Liposome-mediated in Vivo Gene Transfer of Antisense K-ras Construct Inhibits Pancreatic Tumor Dissemination in the Murine Peritoneal Cavity

Kazunori Aoki, Teruhiko Yoshida, Takashi Sugimura, and Masaaki Terada
Genetics Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

ABSTRACT

K-ras point mutation occurs at a characteristically high incidence in human pancreatic cancer. Plasmids expressing antisense (AS), AS-K-ras-LNSX or sense K-ras gene fragment, were first transduced into three human pancreatic cancer cell lines (AsPC-1, MIAPaCa-2, and BxPC-3) by liposome-mediated transfection. A stable expression of antisense or sense K-ras RNA was detected by Northern blot analysis, and Western blot analysis confirmed a reduction of up to 20% of K-ras-specific p21 protein in AsPC-1 cells transduced with AS-K-ras-LNSX. The growth of pancreatic cancer cells with K-ras point mutations (AsPC-1 and MIAPaCa-2) was significantly suppressed after transduction of AS-K-ras-LNSX, although the effect of antisense construct was not found in cells with a wild-type K-ras gene (BxPC-3). Next, to test the efficacy in vivo, AsPC-1 cells were inoculated into the intraperitoneal cavity of nude mice, and 3 days later, the AS-K-ras-LNSX:liposome complex was injected i.p. 3 times. Twenty-eight days after tumor cell inoculation, 9 of 10 control mice developed peritoneal dissemination and/or solid tumors on the pancreas, whereas only 2 of 12 mice treated with AS-K-ras-LNSX showed any evidence of tumors. Although PCR analysis indicated that the injected DNA was delivered to various organs except for the brain, treatment-related toxicity was not observed. This study shows that the liposome-mediated in vivo gene transfer of antisense K-ras construct may be a useful therapeutic strategy for pancreatic cancer.

INTRODUCTION

Pancreatic cancer has continued to be one of the most difficult cancers today with a grave patient prognosis of <10% surviving for 3 years (1, 2). The reasons for the poor prognosis include: (a) the difficulty of early diagnosis due to its anatomical location and lack of specific early symptoms (3, 4); (b) the tendency of the tumor to spread rapidly to the surrounding vital organs (5); (c) the frequent occurrence of metastasis even from a small primary tumor <2 cm in diameter (4, 5); and (d) the poor or negligible response to existing chemo-, radio-, endocrine, or immune therapy (2, 6, 7). On the other hand, pancreatic cancer ranks fifth as a cause of cancer-related mortality in Japan and in the United States (8). In Japan, the death rate for pancreatic cancer has risen sharply from 2.5/100,000 population in 1960 to 9.6/100,000 in 1991 (8). Thus, the development of a new modality of treatment for a pancreatic cancer has been eagerly awaited (6).

K-ras point mutation occurs at a characteristically high incidence in human pancreatic cancer (9-13). About 80-90% of these tumors have been reported to carry the mutation, >95% of which are located in codon 12 (9-12) with the remainder at codon 13 (12). Considering the prominent transforming activity of the mutated K-ras oncogene on NIH3T3 cells, the K-ras mutation could be the major genetic abnormality responsible for transforming the phenotype in human pancreatic cancer (14, 15). However, another line of evidence has suggested that the K-ras mutation could occur in the very early phase of pancreatic ductal carcinogenesis; the mutation has also been found in mucous cell hyperplasia (16-18), and there is no significant difference in the incidence of the K-ras mutation among different stages of the disease (13). Thus, the K-ras point mutation may be related to the initiation of carcinogenesis but not to the malignant progression of the pancreatic cancer.

Furthermore, a full-blown cancer develops only after the accumulation of additional genetic changes during the multistep carcinogenesis. Other genetic alterations of pancreatic cancer reported thus far include: (a) abnormalities of the p53 gene (19-21); (b) loss of expression of the DCC gene (22, 23); (c) somatic mutation of the APC gene (24); (d) overexpression of acidic and basic fibroblast growth factors (25, 26); and (e) microsatellite instability (27). Therefore, although the K-ras mutation is an attractive target for gene therapy of pancreatic cancer, suppression of the mutated K-ras alone may or may not effectively reverse the malignant potential of pancreatic cancer.

