

p16^{INK4a} Expression Adenovirus Vector to Suppress Pancreas Cancer Cell Proliferation¹

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ABSTRACT

The prognoses of pancreatic cancer patients have been miserable even after radical surgery, and adjuvant therapy is necessary to improve the surgical results. p16^{INK4a} (p16) is tight-binding and inhibitory protein for cyclin-dependent kinase 4 to induce G₁ arrest of the cell cycle. p16 gene deletion is frequently identified in human pancreas cancer. The impaired gene function of p16 might be a major factor of the uncontrolled proliferation and malignancy of pancreas cancer cells. In this study, we investigated the effect of adenovirus p16 expression vector for pancreas cancer cell proliferation to clarify whether the vector might be a promising mode to assist the surgical therapy for pancreas cancer. We constructed the adenovirus p16 expression vector AdexCACSp16 by inserting p16 cDNA to a cassette cosmid containing a nearly full-length adenovirus type 5 genome with E1 and E3 deletions. Thereafter, we assessed the activity of AdexCACSp16 to induce p16 gene mRNA expression in pancreas cancer cell line MIAPaCa-2 and to control cell proliferation. AdexCACSp16 induced a high level of p16 gene mRNA expression in MIAPaCa-2 cells with 1 h contact to the cells. The cell proliferation was significantly suppressed by AdexCACSp16 compared with the control adenovirus group. These data indicate that AdexCACSp16 has the potential to induce p16 gene expression and control pancreas cancer cell proliferation and that the adenovirus p16 expression vector AdexCACSp16 might be a possible method of gene therapy to improve the surgical therapeutic results for pancreas cancer.

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INTRODUCTION

The recurrence of pancreas cancer occurs frequently, even after radical surgery, and the prognosis compared with other organs is extremely poor, despite many clinical trials (1). The overall 5-year survival rate of pancreas cancer is <20% and is only ~25% among curative cases (2, 3). These data indicate that there is a limitation on surgical treatment for pancreas cancer and that approaches other than past adjuvant methods are necessary to improve the survival rate.

The deletion of the p16^{INK4a} (p16) gene is identified frequently in human cancer cell lines. The gene MTS1,³ which was sought as the deleted locus in human cancer chromosomes, has been found to be identical with this p16 gene (4, 5). It has been reported that homozygous or heterozygous deletion of the p16 gene is identified frequently in the human cancers with poor prognoses such as pancreatic and esophageal cancer. Caldas *et al.* (6) reported that allelic deletions of the p16 locus were detected in 85% of informative cases and that they found homozygous deletions in 15 (41%) and sequence changes in 14 (38%) among 10 cell lines and 27 xenografts from human pancreas cancer. On the other hand, the p16 deletion is rare in gastric, colon, and hepatocellular carcinomas, whose prognoses are not so poor (7).

p16 is tight-binding protein for CDK4 and negatively regulates the cell cycle through a specific inhibition of the cyclin-CDK4 complex activity. In a condition of p16 gene deletion or its dysfunction, the gap phase (G) in the cell cycle may uncontrollably proceed to synthesis (S) and mitosis (M), and then the cell proliferation will be accelerated. Therefore, the majority of pancreas cancer cells might be in a state in which the cell cycle is unregulated because of deletions or mutations of the p16 gene. These data suggest that this impaired gene function might be a major factor of the uncontrolled cell proliferation and tumor malignancy of human pancreas cancer (8). In this study, we constructed the adenovirus p16 expression vector and then investigated whether it could control the proliferation of pancreas cancer MIAPaCa-2 cells in which the p16 gene was deleted and discussed its significance for the adjuvant therapeutic method in the surgical treatment of pancreas cancer.

