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• BASIC RESEARCH •

The effect of adenovirus expressing wild-type p53 on 5-fluorouracil chemosensitivity is related to p53 status in pancreatic cancer cell lines

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Abstract

AIM: There are conflicting data about p53 function on cellular sensitivity to the cytotoxic action of 5-fluorouracil (5-FU). Therefore the objective of this study was to determine the combined effects of adenovirus-mediated wild-type (wt) p53 gene transfer and 5-FU chemotherapy on pancreatic cancer cells with different p53 gene status.

METHODS: Human pancreatic cancer cell lines Capan-1^{p53mut}, Capan-2^{p53wt}, FAMPAC^{p53mut}, PANC1^{p53mut}, and rat pancreatic cancer cell lines AS^{p53wt} and DSL6A^{p53null} were used for *in vitro* studies. Following infection with different ratios of Ad-p53-particles (MOI) in combination with 5-FU, proliferation of tumor cells and apoptosis were quantified by cell proliferation assay (WST-1) and FACS (PI-staining). In addition, DSL6A syngeneic pancreatic tumor cells were inoculated subcutaneously in to Lewis rats for *in vivo* studies. Tumor size, apoptosis (TUNEL) and survival were determined.

RESULTS: Ad-p53 gene transfer combined with 5-FU significantly inhibited tumor cell proliferation and substantially enhanced apoptosis in all four cell lines with an alteration in the p53 gene compared to those two cell lines containing wt-p53. *In vivo* experiments showed the most effective tumor regression in animals treated with Ad-p53 plus 5-FU. Both *in vitro* and *in vivo* analyses revealed that a sublethal dose of Ad-p53 augmented the apoptotic response induced by 5-FU.

CONCLUSION: Our results suggest that Ad-p53 may synergistically enhance 5-FU-chemosensitivity most strikingly in pancreatic cancer cells lacking p53 function. These findings illustrate that the anticancer efficacy of this combination treatment is dependent on the p53 gene status of the target tumor cells.

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INTRODUCTION

Pancreatic cancer is a very aggressive tumor with a poor prognosis. Unfortunately, the majority of the patients are diagnosed at an advanced disease status and are not suitable for potentially curative resection. Intrinsic limitations of chemotherapy are the endogenous or acquired resistance of tumor cells to anti-cancer drugs and their toxicity to normal tissues, the latter being responsible for the occurrence of severe side effects. For these reasons, new approaches for the treatment of pancreatic cancer patients have to be developed.

The p53 tumor suppressor gene is functionally inactivated in about 50% of all human malignancies including up to 60% of pancreatic cancers[1]. The tumor suppressor activity of p53 is mainly mediated through its ability to induce cell growth arrest or apoptosis in response to a variety of stress signals, whereas a lack of functional p53 usually leads to increased genomic instability, deregulated cell proliferation, accelerated tumor progression, and elevated cellular resistance to anticancer therapy[2]. Expression of wt- p53 has been shown to be required for 5-FU- induced apoptosis and to greatly potentiate 5-FU-cytotoxicity[3,4]. Several reports have suggested that the p53 status of the tumor cells may be an important response determinant to 5-FU- based chemotherapy[5,6]. In contrast, other studies have failed to demonstrate any relationship between p53 expression and 5-FU chemotherapy[7,8]. Gene therapy by adenovirus mediated introduction of the wild-type human p53 gene into tumors that are deficient in functional p53 has shown a significant tumor suppressing effect in preclinical studies and in clinical trials[9-13]. Furthermore, these tumor suppressing activities of p53 reintroduction have been reported to be enhanced by combination with many chemotherapeutic drugs or ionizing radiation in various human tumors[14,15]. However, the influence of the *de novo* expressed exogenous p53 on cellular sensitivity toward DNA-damaging agents is still not clear[16-19].

The goal of the present study was to determine *in vitro* and *in vivo*, whether reintroduction of wt-p53 protein into pancreatic cancer cells could increase the sensitivity to 5-FU chemotherapy. Therefore, we tested pancreatic cancer cells with different p53 status. Our results demonstrate that wt-p53 gene transfer in pancreatic cancer cells with loss of p53 function has a significant impact on 5-FU-chemosensitivity leading to potential tumor regression both *in vitro* and in a pancreatic cancer model of immunocompetent rats.

MATERIALS AND METHODS

Cell lines and culture conditions

The human pancreatic cancer cell lines, Capan-1, Capan-2, PANC1 were provided by the Tumorbank, DKFZ, Heidelberg, Germany. FAMPAC was established in our laboratory from a 43 year old female with familial pancreatic cancer[20]. The rodent pancreatic cancer cell lines, AS (BSp73AS) and DSL6A, were originally derived from a spontaneous pancreatic adenocarcinoma of a BDX rat[21] and from a primary pancreatic carcinoma of an azaserine-treated Lewis rat[22] respectively. All cells were grown

in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 100 mL/L heat inactivated fetal calf serum (FCS, Gibco BRL), 100 units/mL penicillin, 100 μg/mL streptomycin (Gibco BRL) and incubated at 37 ℃ in a humidified atmosphere with 50 mL/L CO_2.

