vided by Dr. Tomoda (National Kyushu Cancer Center, Fukuoka, Japan), and were cultured in DMEM (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS) (ICN Biomedicals, Aurora, Ohio). Human embryonic kidney (HEK) 293 cells were purchased from RIKEN Bioresourse Center (Tukuba, Ibaragi, Japan). Each culture medium contained 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (Gibco-BRL).

Pancreatic cancer tissues were obtained from 22 patients who underwent pancreatectomy for ductal carcinoma at our Department. Other pancreatic malignancies were excluded, such as intraductal papillary mucinous adenocarcinoma, acinar cell carcinoma, and endocrine tumors. Informed consent was obtained from each patient according to our Institutional guidelines. A resected specimen was immediately examined by inspection and palpitation at the operation room. A part of malignant or normal tissues considered was cut by surgical knife and it was divided two pieces. One for the tissue samples to extract RNA was immediately frozen in liquid nitrogen and stored at -80°C, the other was fixed in 10% formalin solution to make a paraffin block and performed with HE staining to evaluate pathologically.

Antibodies

Rabbit polyclonal anti-midkine antibody was kindly provided by Dr. Kadomatsu (Nagoya University School of Medicine, Nagoya, Japan). The following antibodies were purchased; mouse monoclonal anti-E1A (Ad2/Ad5) antibody (clone M73 #05-599) from Upstate Biotechnology (Lake Placid, NY), goat anti-mouse IgG (#62-6500) and HRP-goat anti-mouse IgG (#81-6520) from Zymed Laboratories (South San Francisco, CA), and mouse monoclonal anti-β-actin (clone AC-15 #A-5441) from Sigma (St. Louis, MO). Anti-adenoviral hexon protein antibody was included in the Adeno-X rapid titer kit (BD Biosciences Clontech, Palo Alto, CA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared using TRIZOL Reagent (Life Technologies, Rockville, MD) and cDNA was obtained from 1 μg of total RNA by the random primer method with a First-Strand cDNA Synthesis kit (Pharmacia Biotech, North Peapack, NJ) according to the manufacturer's instructions. Five microliters of first-strand cDNA solution was subjected to the polymerase chain reaction (PCR) with synthetic oligonucleotide primers (NIPPON EGT, Toyama, Japan). For RT-PCR analysis of human adenovirus type 5 E1A, a pair of primers (5'-ATGAGACATATTATCTGCCACGG-3'/5'-TAGACAAACATGCCACAGGTCC-3') was used and PCR was done for 35 cycles at 54°C, yielding a product of 551 base pairs. The reproducibility of the technique and quality of the total RNA were confirmed by amplifying β-actin as well (primers: 5'-GGCATCGTGATGGACTCCG-3'/5'-GCTGGAAGGTGGACAGCGA-3'; product: 613 base pairs).

Quantitative RT-PCR

To assess midkine gene expression, we used quantitative real-time RT-PCR analysis based on the TaqMan fluorescence method, which employs a dual-labeled non-extendable oligonucleotide hydrolysis (TaqMan) probe in addition to the two amplification primers. The probe contains 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye, and 6-carboxytetramethyl-rhodamine (TAMRA) as a quencher for its emission spectrum. During the extension phase of PCR, the probe hybridizes to the target sequence and is then cleaved by the 5' to 3' exonuclease activity of Taq polymerase. The increase in the fluorescence of the reporter is proportional to the amount of specific PCR products, providing highly accurate and reproducible quantification. The level of reporter dye fluorescence is assessed with an automated sequence detector combined with analysis software (ABI Prizm 7700 Sequence Detection System; PE Applied Biosystems, Foster City, CA). Reaction conditions were set according to the manufacturer's protocol. The following primers and TaqMan probe were used for analysis. The midkine-specific primers were 5'-CGACTGCAAGTACAAGTTTGAGAAC-3' (upstream primer) and 5'-TCTCCTGGCACTGAGCATTG-3' (downstream primer), while 5' (FAM)-AAGGCACCCTGAAGAAGGCGCG-(TAMRA) 3' was the TaqMan probe.

The PCR parameters were 95°C for 10 min (for activation of Taq-Polymerase), followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification of β-actin for quality control and normalization was done with the TaqMan β-actin Control Reagent kit (PE Applied Biosystems), which utilizes standard TaqMan probe chemistry.

Western blot analysis

Cells were lysed in RIPA buffer containing 50 mM HEPES (pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride (PMSF), and 20 μg/ml gabexate mesilate, and were incubated on ice for 10 minutes. Then the lysate was sonicated for 10 sec. Total extracts were cleaned by centrifugation at 15,000 rpm for 10 min at 4°C and the supernatants were collected. The protein concentration was measured with the BCA protein assay reagent (Pierce, Rockford, IL). Lysates were resuspended in one volume of gel loading buffer, which contained 50 mM Tris-Hcl (pH 6.7), 4% SDS, 0.02% bromophenol blue, 20% glycerol, and 4% 2-mercaptoethanol, and then were heated at 95°C for 5 min. The extracted protein was subjected to Western blotting. In brief, 50 μg aliquots of protein were size-fractionated in a single dimension by

SDS-PAGE (6–10% gels) and transblotted to 0.45 μm polyvinylidine difluoride membranes (IPVH304F0, Millipore, Billerica, MA) with a semi-dry electroblotting apparatus (Bio-Rad, Richmond, CA).

The blots were then washed three times in TBS with 0.1% Tween-20 (TBST) and incubated for 1 hour at room temperature in blocking buffer (Block Ace, Dainipponseiyaku, Osaka, Japan). Subsequently, the blots were incubated with an appropriate primary antibody for 1 h at room temperature or overnight at 4°C. Excess antibody was removed by washing the membrane with TBST three times for 10 min each. Then the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, followed by an addition of TBST. Reaction products were detected with the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom). The membranes were treated with chemiluminescence reagents according to the manufacturer's protocol, and were exposed to X-ray films for 5–120 sec.

Dual luciferase assay

We prepared the midkne 0.6-luc vector, in which the 609-base pair genomic DNA fragment of the midkne gene was cloned into the pGL2-basic vector (Promega, Madison, WI) and the firefly luciferase gene was included without a promoter sequence[24].

The transcripitional activity of a number of pancreatic cancer cell lines was measured with this dual luciferase reporter assay system (Promega, Madison, WI). Midkine 0.6-luc and a control vector (the renilla luciferase gene fused with the HSV-TK promoter (pRL-TK, Promega, Madison, WI) at a molar ratio of 10:1) were transfected together into target cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were lysed after 2 days and luciferase activity was measured according to the manufacturer's protocol. The relative firefly luciferase activity of each cell lysate was calculated from the level of luminescence.

Adenoviruses and adenoviral transduction analysis

We prepared recombinant adenovirus type 5 containing the 0.6 kb midkne promoter (Ad5MK) for midkne-regulated expression of E1A[24]. Type 5, E1A-deleted, replication-defective adenovirus containing the green fluorescent protein gene (Ad5GFP) was constructed using AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Adenoviruses were propagated in HEK 293 cells, purified by two rounds of cesium chloride density centrifugation, dialyzed, and stored at -70°C. Viral titers were determined with an Adeno-X Rapid Titer Kit (BD Biosciences Clontech, Palo Alto, CA).

To assess the efficiency of adenoviral transduction in human pancreatic cancer cells, we performed fluorescent staining using Ad5GFP. Pancreatic cancer cells were seeded onto coverslips and infected with at recombinant adenovirus at various multiplicities of infection (MOIs). After 48 hours, coverslips were mounted on the glass slides with Vectashield mounting medium (Vector Labolatories, Burlingame, CA), and the cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Assessment of adenoviral replication

First, cells were infected with Ad5MK at various MOIs for 1 h and then the virus was removed. The infected cells were lysed in RIPA buffer to extract proteins after culture for 48 h. The proteins were subjected to SDS-PAGE and expression of E1A protein was analyzed by Western blotting. Next, cells were infected with Ad5MK at 1 MOI for 1 h and the medium was then refreshed. After the cells were cultured for 2 days, the cell lysate was prepared with three cycles of freezing and thawing.

HEK293 cells were infected with serially diluted cell lysates or tissue lysates. After 48 hr, the cells were stained with anti-adenoviral hexon protein antibody by using an Adeno-X rapid titer kit (BD Biosciences Clontech, Palo Alto, CA).

In vitro *cytotoxicity test*

Pancreatic cancer cells were plated into 12-well plates in triplicate at a density of 1.0×10^4 cells/well. After 24- to 36 h of culture, cells were infected with Ad5GFP or Ad5MK at various MOIs for 1 h, and the infecting medium was replaced with complete medium. After 1, 3 and 5 days, the number of viable cells was counted using a cell counter (Coulter Z1, Beckman-Coulter, Fullerton, CA).

Animal study

The animal study was performed in accordance with the guidelines for animal experiments of the Institute of Laboratory Animals at Kyoto University. Six-week-old male BALB/c nude mice were purchased from CLEA Japan (Tokyo, Japan). First, the mice were subcutaneously inoculated with Suit-2 cells (2×10^6/ml) in 100 μl of Hank's balanced salt solution (HBSS) (Gibco-BRL) containing 20% matrigel (BD Biosciences, Bedford, MA). When the tumors reached about 10 mm in diameter, Ad5GFP or Ad5MK (2×10^9 PFU, 0.1 ml/mouse) was injected intratumorally. The mice were sacrificed at 3 or 7 days after adenoviral injection to extract RNA and lysates from the tumors. RT-PCR analysis of human adenovirus type 5 E1A and staining with anti-adenoviral hexon antibody were performed to assess the replication of adenoviruses *in vivo*.

Next, Suit-2 cells (2×10^6/ml) in 500 μl of sterile PBS were inoculated into the peritoneal cavity of BALB/c nude mice to create a peritoneal dissemination xenograft model. The mice were divided into the following 4 groups: (1) an Ad5MK group, (2) an Ad5GFP group, (3) a PBS group, and (4) an untreated group. Ad5MK (2×10^9 PFU, 0.5 ml/mouse), Ad5GFP (2×10^9 PFU, 0.5 ml/mouse) or PBS (0.5 ml/mouse) was administered intraperitoneally at 4 days after the injection of Suit-2 cells. Survival was measured from the start of treatment.

Statistical analysis

Quantitative data are presented as the mean ± SEM. Each *in vitro* experiment was performed independently at least three times. To compare mRNA levels in pancreatic tissue samples, Wilcoxon's rank sum test was used. Survival rates were calculated by the Kaplan-Meier method, and differences between groups were evaluated with the log-rank test and Wilcoxon's test. Statistical analysis was done by using JMP statistical software and statistical significance was considered to be present at $p < 0.05$.

Results

Midkine expression by human pancreatic cancer cell lines

We examined the expression of midkine mRNA in seven human pancreatic cancer cell lines by TaqMan PCR (Table 1). Midkine mRNA expression was strong in AsPC-1 and CFPAC-1 cells, but it was weak in MIAPaCa-2 cells.

