

Original article

Effect of recombinant adenovirus vector mediated human interleukin-24 gene transfection on pancreatic carcinoma growth

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Keywords: interleukin-24; adenovirus; pancreatic carcinoma; gene therapy; angiogenesis; apoptosis

Background Pancreatic cancer is a highly malignant tumor affecting an ever increasing number of patients with a mean 5-year survival rate below 4%. Therefore, gene therapy for cancer has become a potential novel therapeutic modality. In this study we sought to determine the inhibitory effects of adenovirus-mediated human interleukin-24 (AdhIL-24) on pancreatic cancer.

Methods Human interleukin-24 gene was cloned into replication-defective adenovirus specific for patu8988 tumor cells by virus recombination technology. Reverse transcription-polymerase chain reaction and Western blotting analysis were used to determine the expression of human interleukin-24 mRNA in patu8988 cells *in vitro*. Induction of apoptosis by overexpression of human interleukin-24 in patu8988 cells was determined by flow cytometry. *In vivo* efficacy of adenoviral delivery of human interleukin-24 was assessed in nude mice ($n=10$ for each group) bearing patu8988 pancreatic cancer cell lines by determining inhibition of tumor growth, endothelial growth factor and CD34 expression, and intratumoral microvessel density (MVD).

Results The recombinant adenovirus vector AdVGFP/IL-24 was constructed with a packaged recombinant retrovirus titer of 1.0×10^{10} pfu/ml and successfully expressed of both mRNA and protein in patu8988 cells. The AdVGFP/IL-24 induced apoptosis of patu8988 tumor cells *in vitro* and significantly inhibited tumor growth *in vivo* ($P < 0.05$). The intratumoral MVD decreased significantly in the treated tumors ($P < 0.05$).

Conclusion The recombinant adenovirus AdGFP/IL-24 can effectively express biologically active human interleukin-24, which results in inhibition of pancreatic cancer growth.

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Pancreatic cancer is a highly malignant tumor affecting an ever increasing number of patients. In most patients pancreatic cancer is detected during the late stages of illness due to the nonspecific clinical manifestation of the tumors. Only 5%–22% of patients with pancreatic cancer are candidates for surgical resection and the 5-year survival rate is less than 4%. Most patients already have advanced pancreatic carcinoma when diagnosed.^{1,2}

Interleukin-24 (IL-24) is a novel tumor suppressor gene belonging to the IL-10 cytokine superfamily.^{3,4} It was first identified in 1995 by subtraction hybridization of a cDNA library derived from a human HO-1 melanoma cell line treated with interferon (IFN)- β and the protein kinase C activator mezerin.⁵ Overexpression of IL-24 has been shown to induce tumor cell apoptosis *in vitro* in a wide variety of cancer cells including melanoma, glioblastoma, sarcoma, and lung, prostate, breast, cervical, and colorectal cancers.⁶⁻¹² Furthermore, IL-24-induced apoptosis is tumor selective with minimal cytotoxicity observed in normal human cells. This identifies IL-24 as a possible therapeutic agent for effective cancer treatment. Adenovirus can be used to mediate gene transfer to a broad spectrum of cell types and high levels of transgene expression can be obtained at high titers.¹³ It has also been shown that adenovirus (AdV)-mediated IL-24 transgene therapy suppressed tumor growth *in vivo* in various tumor models.^{14,15}

In this study, we constructed a recombinant adenovirus expressing green fluorescent protein (GFP) and the tumor suppressor IL-24 (AdVGFP/IL-24). We determined the therapeutic effect of AdVGFP/IL-24 against a pancreatic carcinoma cell line patu8988 *in vitro* and *in vivo*. We observed that AdVGFP/IL-24 down-regulated vessel expression of vascular endothelial growth factor (VEGF) and CD34, reduced vessel density in tumors, and inhibited tumor growth in nude mice tumor model.

METHODS

Cell lines, reagents, and mice

The human embryonic kidney cell line (QBI-293A) was

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cultured in Dulbecco's modified Eagle's medium (Sigma, Shanghai, China) supplemented with 10% fetal calf serum (FCS). The human pancreatic carcinoma cell line patu8988 was maintained in RP1640 medium containing 10% FCS. Biotin-conjugated anti-CD34, VEGF antibodies, and the anti-mouse IgG antibody were obtained from Santa Cruz (Shanghai, China). BALB/C nude mice were obtained from Shanghai Experimental Animal Center (Shanghai, China) and maintained according to the Animal Research Committee's guidelines at Soochow University.