Adenoviral vectors

The AdEasy system[23] was used for construction and propagation of the human wt- p53 (Ad-p53) adenoviruses. The shuttle vector pAdTrack-CMV and the adenoviral backbone plasmid pAdEasy-1 were kindly provided by Bert Vogelstein (*Howard Hughes Medical Institute and Kimmel Cancer Center at Johns Hopkins University, Baltimore, MD 21 231*). The cDNA coding for human p53 was a gift from Martin Scheffner (*ATV, DKFZ, 69120 Heidelberg, Germany*). The control adenoviral vector expressing cytosine deaminase (Ad-CD) was kindly provided by Zhuangwu Li (*NIH/NCI Bldg.*10/Rm. *12N226, 9000 Rockville Pike, Bethesda, MD 20 892*) and the AdlacZ vector was a gift from Petra Klein-Bauerschmitt (*ATV, DKFZ, 69120 Heidelberg, Germany*). Recombinant adenoviruses were propagated in 293 cells, harvested, aliquoted and stored as described[24]. Adenovirus titer in plaque-forming units (p.f.u.) was determined by plaque formation assays on 293 cells. The multiplicity of infection (MOI) was defined as the ratio of p.f.u. per total number of cancer cells to be infected. Adenoviruses were administered in phosphate buffer (20 mmol/L NaH_2PO_4, pH 8.0, 130 mmol/L NaCl, 2 mmol/L $MgCl_2$, 20 g/L sucrose).

β-galactosidase expression

Pancreatic cancer cells were infected with AdlacZ in a variety of MOI ranging from 1 to 100, in 1 mL of medium in 25-cm^3 tissue culture flasks under routine conditions. After 24 h infected cells were fixed in 960 mL/L ethanol and stained with X-gal solution. The percentage of positively blue stained cells was determined by scoring five random high power fields per 25-cm^3 flask.

Western blotting

The expression of p53 protein was assayed in all six pancreatic cancer cell lines before and after Ad-p53- infection. Cells were transduced with 10^2 MOI Ad-p53 and harvested at 24h. The protein lysates were separated by electrophoresis on a 100 g/L SDS/acrylamide gel and transferred to a PVDF membrane. The membrane was blocked in 200 mL TTBS, 50 g/L skimmed milk, 1 g/L Tween-20 for 1 h, incubated with primary p53 DO7 antibody (DAKO, Cambridgeshire, UK) for 1 h. After washing in TTBS the membrane was incubated with horseradish peroxidase (HRP) conjugated anti-mouse IgG for 45 min. Signals were detected using the enhanced chemiluminescence system (ECL, Amersham Life Science Ltd, Bucks, UK).

WST-1 cell viability assay

Cells were seeded at a concentration of 5-10×10^3 cells/well in 100 μL culture medium into 96-well microtiter plates. After a 12 h incubation period, cells were infected either with Ad-p53 in a variety of MOIs (10^{-2}-10^2) or treated as controls with Ad-CD. After 24 h cells were washed with PBS and treatment with 5-fluorouracil (5-FU, Medac, Hamburg, Germany) at the concentration of 5μL/mL was started for a period of 48 h. All dilutions were performed in culture medium. Cells were washed with PBS and 10 μL/well of the cell proliferation reagent WST-1 (Boehringer Mannheim, Germany) was added in 100 μL cell culture medium for 4 h. Absorbance of the samples was analysed using a bichromatic ELISA reader at 450 nm and 690 nm. Relative proliferation (A/Ao) was defined as the ratio of absorption measured in Ad-p53- infected and/or 5-FU treated cells or Ad-CD- infected cells (A) compared with the absorption measured in untreated control cells (Ao). All experiments were performed in triplicate.

FACS analysis

Cells were plated at a density of 10^5 per 25-cm^3 flask and infected with viral vectors at an MOI of 1 for 24 h. After 24 h cells were washed with PBS and treatment with 5-fluorouracil (5-FU, Medac, Hamburg, Germany) at the concentration of 5 μL/mL was started for a period of 48 h. The cells were harvested and washed with PBS. The apoptotic fraction of treated or untreated cells was determined by FACS analysis (FACSCalibur, Becton Dickinson) following permeabilisation and propidium iodide (PI) staining of the cells. Briefly, 250 μL of PI staining solution (1 g/L TritonX-100, 50 μg/mL PI in PBS, pH 7.4) was added to 5×10^5 cells and analysed within 1 h using the CellQuest software. The proportion of apoptotic cells was calculated for each group: mock infected, Ad-CD, 5-FU, Ad-p53 and Ad-p53+ 5FU. Cell debris and fixation artefacts were excluded by appropriate gating. All experiments were performed in triplicate.

Animal experiments

Male Lewis rats, purchased from Charles River WIGA Laboratories (Sulzfeld, Germany), were kept in isolators and received food and water ad libitum. Animal experiments were performed under NIH and institutional guidelines established for the Animal Care Facility at the University of Heidelberg. A total of 10^6 DSL6A tumor cells were injected in 100 μL of PBS/animal subcutaneously (sc.) into the abdominal wall of 4 wk-old rats. Eight weeks after tumor cell inoculation, when the tumor nodules had an average size of 8 mm×8 mm, the treatment as indicated was started and repeated twice a week for 1 mo. 5-FU (5 mg/kg bm) was given intraperitoneally (ip.) and Ad-p53 or Ad-CD infections (10^8 infective units) were injected intratumorly (it.). The animals were separated by randomisation into 5 groups of 8 rats each namely mock infected, Ad-CD (1×10^8 infectious particles it.), 5-fluorouracil (5 mg/kg bm ip.), Ad-p53 (1×10^8 infectious particles it.) and Ad-p53+5-FU. Tumor size (mm^2) was determined by measuring the largest and smallest diameter as tumor length (mm)×tumor width (mm). When the tumor size reached >600 mm^2, animals were sacrificed. Tumor size and survival rate were documented weekly.

TUNEL assay

Tumor tissues were fixed in 40 g/L neutral formaldehyde or snap frozen in liquid nitrogen and stored at -80 ℃. Sections (5μm) were stained with hematoxylin and eosin using standard procedures. At least 6 representative tumors of each group of treated or untreated animals were analysed for apoptosis. Immunohistochemical detection and quantification of apoptosis in formalin fixed tumor tissue sections were performed using the '*In situ* cell death detection kit, AP' (Roche Diagnostics, Mannheim, Germany). The sections were stained and analysed microscopically according to the manufacturer's instructions.