Next, midkine protein expression was assessed by Western blot analysis (Figure 1A). AsPC-1 and CFPAC-1 cells showed strong expression of midkine protein, whereas BxPC-3, HPAC and Suit-2 cells showed moderate expression. In contrast, expression by PANC-1 cells was weak and MIAPaCa-2 cells showed no midkine band. The extent of midkine protein expression was in parallel to that of midkine mRNA expression. In the following experiments, we therefore designated the MIAPaCa-2 cell line as midkine-negative.

Table 1: Expression of midkine mRNA in human pancreatic cancer cells.

Cell line	Midkine mRNA/β-actin mRNA
AsPC-1	1.19 ± 0.04
BxPC-3	0.37 ± 0.01
CFPAC-1	1.42 ± 0.02
HPAC	0.31 ± 0.01
MIAPaCa-2	0.001 ± 0.0004
PANC-1	0.02 ± 0.001
Suit-2	0.13 ± 0.003

Data are expressed as the mean ± SEM.

Midkine expression in human pancreatic cancer

We assessed the expression of midkine mRNA in 22 pancreatic cancer samples and 18 adjacent non-cancerous pancreatic tissue samples by TaqMan PCR (Figure 1B). The midkine mRNA/β-actin mRNA ratio of pancreatic cancer and non-cancerous pancreatic tissue was 0.60 ± 0.55 and 0.22 ± 0.13, respectively. Expression of midkine mRNA was significantly stronger in pancreatic cancer than in non-cancerous tissue ($p < 0.001$).

Transcriptional activity of the midkine promoter in human pancreatic cancer cells

We investigated the transcriptional activity of the midkine promoter by the dual luciferase reporter assay in AsPC-1, CFPAC-1, MIAPaCa-2, PANC-1, and Suit-2 cells (Table 2). This assay showed that the relative luciferase activity mediated by the 0.6 kb midkne fragment in AsPC-1, PANC-1, and Suit-2 cells was approximately 6 to 20 times greater than that in midkne-negative MIAPaCa-2 cells. Transcriptional activity in MIAPaCa-2 cells was extremly low. The transcriptional activity in each of the cell lines was usually correlated with the expression of midkne, although CFPAC-1 cells showed low transcriptional activity and high midkne expression.

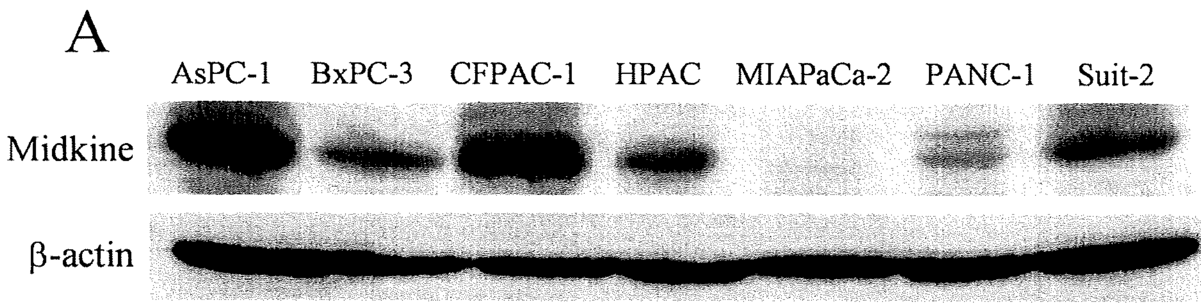
Sensitivity of human pancreatic cancer cell lines to adenovirus infection

We examined the induction of Ad5GFP in human pancreatic cancer cells (Figure 2). Cells were infected with Ad5GFP (1, 10, or 25 MOI) at 16 to 18 h after seeding. After 48 h, GFP-expressing cells were detected by fluorescence microscopy. We found that pancreatic cancer cells exhibited a heterogeneous adenoviral transduction profile. Many of the CFPAC-1 and Suit-2 cells infected at an MOI of 25 expressed GFP, whereas AsPC-1 cells showed far lower adenoviral transduction efficiency. We also found that the number of GFP-expressing cells increased in an MOI-dependent manner.

Ad5MK shows specific replication and infectivity for human pancreatic cancer cell lines

Since the adenoviral infection cycle is completed within 24 h, E1A expression by infected cells after 48 h reflects viral replication. Therefore, to determine the specificity of Ad5MK replication, we used pancreatic cancer cell lines with different levels of midkine expression and then examined E1A expression by Western blotting (Figure 3A). We found that viral replication was dependent on the MOI of infection.

Next, we examined the infectivity of Ad5MK prepared from pancreatic cell line in HEK293 cells by using an anti-adenoviral hexon antibody (Figure 3B). Stained HEK293 cells were found after infection with preparations from midkine-positive cells (PANC-1, Suit-2, AsPC-1, and



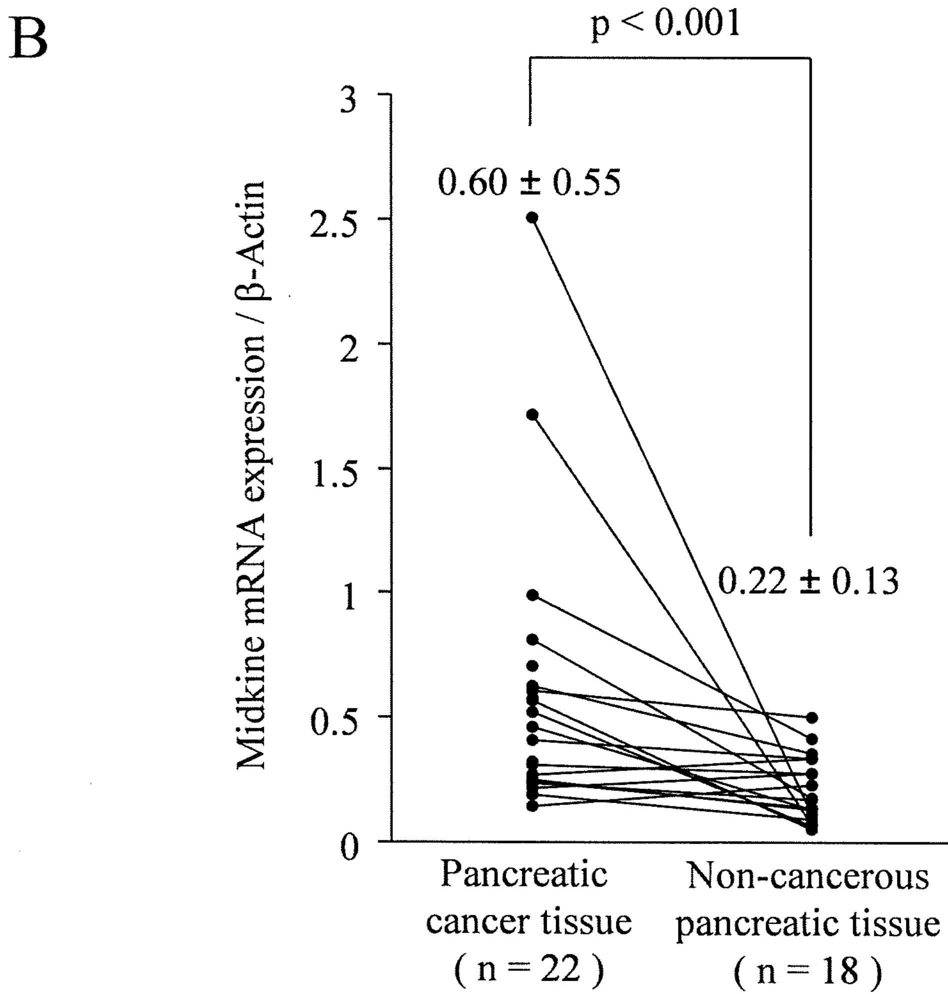


Figure 1
Midkine protein expression by human pancreatic cancer cell lines and human pancreatic cancer tissues. (A) Midkine protein expression by human pancreatic cancer cell lines. AsPC-1 and CFPAC-1 cells showed strong expression of midkine protein, whereas BxPC-3, HPAC, and Suit-2 cells showed moderate expression. In contrast, expression by PANC-1 cells was low and MIAPaCa-2 cells showed no detectable midkine band. (B) Midkine mRNA expression in non-cancerous pancreatic tissues and human pancreatic cancers. Expression of midkine mRNA in the pancreatic cancers was significantly stronger than in the non-cancerous pancreatic tissues ($p < 0.001$).

Table 2: Transcriptional activity of MK promoter in human pancreatic cancer cells.

Cell lines	Relative luciferase activity
AsPC-1	0.57 ± 0.09
CFPAC-1	0.06 ± 0.01
MIAPaCa-2	0.03 ± 0.01
PANC-1	0.16 ± 0.02
Suit-2	0.29 ± 0.01

Relative luciferase activity was defined as firefly luciferase activity per renilla luciferase activity in cancer cells. Data are expressed as the mean ± SEM.

CFPAC-1), whereas there were no stained cells after infection with a preparation from midkine-negative MIAPaCa-2 cells.

Specific cell killing effect of Ad5MK in vitro

We subsequently examined the ability of Ad5MK to kill pancreatic cancer cells (Figure 4). The number of viable cells was counted by using a Coulter counter. Ad5MK showed a much stronger-killing effect against Suit-2 and PANC-1 cells that have moderate and weak midkine expression, respectively, compared with its effect on midkine-negative MIAPaCa-2 cells.

On the other hand, moderate cell-killing effect against CFPAC-1 cells was found in 10 MOI of Ad5MK. CFPAC-1 cells had high midkine expression and moderate efficiency of adenoviral transduction. Transcriptional activity of midkine promoter in CFPAC-1 cells was low, but it was higher compared to that of MIAPaCa-2 cells. This may be the reason why growth suppression of CFPAC-1 cells was observed by 10 MOI of Ad5MK. In contrast, Ad5MK had no effect on AsPC-1 cells as far as the designated conditions, although these cells showed strong midkine expression. This finding may have reflected low efficiency of adenoviral transduction. Ad5GFP had no influence on the growth of any of the cells in this assay compared with normal control cells (data not shown).

Adenoviral replication and anti-tumor effect of Ad5MK in vivo

We assessed adenoviral replication *in vivo* by using a Suit-2 subcutaneous xenograft model of pancreatic cancer.