Specific suppression of the ras oncogene expression has been reported in other systems. The inhibitory effect of anti-H-ras oligonucleotide, antisense RNA, and ribozyme on the neoplastic phenotype has been reported for bladder carcinoma cells and NIH3T3 cells transformed by H-ras oncogene (28-32). The antisense K-ras retroviral construct was useful in suppressing the tumorigenicity of lung cancer in a nude mouse orthotopic transplantation model (33, 34). In this report, we used a plasmid expressing antisense K-ras RNA to inhibit the production of the K-ras-specific p21 protein and found that the antisense strategy can suppress the growth of pancreatic cancer cells with K-ras point mutations in vitro and in vivo. To the best of our knowledge, this study is the first report that an antisense K-ras construct is useful in inhibiting the growth of pancreatic cancer cells. Furthermore, our experience suggests that the direct i.p. injection of the DNA:liposome complex is an efficient way of introducing genes in vivo.

MATERIALS AND METHODS

Cells and Culture Conditions. Three human pancreatic cancer cell lines (AsPC-1, MIAPaCa-2, and BxPC-3) were used in this study. AsPC-1 and BxPC-3 cells were maintained in an RPMI 1640 medium with 10% fetal bovine serum and MIAPaCa-2 cells in DMEM with 10% fetal bovine serum. AsPC-1 has a G to A transition at the second position of K-ras codon 12 (GGT:glycine to GAT:aspartic acid), the MIAPaCa-2 carries a G to C transition at the first position of codon 12 (GGT:glycine to TGT:cysteine), whereas BxPC-3 does not have a mutation on its K-ras gene (35). All three pancreatic cancer cell lines can form tumors on BALB/c nude mice (Charles River Japan, Inc.) upon s.c. or i.p. injections.

Construction of Expression Plasmid. The retroviral vector plasmid LNSX was generously provided by Dr. A. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). The LNSX contains the selectable neomycin phosphotransferase gene, which is expressed from the retroviral LTR, and an inserted DNA is placed in the downstream of an internal SV40 early promoter. A K-ras cDNA fragment spanning from nucleotide 171 in the first exon to nucleotide 517 in the third exon (36) was cloned by reverse transcriptase-PCR from normal human placental mRNA. The ClaI site was

Received 2/21/95; accepted 6/26/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a grant-in-aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, by grants-in-aid for Cancer Research from the Health and Welfare Ministry of Japan, the Ministry of Education, Science and Culture of Japan, and by the Bristol-Myers Squibb Foundation.

2 To whom requests for reprints should be addressed.

The abbreviations used are: LTR, long terminal repeat; AS, antisense; S, sense; DOGS, diocetylamiloglycyloperimine; poly(A)⁺ RNA, polyadenylated RNA.
designed at the 5’ end of the upstream primer and at the AvrII site at the 3’ end of the downstream primer to obtain a K-ras cDNA fragment in antisense orientation, whereas the engineered restriction sites were reversed for the sense construct. The LNSX was digested with CiaI and AvrII, and the recombinant plasmid clone was constructed by subcloning the 347-bp K-ras cDNA fragment in antisense or sense orientation (Fig. 1). The K-ras expression unit of the antisense and sense plasmids was sequenced by the dideoxynucleotide chain termination method.