MATERIALS AND METHODS

LacZ Expression. Prior to p16 expression vector experiment, we set up a LacZ expression experiment to investigate whether the adenovirus vector works well enough to express the gene in pancreas cancer MIAPaCa-2 cells. Adenovirus LacZ expression vector (Adex1CALacZ) was a generous gift from Dr. I. Saito (The Institute of Medical Science, The University of

³ The abbreviations used are: MTS1, multiple tumor suppressor 1; CDK, cyclin-dependent kinase 4; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MOI, multiplicity of infection.

Tokyo, Tokyo, Japan) (9). We introduced Adex1CALacZ into MIAPaCa-2 for 60 min and washed the cells twice with PBS, after which the cells were incubated. For cell staining, 3 days after infection, the cells were washed twice with PBS again, fixed with 0.25% glutaraldehyde, and stained with 0.1% X-gal (10).

Construction of Adenovirus p16 Expression Vector.

As the first step of construction of adenovirus p16 expression vector, we inserted p16 cDNA into the *Swa*I site of a cassette cosmid containing a nearly full-length adenovirus type 5 genome with E1 (nucleotides 454-3328) and E3 (nucleotides 28592-30470) deletions through an *in vitro* package (Stratagene). The inserted p16 sequence was cut out from p16 cDNA by a PCR procedure as the size of three bases upstream to the starting codon and 31 bases downstream from the stop codon, fitting Kozak's rule (11), to improve gene expression. In the cassette cosmid into which p16 had been inserted, the *p16* gene could be of right or leftward orientation. The efficiency of expression was known to be better in leftward orientation; therefore, we selected the leftward-directed cassette cosmid by restriction-enzyme digestion (9).

To produce a recombinant adenovirus (AdexCACSp16), the expression cosmid and adenovirus DNA-terminal protein complex were cotransfected into 293 cells by calcium phosphate precipitation after the digestion of adenovirus DNA-terminal protein complex by EcoT22I (TaKaRa, Otsu, Japan). Recombinant adenovirus was isolated and expanded in 293 cells, and then the viral solutions were stored at -80°C . The virus titer was determined by plaque assay using 293 cells as described (12).

Cell Culture and Infection of Adenovirus Vectors.

The human pancreas cancer cell line MIAPaCa-2 was obtained from the Japanese Cancer Research Resources Bank. We also used human embryonic kidney cell line 293 cells, which were obtained from American Type Culture Collection, for the adenovirus recombination. These two cell lines were cultured in DMEM containing 10% fetal bovine serum.

The pancreas cancer cell MIAPaCa-2 was seeded and cultured in 96-well plates at a density of 2×10^5 cells/1.0 ml of medium supplemented with 10% fetal bovine serum for 24 h. Immediately before infection, the culture medium was removed from the wells, and then the suspension of adenovirus vectors [AdexCACSp16, Adex1CALacZ, and Adex1w1 (control virus)], at MOI of 30, was plated onto the cells of the monolayer. The cell-vector contact as infection was kept for 1 h, and subsequently the cells were incubated by adding DMEM medium.

Northern Blot Hybridization. We evaluated the activity of adenovirus p16 expression vector to express *p16* gene mRNA in the pancreas cancer cell line MIAPaCa-2 by Northern blot hybridization. Total RNA was obtained from MIAPaCa-2 cells dishes at 24 h incubation after infection. The RNA extraction was carried out following the guanidinium thiocyanate/CsCl procedure involving high-speed centrifugation (13). The cells were mixed with guanidinium thiocyanate solution and homogenized at 4°C . Each 1-ml homogenate had 0.4 g of CsCl added to it and was then layered onto a 0.6-ml cushion of 5.7 M CsCl with 0.1 M EDTA. This solution were centrifuged at 45,000 rpm for 12 h at 20°C , and then RNA pellets were dissolved in a