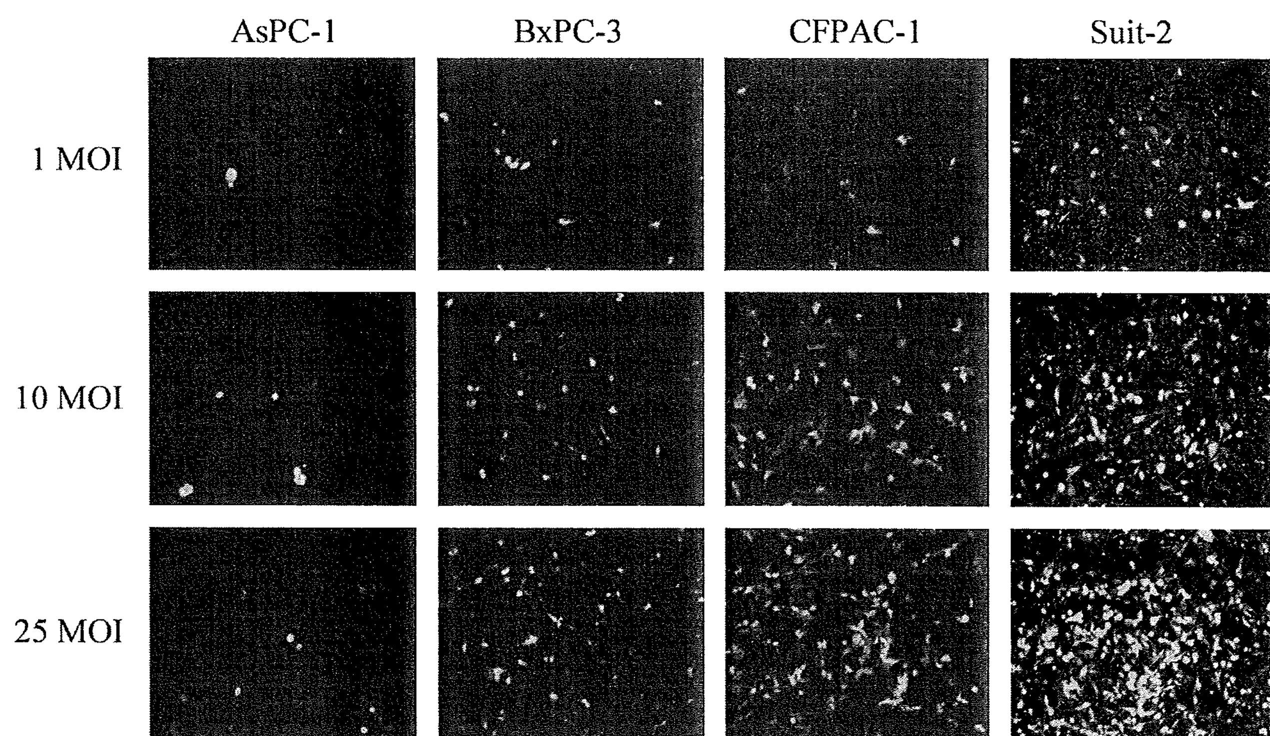


Figure 2
Sensitivity of human pancreatic cancer cell lines to adenovirus infection. Pancreatic cancer cells were infected with Ad5GFP (1, 10 or 25 MOI) at 16 to 18 h after seeding. After 48 h, GFP-expressing cells were detected by fluorescence microscopy. (Original magnification × 100).

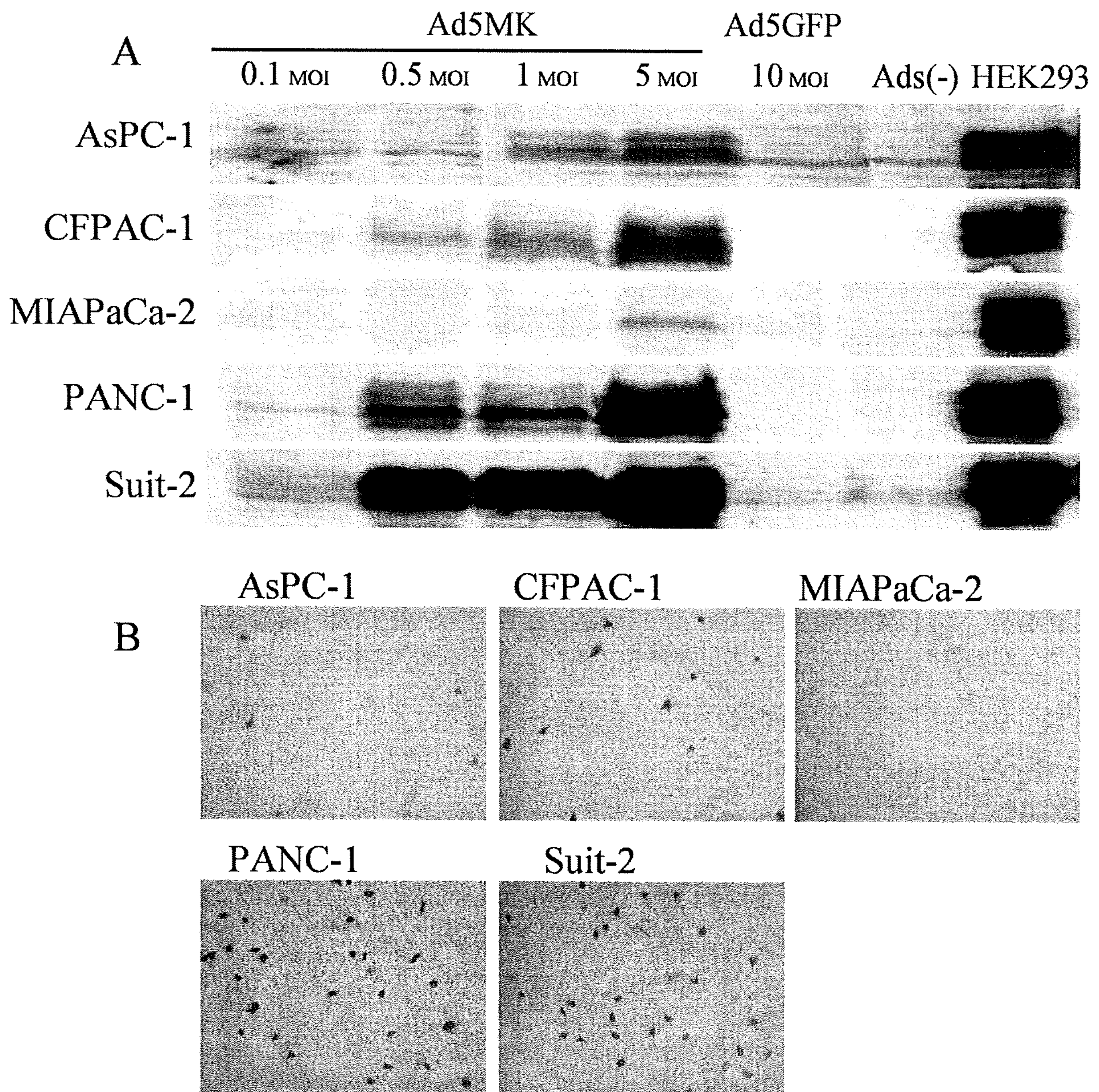


Figure 3
Replication specificity and infectivity of Ad5MK for human pancreatic cancer cell lines. (A) E1A protein expression by pancreatic cancer cells. Pancreatic cancer cell lines were infected with Ad5MK showing different levels of midkine expression, Ad5GFP alone, or the vehicle. (B) Adenoviral hexon staining. HEK293 cells were treated with preparations of Ad5MK-infected pancreatic cancer cells. After 48 hr, HEK293 cells were stained with anti-adenoviral hexon antibody. (Original magnification ×100)

RNA and tumor lysates were obtained from the subcutaneous xenografts after injection of adenoviruses. RT-PCR analysis for human adenovirus type 5 E1A showed that E1A expression could be detected in the Ad5MK groups (Figure 5A). On the other hand, no band was seen in the untreated and Ad5GFP groups. After staining with anti-adenoviral hexon antibody, positive cells were only found in the Ad5MK groups, whereas there were no positive cells in the untreated and Ad5GFP groups (Figure 5B).

Finally, we assessed the anti-tumor effect in a Suit-2 intraperitoneal xenograft model after the intraperitoneal injec-

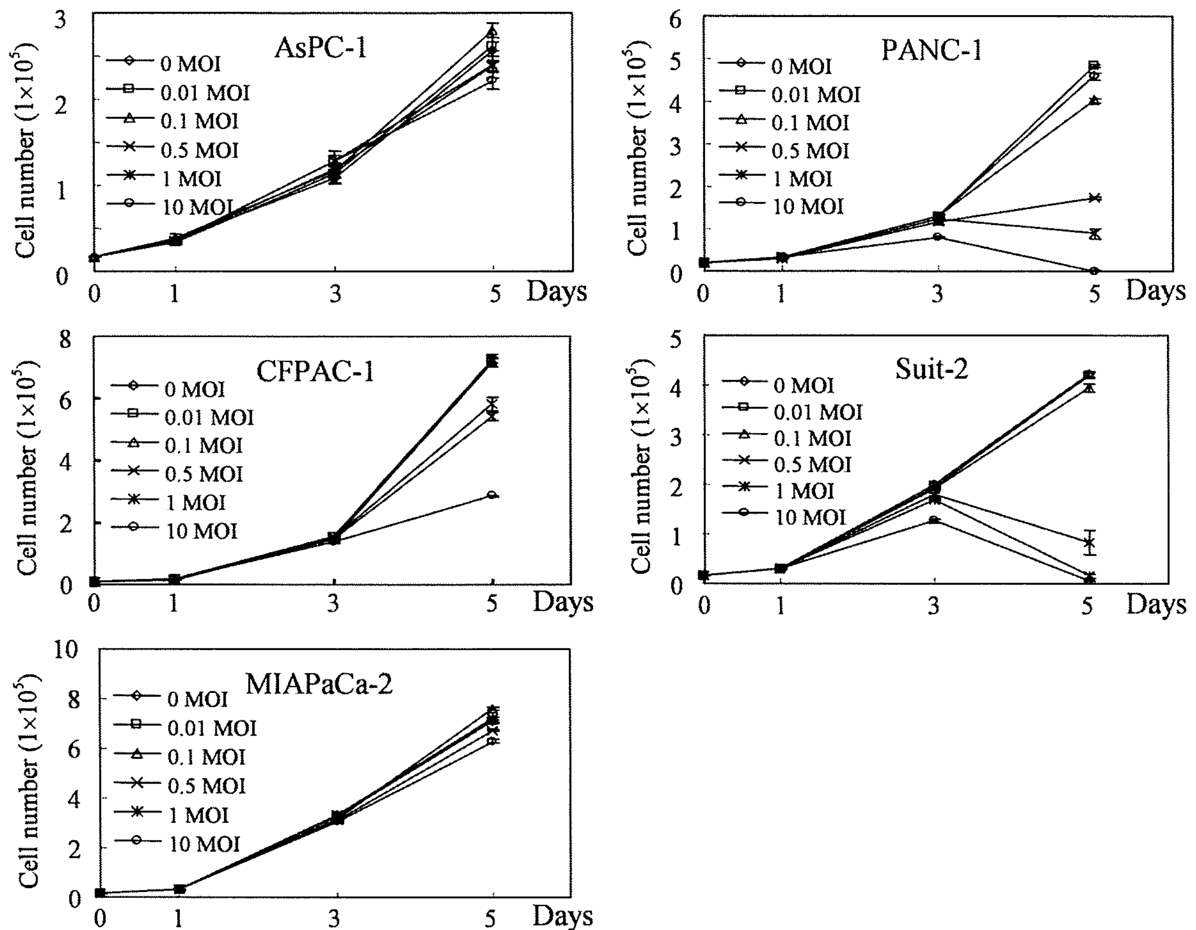


Figure 4
***In vitro* cytotoxicity of Ad5MK.** Pancreatic cancer cells were plated in triplicate in 12-well plates at 1.0×10^4 cells/well. After 24 to 36 h, the cells were infected with Ad5GFP or Ad5MK at various MOIs. After 1, 3, and 5 days, the number of viable cells was counted.

tion of Ad5MK (Figure 6). Following the inoculation of 2×10^6/ml Suit-2 cells into the peritoneal cavity of nude mice, 2×10^9 PFU of Ad5MK or Ad5GFP, or 500 μl of PBS, was administered intraperitoneally. In this intraperitoneal xenograft model, our preliminary study revealed that untreated mice died of peritoneal dissemination with bloody ascites after approximately 2 to 3 weeks. Almost all of the mice in the untreated, PBS, and Ad5GFP groups died within 35 days, whereas almost all of the mice in the Ad5MK group survived for more than 50 days. There was a statistically significant difference of the survival time between the Ad5MK group and the other group.

