

Oncolytic Virotherapy as a Novel Strategy for Pancreatic Cancer

Makoto Sunamura, MD,* Hirofumi Hamada, MD,† Fuyuhiko Motoi, MD, Masaru Oonuma, MD, Hisashi Abe, MD, Yukoh Saitoh, MD, Toru Hoshida, MD, Shigeru Ottomo, MD, Noriyuki Omura, MD, and Seiki Matsuno, MD

Abstract: We have developed a novel gene therapy that targets genetic alterations in pancreatic cancer using oncolytic replication-selective adenoviruses in tumor cells. E1B-55kDa-deleted adenovirus (AxE1AdB) can selectively replicate in *TP53*-deficient human cancer cells but not cells with functional *TP53*. Consecutive injection with AxE1AdB markedly inhibited the growth of human pancreatic tumors in severe combined immunodeficiency disease mice. Furthermore, AxE1AdB displayed the ability to enhance gene expression as a virus vector. It is reported that uracil phosphoribosyl transferase (UPRT) overcomes 5-FU resistance. The therapeutic advantage of a replication-selective adenovirus that expresses UPRT (AxE1AdB-UPRT) was thus evaluated in an intraperitoneum-disseminated tumor model. Combined treatment with 5-FU and AxE1AdB-UPRT dramatically reduced the disseminated tumor burden without causing toxicity in normal tissues. We also clarified the process of AxE1AdB-inhibited tumor angiogenesis through the preserved E1A region: an adenoviral E1A protein binds to pRB, forcing the quiescent cell into the S phase. We constructed a double-mutant, replication-selective adenovirus (AxdAdB-3) containing a mutation in the RB-binding motif of the E1A region and a deletion of large E1B-55kDa. AxdAdB-3 swiftly induced cancer cell death in vitro and showed a potent antitumor effect in vivo. These results strongly suggest that AxdAdB-3 possesses a wider therapeutic potential than previously believed, given that most pancreatic cancers have abnormalities in both the *TP53* and RB pathways.

Key Words: gene therapy, replication-selective adenovirus, *TP53*, RB, uracil phosphoribosyl transferase

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Although gene therapies have proven effective against pancreatic tumors in in vivo experiments,^{1–4} sufficient therapeutic effects have not been established in clinical trials. Cur-

rently, adenovirus vectors that lack genes essential to viral replication are the most favorable gene therapy tools in the clinical treatment of cancer. The major difficulties of this approach involve transfecting adenovirus into every cancer cell of a tumor and effectively inducing gene expression. These limitations result in an incomplete antitumor effect and subsequent regrowth of tumors. Increasing the titer of the adenovirus is an alternative method that increases gene expression in solid tumors, including those of pancreatic cancer. At the same time, however, this strategy brings with it the adverse effects of adenovirus gene therapy.

In an attempt to increase the antitumor effect and efficiency of gene expression and delivery, various groups have experimented with replication-competent viruses. Adenovirus E1 gene products, in addition to transactivating other early gene promoters, prepare the cellular environment for optimal viral replication by associating with a number of key cell cycle proteins. Replication-selective viruses may overcome the limitations of gene transfer of conventional adenoviral vectors. Viral replication in a small fraction of tumor cells leads to amplification and extension of the antitumor effect of gene expression. Cell death is due exclusively to viral replication and cell lysis.

In this paper, we introduce a novel therapeutic strategy for pancreatic cancer involving an oncolytic replication-selective adenovirus. It is demonstrated that oncolytic replication-selective adenoviruses are also useful as vectors of anti-tumor genes because they significantly increase gene expression in tumors only.

REPLICATION-SELECTIVE ADENOVIRUS TARGETING *TP53* ABNORMALITY

A malignant tumor develops in a multistep process that results from the mutation of several specific genes involved in the control of cell growth and programmed cell death. The *TP53* gene is mutated in more than half of human tumors,⁵ which indicates that it plays a key role as a tumor suppressor. Because *TP53* is functionally inactivated in many human tumors, including pancreatic tumors,⁶ and because the prognosis of patients with LOH on 17p is worse when pancreatic cancer is present,⁷ transduction of the wild-type *TP53* gene into can-

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From the *Division of Gastroenterological Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan; and the †Department of Molecular Medicine, Sapporo Medical College, Sapporo, Japan.

Reprints: Makoto Sunamura, MD, PhD, Division of Gastroenterological Surgery, Tohoku University, Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai, 980-8574 Japan (e-mail: msun-thk@umin.ac.jp).