Reverse transcriptase- PCR analysis

From snap frozen tumor tissue total RNA was extracted using RNAzol (Cinna/Biotech, Friendswood, TX). cDNA was then generated using the cDNA cycle kit for RT-PCR (Invitrogen, San Diego, CA, USA). To assay expression of the p53 transgene from Ad-p53 in infected tumors, the following primers were generated to amplify a 294 bp product which is specific for Ad-p53: forward primer: 5' GGTGCATTGGAACGCGGA TT 3', reverse primer: 5' GGGGACAGAACGTTGTTTTC3'. PCR was performed by an initial heating step at 95 ℃ for 5 min, followed by 35 cycles at 95 ℃ for 1 min, at 58 ℃ for 1 min and at 72 ℃ for 1 min and a final extention step at 72 ℃ for 5 min. The PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining and UV light.

Statistical analysis

Statistical analysis was performed using SPSS software (Release 11.0.1, SPSS Inc.). The rate of cell viability and the rate of

apoptotic cells were expressed as mean±SE and 95% confidence interval. The 95% confidence intervals of the means were used to analyse the rate of cell viability and the rate of apoptotic cells between two groups of each cell line. Differences between means without overlap of the confidence intervals were described as statistically significant. Tumor size in rats was given as mean±SD. Normal distribution of tumor size in different groups was judged by the Shapiro-Wilk test. ANOVA was performed to analyse the therapeutic effects after 4 cycles and at study end between the groups with respect to tumor size. Two-sided *P*-values were reported and an effect was considered statistically significant at $P<0.05$.

RESULTS

Adenovirus mediated gene transfer efficacy in pancreatic cancer cells

The efficacy of Ad-p53 gene transfer into pancreatic cancer cell lines was assessed as the percentage of X-gal stained positive cells 24 h after infection with AdlacZ. There was a variation in transduction efficacy as summarised in Table 1. An MOI of 50 resulted in 73-95% of cells stained blue in the tested cell lines.

Table 1 p53 status, transduction efficacy and IC_{50} value of 5-FU in pancreatic cancer cell lines; mean±SD

	Cell line	p53 status	β-gal[1]%	5-FU-IC_{50} value[1] μg / mL
Human				
	Capan-1	159 GCC→GTC	92 (4.5)	8.25 (0.45)
	Capan-2	wild type	95 (7.2)	5.75 (0.55)
	FAMPAC	175 CGC→CAT	82 (7.8)	8.00 (0.45)
	PANCL	273 CGT→CAT	82 (7.8)	9.50 (0.75)
Murine rat				
	AS	wild type	73 (5.3)	6.55 (0.45)
	DSL6A	Null	83 (6.2)	7.75 (0.55)

[1]β-gal was the percentage of X-gal positive cells 24 h after AdlacZ infection at a multiplicity of infection of 50. [2]IC_{50} values were defined for each cell line as the 5-FU concentration leading to 50% growth inhibtion. Results are expressed as mean±SD from three independent experiments.

p53 status and p53 protein expression after Ad-p53 infection in vitro

To determine the p53 status of the tested cell lines, we reviewed the literature (Capan-1[25], Capan-2[25], PANC1[26]) or analysed cell samples (FAMPAC[27]) for mutations in exons 5-8 of p53. For the rat pancreatic cancer cell lines, DSL6A and AS, the p53 status was determined by PCR-SSCP analysis followed by direct sequencing[28]. Despite repeated attempts, the DSL6A cell line failed to give rise to amplified products for tested exons 5-8 of p53, suggesting a homozygous deletion of the loci. All exons could be amplified from normal control DNA of AS cells. The mutation status of the pancreatic cancer cell lines is outlined in Table 1.

The expression of p53 protein was confirmed by Western blot analysis. The antibody used reacted with both wild type and mutant p53. This was done to confirm the expression after Ad-p53 infection and to establish the baseline protein expression patterns. These *in vitro* experiments demonstrated for all cell lines detectable amounts of p53 protein 24 h after infection with Ad-p53 indicating an efficient translation. As indicated in Figure 1 the quantity of p53 was increased following infection with Adp53, with the exception of Capan-1 and FAMPAC cell lines showing a strong baseline overexpression of mutated p53.

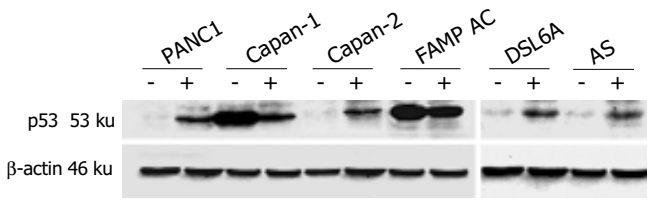

Figure 1 Expression of p53 protein before (-) and after infection with Ad-p53 (+) in pancreatic cancer cell lines.

Effect of p53 gene transduction on in vitro pancreatic tumor cell proliferation

To establish defined conditions for the *in vitro* experiments, all six pancreatic cancer cell lines were transduced with different multiplicity of infection (MOI = p.f.u./cell) of the Ad-p53 vector in the range from 10^{-2} to 10^{2}. To test the effect of the p53 recombinant adenoviruses on tumor cell growth, we assayed the cell viability using a colorimetric cell proliferation test. Infection with the control vector Ad-CD had no significant effect on cell growth (data not shown). In contrast, we observed that Ad-p53 infection dose dependently resulted in significantly decreased cell viability. A significant inhibitory effect on tumor cell growth was achieved at MOI ³ 1 in all tested cell lines with an alteration of p53, whereas lower levels of Ad-p53 virus particles had no effect. Capan-2 cells containing wt-p53, were less sensitive and a similar inhibitory effect was only achieved with a higher MOI of 10. The rat pancreatic cancer cell line, AS containing wt-p53, revealed only a small effect on tumor growth (Figure 2).