**DNA Transduction.** The antisense (AS-K-ras-LNSX) or sense (S-K-ras-LNSX) K-ras expression plasmid was transduced into the three human pancreatic cancer cell lines by liposome-mediated transfection. For the transfection to AsPC-1 and MIAPaCa-2 cells, cationic liposomes containing 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate and dioloyl phosphatidylethanolamine (lipofectamine; Gibco-BRL) were used. Because lipofectamine was toxic to the BxPC-3 cells, the cells were transfected by use of a cationic lipopolyamine containing DOGS (BIOSEPRA). Forty-eight h after transfection, G418 selection was started. For AsPC-1 and BxPC-3 cells, several G418-resistant colonies were isolated. The remaining colonies were pooled and grown as a mixture for additional analysis in AsPC-1 cells. For MIAPaCa-2 cells, all colonies were pooled and used for analysis. Cells transduced with the AS-K-ras-LNSX were suffixed with “AS”, and those transduced with the S-K-ras-LNSX with “S”; AsPC-1-AS or AsPC-1-S, MIAPaCa-2-AS or MIAPaCa-2-S, and BxPC-3-AS or BxPC-3-S are AsPC-1, MIAPaCa-2, and BxPC-3 cells transduced with either AS-K-ras-LNSX or S-K-ras-LNSX, respectively. AsPC-1-AS (4.8), AsPC-1-AS (4.3), AsPC-1-AS (4.4), AsPC-1-S (5.8), BxPC-3-AS (4.2), BxPC-3-AS (4.4), and BxPC-3-S (5.1) are the single cell clones of AsPC-1 and BxPC-3 cells transduced with AS-K-ras-LNSX or S-K-ras-LNSX.

**PCR and Southern Blot Analysis of Genomic DNA from Transduced Cells.** Total genomic DNA was extracted from the parental and transduced AsPC-1 cells. One μg of DNA was subjected to PCR analysis by using two primers specific to the LNSX sequence (LNSX-L, GGGTACCCGATTC-CCATA; LNSX-R, GAACCCACATATCGAAGCGG), which should yield a 422-bp fragment. We performed PCR in 30 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 2 min. The PCR products were electrophoresed on a 2% agarose gel and stained by ethidium bromide. The stable integration and expression of the 3.5-kb antisense or sense K-ras transcript was confirmed by Northern blot analysis.

**Expression of Antisense or Sense K-ras RNA in Transduced Cells.** Poly(A)+ RNA was size fractionated on a 1.5% denaturing agarose gel, transferred onto a nitrocellulose transfer membrane, and hybridized with a strand-specific RNA probe. A 372-bp K-ras cDNA fragment spanning the first and second exon sequences was amplified by PCR and subcloned into a Bluescript vector. Sense and antisense RNA probes were synthesized by using either a T7 or T3 bacteriophage RNA-dependent RNA polymerase. The hybridization and washing were performed under the same conditions as the Southern blot analysis.

**Western Blot Analysis.** The parental or transduced AsPC-1 and BxPC-3 cells were lysed in RIPA buffer [10 mM Tris-HCl (pH 7.4), 1% deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin]. Eighty μg of the parental or transduced AsPC-1 cell lysates and 100 μg of the parental or transduced BxPC-3 cell lysates were heated at 90°C for 5 min, size fractionated by 8–16% SDS-PAGE (TEFCO), and electroblotted onto polyvinylidene difluoride membranes (Millipore). The K-ras protein was detected by a K-ras-specific p21 mAb (OncoGene Science; Ref. 37) by using an enhanced chemiluminescence system (Amersham).

**In vivo Growth of Pancreatic Cancer Cells.** The parental and transduced pancreatic cancer cells (AsPC-1, MIAPaCa-2, and BxPC-3) were seeded at 10⁶ cells/well and grown in 6-well plates for 7–9 days in duplicate. Cells were harvested and counted by trypan blue exclusion on different days.

**Effect of AS-K-ras-LNSX: Liposome Complex In vivo.** i.p. inoculation of 6 × 10⁵ AsPC-1 cells into the nude mice resulted in the peritoneal dissemination and formation of tumor nodules in pancreatic regions within 28 days. Twenty-two male BALB/c nude mice received i.p. injection of AsPC-1 cells, and 3 days later, 12 of the mice were injected i.p. with 100 μg of AS-K-ras-LNSX complexed with 400 nmol of DOGS lipopolyamine (38) at 12 h-intervals for a total of 3 times. As control, S-K-ras-LNSX complexed with DOGS was used in 10 of the mice. Twenty-eight days after the injection of AsPC-1 cells, the mice were sacrificed and examined for whether they developed peritoneal dissemination and tumors in the pancreas. Three-mm histological sections were made from the brain, lung, liver, pancreas, spleen, kidney, testis, small intestine, and colon and analyzed for the presence of tumor and for a pathological change.