Construction of recombinant adenoviral vectors

The sense primer (5'-ATGGATATCATGCAGGGCCAA-GAATTCCACTT-3') and the antisense primer (5'-GC-ACTCGAGTCAGAGCTTGTAGAATTC-3') were used for cloning the human IL-24 gene directly from RNA of IFN- γ -treated human peripheral blood mononuclear cells by using reverse transcription-polymerase chain reaction (RT-PCR). The cloned IL-24 cDNA fragment was ligated into pAd-Track-GFP vector expressing GFP to form pAd-Track-GFP/IL-24 expressing both GFP and human interleukin (hIL)-24. The resultant pAdGFP/IL-24 and pAdGFP plasmid vectors were purified from transfected BJ5183 cells, then linearized by Pac I digestion. The resulting digest was transfected into the QBI-293A cells by lipofectamine, leading to formation of the recombinant adenoviruses AdVGFP expressing GFP and AdVGFP/IL-24 expressing both GFP and hIL-24. The AdVGFP/IL-24 were amplified in QBI-293A cells, purified by cesium chloride ultracentrifugation, and stored at -80°C .

Transfection of patu8988 cells with AdVGFP/IL-24

The human pancreatic carcinoma cell line patu8988 in exponential growth were seeded onto a 6-well plate at 1×10^5 cells per well and incubated for 48 hours. The cells were allowed to grow to 80% confluence and medium removed. The mixtures were incubated for 12 hours with viral solutions. Transfections were stopped by replacing viral solutions with fresh medium. Two days after culturing, fluorescence microscopy was used to observe the expression of GFP in infected patu8988 cells.

IL-24 gene expression in patu8988 cells

Two weeks after transfection, total RNA was extracted separately from patu8988 cells (AdVGFP/IL-24-transfected cells, AdVGFP-transfected cells, and nontransfected cells). Expression of IL-24 mRNA and β -actin mRNA as internal control was detected by RT-PCR. The protocol of PCR reaction system was: denaturation (94°C , 2 minutes); 30 cycles of denaturation (94°C , 50 seconds), annealing (55°C , 50 seconds), and extension (72°C , 55 seconds); and final extension (72°C , 10 minutes). RT-PCR products were electrophoresed on a 15% agarose gel and photographs taken. The integral optical densities of the electrophoretic lanes containing hIL-24 and β -actin in each group were detected by a GDS8000 pathology image processing system (Ultra-Violet Products, Cambridge, UK) and the ratio (integral optical density of hIL-24)/(integral optical

density of β -actin) calculated. The calculated data represented the expression of IL-24 mRNA in each group.

Western blotting analysis

The patu8988 cells were cultured, infected, and harvested. Total cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked by incubation for 2 hours at room temperature with 3% (w/v) nonfat dry milk in phosphate-buffered saline (PBS). The membrane was incubated with primary antibody anti-IL-24 (1:2000), for 2 hours in blocking solution at room temperature. All of the membranes were then washed and incubated for 1 hour at room temperature, and incubated overnight with alkaline phosphatase conjugated secondary antibody (Sigma, 1:10 000).

Apoptotic cell detection by flow cytometry

The patu8988 cells (5×10^5) were cultured. After reaching 70% confluence, the cells were infected with AdVGFP and AdVGFP/IL-24. Forty-eight hours later, cells were harvested by 0.25% trypsinization, washed with PBS, and fixed with 70% ethanol for overnight. Cells were resuspended in 1 ml of PBS containing 1 mg/ml of RNaseA and 0.5 mg/ml propidium iodide (Sigma, Oakville, Ontario, Canada). After 30 minutes of incubation, cells were analyzed by flow cytometry.

