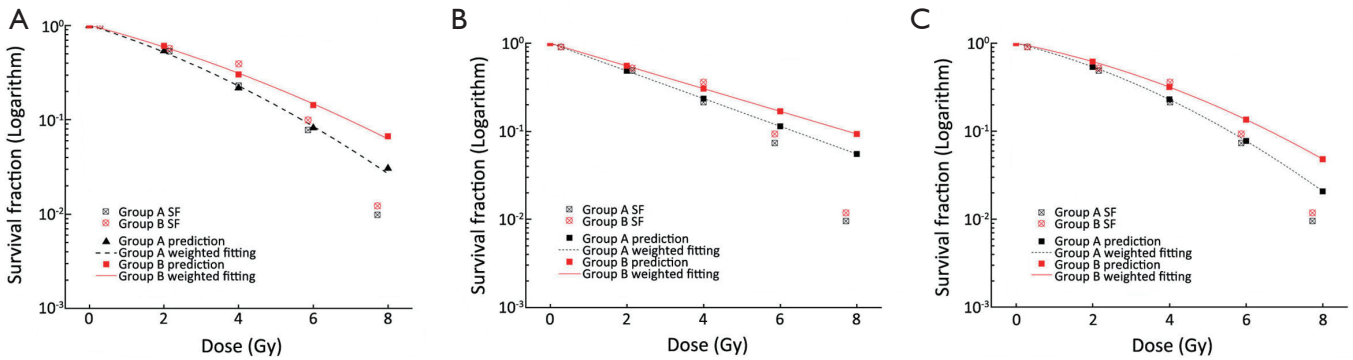

Figure 2 The cell survival curves fitting with three models separately. (A) The SHMT model; (B) The mSHMT model; (C) The L-Q model. Group A, cells are infected with rAd-p53 solution at 1:100 MOI; group B, control group; SF, survival fraction.

Compared to the SHMT and mSHMT models, the L-Q model appeared to give a better fit. *Figure 2* demonstrates the prediction of SF with the three different models. Cell survival curves were plotted, and the curve for group A (rAd-p53 infected) was consistently shifted to the left compared to the curve for group B, which suggested that transfection of rAd-p53 enhances the radiosensitivity of SW1990 cells.

Discussion

Pancreatic cancer is associated with a relatively poor prognosis, prompting research and clinical trials for new and improved therapies (11-14). Gene therapy has emerged as a promising treatment modality in oncology, with more than one thousand clinical trials being conducted worldwide (15). Previous researches on rAd-p53 had been published for esophageal squamous cell carcinoma (16), non-small cell lung cancer (17), ovarian cancer (18), hepatocellular cancer (19), and nasopharygeal cancer (9). However, the applicability and mechanism of action of gene therapy in pancreatic cancer has not been fully elucidated. In this study, we sought to evaluate the effect of rAd-p53 in a pancreatic cancer cell line with mutant *p53*. We succesfully achieved adenovirus-mediated expression of *p53* in SW1990 cells and showed that this expression of wild-type P53 sensitizes the cells to clinically relevant doses of radiation.

We found that rAd-p53 was capable of infecting SW1990 cells, which was similar to previous reports for lung adenocarcinoma cells (20). The infected group showed increased expression of both P21 and Bax compared to the control group. P21 is tightly controlled by P53 and plays a crucial role in mediating growth arrest when cells are exposed to DNA damaging agents, such as radiation. The expression of Bax is upregulated by P53, and Bax has been shown to be involved in P53-mediated apoptosis. The increase in P53 protein expression was time-dependent, with the group at 96 h achieving the highest level of expression. This is in contrast to an earlier study using A549 lung adenocarcinoma cells, which showed no significant difference in P53 expression when comparing the 6 h group to the 24 h group (20). Such difference may be due to the different cancer cell lines used. Further studies need to be performed to identify the peak of P53 expression following infection of this pancreatic cancer cell line.

We found that transfection of SW1990 cells with wild-type *p53* increases the radiosensitivity of this cell line, and the L-Q model (sensitizing enhancement ratio, 1.228) gives the best fit for the SF data (*Table 5*). Our results of successful transfection are similar to the results published by Merlin *et al*. (21), who used a non-viral technique, and Mercadé *et al*. (22), who used a viral technique with pro-drug activating enzymes.

This work adds to the body of literature exploring gene therapy in pancreatic cancer. There are still several important questions that must be answered before gene therapy can become a standard treatment option. Adenoviral-mediated expression of P53 (rAd-p53) is currently licensed for clinical use in China. To date, it is unclear whether P53 is an optimal target in pancreatic cancer, and whether there are pre-treatment predictive markers for response. The use of an adenovirus as a delivery system may also pose certain problems. Patients may require multiple intratumoral injections of the virus, as has been previously shown for oral cancer (23). This is primarily

die to the fact that the efficacy of expression is quite low when administered intravenously. Additionally, there are safety concerns related to the host immune response (24). However, to date, rAd-p53 has been shown to be associated with only a relatively low toxicity profile (fever) in an earlier trial (10). The experiment should also be performed on other pancreatic cancer cell lines.

In conclusion, the results support the development of gene replacement therapy for pancreatic cancer with rAd-p53 and provide an important preclinical foundation for further work.

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