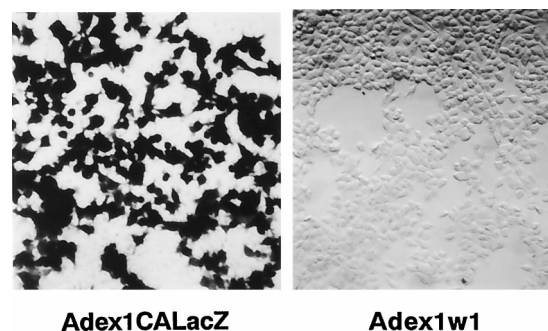


Fig. 1 Adenovirus LacZ expression vector (Adex1CALacZ) mediated LacZ expression in MIAPaCa-2 cells. *Adex1CALacZ* panel, the X-gal staining of MIAPaCa-2 cells 3 days after infection of Adex1CALacZ. Approximately 100% of MIAPaCa-2 cells were stained with X-gal at MOI 30. *Adex1w1* panel, X-gal staining of MIAPaCa-2 cells 3 days after infection of control vector Adex1w1. No cells expressed LacZ. It could be confirmed that *LacZ* gene expression was induced by adenovirus expression vector (Adex1CALacZ) in pancreas cancer MIAPaCa-2 cells.

buffer of 10 mM Tris-HCl, 5 mM EDTA, and 1% SDS and then purified through the procedure using chloroform/*n*-butyl alcohol and ethanol.

The Northern blots hybridization was prepared with 10 μg of total RNA obtained from MIAPaCa-2 cells. The electrophoresis of RNA was performed on 1.0% agarose gels prepared in MOPS buffer (0.02 M morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0) containing 2.2 M formaldehyde (14). After the identification of 28S and 18S RNA stained by ethidium bromide, the denatured RNA was transferred to the nylon membrane filters (Amersham International, Buckinghamshire, United Kingdom). We used p16 cDNA as the *p16* gene probe (15). The filters were incubated with this ^{32}P -labeled probe for 15 h in a solution containing 50% formamide, $5\times$ saline and sodium citrate, $5\times$ Denhardt's solution, and 20 mg/ml denatured salmon sperm DNA after prehybridization. The filter was washed in $2\times$ SSC containing 0.1% SDS at 60°C and then exposed to X-ray film.

Effect of AdexCACSp16 on Cell Proliferation. The MIAPaCa-2 cells were seeded in 48-well plates and infected with adenovirus vectors (AdexCACSp16 and Adex1w1) at MOI of 30. The number of the living cells was counted before infection, on the first day, third day, fifth day, and seventh day after infection. Thereafter, we compared the number of cells between the groups of AdexCACSp16, Adex1w1, and no virus by statistical analyses, and then we assessed the effect of the p16 expression vector on MIAPaCa-2 cell proliferation. As a statistical analysis, we used ANOVA corrected for repeated measures to analyze the data for the difference between the three groups. Post hoc analysis was performed at individual time points using Tukey's test with a computer statistical package. $P < 0.05$ was considered significant.

RESULTS

LacZ Expression. As shown in Fig. 1, nearly 100% of MIAPaCa-2 cells were stained with X-gal at MOI 30 on 3 days after infection of Adex1CALacZ. It could be confirmed that the

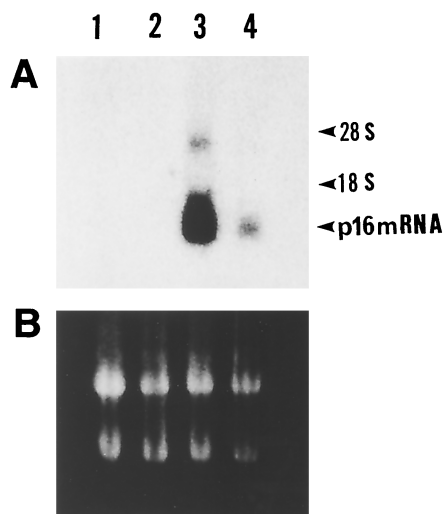


Fig. 2 Northern blot hybridization to identify p16 mRNA expression in MIAPaCa-2 cells induced by AdexCACSp16. **A:** Lane 1, no virus; Lane 2, control virus (Adex1w1); Lane 3, AdexCACSp16 of MOI 30; Lane 4, AdexCACSp16 of MOI 3. The p16 mRNA expression could not be identified in the lanes with no virus and control virus (Adex1w1), as shown in Lanes 1 and 2. However, a high level of p16 mRNA expression could be detected at the expected position in MIAPaCa-2 to which AdexCACSp16 had been added, as shown in Lanes 3 and 4. **B:** ethidium bromide stainings of RNA.