**Distribution of Injected DNA.** To characterize the distribution of i.p. injected DNA in vivo, total genomic DNA was extracted from the brain, lung, heart, liver, pancreas, spleen, kidney, testis, stomach, small intestine, colon, skeletal muscle, and bone marrow of 3 mice given injections with AS-K-ras-LNSX. One μg of DNA was subjected to PCR analysis by using LNSX-specific primers (LNSX-L and LNSX-R). The KpnI-digested genomic DNA from the liver, spleen, stomach, and testis was also analyzed by Southern blot analysis as described above.

**RESULTS**

**Detection of LNSX Sequence by PCR and Southern Blot Analysis in Transduced Cells.** Genomic DNA was extracted from three AsPC-1-AS clones, one AsPC-1-S clone and the parental AsPC-1 cells. A 422-bp segment of the LNSX sequence was detected in the transduced AsPC-1 cells, but not in the parental AsPC-1 cells, by PCR using probes specific to LNSX (Fig. 2A). Southern blot analysis was performed on the KpnI-digested DNA (Fig. 2B). KpnI cuts the LNSX plasmid with antisense or sense K-ras gene at two sites, generating a 3378-bp fragment that represents a nearly full-length K-ras-LNSX. The major band of 3.4 kb was detected in the transduced AsPC-1 cells, which suggests the presence of an intact K-ras-LNSX. The few additional bands and their relative intensity to the internal 3.4-kb band suggest the presence of multiple copy inserts.

**Expression of Antisense or Sense K-ras RNA in Transduced Cells.** Poly(A)+ RNA was extracted from the parental cells, cloned AsPC-1 and BxPC-3 cells transduced with AS- or S-K-ras-LNSX. Northern blot hybridization with sense or antisense K-ras RNA probe detected a stable expression of the 3.5-kb antisense or sense K-ras RNA, which is considered to be the read-through transcript from the 5’ LTR (Fig. 3). Reprobing the filter with the β-actin DNA probe showed that each RNA sample was loaded in an equal amount.

**Inhibition of K-ras-specific p21 Protein in AS-K-ras-LNSX-transduced Cells.** As shown in Fig. 4, Western blot analysis using a K-ras-specific p21 mAb showed a reduction of the K-ras p21 protein.

![Fig. 1. The K-ras expression plasmid used in this study. A 347-bp K-ras cDNA fragment containing the first and second exons was subcloned into the LNSX plasmid in antisense or sense orientation. 3V, SV40 early promotor and enhancer; neo, bacterial neomycin phosphotransferase.](image-url)
Fig. 2. PCR and Southern blot analyses for presence of the LNSX sequence in the transduced AsPC-1 cells. A, genomic DNA of a cloned AsPC-1 cell transduced with AS- or S-K-ras-LNSX was extracted and amplified by PCR with primers specific for LNSX. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Lane 1, dX174/HindII ladder; Lanes 2-4, cloned AsPC-1-AS cells (Lane 2, clone 4.8; Lane 3, clone 4.3; Lane 4, clone 4.4); Lane 5, cloned AsPC-1-S cells (clone 5.8); Lane 6, parental AsPC-1 cells; Lane 7, LNSX plasmid DNA. B, Southern blot analysis of genomic DNA digested with KpnI and hybridized with a 32P-labeled LNSX plasmid probe. Representative cases of AS- or S-K-ras-LNSX-transduced clone are shown. Lane 1, AS-K-ras-LNSX plasmid digested with KpnI (22.5 pg); Lane 2, parental AsPC-1 cells; Lane 3, AsPC-1-AS cells (clone 4.8; Lane 6, clone 4.3; Lane 7, clone 4.4); Lane 8, cloned AsPC-1-S cells (clone 5.8).