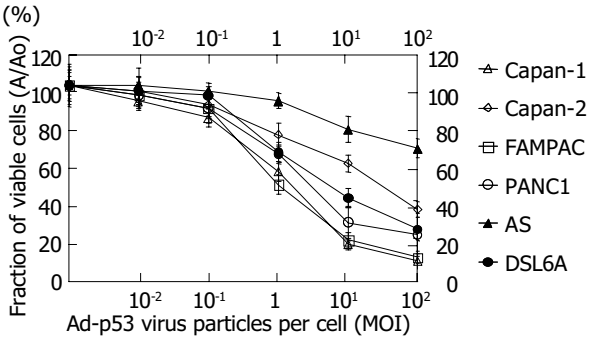

Figure 2 Decreased *in vitro* growth rate in pancreatic cancer cell lines 24 h after infection with Ad-p53. Data points represent means from 9 samples of viable cells in three independent experiments; bars, SD.

Efficacy of combined Ad-p53 and 5-FU therapy on tumor cell growth in vitro

To investigate whether the reintroduction of wt-p53 protein could enhance the tumor inhibitory effect of 5-FU treatment, all six pancreatic cancer cell lines were infected with Ad-p53 at MOI 1, which revealed an intrinsic antiproliferative activity by itself. 5-FU at the concentration of 5 μg/mL was chosen for the combination experiments and was given 24 h after Ad-p53 infection. In preliminary dose escalation studies, the IC_{50} values of each cell line were determined and listed in Table 1. Low dose FU (5 μg/mL) given alone reduced cell viability most significantly to wt-p53 containing Capan-2 cells and AS cells compared to the other tested cell lines. The antiproliferative effects of the combination of Ad-p53 and 5-FU were next evaluated and non-functional p53 pancreatic cancer cell lines were most sensitive to the cytotoxic action of the combined treatment. This growth inhibitory effect was highly significant compared to either administration of p53 gene transduction or 5-FU treatment alone. For Capan-2 cells containing wt-p53, the combined antiproliferative effect of the two drugs was only 9%

Table 2 *In vitro* growth of different pancreatic cancer cell lines after indicated treatment [1]measured by WST-1 cell viability assay[2]; % mean (95% CI)

	Cell line	Control	Ad-CD-control	Ad-p53	5-FU	Ad-p53+5-FU
Human						
	Capan-1	97.8 (96.0-99.5)	91.9 (86.6-97.6)	55.0 (51.3-58.6)	72.9 (70.4-74.4)	30.7 (28.9-32.4)
	Capan-2	97.9 (95.7-100.0)	91.3 (88.2-94.5)	75.0 (71.3-78.6)	55.6 (53.1-58.0)	51.2 (48.0-54.4)
	FAMPAC	98.0 (95.9-100.1)	93.3 (90.5-96.2)	48.6 (44.7-52.4)	70.6 (68.0-73.1)	31.7 (29.6-33.8)
	PANCL	98.4 (97.1-99.8)	95.0 (92.2-97.8)	64.7 (59.8-69.5)	73.9 (71.6-76.2)	33.8 (31.5-36.0)
Murine rat						
	AS	99.0 (97.6-100.4)	97.9 (95.7-100.0)	92.3 (88.9-95.8)	56.8 (52.2-61.3)	53.1 (48.2-58.0)
	DSL6A	98.8 (97.5-100.0)	94.0 (90.8-97.2)	64.2 (60.5-67.9)	69.4 (66.0-72.9)	32.8 (29.3-36.2)

[1]Cells were either infected with Ad-CD control or Ad-p53 (MOI 1) 24 h prior±5FU chemotherapy (5 μg / mL) given for 48 h. [2]The data represents the ratio of the colorimetric values of viable cells treated as indicated (A) compared with the untreated control cells (Ao). Results are expressed as mean with 95% confidence interval (CI) from more than 9 samples in three independent experiments.

more than with 5-FU chemotherapy alone. In wt-p53 containing AS cells, Ad-p53 infection could not enhance the 5-FU antiproliferative action to a significant level (Table 2).

Effect of Ad-p53 gene transduction and 5-FU treatment on apoptotic cell death in vitro

To determine whether Ad-p53 mediated increase of 5-FU cytotoxic action was due to enhanced apoptosis, we analyzed DNA-fragmentation by fluorometric analysis of propidium iodide stained pancreatic cells. Cells treated with 5-FU (5 μg/mL) showed a percentage of dead cells between 8% and 16%, which was slightly above that of native cells or cells infected with the Ad-CD control vector. In cells transduced with the p53 gene (MOI 1), the percentage increased up to 25%. p53 gene transduction followed by 5-FU chemotherapy, caused a marked increase of apoptotic cell death. These findings were consistent showing the highest apoptotic rate for the pancreatic cancer cell lines with an altered p53 (47-58%), and the lowest apoptotic rate in wt-p53 containing cell lines (18-20%) as indicated in Figure 3.

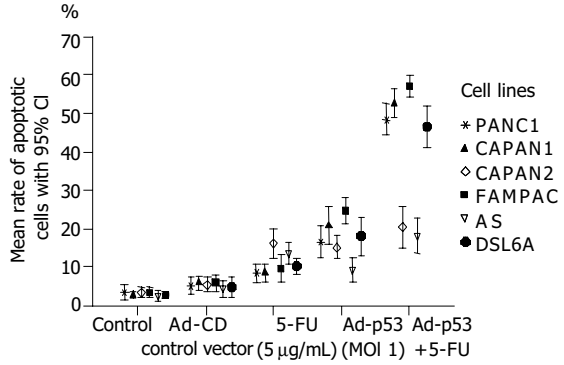

Figure 3 Percentage of apoptotic cells of human and rat pancreatic carcinoma cell lines either infected with Ad-CD control vector or Ad-p53 (MOI 1) 24 h prior±5-FU chemotherapy (5 μg / mL) given for 48 h. Points represent means from 9 samples in three independent experiments; bars, 95% CI.