Discussion

Conditionally replicative adenoviruses, which show tumor-specific replication and oncolysis, are a promising new treatment modality for malignancies resistant to conventional therapies [25,26]. In this study, we demonstrated a possible strategy for pancreatic cancer based on Ad5MK replication-competent adenovirus. Midkine expression is increased in various human tumors, including gastrointestinal cancers [19-22]. We showed that most human pancreatic cancer cell lines (6 out of 7 lines tested) express midkine to some extent and that the level of midkine mRNA expression in pancreatic cancer tissues is significantly higher than in non-cancerous pancreatic tissues. These findings suggested that the midkine promoter may be a potential candidate for safe suicide gene therapy targeting pancreatic cancer.

We next examined the promoter activity of midkine in a dual luciferase reporter assay. A 2.3-kb fragment from the 5' region of the midkine gene contains the elements responsible for promoter activity [27]. Within this 2.3-kb

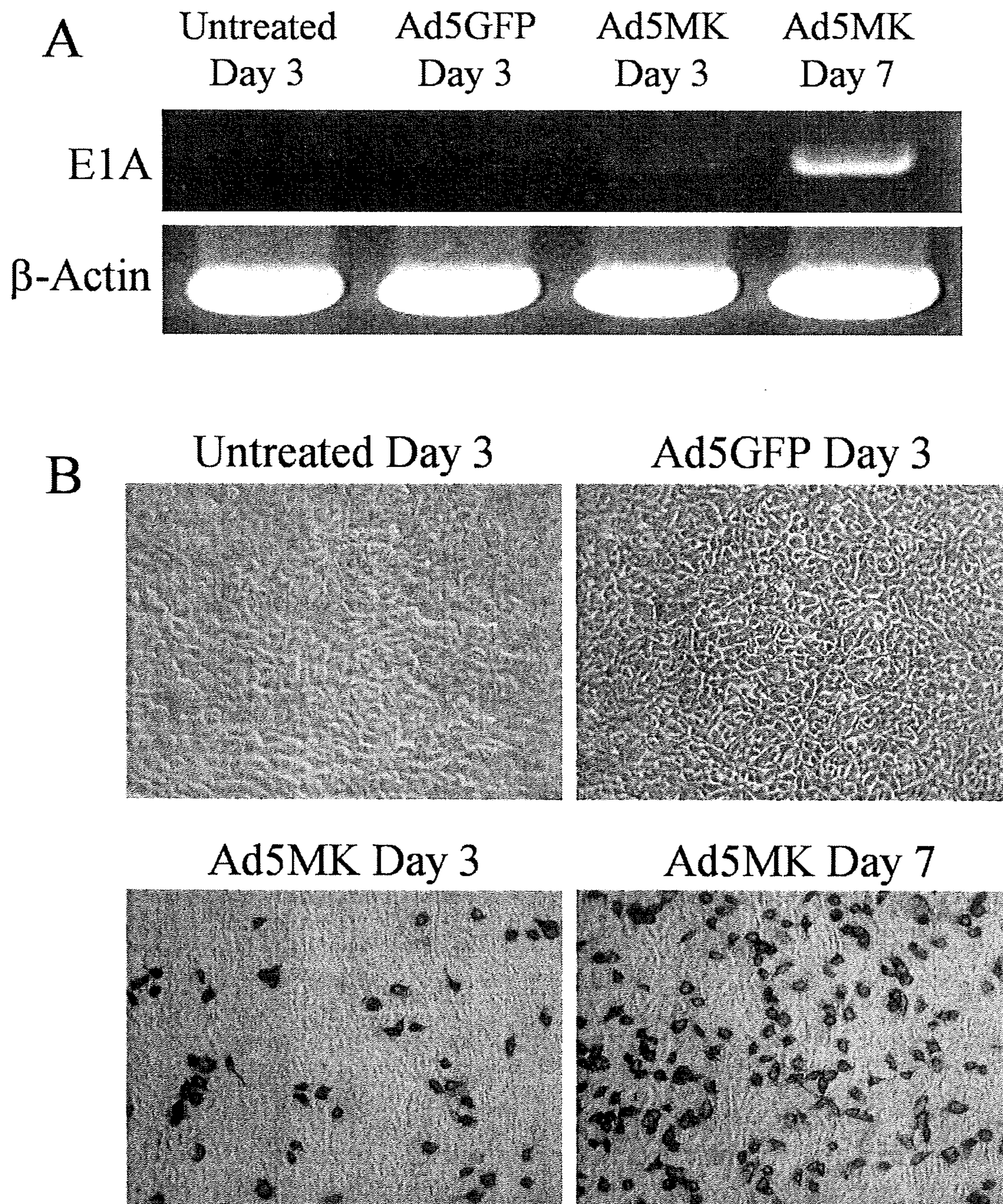


Figure 5
Replication of adenovirus in a Suit-2 subcutaneous xenograft model of pancreatic cancer.(A) Human adenovirus type 5 E1A expression. RNA was extracted from subcutaneous tumors after injection of adenoviruses. Expression of E1A was analyzed by RT-PCR. (B) Adenoviral hexon staining. HEK293 cells were infected with Ad5MK prepared from subcutaneous xenografts after injection of adenoviruses. After 48 hr, HEK293 cells were stained with anti-adenoviral hexon antibody. Positive cells were only found in the Ad5MK groups, whereas there were no positive cells in the untreated and Ad5GFP groups. (Original magnification ×100)

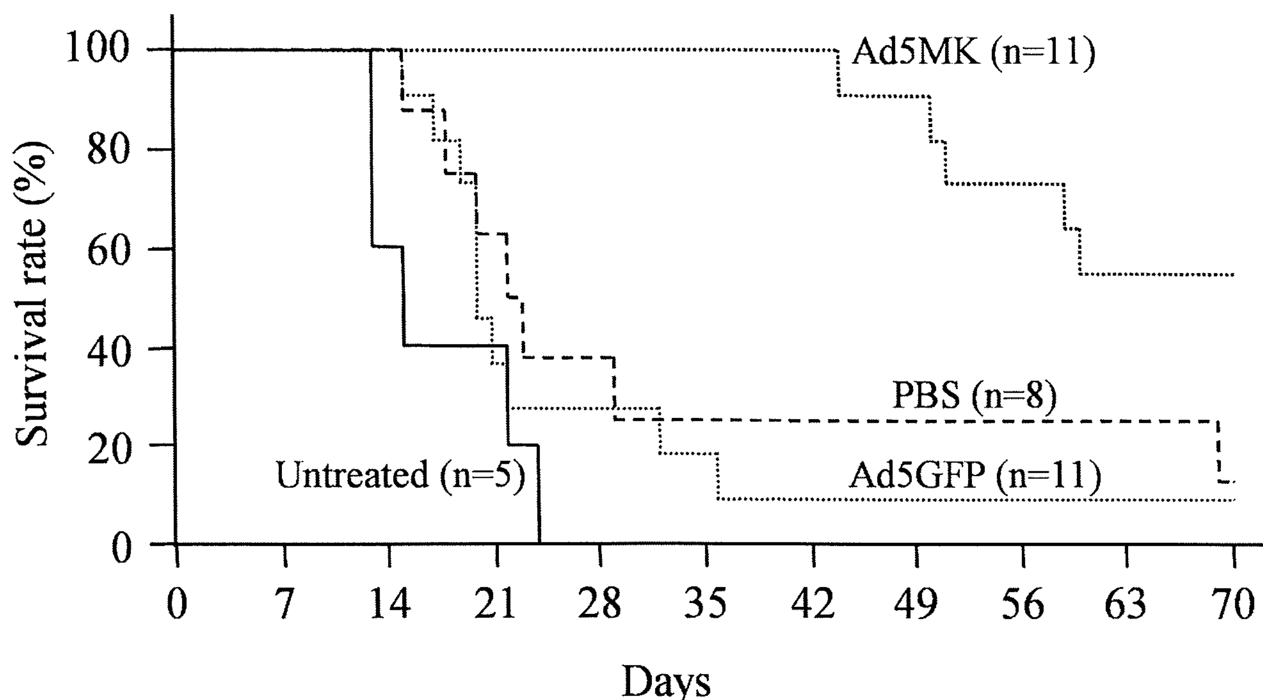


Figure 6
Anti-tumor effect of Ad5MK in a Suit-2 intraperitoneal xenograft model. After intraperitoneal inoculation of 2 × 10^6/ml Suit-2 cells in nude mice, 2 × 10^9 PFU of Ad5GFP or Ad5MK, or 500 μl of PBS, was administered intraperitoneally. There was a significant difference of survival time between the Ad5MK group and the other groups (p = 0.0002 Log-rank test and p = 0.0002 by Wilcoxson's test).

fragment, Yoshida et al. have demonstrated the presence of a cis-element with strong promoter activity located at the -559/50 region using the CAT assay, and they also reported that its midkine promoter activity in tumor cells was comparable to that of the SV40 early promoter [28]. Therefore, we examined the promoter activity of this 0.6-kb fragment in human pancreatic cancer cells by using the dual luciferase reporter assay. We showed that the relative luciferase activity in AsPC-1, PANC-1, and Suit-2 cells was greater than that in midkne-negative MIAPaCa-2 cells. Transcriptional activity in the cell lines was generally parallel to the level of midkne expression. In CFPAC-1 cells, however, the level of midkne expression did not correspond well with the transcripitional activity of the midkne promoter. We cannot explain this discrepancy, but it has been reported that the level of endogenous midkne expression and its promoter activity are not always correlated with each other because of various factors such as negative and positive regulatory elements residing outside the region analysed [29]. Another possible explanation may be differences between cell lines with respect to regulation of the proteins involved in transcription [29].