Fig. 3. Northern blot analysis for presence of sense or antisense K-ras RNA. Poly(A)+ RNA were extracted from the parental cells, cloned AsPC-1 and BxPC-3 cells transduced with AS- or S-K-ras-LNSX. Two µg of size-fractionated poly(A)+ RNA were transferred onto a nitrocellulose membrane and hybridized with a 32P-labeled LNSX plasmid probe. Representative cases of AS- or S-K-ras-LNSX-transduced clone are shown. A, Lane 1, parental AsPC-1 cells; Lanes 2-4, cloned AsPC-1-AS cells (Lane 2, clone 4.8; Lane 3, clone 4.3; Lane 4, clone 4.4); Lane 5-6, cloned BxPC-3-AS cells (Lane 5, clone 4.8; Lane 6, clone 4.3). B, Lane 1, parental AsPC-1 cells; Lane 2, cloned AsPC-1-AS cells (clone 4.4); Lane 3, parental BxPC-3 cells; Lane 4, cloned BxPC-3-S cells (clone 5.8).

Fig. 4. Western blot analysis of K-ras-specific p21 protein. Protein extracts were prepared from the parental and transduced cells (AsPC-1 and BxPC-3). For AsPC-1 cells and their derivatives, 80 µg of the cell lysates were loaded on the gel, except Lanes 2-4. One hundred µg of the proteins were analyzed for BxPC-3 and its transduced counterparts. The cell lysates were size fractionated by 8–15% SDS-PAGE, and Western blot analysis was performed by using the K-ras-specific p21 mAb. Lanes 1-4, 20, 26.7, 16, and 8 µg of the parental AsPC-1 cell lysates, respectively; Lanes 5-7, cloned AsPC-1-AS cells (Lane 5, clone 4.8; Lane 6, clone 4.3; Lane 7, clone 4.4); Lane 8, pooled AsPC-1-AS cells; Lane 9, cloned AsPC-1-S cells (clone 5.8); Lane 10, pooled AsPC-1-S cells; Lane 11, parental BxPC-3 cells; Lanes 12-13, cloned BxPC-3-AS cells (Lane 12, clone 4.2; Lane 13, clone 4.4); Lane 14, cloned BxPC-3-S cells (clone 5.1).

in the cloned AsPC-1-AS cells. Furthermore, in pooled AsPC-1-AS, the K-ras p21 protein was also decreased. When the band intensities are compared with the reference lanes where different amount of the parental AsPC-1 cell lysates were loaded (Lanes 1-4), it is estimated that in the AsPC-1-AS cells the K-ras p21 protein was reduced up to 20% of that of the parental AsPC-1 cells (Lane 7). The parental AsPC-1 cells and AsPC-1-S cells showed the same level of the K-ras p21 expression (Lanes 1, 9 and 10). There was no significant difference in the expression of K-ras p21 protein among the parental BxPC-3 cells, cloned BxPC-3-AS cells, and BxPC-3-S cells (Lanes 11-14).

Growth Suppression of AS-K-ras-LNSX-transduced Cells. The various clones of AsPC-1 cells expressing antisense K-ras RNA showed variable growth retardation compared with those of AsPC-1-S and the parental AsPC-1 cells (Fig. 5A). AsPC-1-AS clones showing a prominent growth suppression had a flat, nonreflective morphology (data not shown). To examine whether apoptotic cell death could be induced in AsPC-1-AS, the cells were seeded onto tissue culture chamber slides (Lab-Tek) and stained for DNA with 2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole (bis-BENZIMIDE; Sigma Chemical Co.) fluorochrome. However, chromatin condensation and fragmentation of nuclei were not evident in AsPC-1-AS (data not shown).