In vivo effects of combined treatment on tumor growth in rat pancreatic cancer

Subcutaneous DSL6A tumors generated in 4-wk-old male Lewis rats were treated by intratumoral injection with adenoviral vectors and/or 5-FU chemotherapy given intraperitoneally twice a week for 1 mo. Details of the onset and cessation of treatment were indicated in the methods section. To determine if p53 recombinant adenoviruses could induce p53 mRNA expression, RT-PCR analysis was performed in the generated DSL6A tumors. Non- infected and Ad-p53 infected tumors were removed and p53 transgene expression was confirmed 24 h after the last injection. As shown in Figure 4, a 294 bp band was detected only in Ad-p53 infected tumors.

Tumor growth was measured regularly during the 4 wk following treatment. Animals in both control groups showed an extensive tumor proliferation, and after 6 cycles of therapy tumor size was above 600 mm^2, so that treatment was stopped and animals were killed according to the protocol. During the first four cycles of treatment, in animals with 5-FU monotherapy tumor growth was significantly reduced compared to the controls (157±54 mm^2 *vs* 410±65 mm^2, $P<0.0001$). Ad-p53 treatment alone (72±19 mm^2, $P<0.0001$) or the combination Ad-p53 plus 5-FU (61±8 mm^2, $P<0.0001$) demonstrated local tumor growth control. However, within the following four cycles of therapy we observed an increasing tumor size in animals treated only with 5-FU (580±58 mm^2) or Ad-p53 (347±52 mm^2) alone compared to the combination of Ad-p53 plus 5-FU (67±14 mm^2, $P<0.0001$). As shown in Figure 5, the combination of Ad-p53 and 5-FU demonstrated the most potent inhibitory effect on tumor growth, but none out of the 8 animals was tumor free at the end of the treatment.

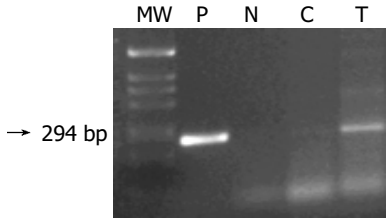

Figure 4 Gel electrophoresis for PCR products from DSL6A tumors removed from Lewis rats 24 h following the last treatment. (P) positive control, vector DNA; (N) negative control (RT minus control); (C) Ad-CD control virus treated tumor (Ad-CD); (T) Ad-p53 infected tumor.

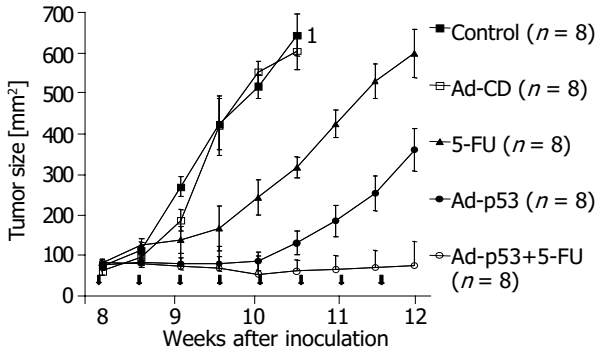

Figure 5 Efficacy of Ad-p53 infection and 5-FU as single agents *vs* the combination of both on tumor growth of Lewis rats challenged with DSL6A tumor cells. Arrowheads indicate treatment application. [1]All animals were killed after 6 cycles of therapy because the tumor size was above 600 mm^2.

Detection of apoptosis in tumor tissues after treatment

Since our *in vitro* data indicated that the tumor- inhibitory effects of the combined treatment of Ad-p53 plus 5-FU were significantly mediated by induction of apoptosis, we assessed the tumor tissue sections for DNA degradation using the TUNEL assay. Analyses of tumor sections of each group revealed a slight increase of apoptotic cells in rats treated with 5-FU compared to both control groups, mock or Ad-CD infected. Adenoviral p53 gene transduction significantly increased the number of apoptotic cells in comparison to single 5-FU chemotherapy. As shown in Figure 6, *in situ* quantification revealed the highest number of cells undergoing apoptosis in tumors treated with Ad-p53 plus 5-FU.

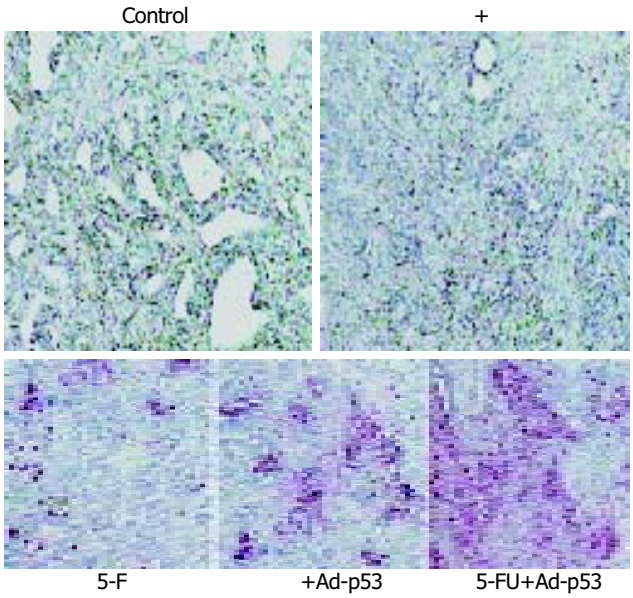

Figure 6 Ad-p53 mediated sensitisation of tumors to apoptosis in response to 5-FU *in vivo*. Formalin-fixed tissue sections were analyzed by TUNEL labelling. Original magnification ×200.