LacZ gene expression was induced in almost all cells by Adex1CALacZ, and the adenovirus expression vector constructed in this system works well enough to express the gene in pancreas cancer cells MIAPaCa-2. The adenovirus-mediated p16 vector of the subsequent experiment could be expected to efficiently express p16 protein in MIAPaCa-2 cells.

Northern Blot Hybridization. Fig. 2 shows Northern blot hybridization to detect p16 mRNA expression in MIAPaCa-2 cells induced by AdexCACSp16. Fig. 2, Lanes 1 and 2, indicated p16 mRNA expression in MIAPaCa-2 cells with no virus (medium) and with control virus (Adex1w1), respectively. Because MIAPaCa-2 cells deleted *p16* gene homozygously (6), p16 mRNA expression could not be identified in the no-virus and control-virus (Adex1w1) lanes. However, a high level of p16 mRNA expression could be detected at the expected position of Fig. 2, Lanes 3 and 4, which represented the RNA extracted from MIAPaCa-2 cells after the incubation for 24 h subsequently to 1 h of infection with vector (AdexCACSp16) at MOI 30 and MOI 3, respectively.

Cell Proliferation. Fig. 3 shows the cell proliferation curves of MIAPaCa-2 cells with AdexCACSp16, Adex1w1 (control virus), and control (no virus). The cell number had increased during the incubation in those three groups; however, the cell proliferation curve of the AdexCACSp16 group was significantly lower than the Adex1w1 group when the comparisons were run using ANOVA ($P < 0.05$). This result indicated that p16 expression vector (AdexCACSp16) could suppress the cell proliferation of MIAPaCa-2.

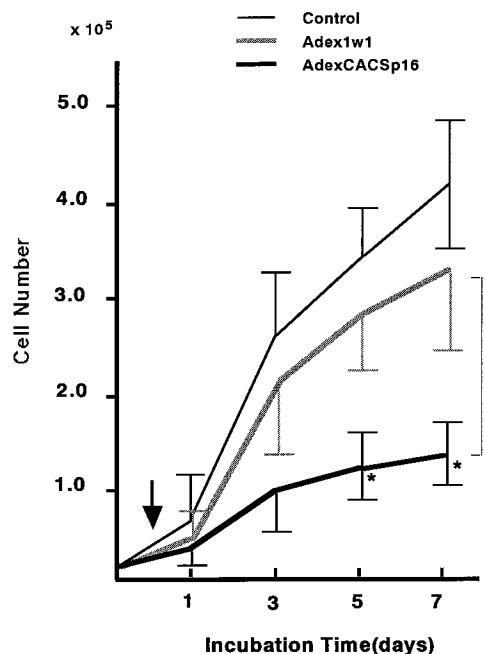


Fig. 3 The cell proliferation curves of MIAPaCa-2 cells with AdexCACSp16, Adex1w1, and control (no virus). Cell proliferation was significantly suppressed by the p16 expression vector (AdexCACSp16), as compared with the control adenovirus group (Adex1w1). Arrow, time point of adding the vectors. Data are shown as means; bars, SD. *, $P < 0.05$, AdexCACSp16 compared with Adex1w1.

DISCUSSION

The postsurgical prognoses of pancreas cancer are extremely poor. In our Department of Surgery, Chiba University Hospital, the 1-year survival rate is ~39%, and the 5-year survival rate is <15%; these data are close to the average of the Japanese surgical society (2, 3). These data indicate that there is a limitation to surgical treatment for pancreas cancer. The clinical trials, such as radiation and chemotherapy, have been carried out in a large number of hospitals to improve postoperative prognoses; however, such approaches have never yielded good results as compared with cases of cancer in other organs (1). Therefore, some other therapeutic modes to assist surgical treatment are necessary for the improvement of the survival rate of pancreas cancer patients.