For the two human pancreatic cancer cell lines (AsPC-1 and MIAPaCa-2), the growth rates of each pooled cells transduced with AS-K-ras-LNSX were compared with those of the parental or S-K-ras-LNSX-transduced cells. AsPC-1-AS and MIAPaCa-2-AS cells showed growth retardation, although the growth inhibition was less marked in MIAPaCa-2-AS cells (Fig. 5, B and C). However, in BxPC-3 cells, which have the wild-type K-ras gene, the growth rate of AS-K-ras-LNSX-transduced clones was not significantly different from that of S-K-ras-LNSX-transduced clones and the parental cells (Fig. 5D), in spite of the stable expression of the antisense K-ras transcripts (Fig. 3A).

To investigate whether the AS-K-ras-LNSX-transduced cells can exert a bystander effect on nontransduced cells, 0.5 × 10⁶ cloned AsPC-1-AS cells were cocultured with an equal number of the parental AsPC-1 cells in 6-well plates. The growth curve of the mixture was
Fig. 5. *In vitro* growth curve of transduced cells. Cells (1 × 10⁴) were seeded in 6-well plates and grown for 5–10 days. A. growth of cloned AsPC-1-AS cells was compared with AsPC-1-S and parental AsPC-1 cells. B and C, growth of the mixed populations of the transduced and parental cells. B, AsPC-1 cells; C, MIA PaCa-2 cells. D, growth curve of the parental BxPC-3, cloned BxPC-3-AS, and cloned BxPC-3-S cells.

intermediate between those of the AsPC-1-AS and parental AsPC-1 cells (data not shown), suggesting the absence of a bystander effect.

**Inhibition of Peritoneal Dissemination by AS-K-ras-LNSX: Liposome Complex.** The liposome-mediated direct *in vivo* gene transfer was examined in the peritoneal dissemination model of nude mice. AsPC-1 cells were inoculated into male nude mice i.p., and 3 days later, AS- or S-K-ras-LNSX complexed with DOGS lipopolyamine was injected into the peritoneal cavity. The mice were sacrificed 28 days after tumor injection, and 9 of 10 control mice injected with S-K-ras-LNSX were found to have developed peritoneal dissemination and/or tumor formation on the pancreas or at the hepatic hilus. In contrast, 10 of 12 mice treated with AS-K-ras-LNSX were free of tumors by careful inspection (Table 1). Representative specimens from these experiments are shown in Fig. 6. Histological examination of the small intestine, colon, and pancreas of mice treated with AS-K-ras-LNSX showed no evidence of AsPC-1-derived tumor formation.

**Distribution of Injected DNA and Organ Toxicity.** PCR analysis of DNA from the brain, lung, heart, liver, pancreas, spleen, kidney, testis, stomach, small intestine, colon, skeletal muscle, and bone marrow showed that the injected DNA was present in multiple organs except for the brain at the 24th day after injection (Fig. 7A; Table 2). The sensitivity of the PCR analysis was estimated to be about 1 copy/10²–10³ genomes. However, Southern blot analysis of genomic DNA extracted from the liver, spleen, stomach, and testis failed to detect the plasmid DNA by *KpnI* digestion, which should excise out the internal 3378-bp fragment from either the episomal or integrated K-ras-LNSX plasmid (Fig. 7B). Treatment-related toxicity was not observed in any treated mice; hematopoietin- and eosin-stained histological sections from treated mice revealed no pathological change, and organ weight was not significantly different between control and treated mice. Furthermore, treated mice appeared normal from the time we injected the DNA:liposome complex until we sacrificed them.
Table 1  Tumors in the peritoneal cavity of the mice administered with AS- or
S-K-ras-LNSX: liposome complex

Twenty-two Balb/c nude mice were given i.p. injections with $6 \times 10^5$ AsPC-1 cells at
day 0, and 12 of the mice were administered with AS-K-ras-LNSX:liposome complex
three times at 12-h intervals at days 3–4. As control, S-K-ras-LNSX:liposome complex
was used in the other 10 mice. The mice were sacrificed at day 28 and examined for
evidence of the tumor in the peritoneal cavity.