DISCUSSION

A number of previous studies have shown that the restoration of wt-p53 may increase or decrease drug sensitivity, indicating that p53 status may influence the response of human cancers to chemotherapy in a cell- and drug specific manner[3,16,17,29]. However, what is currently lacking is critical information on the cytotoxicity and target response to such a combined modality therapy. In this study we investigated a panel of human pancreatic cancer cell lines with well characterised defects in the p53 gene to identify differences following treatment with Ad-p53 vector and 5-FU chemotherapy. Because high concentrations of p53 were difficult to reach in patients by vectors available so far, and high doses of 5-FU could not be used *in vivo* due to their side effects, we evaluated the efficacy of low concentrations of p53 infection and low doses of 5-FU.

In our *in vitro* study infection with Ad-p53 alone produced tumor growth suppression in all pancreatic cancer cell lines ranging from highly significant to almost absent. The most prominent growth inhibitory effect was observed in the pancreatic cancer cell lines with an alteration of the p53 function. These findings may not only relate to the endogenous p53 status, but also to differences in transduction efficiency of cell lines to the expression of β3 integrins and coxsackie virus and adenovirus receptors (CAR) on tumor cells[30]. Ad-p53 infection at a low concentration (MOI 1) induced only a small number of apoptotic cells in the tested pancreatic cancer cell lines. These results are in agreement with those of other authors showing that levels of p53 expression that inhibited proliferation may not be sufficient by themselves to induce cell death[10,31-33]. The reduced cell proliferation observed after Ad-p53 infection might be rather attributed to a persistent S phase depletion and G0/G1 accumulation without induction of apoptosis.

However, the key question in our study was to correlate the exogenous p53 expression with the response to 5-FU based chemotherapy. Like a number of other studies the p53 replacement into tumor cell lines with altered p53, revealed the most effective tumor growth inhibition[9-12]. Our data clearly support other *in vitro* studies, which have reported that loss of p53 function could reduce cellular sensitivity to 5-FU[3,4,34]. These synergistic effects are less conclusive in cases of p53 transgene overexpression in wt-p53 tumors. Only a small, but not significant advantage of the combination regimen was achieved in the human Capan-2^{p53wt} cells, whereas the murine AS^{p53wt} cells failed to such a treatment. Since from the clinical point of view apoptotic death is the final outcome that can define the success of therapy, we screened for the induction of apoptosis in relation to the different treatment schedules. Our data demonstrated, that the combination regimen of Ad-p53 plus 5-FU was able to significantly increase the number of apoptotic cells in the pancreatic tumor cell lines with an altered p53 gene. Likewise to our findings some reports have shown, that high level expression of p53 could cause some cancer cells to undergo apoptosis, whereas others simply undergo prolonged cell cycle arrest[10,31]. To overcome the resistance to p53 mediated apoptosis several reports have shown a significant advantage of Ad-p53 gene therapy in combination with DNA damaging agents[10,14,15,32].

A foremost concern with such a strategy is whether sufficient chemosensitisation can be accomplished with relatively few supplemental gene therapy treatments during 5-FU based chemotherapy. Experimental studies reported a rate of gene transduction between 5% and 15% using immunohistochemical analysis[9]. Therefore we used a subcutaneous pancreatic tumor model to allow assessment of the efficacy of this approach. In immunocompetent Lewis rats, combination of intratumoral Ad-p53 infection of implanted pancreatic DSL6A^{p53null} tumors with additional systemic 5-FU chemotherapy resulted in a significant retardation of tumor growth. These combined effects clearly exceeded those achieved by administration of either agent alone. In contrast to all other treatment modalities, the combination regimen Ad-p53 plus 5-FU revealed no further *in vivo* tumor growth during the time of treatment. The reason for incomplete tumor regression may be related to the low p53 transduction rate and limited spread within the tumor. Our *in vivo* data point to an enhancement of 5-FU mediated apoptosis in DSL6A tumors as a result of adenoviral mediated p53 gene transfer. This is in line with our *in vitro* findings, which strongly suggest that the sensitisation is mediated by enhancing the apoptotic response of neoplastic cells to 5-FU treatment. Similar anticancer therapy approaches using combined administration of adenovirus mediated p53 gene therapy and chemotherapeutic drugs have been evaluated. In some tumors, inactivation of p53 correlated with an increased resistance to radiation and several antineoplastic drugs and transduction of the wt-p53 gene increased the sensitivity[3,16,17,32]. In contrast, in other tumors the expression of exogenous wt-p53 reduced sensitivity, while the disruption of p53 function increased sensitivity[35-37]. A number of clinical studies have found that p53 overexpression, a surrogate marker for p53 mutations, correlated with resistance to 5-FU[5,6,38,39], but other studies found no correlation[40,41]. Such conflicting findings might in part, be due to the wide variation in immunohistochemical protocols, and the use of different antibodies to detect p53. Other techniques, such as DNA sequencing, might provide a more objective assessment of the p53 status. At present, despite the *in vitro* evidence for p53 involvement in downstream signalling in response to 5-FU, the clinical value of p53 as a

predictive marker for 5-FU based chemotherapy remains a matter for debate.