In vivo experiment

Male BALB/c nude mice ($n=10$ for each group) were inoculated subcutaneously in the flank with 1×10^7 patu8988 cells. Two weeks later, when the tumors were 0.2–0.3 cm, the mice were injected intratumorally with PBS or AdVGFP or AdVGFP/IL-24 (1×10^7 pfu/50 μl) every day for 5 days and monitored daily for tumor growth. The tumor size = $ab^2/2$, where a is the larger and b is the smaller of the two dimensions. For immunohistochemical analysis, the VEGF and CD34 (1:1000) were measured with immunohistochemistry kit. The brown staining in or around the nucleus was taken as positive immunoreactivity for VEGF and CD34. One lumen of blood vessels was assessed as one new blood capillary.

Statistical analysis

Statistical analysis was performed with SPSS 10.0 software. Data were expressed as mean \pm standard deviation (SD). Statistically significant differences were determined by Student's t test and one-way analysis of variance (ANOVA) as appropriate.

RESULTS

Construction of the adenoviral vector AdVGFP/IL-24

The recombinant adenoviral vector was validated by restriction endonuclease analysis (Figure 1) and DNA sequence analysis. The results demonstrated that the hIL-24 cDNA sequence was consistent with the GenBank sequence. We concluded that the recombinant adenoviral vector AdVGFP/IL-24 was constructed successfully.

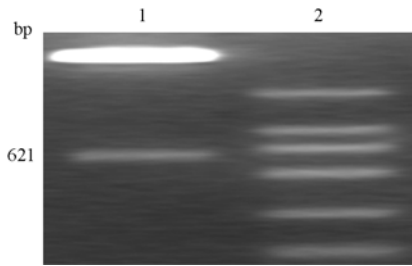


Figure 1. Verification of adenoviral plasmid vector pAdTrack-CMV-hIL-24 by means of the restriction endonucleases Sal I and EcoR V. Lane 1: pAdTrack-CMV-hIL-24 digested by Sal I and EcoR V; lane 2: DNA marker.

Transfection of patu8988 cells with AdVGFP/IL-24

To assess the optimal multiplicity of infection (MOI) for maximal transgene expression, patu8988 cells were infected with AdVGFP/IL-24 at various MOIs and examined by fluorescence microscopy. At an MOI of 10 and higher, more than 95% of the patu8988 tumor cells transfected with AdVGFP/IL-24 were GFP positive (Figure 2). Therefore, an MOI of 10 was selected as the optimal dose for transfection of patu8988 tumor cell line.

IL-24 gene expression in patu8988 cells

The results of the RT-PCR analysis demonstrated that IL-24 mRNA was stably expressed in patu8988 cells infected with AdVGFP/IL-24. There was no expression of IL-24 mRNA in patu8988 infected with AdVGFP or PBS. The expression of β-actin was not observably altered with any of the transfections (Figure 3).

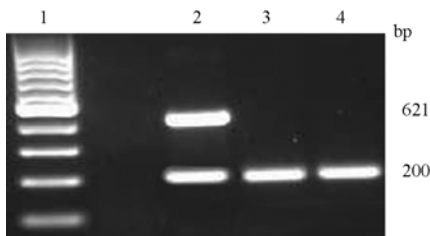


Figure 3. Expression of IL-24 mRNA in patu8988 cells detected by RT-PCR. Lane 1: marker; lane 2: AdVGFP/IL-24 infected cells; lane 3: AdVGFP infected cells; lane 4: Uninfected patu8988 cells.

Western blotting analysis

To investigate the expression in AdVGFP/IL-24-

transfected patu8988 cells, we examined the expression of hIL-24 by Western blotting. Expression of hIL-24 was observed only in the AdVGFP/IL-24-transfected patu8988 cells (Figure 4), demonstrating the AdVGFP/IL-24-mediated effective infection and specific expression of hIL-24 gene in patu8988 cells.

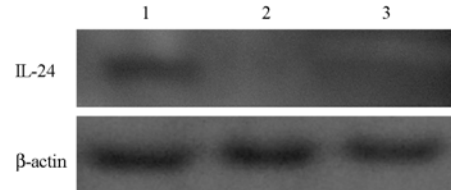


Figure 4. Expression of IL-24 mRNA in patu8988 cells detected by Western blotting analysis. Lane 1: AdVGFP/IL-24 transfected cells; lane 2: AdVGFP transfected cells; lane 3: Untransfected cells.