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cer cells using a virus vector is an attractive therapeutic strategy for pancreatic cancer. However, one of the limitations of this strategy and other cancer gene therapies is the low efficiency at which therapeutic genes can be delivered in vivo, especially into solid tumors.

To replicate, small DNA tumor viruses encode proteins to shut down the *TP53* gene. In an adenovirus, the E1B-encoded proteins E1B-19kDa and E1B-55kDa are responsible for turning off *TP53*. The former acts downstream of TP53 to prevent apoptosis, whereas the latter physically associates with TP53 to prevent TP53-mediated transactivation.⁸ The E1B-55kDa protein binds to TP53 and prevents it from stimulating the promoters of growth arrest genes such as P21 and GADD45.^{9,10}

The widely used adenovirus vector with a deletion of E1 (essential to replication) is unable to replicate in infected cells. Several research groups have demonstrated that when the E1A-deleted adenovirus vector is linked to a plasmid expressing the E1A gene, it is able to replicate by transcomplementation of the E1A gene product. An adenovirus vector containing the E1A-expressing plasmid can amplify adenovirus vector-mediated transduced genes such as luciferase,¹¹ thymidine kinase,¹² and lacZ.¹³ If the E1B-55kDa-deleted adenovirus were used as a helper virus, it would also be able to amplify the effects of an adenovirus vector carrying a therapeutic gene. To develop further strategies of gene therapy for cancer, we constructed the E1B-55kDa-deficient adenovirus (AxE1AdB) and examined the effects of a combination of this mutant adenovirus and other adenovirus vectors on pancreatic cancer cell lines.¹⁴ AxE1AdB produced a stronger oncolytic effect against pancreatic cancer cell lines when compared with *TP53* gene therapy using a replication-incompetent adenovirus vector. Co-infection with AxE1AdB and the E1-deficient adenovirus expressing the reporter *lacZ* gene resulted in the replication of both viruses and a marked increase in reporter gene expression in pancreatic cancer cells without TP53 function. Pancreatic cancer cells infected with both the E1B-55kDa-deficient adenovirus and the adenovirus vector for human interleukin-2 (AxCAhIL-2) produced 110 times more IL-2 than cells infected with AxCAhIL-2 alone. Moreover, injection of the pancreatic cells with AxE1AdB and AxCAhIL-2 resulted in a complete regression of the established tumors (Fig. 1).

It is reported that uracil phosphoribosyl transferase (UPRT) overcomes 5-FU resistance by catalyzing the synthesis of 5-fluorouridine monophosphate (FUMP) from uracil and phosphoribosylpyrophosphate (PRPP). The antitumor effect of 5-FU is enhanced by augmenting 5-fluorodeoxyuridine monophosphate (FdUMP) (converted from FUMP), which inhibits thymidylate synthetase (TS). We studied the effectiveness of gene therapy using adenovirus-mediated UPRT in overcoming the 5-FU resistance seen in pancreatic cancer.¹⁵ Transduction of the UPRT gene resulted in an increase of FdUMP and subsequent sensitivity of various pancreatic can-

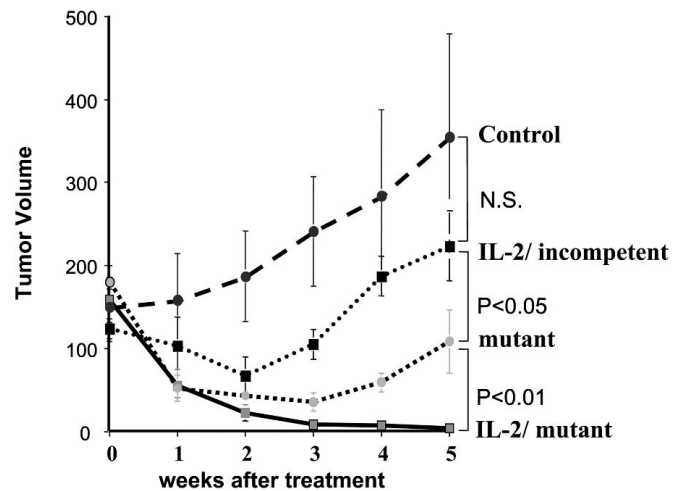


FIGURE 1. Tumor growth in SCID mice was treated with AxE1AdB and AxCAhIL2. Treatment with AxE1AdB plus AxCAhIL2 caused significant tumor regression compared with AxCAhIL2 alone or AxE1AdB alone.