In 1993, the *p16* gene was cloned by Serrano *et al.* (15), using the two-hybrid system to identify gene products that bound to CDK4. The *p16* gene product was found to bind and inhibit cyclin D1-CDK4 or cyclin D2-CDK4 complex activity, which is involved in regulation of the G₁ checkpoint in the cell cycle. Thereafter, by identifying the region most frequently deleted in the wide variety of malignant cell lines, especially melanoma, a candidate tumor suppressor gene was indicated in the locus of the 9p21-p22 region. The gene from this locus was found to encode the p16 protein as the CDK inhibitor. Therefore, the gene *MTS1* was shown to be identical with *p16* (4, 16). The most common mechanism for inactivation of this gene in human cancers is considered to be homozygous deletion. However, in a smaller proportion of tumors and tumor cell lines, intragenic mutations occur with heterozygous deletion (17). Using cell lines and xenografts from human pancreas

cancer, Caldas *et al.* (6) reported that allelic loss of at least one copy of 9p was detected in 85% of informative cases and that homozygous deletions could be detected in 41% among cell lines and xenografts in the *MTS1* locus. If methylation to silence the transcription of the *p16* gene is involved in those mechanisms, the inactivation of the *p16* gene occurs in 98% of human pancreas carcinoma (18). This great frequency of the inactivation of p16, especially caused by homozygous deletion, suggests that abnormal regulation of CDK may play an important role in the malignant potential of pancreas carcinoma.

In this study, we constructed the adenovirus p16 expression vector (AdexCACSp16) to transfect the *p16* gene into MIAPaCa-2, a pancreas cancer cell line, which lacks functional p16. Northern blot hybridization did not indicate any *p16* gene mRNA expression in the RNA of MIAPaCa-2 before the transfection of AdexCACSp16 attributable to homozygous deletion of p16 in MIAPaCa-2. In contrast, AdexCACSp16 induced a high level of *p16* gene mRNA expression in MIAPaCa-2 cells by contact for 1 h with the cells. This result proved that AdexCACSp16 was successfully constructed as a p16 expression vector by the system applied in this study. Fig. 3 shows the cell proliferation curves of MIAPaCa-2 cells with or without AdexCACSp16 infection. The proliferation of the cells was significantly suppressed by AdexCACSp16 compared with the control adenovirus group (Adex1w1). The p16 protein induced by this vector might function to inhibit the cyclin-CDK4 complex. This inhibition might induce G₁ arrest of MIAPaCa-2 cells, which led to the suppression of cell proliferation. These results also suggest that *p16* gene deletion may play some important roles in the dysregulated cell cycle of pancreatic carcinoma and that the Rb/p16 pathway might be recovered to some extent by the p16 expression vector in such cells.

Pancreas cancer cells have invasive activity as a clinical characteristic, which has been considered to be the cause of the frequent liver metastasis and locus recurrence associated with pancreas cancer. Chintala *et al.* (19) reported that p16 adenovirus expression vector suppressed the glioma invasion *in vitro*. This action of the p16 expression vector may play an important role in inhibiting the spread of pancreas cancer. Pancreas cancer most frequently causes liver metastasis. If the p16 expression vector could reduce the invasive activity of pancreas cancer cells, it might be possible for the vector to suppress the liver metastasis by injection into the portal vein (20), although further studies, such as *in vivo* testing, are necessary to clarify these effects.

In conclusion, the p16 expression vector AdexCACSp16 has a powerful potential to induce *p16* gene expression and is able to suppress pancreas cancer cell proliferation. These results indicate that the adenovirus p16 expression vector might be a possible method of gene therapy, in combination with anticancer drugs to supplement the surgical therapy of pancreas cancer.

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