Flow cytometry analysis

AdVGFP/IL-24 transfection induced alterations of cell cycling *in vitro* with a reduction in S phase and G₂/M phase arrest. The flow cytometric analysis indicates that patu8988 cells infected with AdVGFP/IL-24 arrest in G₂/M with a reduction in the number of cells in S phase. These effects on cell cycle were not observed in patu8988 cells transfected with AdVGFP or PBS (Figure 5).

AdVGFP/IL-24 suppresses tumor growth *in vivo*

Treatment of nude mice bearing patu8988 tumor cells with AdVGFP/IL-24 induced a significant reduction in tumor growth as compared with mice treated with AdVGFP or PBS (Figure 6; (896.24±17.25) mm³ vs (1786.36±24.20) mm³, (1821.30±22.35) mm³, P < 0.05). These data indicate that AdVGFP/IL-24 can efficiently induce tumor growth suppression *in vivo*.

The expression of VEGF, CD34 in the tumor

As a possible explanation for the AdVGFP/IL-24-induced reduction in tumor growth, we assessed the ability of AdVGFP/IL-24 to alter tumor expression of VEGF and CD34. AdVGFP/IL-24 treatment significantly down-regulated expression of VEGF and CD34 in tumors infected with AdVGFP/IL-24 compared with AdVGFP and PBS (Figure 7). These data indicate that down-regulation of tumor VEGF and CD34 expression is a

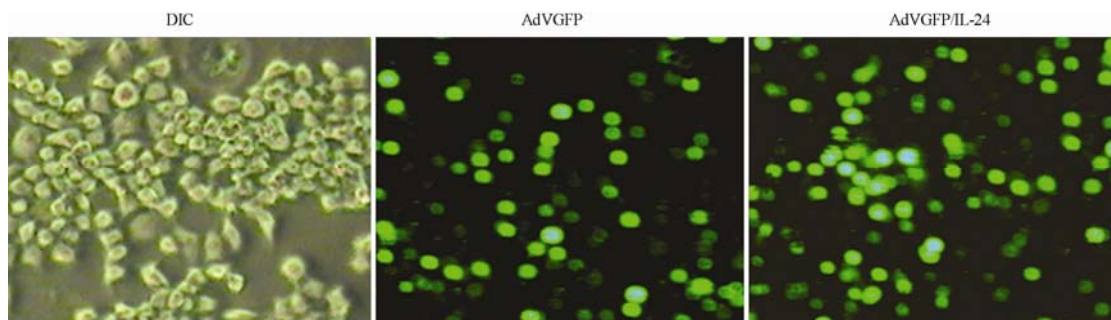


Figure 2. The expression of human interleukin (hIL)-24 in patu8988 cells infected with AdVGFP/IL-24. Patu8988 cells were imaged by differential interference contrast (DIC) and fluorescence microscopy following infection with AdVGFP/IL-24 and AdVGFP.

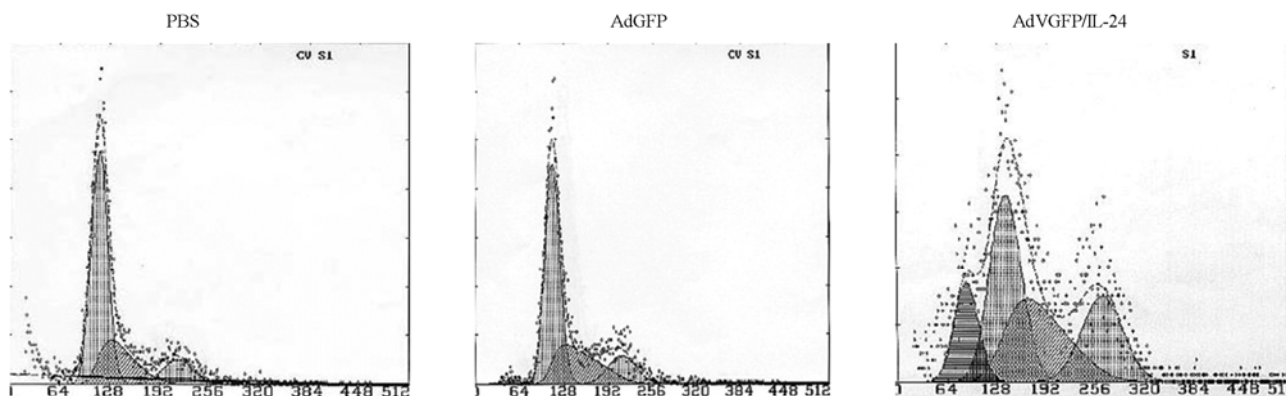


Figure 5. FCMs of the patu8988 cells. AdVGFP/IL-24 induced patu8988 cells to apoptosis *in vitro*.