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Tumor on the mesentery</th>
<th>Tumor on the pancreas</th>
<th>Tumor on the hepatic hilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice given injections of AS-K-ras-LNSX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>No. of mice with tumor/total</td>
<td>2/12*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice given injections of S-K-ras-LNSX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>+*</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>No. of mice with tumor/total</td>
<td>9/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Tumors on the mesentery and pancreas were evaluated on the basis of the maximum size and number as follows: +, $<3$ mm in size and $<3$ in number; ++, $3–10$ mm in size or $3–10$ in number; ++++, $>10$ mm in size or $>10$ in number.

*P < 0.005 compared to the S-K-ras-LNSX-injected group by $\chi^2$ analysis.

DISCUSSION

Plasmids expressing antisense or sense K-ras RNA were transduced to the human pancreatic cancer cells by liposome-mediated transfection. The expressed antisense K-ras RNA could effectively block the synthesis of the K-ras p21 protein, and the growth of the AS-K-ras-LNSX-transduced cells was inhibited in vitro in pancreatic cancer cells with K-ras point mutation. In the nude mouse peritoneal dissemination model of the pancreatic cancer, the liposome-mediated in vivo gene transfer of the antisense K-ras expression plasmid significantly suppressed tumor development in the peritoneal cavity. Although the injected DNA was delivered to various organs, no evidence of treatment-related toxicity was observed.

Cloned AsPC-1-AS (4.4), which had the greatest reduction of the K-ras-specific p21 protein, showed the slowest cell growth in vitro. The reduction of the K-ras p21 protein appeared to be proportional to the growth inhibition attained by each clone of the transduced pancreatic cancer cells with K-ras mutations (Figs. 4 and 5). No evidence of increased apoptosis in AsPC-1-AS was found by bisBENZIMIDE fluorochrome staining. Thus, the mechanism of the population growth suppression may not be the direct killing effect of the antisense K-ras RNA but the slowing down of the cell growth induced by the antisense message. We also investigated whether the transduced cells could alter the growth of the surrounding nontransduced populations. The coculture of the AsPC-1-AS and the parental AsPC-1 cells did not show the growth inhibitory effect of the transduced cells on the nontransduced cells. In the absence of the significant bystander effect, the gene transfer into as many cancer cells as possible should be crucial for the therapeutic effects of this strategy.

The BxPC-3 cells had no mutation on the K-ras gene, and transduction of BxPC-3 cells with AS-K-ras-LNSX did not affect the...
expression of K-ras p21 protein nor the growth of the cells. Because we have not analyzed other pancreatic cancer cells without the K-ras mutation, we do not know if the resistance of the therapy is unique to the BxPC-3 cells. It should be, however, pointed out that neither gross nor histological abnormality was detected in normal organs of the mice given injections with AS-K-ras-LNSX. Additional study is needed to determine whether our antisense K-ras transcript strategy is specific to the mutated p21 K-ras production. It is also necessary to elucidate the molecular mechanism involved in decrease of K-ras p21 expression.

By Northern blot analysis, a stable expression of antisense or sense K-ras RNA was detected by the strand-specific RNA probes. However, the size of major antisense transcript was 3.5 kb, which was considered to be produced by read-through from 5’ LTR in the LNSX. The most frequently detected mutation is a GGT to GAT transition at codon 12 (11, 12). On the other hand, Smit et al. (39) showed that mutations of this codon to TGT, GTT, and GAT occurred essentially at an equal incidence in the Netherlands. In this study, AsPC-1 cells had a GGT to GAT transition, and MIA PaCa-2 cells had a GAT to TGT. The growth of AsPC-1-AS and MIA PaCa-2-AS was inhibited for both compared with those of controls, suggesting that the transduction of antisense K-ras construct is useful in suppressing the proliferation of pancreatic cancer cells irrespective of the type of their K-ras mutation. This point may give an advantage to our antisense strategy against the other approach to target a specific mutated K-ras mRNA such as the ribozyme method, which cleaves only 3’ to XUX°C sequences (40).