Because the efficiency of adenoviral transfection varies between cell lines, it was essential to examine the infectivity of Ad5GFP for multiple human pancreatic cancer cell lines. Adenoviral transduction efficiency was far lower for AsPC-1 than the other cell lines. Adenoviral replication in AsPC-1 cells was lower than in any other midkine-positive cell line and no killing of AsPC-1 cells was observed at 10 MOI, although these cells showed high midkine expression and transcriptional activity. Thus, we consider that adenoviral transduction efficiency as well as transcriptional activity is critical for adenovirus-mediated gene therapy. In pancreatic cancer cells, the combined efficiency of transduction and transcription determines the actual cell-killing effect of Ad5MK.

When clinical application is considered, not only anti-tumor activity but also toxicity for normal tissues should be taken into account. In this regard, Ad5MK showed specific cytotoxicity for midkine-positive cells both *in vitro* and *in vivo*. Despite the target selectivity of Ad5MK, weak E1A expression was found in midkine-negative cells after infection with 5 MOI of Ad5MK. Type 5 adenoviruses are commonly used in viral therapy experiments, but show

species-specific replication, and it has been reported that these adenoviruses do not replicate in mice or rats. There are no suitable animal models apart from the cotton rat to assess the toxicity of conditionally replicative adenoviruses, but replicative viruses have already shown at least low-level viral production and/or systemic toxicity in clinical trials[25,30,31]. The precise requirements for selective targeting to prevent damage to normal tissues *in vivo* need to be clarified in the future.

We demonstrated an anti-tumor effect of Ad5MK on midkine-positive tumor cells *in vivo* as well as *in vitro*. Our animal studies showed that mice treated with Ad5MK survived for significantly longer than the other groups. Even in the Ad5MK group, however, half of the mice died due to peritoneal dissemination after about 50 days. This may have been due to an inadequate dose of adenovirus or because of the regrowth of cancer cells with resistance to adenovirus-mediated gene therapy. In this study, we only gave a single dose of adenovirus intraperitoneally after tumor cell inoculation. In the future, we should investigate the optimum volume and number of adenovirus doses to improve the efficacy of Ad5MK treatment. Another way to improve the results could be employment of fiber-modified adenoviruses, which can enter cancer cells resistant to conventional adenoviral gene transfer in a coxsackievirus and adenovirus receptor (CAR)-independent manner.

Conclusion

Midkine expression was increased in pancreatic cancer cell lines and pancreatic cancer tissues. Ad5MK showed specific targeting of and cytotoxicity for midkine-positive cells both *in vitro* and *in vivo*. These results suggest that replication-competent adenoviruses based on the midkine promoter might have the potential to be used in gene therapy for pancreatic cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ET conceived of the study and performed experiments on adenoviruses. RD conceived of the study, and participated in its design and coordination and helped to draft the manuscript. KK conceived of the study and performed experiments on mice. TM conceived of the study and performed experiments on mice. DI conceived of the study and performed experiments on pancreatic cancer cell lines. MK conceived of the study and performed experiments on pancreatic cancer cell lines. AK conceived of the study and performed experiments on pancreatic cancer cell lines. KN conceived of the study and performed experiments on mice. TI conceived of the study and performed experiments on mice. TM conceived of the study and performed experiments on pancreatic cancer cell lines. MW conceived of the study, and participated in its design and coordination and helped to draft the manuscript. MT conceived of the study, and participated in its design and coordination and helped to draft the manuscript. SU conceived of the study, and participated in its design.

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Adenovirus vectors with chimeric type 5 and 35 fiber proteins exhibit enhanced transfection of human pancreatic cancer cells

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Abstract. Adenovirus (Ad) vectors are widely used for gene transfer. Efficient gene transfer into malignant cells is an important requirement for anticancer gene therapy, but transgene expression after transfer with adenoviral vectors varies among different cancer cell lines. Recently, Ad vectors containing chimeric type 5 and 35 fiber proteins have been developed. We evaluated the expression of coxsackie and adenovirus receptor (CAR), as well as integrins αV, ß3 and ß5, in seven human pancreatic cancer cell lines and assessed the relationship between expression of these molecules and Ad transfection efficiency. We compared the transfection efficiency of a conventional type 5 Ad vector (Ad5GFP) with that of an Ad vector containing chimeric type 5 and 35 fiber proteins (Ad5/35GFP), which expressed green fluorescent protein (GFP) driven by the cytomegalovirus promoter. There was strong CAR expression by AsPC-1, CFPAC-1 and PANC-1 cells, whereas the other cell lines showed weak expression. There was strong integrin ß3 expression by MIAPaCa-2, PANC-1 and Suit-2 cells, but expression by AsPC-1, BxPC-3, CFPAC-1 and HPAC cells was weak. Transfection efficiency of the vectors for human pancreatic cancer cell lines was not directly related to the CAR or integrin expression. However, transfection by Ad5/35GFP was significantly greater than by Ad5GFP at MOIs of 10 and 25 in all five human pancreatic cell lines. In conclusion, the Ad5/35GFP vector mediates more efficient gene transfer to human pancreatic cancer cells. These results may have implications for improving the efficiency of Ad-mediated gene transfer and developing adenoviral vectors.

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Key words: pancreatic neoplasms, conditionally replicative adenovirus vectors, fiber chimeric Ad5/35

Introduction

Adenovirus (Ad) has a linear double-stranded DNA genome of ~35,000 base pairs. The DNA is packaged in an icosahedral capsid protein, which is 70-100 nm in diameter. Each virion (viral particle) consists of a DNA core surrounded by a protein shell composed of 252 subunits called capsomeres, 240 of which are hexons and 12 of which are pentons. A rod-like structure called a fiber protrudes from the base of each penton (1,2). At least 51 serotypes of human Ad have been identified and classified into six distinct subgroups (A-F).

Among them, type 2 and type 5 (which belong to subgroup C) have been the most extensively studied and type 5 (Ad5) has been widely used as a vector for gene therapy because of its ability to infect a wide variety of cells, propagate at a high titer *in vitro* and support efficient expression of transgenes.

Infection of susceptible cells by Ad involves two distinct steps. In the first step, high-affinity binding of the virus to its primary receptor on the cell surface, the coxsackie and adenovirus receptor (CAR), occurs via the C-terminal knob domain of the fiber protein (3-5). In the second step, interaction between the RGD (Arg-Gly-Asp) motifs of the penton bases and secondary host cell receptors (αVß3 and αVß5 integrins) facilitates internalization of the virus via receptor-mediated endocytosis (6-8). Therefore, deficiency of CAR and/or αVß3 and αVß5 integrins will limit the gene transfer capacity of Ad5-based vectors.

Ad serotype 35 (Ad35), which belongs to subgroup B, has primarily been isolated from the kidneys and lungs of renal transplant patients (9). Ad35 has shorter fiber proteins (7 repeats of the ß sheet) than Ad5 (22 repeats of the ß sheet) and shows a different pattern of tropism from that of Ad5. CD46 is a receptor for Ad35 that is ubiquitously expressed in human cells (10,11). Ad vectors containing chimeric type 5 and 35 fiber protein have been developed (12,13). The Ad5/35 vector can transduce CAR-negative cell lines and various human cell lines more effectively than the Ad5

vector. Therefore, the Ad5/35 vector could be a promising candidate for efficient gene transfer to human cancer cell lines.

In the present study, to evaluate the possibility of developing gene therapy for pancreatic cancer, we examined the expression of CAR, as well as αV, ß3 and ß5 integrins, by human pancreatic cancer cell lines. Then we evaluated the ability of the Ad5/35 vector to transfer genes to these human pancreatic cell lines in comparison with that of the Ad5 vector.

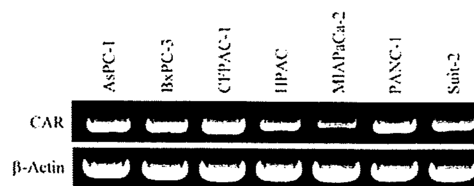

Figure 1. Expression of CAR mRNA by human pancreatic cancer cells. CAR mRNA was detected in all cell lines. ß-Actin was used as the internal control.

Materials and methods

Cell culture. Seven human pancreatic cancer cell lines were used. AsPC-1, BxPC-3, CFPAC-1, HPAC, MIAPaCa-2 and PANC-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in the medium recommended by the ATCC at 37˚C under a humidified atmosphere of 5% CO_2. Suit-2 cells were kindly provided by Dr H. Tomoda (National Kyushu Cancer Center, Fukuoka, Japan) and were cultured in DMEM (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS) (ICN Biomedicals, Aurora, OH). Human embryonic kidney (HEK) 293 cells were purchased from RIKEN Bioresourse Center (Tukuba, Japan). Each medium contained 100 U/ml of penicillin and 0.1 mg/ml streptomycin (Gibco-BRL).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using TRIzol Reagent (Life Technologies, Rockville, MD). Then cDNA was prepared by random priming from 1 μg of total RNA using a first-strand cDNA synthesis kit (Pharmacia Biotech, North Peapack, NJ) according to the manufacturer's instruction. Five microliters of first-strand cDNA solution was subjected to the polymerase chain reaction (PCR) with synthetic oligonucleotide primers (Nippon EGT, Toyama, Japan). The sequences of the primers used in this study were as follows: CAR, forward: 5'-GCAGGAGCCTTATAGGAA CTTTG-3', reverse: 5'-GGACCCCAGGGATGAATGAT-3' Integrin αV forward: 5'-GAAGATGTTGGGCCAGTTGT-3', reverse: 5'-TCCACAACCCAAAGTGTGAA-3', Integrin ß5 forward: 5'-AGCACCAAGAGAGATTGCGT-3', reverse: 5'-GGTGTTTCCACACTCTGGCT-3', ß-actin, forward: 5'-GGCATCGTGATGGACTCCG-3', reverse: 5'-GCTGGA AGGTGGACAGCGA-3'. The products were 195, 381, 246 and 613 base pairs in size, respectively.

The reproducibility of the technique and quality of the total RNA was confirmed by using ß-actin primers. Sequences were amplified by 30 PCR cycles (30 sec of denaturation at 94˚C, 30 sec of annealing at 57˚C and 1 min of extension at 72˚C), followed by final extension at 72˚C for 10 min. Then the PCR products were analyzed on 2% agarose gel.

Quantitative RT-PCR. To monitor CAR gene expression, we performed quantitative real-time RT-PCR analysis based on the Taq Man fluorescence method. This method uses a dual-labeled non-extendable oligonucleotide hydrolysis (Taq Man) probe in addition to the two amplification primers. The probe contains 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye, and 6-carboxytetramethyl-rhodamine (TAMRA) as a quencher for light emission. During the extension phase of PCR, the probe hybridizes to the target sequence and is then cleaved by the 5' to 3' exonuclease activity of Taq polymerase. The increase in the fluorescence signal of the reporter is proportional to the amount of the specific PCR product, providing highly accurate and reproducible quantification. The increase of reporter dye emission is detected by an automated sequence detector combined with analysis software (ABI Prizm 7700 Sequence Detection System; PE Applied Biosystems, Foster City, CA). The reaction conditions were set according to the manufacturer's protocol. The following primers and Taq Man probe were used for analysis. The specific primers for CAR were: 5'-CGACTGCAAGTACAA GTTTGAGAAC-3' (forward primer), 5'-TCTCCTGGCACT GAGCATTG-3' (reverse primer), 5' (FAM)-AAGGCACCC TGAAGAAGGCGCG-(TAMRA) 3' (Taq Man probe). The cycle parameters were heating at 95˚C for 10 min (for activation of Taq-Polymerase), followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Detection of ß-actin RNA for assessment of quality and normalization was done with the Taq Man ß-actin Control Reagent kit (PE Applied Biosystems), which utilizes standard Taq Man probe chemistry.