In conclusion, our results confirm the feasibility of sensitising pancreatic cancer cells to 5-FU chemotherapy *in vivo* using adenovirus mediated p53 gene therapy. In tumors, where sustained expression of wt-p53 cannot be achieved or the levels of expression are insufficient to significantly induce apoptosis, wt-p53 can be used as a therapeutic agent in combination with conventional antineoplastic drugs. Our results have confirmed that this enhanced cytotoxicity is mostly provided by driving significant numbers of neoplastic cells into apoptosis. Therefore, the assessment of endogenous p53 status by DNA sequencing, which may correlate with the response to 5-FU chemotherapy may have an important role in defining patients who are most likely to benefit from 5-FU chemotherapy or combination treatment in the future[42].

REFERENCES

1 **Ghaneh P,** Kawesha A, Evans JD, Neoptolemos JP. Molecular prognostic markers in pancreatic cancer. *J Hepatobiliary Pancreat Surg* 2002; **9**: 1-11

2 **Levine AJ.** p53 the cellular gatekeeper for growth and division. *Cell* 1997; **88**: 323-331

3 **Lowe SW,** Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; **74**: 957-967

4 **Bunz F,** Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Inves* 1999; **104**: 263-269

5 **Lenz HJ,** Hayashi K, Salonga D, Danenberg KD, Danenberg PV, Metzger R, Banerjee D, Bertino JR, Groshen S, Leichman LP, Leichman CG. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. *Clin Cancer Res* 1998; **4**: 1243-1250

6 **Ahnen DJ,** Feigl P, Quan G, Fenoglio-Preiser C, Lovato LC, Bunn PA Jr, Stemmerman G, Wells JD, Macdonald JS, Meyskens FL Jr. Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a southwest oncology group study. *Cancer Res* 1998; **58**: 1149-1158

7 **Yeh KH,** Shun CT, Chen CL, Lin JT, Lee WJ, Lee PH, Chen YC, Cheng AL. Overexpression of p53 is not associated with drug resistance of gastric cancers to 5-fluorouracil-based systemic chemotherapy. *Hepatogastroenterology* 1999; **46**: 610-615

8 **Cascinu S,** Catalano V, Aschele C, Barni S, Debernardis D, Gallo L, Bandelloni R, Staccioli MP, Baldelli AM, Brenna A, Valenti A, Muretto P, Catalano G. Immunohistochemical determination of p53 protein does not predict clinical response in advanced colorectal cancer with low thymidylate synthase expression receiving a bolus 5-fluorouracil-leucovorin combination. *Ann Oncol* 2000; **11**: 1053-1116

9 **Ghaneh P,** Greenhalf W, Humphreys M, Wilson D, Zumstein L, Lemoine NR, Neoptolemos JP. Adenovirus-mediated transfer of p53 and p16 (INK4a) results in pancreatic cancer regression *in vitro* and *in vivo*. *Gene Ther* 2001; **8**: 199-208

10 **Sasaki Y,** Morimoto I, Ishida S, Yamashita T, Imai K, Tokino T. Adenovirus-mediated transfer of the p53 family genes, p73 and p51/p63 induces cell cycle arrest and apoptosis in colorectal cancer cell lines: potential application to gene therapy of colorectal cancer. *Gene Ther* 2001; **8**: 1401-1408

11 **Lang FF,** Bruner JM, Fuller GN, Aldape K, Prados MD, Chang S, Berger MS, McDermott MW, Kunwar SM, Junck LR, Chandler W, Zwiebel JA, Kaplan RS, Yung WK. PhaseI trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results. *J Clin Oncol* 2003; **21**: 2508-2518

12 **Shimada H,** Matsubara H, Ochiai T. p53 gene therapy for esophageal cancer. *J Gastroentero* 2002; **37**: 87-91

13 **Buller RE,** Shahin MS, Horowitz JA, Runnebaum IB, Mahavni V, Petrauskas S, Kreienberg R, Karlan B, Slamon D, Pegram M. Long term follow-up of patients with recurrent ovarian cancer after Ad p53 gene replacement with SCH 58500. *Cancer Gene Ther* 2002; **9**: 567-572

14 **Spitz FR,** Nguyen D, Skibber JM, Meyn RE, Cristiano RJ, Roth JA. Adenoviral-mediated wild-type p53 gene expression sensitizes colorectal cancer cells to ionizing radiation. *Clin Cancer Res* 1996; **2**: 1665-1671

15 **Nishizaki M,** Meyn RE, Levy LB, Atkinson EN, White RA, Roth JA, Ji L. Synergistic inhibition of human lung cancer cell growth by adenovirus-mediated wild-type p53 gene transfer in combination with docetaxel and radiation therapeutics *in vitro* and *in vivo*. *Clin Cancer Res* 2001; **7**: 2887-2897

16 **Gjerset RA,** Turla ST, Sobol RE, Scalise JJ, Mercola D, Collins H, Hopkins PJ. Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. *Mol Carcinog* 1995; **14**: 275-285

17 **Blagosklonny MV,** el-Deiry WS. *In vitro* evaluation of a p53-expressing adenovirus as an anti-cancer drug. *Int J Cancer* 1996; **67**: 386-392

18 **Zeimet AG,** Marth C. Why did p53 gene therapy fail in ovarian cancer? *Lancet Oncol* 2003; **4**: 415-422

19 **Lain S,** Lane D. Improving cancer therapy by non-genotoxic activation of p53. *Eur J Cancer* 2003; **39**: 1053-1060

20 **Eisold S,** Ryschich E, Linnebacher M, Nauheimer D, Wild A, Bartsch DK, Büchler MW, Schmidt J. Characterisation of a new human pancreatic cancer cell line, FAMPAC, with a hereditary background. *Cancer* 2004; **100**: 1978-1986

21 **Matzku S,** Komitowski D, Mildenberger M, Zoller M. Characterization of BSp73, a spontaneous rat tumor and its *in vivo* selected variants showing different metastasizing capacities. *Invasion Metastasis* 1983; **3**: 109-123