cer cells to 5-FU. Although in vivo gene transduction of UPRT followed by administration of 5-FU resulted in regression of intraperitoneal pancreatic tumors, the high dose of adenovirus needed to obtain a complete reduction of the tumors produced adverse effects, including severe diarrhea with dehydration. FdUMP, which is converted from 5-FU in normal mucosal cells, inhibits cell growth, thereby causing gastrointestinal toxicity. We manufactured a replication-competent adenovirus expressing UPRT (AxE1AdB-UPRT). As expected, selective replication and amplification of the UPRT gene did occur in cells with abnormal *TP53* genes. In contrast, human fibroblast cells with normal *TP53* genes appeared to be resistant to the replication of AxE1AdB-UPRT. The restricted replication-competent adenovirus augmented the antitumor effect without producing adverse effects, in contrast to a replication-incompetent adenovirus.

This replication-selective adenovirus is useful in laparoscopic examination and aids in the diagnosis of tumor staging. We confirmed its efficacy using a mouse model of intraperitoneally disseminated pancreatic cancer. Injection of this virus and the GFP-expressing adenovirus vector made it possible to detect peritoneal dissemination and metastasis to lymph nodes, which were stained with green color and observable under fluorescence, as shown in Figure 2.

ANTIANGIOGENESIS EFFECT OF E1B-55kDa-DELETED ADENOVIRUS

E1A proteins, which are divided into 2 subtypes, bind to various cellular proteins such as CREB-binding protein (CBP)¹⁶ and p300,¹⁷ and have a significant role in the inhibition of angiogenesis. Aggressive tumors, including those of

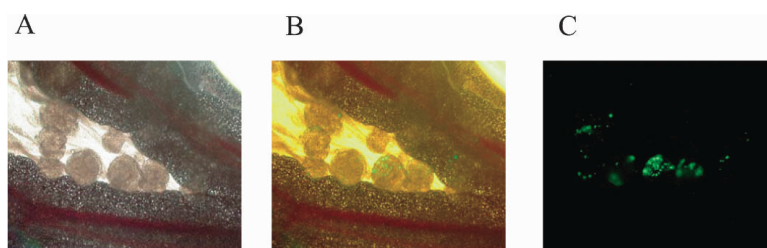


FIGURE 2. Detection of the abdominal dissemination of tumors. Disseminated pancreatic tumors (A) were stained with green color under fluorescence (C). B: Image was taken under light and fluorescence.

pancreatic cancer, often have an insufficient blood supply, partly because tumor cells grow faster than endothelial cells and partly because the newly formed vascular supply is disorganized. When tumor cells are exposed to hypoxia, hypoxia-inducible factor-1 α (HIF-1 α) is stabilized and activated to promote the transcription of several genes, such as vascular endothelial growth factor (VEGF).¹⁸ In the presence of E1A hypoxia-induced VEGF, however, the binding of E1A to p300/CBP inhibits mRNA synthesis.

We speculated that the replication-selective adenovirus has an inhibitory effect on tumor angiogenesis through E1A-mediated inhibition of the p300 function. To analyze this antiangiogenesis property, we infected pancreatic cancer cells with several mutant constructs and demonstrated that the oncolytic replication-selective adenovirus (AxE1AdB) inhibits the production of VEGF in vitro and neovascularization in vivo.¹⁹ VEGF and HIF-1 α expression in various cancer cells in hypoxia was confirmed. Under hypoxic conditions, there was less HIF-1 α protein in cancer cells infected with AxE1AdB than in cells infected with the mutant E1A-type adenovirus. In vivo, the cancer cells infected with AxE1AdB were significantly inhibited in contrast to the angiogenesis in flank skin and microvessel count by immunohistochemical staining of mutant E1A CD31. These results suggest that the E1A region preserved in the adenovirus AxE1AdB inhibited tumor angiogenesis not only by binding with p300 but also by participating in the degradation process of HIF-1 α protein under hypoxic conditions. Since several groups have developed new oncolytic replication-selective adenoviruses, the findings from this study on E1A have important and immediate implications for future projects in developing and refining gene therapy for cancer.