Immunocytochemistry. Pancreatic cancer cells grown on glass slides were washed with TBS, then fixed with 4% paraformaldehyde in TBS for 10 min, washed again with TBS, permeabilized in 0.2% Triton X-100 in TBS for 5 min and washed and blocked with TBS containing 1% BSA. Fixed and permeabilized cells were incubated with a monoclonal anti-CAR RmcB antibody (#05-644, Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature, rinsed twice with TBS and then incubated for 30 min with the secondary antibody (goat anti-mouse IgG) (#62-6500, Zymed Laboratories, South San Francisco, CA) at room temperature. Cells were washed 3 times with TBS and incubated for 20 min with Cy3-conjugated donkey anti-goat IgG antibody (#Ap180C, Chemicon International, Temecula, CA). After the final wash, a coverslip was mounted on each slide with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Then the cells were examined under a fluorescene microscope (Olympus, Tokyo, Japan).

Flow cytometry. Cells were briefly trypsinized, washed with PBS and incubated for 1 h at 4˚C with primary antibodies targeting CAR and integrin ß3 in PBS containing 2% FBS. Mouse monoclonal anti-CAR antibody was used at a dilution of 1:100. Then the cells were washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (#A1102, Molecular Probes, Eugene, OR) at 1:1,000 for 45 min at 4˚C. Cells were washed again with PBS and analyzed by flow

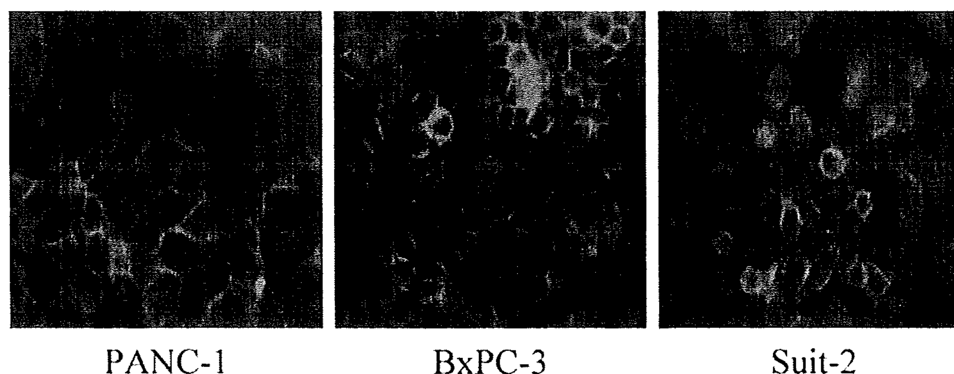

Figure 2. Membrane expression of CAR on pancreatic cancer cells. Cells were grown on glass slides, fixed with methanol and immunostained with an anti-CAR monoclonal antibody (RmcB) and a Cy3-conjugated donkey anti-mouse antibody. Confocal images of the pancreatic cancer cell lines PANC-1, BxPC-3 and Suit-2 are shown.

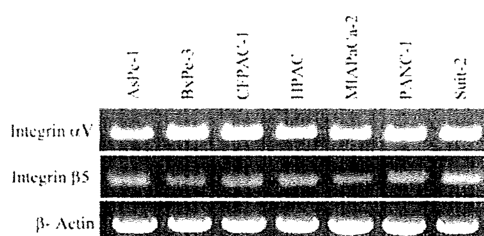

Figure 3. Expression profile of integrin αV and ß5 mRNA in human pancreatic cancer cell lines. ß-Actin was used as the internal control.

Table I. Relative mRNA levels of CAR in human pancreatic cancer cells.

Cell lines	CAR mRNA/ß-actin mRNA (mean ± SE)
AsPC-1	0.130±0.017
BxPC-3	0.100±0.009
CFPAC-1	0.087±0.004
HPAC	0.027±0.003
MIAPaCa-2	0.012±0.001
PANC-1	0.071±0.003
Suit-2	0.023±0.001

cytometry on a FACSCalibur using Cell Quest software (Becton-Dickinson, Franklin, NJ).

Adenovirus-mediated gene transduction of human pancreatic cancer cells. A type 5, E1A-deleted, replication-defective Ad vector containing the green fluorescent protein gene (Ad5GFP) was constructed with the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The Ad5/35 GFP vector was purchased from Zymed Laboratories Inc. Each Ad was propagated in HEK 293 cells, purified by two rounds of cesium chloride density centrifugation, dialyzed and stored at -70˚C. Viral titers were determined with an Adeno-X Rapid Titer kit (BD Biosciences Clontech, Palo Alto, CA).

Transfection efficiency for human pancreatic cancer cells was compared between the Ad5GFP and Ad5/35 GFP vectors. Cancer cells were transfected with Ad5GFP or Ad5/35 GFP at various multiplicities of infection (MOI) for 16 to 18 h after seeding. At 48 h after transfection, GFP expression in the cells was determined by FACS analysis.

Statistical analysis. Results are presented as the mean ± SEM for quantitative experiments and each experiment was performed at least three times independently. To compare GFP expression by flow cytometry, the t-test was used. Statistical analysis was done with JMP statistical software and significance was considered to be present at $p<0.05$.

Results

Expression of CAR and integrins by pancreatic cancer cell lines. Expression of CAR mRNA by the human pancreatic cancer cell lines is shown in Fig. 1. This mRNA was detected in all of the cell lines tested by RT-PCR. The CAR mRNA/ß-actin mRNA expression ratio is shown in Table I for each cell line.

Next, we performed immunocytochemistry and flow cytometry to determine the surface expression of CAR by the cells lines, because CAR is a membrane-associated protein. Immunofluorescence was observed on the cell membrane and not in the cytoplasm. Fig. 2 shows typical membrane expression of CAR by AsPC-1, CFPAC-1 and PANC-1 cells.

We also examined the expression of integrins αV and ß5 by human pancreatic cancer cells. The mRNA of integrins αV and ß5 was expressed in all cell lines tested (Fig. 3).

The relative expression of CAR and integrin ß3 was measured by flow cytometry. There was strong expression of CAR by AsPC-1, CFPAC-1 and PANC-1 cells, while expression by BxPC-3, HPAC and Suit-1 and MIAPaCa-2 cells was weak (Fig. 4). Protein expression was identical to mRNA expression for each cell line. There was strong integrin ß3 expression by MIAPaCa-2, PANC-1 and Suit-2 cells, while expression by AsPC-1, BxPC-3, CFPAC-1 and HPAC cells was weak (Fig. 5). The mean fluorescence intensity (MFI) of CAR and integrin ß3 expression is summarized in Table II.

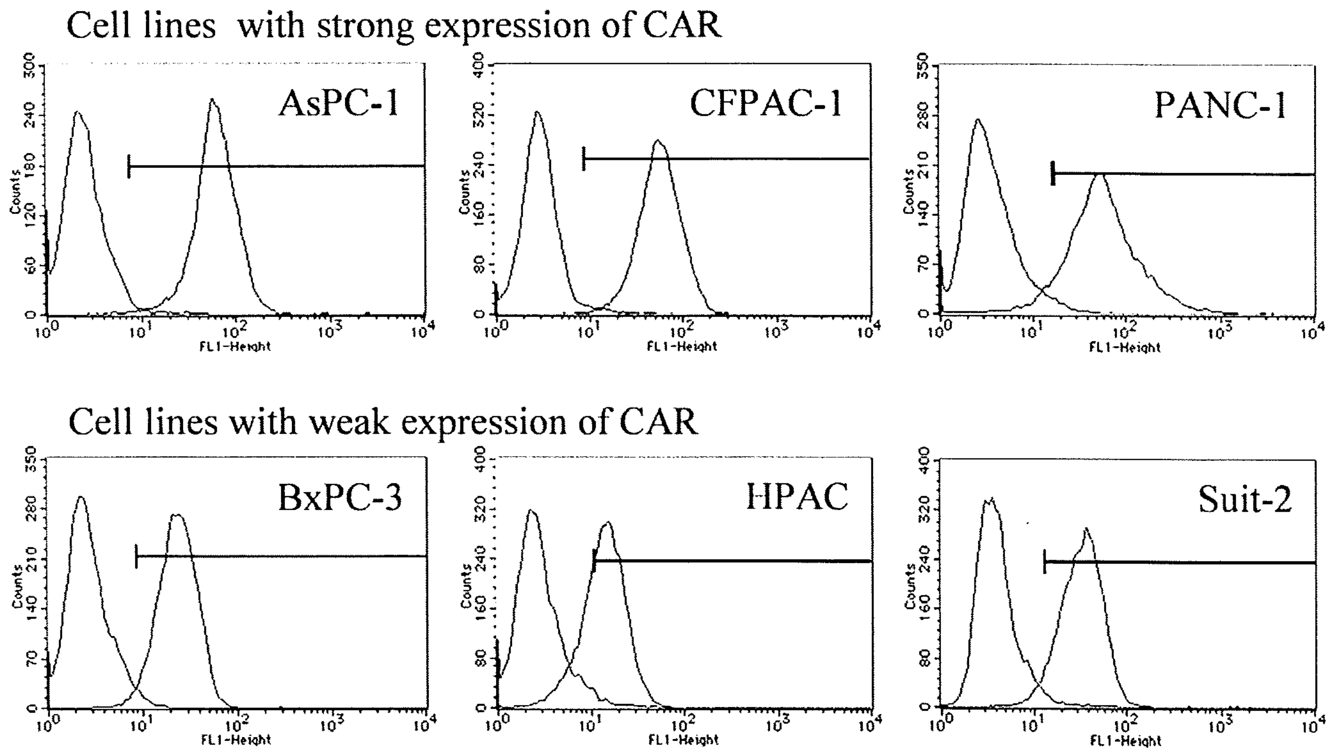

Figure 4. Flow cytometric analysis of the cell membrane expression of CAR protein after immunostaining with RmcB (anti-CAR antibody). AsPC-1, CFPAC-1 and PANC-1 cells show strong expression of CAR (upper row), while BxPC-3, HPAC and Suit-1 show weak expression (lower row).