22 **Pettengill OS,** Faris RA, Bell RH Jr, Kuhlmann ET, Longnecker DS. Derivation of ductlike cell lines from a transplantable acinar cell carcinoma of the rat pancreas. *Am J Pathol* 1993; **143**: 292-303

23 **He TC,** Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 1998; **95**: 2509-2514

24 **Gerard R,** Meidell RS. Adenovirus vectors. In: Glover DM, Hames BD (eds). DNA cloning 4. A practical approach. *Oxford University press: Oxford* 1996: 285-306

25 **Berrozpe G,** Schaeffer J, Peinado MA, Real FX, Perucho M. Comparative analysis of mutations in the p53 and K-ras genes in pancreatic cancer. *Int J Cancer* 1994; **58**: 185–191

26 **Moore PS,** Sipos B, Orlandini S, Sorio C, Real FX, Lemoine NR, Gress T, Bassi C, Klöppel G, Kalthoff H, Ungefroren H, Löhr M, Scarpa A. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-*ras*, *p*53, *p*16 and *DPC4/Smad*4. *Virchows Arch* 2001; **439**: 798-802

27 **Erber R,** Conradt C, Homann N, Enders C, Finckh M, Dietz A, Weidauer H, Bosch FX. TP53 DNA contact mutations are selectively associated with allelic loss and have a strong clinical impact in head and neck cancer. *Oncogene* 1998; **16**: 1671-1679

28 **Hirayama Y,** Wakazono K, Yamamoto M, Kitano M, Tatematsu M, Nagao M, Sugimura T, Ushijima T. Rare mutations of *p*53, Ki-*ras*, and β-*catenin* genes and absence of K-*sam* and c-*erb*B-2 amplification in *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced rat stomach cancers. *Mo Carcinog* 1999; **25**: 42-47

29 **Mohiuddin M,** Chendil D, Dey S, Alcock RA, Regine W, Mohiuddin M, Ahmed MM. Influence of p53 status on radiation and 5-flourouracil synergy in pancreatic cancer cells. *Anticancer Res* 2002; **22**: 825-830

30 **Pearson AS,** Koch PE, Atkinson N, Xiong M, Finberg RW, Roth JA, Fang B. Facrors limiting adenovirus-mediated gene transfer into human lung and pancreatic cancer cell lines. *Clin Cancer Res* 1999; **5**: 4208-4213

31 **Kusumoto M,** Ogawa T, Mizumoto K, Ueno H, Niiyama H, Sato N, Nakamura M, Tanaka M. Adenovirus-mediated p53 gene transduction inhibits telomerase activity independent of its effects on cell cycle arrest and apoptosis in human pancreatic cancer cells. *Clin Cancer Res* 1999; **5**: 2140-2147

32 **Biroccio A,** Bufalo DD, Ricca A, D'Angelo C, D'Orazi G, Sacchi A, Soddu S, Zupi G. Increase of BCNU sensitivity by wt-p53 gene therapy in glioblastoma lines depends on the administration schedule. *Gene Ther* 1999; **6**: 1064-1072

33 **Longley DB,** Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003; **3**: 330-338

34 **Longley DB,** Boyer J, Allen WL, Latif T, Ferguson PR, Maxwell PJ, McDermott U, Lynch M, Harkin DP, Johnston PG. The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. *Cancer Res* 2002; **62**: 2644-2649

35 **Parsels LA,** Zellars RC, Loney TL, Parsels JD, Clarke MF, Merchant AK, Lawrence TS, Maybaum J. Prevention of fluorodeoxyuridine-induced cytotoxicity and DNA damage in HT29 colon carcinoma cells by conditional expression of wild-type p53 phenotype. *Mol Pharmacol* 1997; **52**: 600-605

36 **Brachman DG,** Beckett M, Graves D, Haraf D, Vokes E, Weichselbaum RR. p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines. *Cancer Res* 1993; **53**: 3667-3669

37 **Fan S,** Smith ML, Rivet DJ 2nd, Duba D, Zhan Q, Kohn KW, Fornace AJ Jr, O'Connor PM. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res* 1995; **55**: 1649-1654

38 **Liang JT,** Huang KC, Cheng YM, Hsu HC, Cheng AL, Hsu CH, Yeh KH, Wang SM, Chang KJ. p53 overexpression predicts poor chemosensitivity to high-dose 5-fluorouracil plus leucovorin chemotherapy for stage IV colorectal cancers after palliative bowel resection. *Int J Cancer* 2002; **97**: 451-457

39 **Nio Y,** Dong M, Uegaki K, Hirahara N, Minari Y, Sasaki S, Takamura M, Iguchi C, Tamura K. p53 expression affects the efficacy of adjuvant chemotherapy after resection of invasive ductal carcinoma of the pancreas. *Anticancer Res* 1998; **18**: 3773-3779

40 **Paradiso A,** Ranieri G, Simone G, Silvestris N, Costa A, De Lena M, Leone A, Vallejo C, Lacava J. mdm2-p53 Interaction: lack of correlation with the response to 5-fluorouracil in advanced colorectal cancer. *Oncology* 2002; **62**: 278-285

41 **Elsaleh H,** Powell B, Soontrapornchai P, Joseph D, Goria F, Spry N, Iacopetta B. p53 gene mutation, microsatellite instability and adjuvant chemotherapy: impact on survival of 388 patients with Dukes' C colon carcinoma. *Oncology* 2000; **58**: 52-59

42 **Barratt PL,** Seymour MT, Stenning SP, Georgiades I, Walker C, Birbeck K, Quirke P. UKCCCR AXIS trial collaborators. Adjuvant X-ray and fluorouracil infusion study. DNA markers predicting benefit from adjuvant fluorouracil in patients with colon cancer: a molecular study. *Lancet* 2002; **360**: 1381-1391

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