The i.p. injection of the AS-K-ras-LNSX:liposome complex significantly suppressed the formation of peritoneal and pancreatic tumors. The gene transfer by liposome in vivo has some restraint, such as low transduction efficiency and transient nature of the expression (41). In this study, however, the large amount of injected DNA to tumor cells might have enabled the efficient in vivo gene transfer. Liposome-mediated in vivo gene transfer may have a number of advantages over the viral infection-mediated approach as follows (41).

To address the possibility of inadvertent transduction of normal tissues, we analyzed the organ distribution of plasmid DNA administered into the peritoneal cavity of mice, and PCR analysis showed that the injected DNA was delivered to various organs except for the brain. Liposomes injected into the peritoneal cavity have been shown to enter the lymphatics, and then the blood circulation (41–43), to be distributed to the extraperitoneal organs, precluding the entry to the brain probably due to the blood brain barrier. However, there was no remarkable change in hematoxylin- and eosin-stained histological sections of the major organs of the treated mice. It should be noted that the potential for germline transduction is currently a safety concern for all in vivo gene transfer. In this study, PCR analysis, but not Southern blot analysis, showed the presence of the injected DNA in the testis. Zhu et al. (44) have demonstrated that chloramphenicol acetyltransferase activity could be detected in the ovaries of mice given i.v. injections with the DNA:liposome complex containing a chloramphenicol acetyltransferase expression plasmid (44). Thus, although our strategy has suggested a potential clinical efficiency of the gene therapy strategy, additional examinations are required to define in which type of cells the injected DNA is present in tissues, how long DNA stays in the testis and ovary, and whether DNA is integrated into the genome of the spermatogonia and oocytes.

It has been reported that after the inoculation of cancer cells into the peritoneal cavity of nude mice, the first adhesion of cancer cells to peritoneal mesothelium takes place on days 5–7, and the cancer cells start proliferating and infiltrating the muscle layer on days 9–11 (45, 46). The administration of AS-K-ras-LNSX at days 3–4 might inhibit the

| Organ       | Brain | Lung  | Heart | Liver | Pancreas | Spleen | Kidney | Testis | Stomach | Small intestine | Colon | Skeletal muscle | Bone marrow |
|-------------|-------|-------|-------|-------|----------|--------|--------|--------|---------|---------|---------|---------|--------------|------------|

*a The number of mice that tested positive relative to the total number analyzed.
initial adherence of AsPC-1 cells to the peritoneal mesothelium and pancreas. The peritoneal dissemination and locoregional recurrence are the most frequent modes of recurrence after surgical resection of pancreatic cancer (47, 48). Therefore, the prevention and treatment for peritoneal dissemination are one of the most important therapeutic targets for pancreatic cancer. Because this study suggested that the i.p. injection of the antisense K-ras expression construct complexed with liposome is very effective in an early stage of carcinomatous peritonitis, this strategy might be useful as an adjuvant therapy at the time of the surgical resection of the pancreatic cancer. Furthermore, our preliminary experiment showed that the administration of the AS-K-ras-LNSX:liposome complex at day 14–15 could suppress the development of peritoneal dissemination of AsPC-1 cells (data not shown), suggesting this antisense strategy is also potentially useful for the treatment of the existing carcinomatous peritonitis.

ACKNOWLEDGMENTS

We thank Dr. A. Dusty Miller for providing vector LNSX; Dr. Ken Yamaguchi for AsPC-1 and BxPC-3; and the Japanese Cancer Research Resources Bank for MIAPaCa-2. We are also grateful to Drs. Makoto Tsukamoto and Takahiro Ochiya for discussions and technical advice.

REFERENCES


Liposome-mediated *in Vivo* Gene Transfer of Antisense K-*ras* Construct Inhibits Pancreatic Tumor Dissemination in the Murine Peritoneal Cavity

Kazunori Aoki, Teruhiko Yoshida, Takashi Sugimura, et al.


**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/17/3810

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.