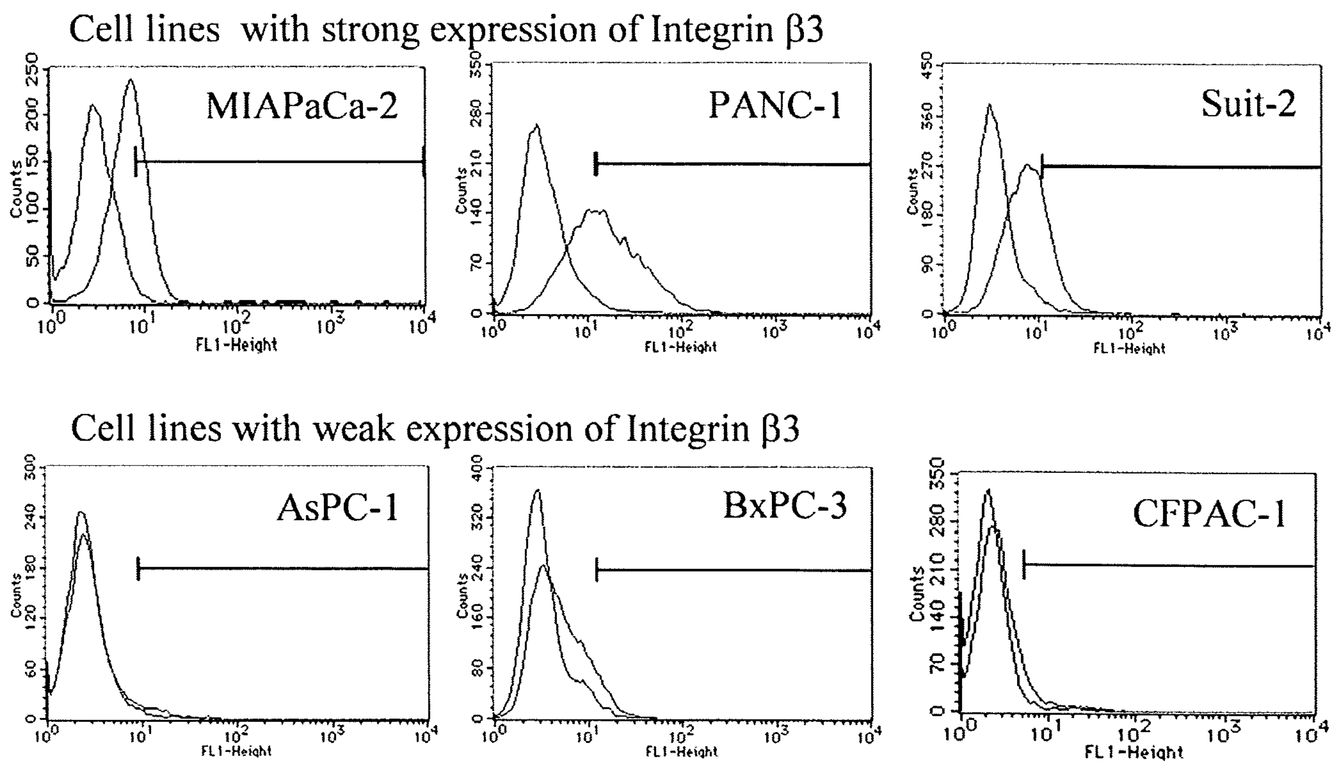

Figure 5. Flow cytometric analysis of the expression of integrin ß3 by human pancreatic cancer cell lines after staining with a primary anti-integrin ß3 monoclonal antibody followed by a fluorescein-conjugated goat anti-mouse secondary antibody. MIAPaCa-2, PANC-1 and Suit-2 cells show strong expression of integrin ß3 (upper row), while AsPC-1, BxPC-3 and CFPAC-1 cells show weak expression (lower row).

Sensitivity of human pancreatic cancer cell lines to infection with adenovirus type 5 and chimeric type 5/35. The induction efficacy of Ad5GFP vector into human pancreatic cancer cells was examined. Cancer cells were infected with Ad5GFP at a MOI of 1, 10, 25, 50 and 100 at 16 to 18 h after seeding. After 48 h, GFP-expressing cells were detected by flow cytometry.

Figure 6. Human pancreatic cancer cells were seeded into a 6-cm plate and the Ad5GFP vector (1, 10, or 100 MOI) was added at 16 to 18 h after seeding. After 48 h, GFP expression was measured by flow cytometry. Data represent the mean ± SE of three experiments.

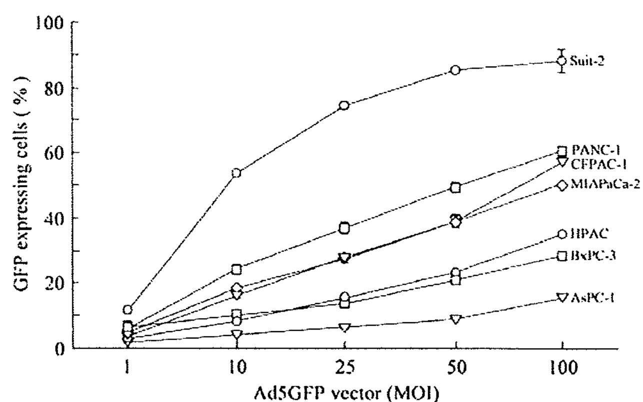

Figure 7. MOI-dependent increase of GFP expression by human pancreatic cancer cells infected with Ad5GFP at various MOIs. In each cell line, GFP expression increased in a MOI-dependent manner. Data are the mean ± SE of three experiments.

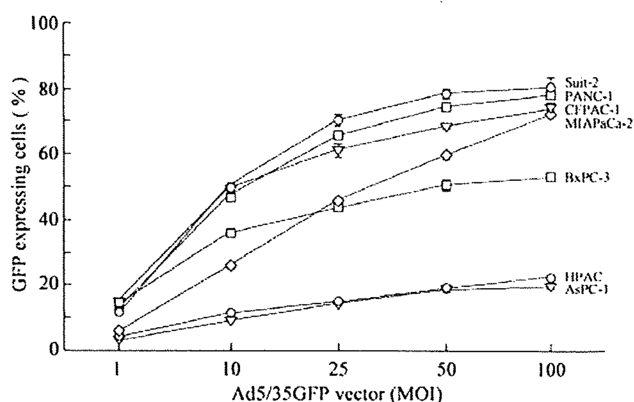

Figure 8. MOI-dependent increase of GFP expression by human pancreatic cancer cells infected with Ad5/35GFP at various MOIs. In each cell line, GFP expression increased in a MOI-dependent manner. Data are the mean ± SE of three experiments.

The relative mean fluorescence intensity increased in a MOI-dependent manner. As shown in Fig. 6, GFP expression driven by the Ad5GFP vector in AsPC-1, MIAPaCa-2 and Suit-1 cells increased as the MOI increased.

The transfection efficiency of the Ad5 and Ad5/35 vectors was also cell-specific. GFP expression at 48 h after infection by Ad5GFP and Ad5/35GFP is summarized in Figs. 7 and 8. Both vectors induced a dose-dependent increase of GP

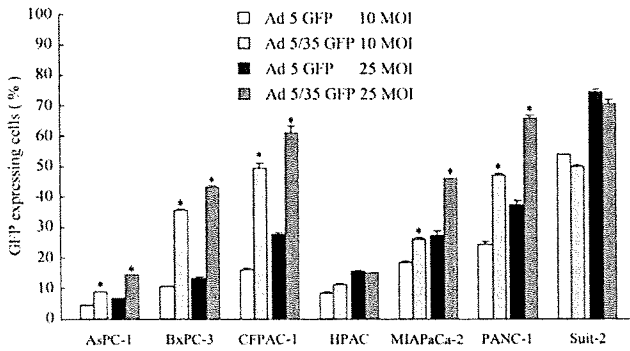

Figure 9. Comparison of the transfection efficiency of the conventional Ad5 vector Ad5GFP and chimeric Ad5/35GFP vector in human pancreatic cancer cell lines. Data are the mean ± SE of three experiments. Asterisks indicate a significant difference compared with the corresponding MOI of Ad5GFP.

Table II. The cell surface expression of CAR and integrin ß3 in human pancreatic cancer cell lines.

Cell lines	Mean fluorescence intensity (MFI)	
	CAR	Integrin ß3
AsPC-1	23.3±0.8	1.03±0.02
BxPC-3	8.5±0.1	1.05±0.01
CFPAC-1	23.6±0.4	1.12±0.02
HPAC	4.9±0.1	1.14±0.01
MIAPaCa-2	3.8±0.1	2.13±0.08
PANC-1	15.4±0.1	4.25±0.09
Suit-2	7.7±0.1	2.10±0.02

expression by all cell lines. However, at the same MOI (e.g. 10 or 25), the percentage of GFP-positive cells was significantly higher with the chimeric type Ad5/35GFP vector than the Ad5GFP vector, except in the case of HPAC and Suit-2 cells (Fig. 9).

Discussion

It has been reported that two cell surface events, attachment and internalization, are required for Ad to enter host cells (7). First, the viral fiber protein attaches to the CAR on the surface of a host cell (3) and then the virus enters the cell through interaction of its penton base with integrins αVß3 and αVß5 on the host cell membrane (7). Therefore, the expression of these cell surface proteins and their correlation with viral transfection efficiency have been investigated and it has been reported that integrins αVß3 (14,15), αVß5 (16,17) and αVß1 (18) are important for efficient gene transfer as well as CAR (19,20). Similarly, Qin *et al* (21) have reported that CAR expression *in vitro* is correlated with susceptibility to Ad infection.

Pearson *et al* (22) have reported that a positive association between the level of integrin ß3 expression and efficiency of Ad gene transfer. They postulated that although CAR and integrins αVß5, αV and ß1 are all required for the entrance of adenoviral vectors into host cells, these proteins may not be the limiting factor once a minimum level of expression is present. Apart from the expression patterns of these molecules, it has been stated that characterization of molecules having a known association with viral entry into cells is important to improve adenovirus-mediated gene transfer.

Ad vectors containing chimeric type 5 and 35 fiber protein have been developed (12,13). The Ad5/35 vector can transduce CAR-negative cell lines and various human cell lines more effectively than the Ad5 vector, so this vector could be a promising candidate for efficient gene transfer to human cancer cell lines. The group B serotype Ad35 virus recognizes a different receptor from CAR (23), which has been identified as CD46 (10,24), a molecule that is ubiquitously expressed by human cells (11). The Ad5/35 chimeric vector is identical with the Ad5 parent vector, except for the difference of fiber construction. Because both vectors used in this study contained a GFP reporter transgene driven by the cytomegalovirus promoter, differences of transfection efficiency and transgene expression should be related to the influence on the viral vector of this change in the fiber protein.

In this study, we evaluated the transfection efficiency of Ad5 and Ad5/35 using several human pancreatic cancer cell lines and found that the chimeric Ad5/35 vector could transfect the cell lines more efficiently than the conventional Ad5 vector. For example, at 25 MOI, 43.5% of BxPC-3 cells expressed GFP after transfection with Ad5/35GFP, while the conventional Ad5GFP vector only achieved GFP expression in 13.5% of BxPC-3 cells. There were only two cell lines (HPAC and Suit-2) for which the transfection efficiency was equal between Ad5 and Ad5/35.