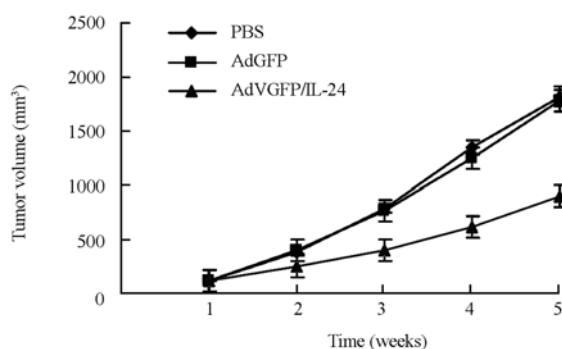


Figure 6. Inhibition of tumor growth *in vivo*. The growth inhibition of tumors infected with AdVGFP/IL-24 in nude mice vs PBS and AdVGFP.

possible mechanism by which AdVGFP/IL-24 inhibits tumor growth *in vivo*. Further, the intratumoral microvessel density (MVD) decreased significantly in the tumors treated with AdVGFP/IL-24 as compared with those treated with AdVGFP or PBS (6.7 ± 1.2 vs 19.5 ± 1.7 , 17.6 ± 2.1 , $P < 0.05$).

DISCUSSION

Pancreatic carcinoma is one of the most common malignancies in the world and in recent years its incidence has been increasing. Early-stage diagnosis of pancreatic cancer is very difficult, resulting in poor prognosis and a 5-year survival rate of only 1%–3%. Non-operative therapy, such as radiation, chemotherapy, and gene therapy have played an important role in the treatment of patients with pancreatic carcinoma. Until now, the effect of adenovirus-mediated human interleukin-24 on the patients had not been reported.

Melanoma differentiation-associated gene-7 (MDA-7) was discovered in 1995, and identified by subtractive hybridization of melanoma cells following treatment with IFN- β and mezerein, which caused terminal differentiation and growth arrest. MDA-7 is a secreted protein with cytokine-like properties belonging to the IL-10 cytokine family (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26). Based on this consideration, MDA-7 was renamed IL-24.¹⁶

AdVIL-24-mediated cancer gene therapy has been demonstrated to be cytotoxic *in vitro* to various tumor cells. The *in vivo* tumor-suppressive effect of AdVIL-24 has been demonstrated in various animal tumor models. This antitumor effect of hIL-24 is independent of classic tumor suppressor genes, such as p53, Rb, and p16.¹⁷⁻¹⁹ In this study, we constructed an adenovirus containing both GFP and IL-24. Our data demonstrated that infection of tumor cells with AdVGFP/IL-24 at a MOI of 10 resulted in GFP expression in more than 95% of the cells, indicating that this construct exhibits high infectivity. Our data also demonstrated that the AdVGFP/IL-24 could replicate in patu8988 tumor cells.

The decision of cells to differentiate is commonly made in the G₁ phase of the cell cycle.²⁰ The regulation of cells entering from the G₁ phase of the cell cycle into S phase is particularly important, as the cells normally must pass through a restriction point in late G₁ to progress to the S phase. In this study, the AdVGFP/IL-24 was demonstrated to also induce *in vitro* apoptosis of patu8988 cells. Treatment of patu8988 cells with AdVGFP/IL-24, but not AdVGFP or PBS, resulted in a significant reduction in S phase concomitant with an increase in G₂/M phase, which is consistent with a previous report.²¹

Tumor growth relies on angiogenesis, the formation of new blood vessels, to receive an adequate supply of oxygen and nutrients.²² Angiogenesis in pancreatic carcinoma is based on the same fundamental principles of activation, proliferation, and migration of endothelial cells. Over-expression of angiogenic genes such as VEGF and CD34 has been shown to be associated with enhanced tumorigenicity and tumor metastatic potential.^{23,24} VEGF is important to the growth of many solid tumors conferring survival advantage by inducing vascular formation via stimulation of endothelial cells.²⁵ CD34 is a cell surface marker of progenitor cells, and is frequently used as a marker of new vessel. In this study, we demonstrated that *in vivo* expression of the angiogenesis-associated molecules CD34 and VEGF was significantly downregulated in tumors by AdVGFP/IL-24 treatment compared with