E1A-MUTATED ADENOVIRUS

The E2F family of transcription factors is required for transcription of several genes involved in DNA and deoxy-nucleotide synthesis by inducing the G1-S transition of cell cycle.²⁰ The binding of 2 proteins, RB and a related protein p107, to E2F inhibits its ability to activate transcription. The RB protein was initially identified as the product of the prototype tumor suppressor gene *RB*.²¹ The underphosphorylated form of RB may act as a growth suppressor by blocking exit from the G0 or G1 phase. Phosphorylation of RB inhibits its

growth suppression function, allowing the cell to enter the S phase. The mechanisms of action of the *RB* gene have been clarified through studies of the E1A oncogene of human adenovirus type 5. The binding of proteins, such as adenovirus E1A or SV40 large T-antigen gene products, to RB negates the requirement of RB phosphorylation and allows quiescent cells to enter the cell cycle.

Recently, another adenovirus with mutation in the E1A region was reported to be an alternative mode of cancer therapy. It is hypothesized that an adenovirus with a deletion in the RB-binding (CR2) region of E1A or with mutations in the p300-binding region (CR1) of E1A selectively replicates in cancer cells with defects in the RB pathway (eg, *RB* mutation, *P16* loss, cyclin D amplification).

We expected that an E1A mutant adenovirus unable to bind pRB would be selectively replicated in tumor cells with dysregulated cell cycles but would not be replicated in normal cells, which have tightly regulated cell cycles. We constructed a double-mutant, replication-selective adenovirus (AxdAdB-3) that contained a mutation in the RB-binding motif of the E1A region and the same E1B-55kDa deletion as AxE1AdB.²² The effect of AxdAdB-3 on pancreatic cancers was evaluated both in vitro and in vivo. AxdAdB-3 induced cancer cell death efficiently in vitro and had a more potent antitumor effect in vivo (Fig. 3). These results strongly suggest that AxdAdB-3 has great therapeutic potential since most pancreatic cancers have abnormalities in both the TP53 and/or RB pathways and may be a promising new tool in gene therapy.

DISCUSSION

Cancer therapy employing viral replication has been previously reported. Patients with cervical cancer have been treated with direct injection of wild-type adenovirus,²³ which then replicated and destroyed cancer cells. However, wild-type viruses are able to replicate in both tumor cells and normal cells, and systemic manifestation of viral disease was observed in some immunocompromised patients. The E1B-55kDa-deleted adenovirus ONYX-015 (ONYX Pharmaceuticals, Richmond, CA) can selectively replicate in tumor cells²⁴ without requiring any additional procedures. ONYX-015 is able to replicate in and lyse TP53-deficient human tumor cells but not cells with functional TP53. Moreover, the released viruses infect neighboring cells, and their subsequent proliferation re-

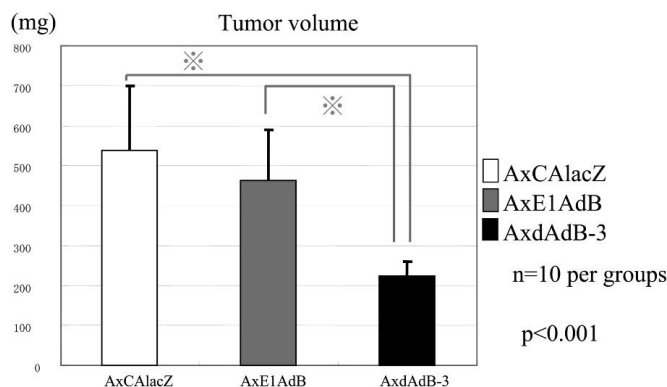


FIGURE 3. Therapeutic effect of oncolytic replication–selective adenovirus AxAdB3. Tumor weight in animals treated with AxAdB3 was significantly reduced when compared with the control (AxCAIacZ) and AxE1AdB groups.

sults in a destructive cycle. This is characteristic of mutant adenoviruses, distinguishing their use in cancer therapy from other gene therapy approaches, which are limited due to the nonreplicating character of conventional virus vectors. Phase I clinical testing of ONYX-015 began in April 1996 in patients with head and neck cancer²⁵ and has continued in patients with pancreatic cancer²⁶ and liver metastasis of colorectal cancer.²⁷

There are several theoretical advantages to using oncolytic replication–selective adenoviruses over replication-defective adenoviruses in cancer gene therapy. Our studies have clarified that replication-competent adenoviruses are not only strong weapons in and of themselves but that they are also useful carriers of genes that possess antitumor activity because they are virus vectors specific to tumors without normal TP53 function or intact RB pathways. Whether these experimental results are universally valid requires confirmation in future clinical trials.

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