Improvement of transfection efficiency by chimeric vectors such as Ad5/35 has also been reported for other cells. After modification of the viral fiber (such as Ad5/3 or Ad5/35), better transfection efficiency is seen for ovarian cancer cells and hematopoietic stem cells compared with that of the parent Ad5 vector (25,26). These chimeric vectors bind to other receptors than CAR and enter the cell via an integrin-independent pathway (23,27). The Ad5/35 chimeric vector has been shown to infect both CAR-positive and CAR-negative hematopoietic stem cells efficiently and to mediate high levels of transgene expression (28).

As expected, we found no direct relationship between Ad transfection efficiency and expression of CAR or integrin ß3. Interestingly, the vectors had low efficiency for AsPC-1 cells with almost no integrin ß3 expression. In conclusion, the Ad5/35 chimeric vector shows better transfection efficiency for human pancreatic cancer cells than that of Ad5 independent of the cell surface expression of molecules such as CAR and integrins. Modification of the viral fiber protein significantly improves the susceptibility of some pancreatic cancer cell lines that were not targeted by the conventional Ad5 vector. The enhancement of transgene expression that was achieved by infecting previously refractory cells suggests that modified fiber vectors may be useful for further studies of gene delivery to cancer cells.

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Radiation Enhances Adenoviral Gene Therapy in Pancreatic Cancer via Activation of Cytomegalovirus Promoter and Increased Adenovirus Uptake

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Abstract **Purpose:** Adenovirus-mediated gene therapy combined with radiation is expected to be a new approach to treat pancreatic cancer. However, there are no reports of definitive effects of radiation on adenovirus-mediated gene therapies. In the present study, we investigated the effect of radiation on the transduction efficiency of an adenovirus-based gene therapy.

Experimental Design: We used adenovirus expressing NK4 (Ad-NK4), an antagonist for hepatocyte growth factor, as a representative gene therapy. Pancreatic cancer cells preinfected with Ad-NK4 were irradiated, and NK4 levels in culture media of these cells were measured. We investigated cytomegalovirus (CMV) promoter activity and uptake of adenovirus in these cells. To examine the effect of radiation *in vivo*, Ad-NK4 was given to irradiated subcutaneous tumors in nude mice, and NK4 levels in tumors were measured.

Results: NK4 levels in culture media of irradiated cells were 4.5-fold ($P < 0.01$) higher than those of nonirradiated cells. Radiation enhanced activation of the CMV promoter and adenovirus uptake ($P < 0.01$), leading to increased levels of NK4. We found that activation of p38 mitogen-activated protein kinase and up-regulation of dynamin 2 may be involved in the radiation-induced activation of the CMV promoter and adenovirus uptake, respectively. NK4 levels in irradiated tumors were 5.8-fold ($P = 0.017$) higher than those in nonirradiated tumors.

Conclusions: The present findings suggest that radiation significantly improves the efficiency of adenovirus-mediated gene transfer in pancreatic cancer and probably contributes to decreasing the dose of adenovirus required for gene transfer and controlling side effects of adenovirus infection in nonirradiated normal tissue.

On the basis of recent advances in our understanding of the molecular biology of a variety of cancers (1-4), molecular therapies, which target tumor-specific pathways and interfere with key regulatory cellular functions, including proliferation, differentiation, metastasis, and survival of cancer cells, have been extensively studied (5, 6). Many researchers have used monoclonal antibodies, specific antagonists, or specific small molecule inhibitors as antitumor agents against cancer-associated genes. However, monoclonal antibodies are expensive, and small molecule inhibitors have low specificity. These agents also induce allergic reaction, such as skin rash (5). The use of viral vectors, which have high gene transfer efficiencies, is one approach for molecular therapy and is often useful for expressing an antagonist of a target protein. Adenovirus-based vectors are often used due to their high transduction efficiency and high levels of transient expression of the transfected gene (7).

Pancreatic cancer is a leading cause of cancer-related death in industrial countries (8, 9). Most patients with pancreatic cancer have poor outcomes because early diagnosis is difficult and because conventional therapies have limited effectiveness (10). Recently, advances in our understanding of the genetics and epigenetics of pancreatic cancer revealed that alterations of several tumor-related genes, including *K-ras*, *p53*, *matrix metalloproteinase*, *hepatocyte growth factor* (*HGF*), and *epidermal growth factor receptor* (11-16), may underlie the aggressiveness of this neoplasm and its resistance to conventional therapies (6). Therefore, molecular therapies for pancreatic cancer are promising new approaches to treat this often fatal disease. Investigators have used adenovirus-mediated gene transfer to treat pancreatic cancer and reported that adenovirus-mediated gene therapy inhibited progression of pancreatic cancer *in vivo* and *in vitro* (17, 18). However, clinical trials revealed that it is

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difficult to eradicate pancreatic tumors with adenovirus-mediated gene therapy alone (19, 20). There is also concern that the dose of adenovirus necessary to achieve therapeutic effectiveness may have some significant toxicity. Therefore, adenovirus alone may not be an effective treatment for cancer, and it may be necessary to combine adenovirus-mediated gene therapies with conventional treatments to maximize the antitumor effects for pancreatic cancer.

Recently, the combination of radiotherapy and adenovirus-mediated gene therapy has been reported to be effective for cancer treatment. Shi et al. (21) reported that adenovirus-mediated gene therapy targeting endostatin enhanced the antitumor effect of radiation therapy in colorectal cancer. Similarly, Geoerger et al. (22), Portella et al. (23), and Rogulski et al. (24) reported that ONYX-015, an E1B-55-kDa gene-deleted adenovirus that replicates selectively in and lyses tumor cells with abnormalities in p53 function, combined with radiation therapy is a promising strategy for treatment of gliomas and thyroid cancers and that there are synergistic effects with such combination therapies. Previously, we reported that gene therapy with an adenovirus vector expressing NK4 (Ad-NK4), which acts as an HGF antagonist, could be a viable option for treatment of pancreatic cancer (25-27). More recently, we reported that radiation therapy enhances the invasiveness of pancreatic cancer cells via the activation of the HGF receptor c-Met and that NK4 inhibits this radiation-enhanced invasiveness (28, 29), suggesting that a combination of radiation therapy and NK4 gene therapy may be a viable strategy for treatment of pancreatic cancer. However, the effects of radiation on features of adenovirus-mediated gene therapies, such as adenovirus uptake and efficiency of target gene expression, have remained unknown.

In the present study, to investigate the effect of radiation on the efficiency of transfer and expression of a target gene, we examined the effect of radiation on NK4 expression by an adenovirus-based vector (Ad-NK4) as a representative gene therapy. We found that radiation increased expression of NK4 via enhanced activation of the cytomegalovirus (CMV) promoter, which is commonly used as the target gene promoter in adenovirus vectors, and we also found that radiation enhanced

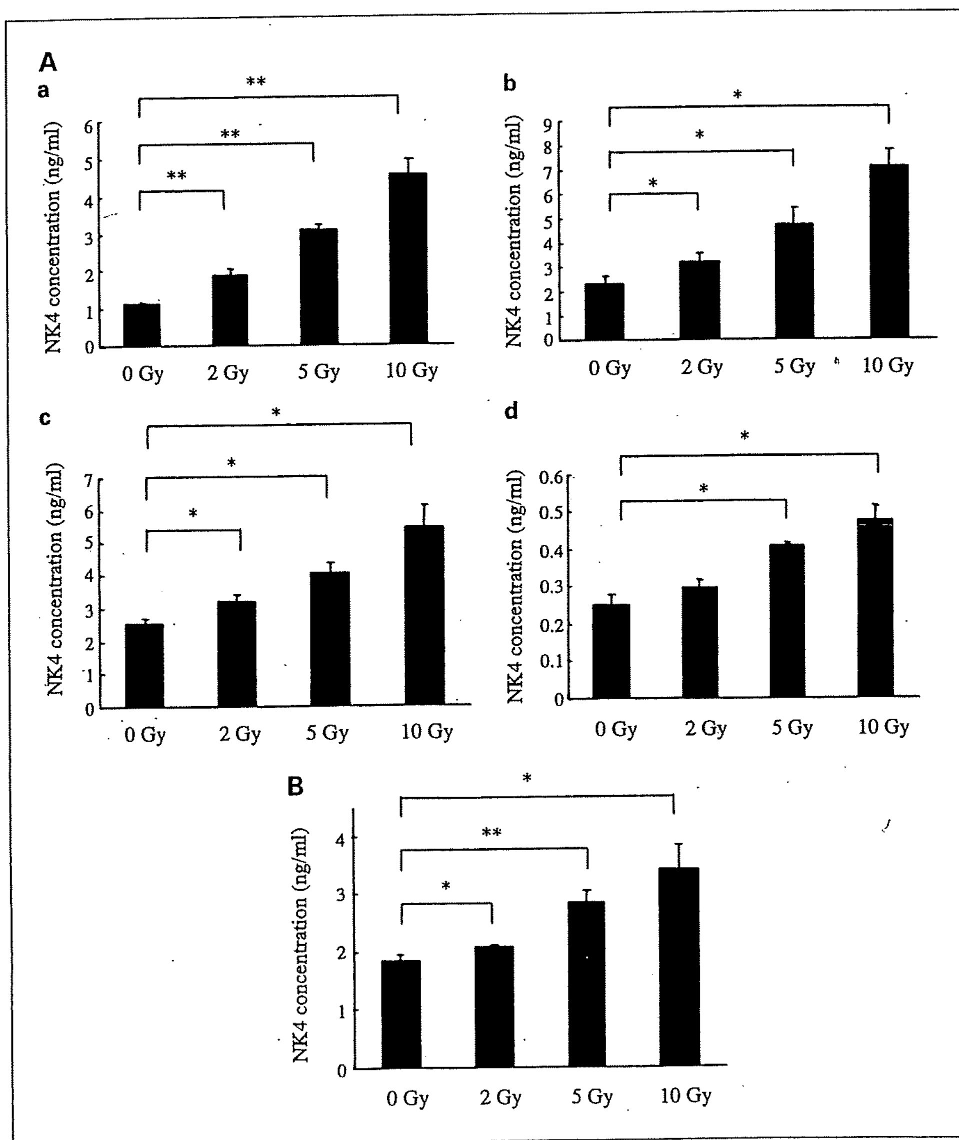

Fig. 1. Radiation significantly increases NK4 expression in Ad-NK4-infected cells. *A*, pancreatic cancer cells were irradiated and infected with Ad-NK4 at MOI of 10 at 24 h after radiation. NK4 levels in culture media were measured by ELISA on postinfection day 2. *a*, SUIT-2; *b*, KP-1N; *c*, PANC-1; and *d*, AsPC-1. Columns, mean of three independent samples; bars, SD. **, $P < 0.01$; *, $P < 0.05$. *B*, proteins were isolated from irradiated SUIT-2 cells infected with Ad-NK4 as described above on postinfection day 2, and NK4 concentrations were determined by ELISA.