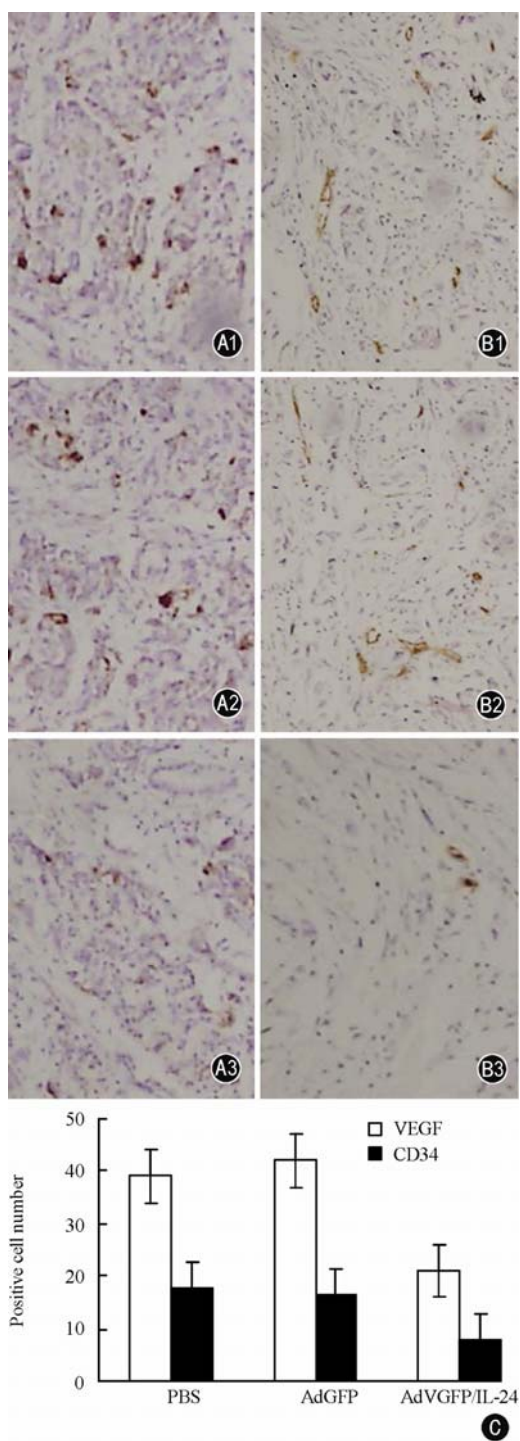


Figure 7. Immunohistochemical staining of VEGF and CD34 in tumor tissues (Original magnification $\times 200$). **A1:** Expression of VEGF in PBS group; **A2:** Expression of VEGF in AdVGFP group; **A3:** Expression of VEGF in AdVGFP/IL-24 group; **B1:** Expression of CD34 in PBS group; **B2:** Expression of CD34 in AdVGFP group; **B3:** Expression of CD34 in AdVGFP/IL-24 group; **C:** Results of VEGF and MVD. AdVGFP/IL-24 group was compared with PBS group and AdVGFP group.

AdVGFP-treated tumors. Further, AdVGFP/IL-24 treatment resulted in reduced vascular density within tumors. These data indicate that down-regulation of tumor VEGF and CD34 expressions is a possible mechanism underlying AdVGFP/IL-24-mediated

inhibition of pancreatic carcinoma tumor growth *in vivo*.

In summary, AdVGFP/IL-24 can replicate in patu8988 cells and induce cellular apoptosis. It also down-regulates expression of angiogenic genes VEGF and CD34, resulting in reduced tumor vessel formation. In an animal model, AdVGFP/IL-24 gene therapy significantly inhibited pancreatic carcinoma growth and prolonged the survival period of the tumor-bearing mice. Therefore, adenovirus-mediated human interleukin-24 gene therapy may serve as a novel therapeutic method for pancreatic carcinoma.

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