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Antisense Therapy Specific to Mutated K-*ras* Gene in Hamster Pancreatic Cancer Model. Can it Inhibit the Growth of 5-FU and MMC-resistant Metastatic and Remetastatic Cell Lines?

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Abstract. *K-ras point mutation at codon 12 has a relationship greater than 90% with pancreatic cancer. Cancer therapy should also include the treatment of metastatic disease because it is known that the properties of metastatic cells may vary considerably from those of the primary tumor. Aim: To clarify if the same drugs, which can inhibit the tumor growth in the parental cell line, can inhibit the pancreatic metastatic and remetastatic cell lines at the same concentrations and to compare the inhibition with antisense oligonucleotides mismatched to K-ras gene, in Syrian golden hamsters. Materials and Methods: HaP-T1, a BHP-induced hamster pancreatic cancer cell line, MS-PaS-1 (a metastatic cell line established from "return trip" metastases from the liver to the pancreas) and MS-PaS-2 named as a "remetastatic cell line", i.e., metastases from MS-PaS-1, were used. MTT and MTT-agarose assays were performed, using 5-Fluorouracil (5-FU), Mitomycin C (MMC) and antisense oligonucleotide specific to K-ras oncogene. Results: The inhibitory concentration (IC_{50}) of 5-FU, which inhibited HaP-T1, had to be increased by 50-fold to inhibit MS-PaS-1 and 100-fold to inhibit MS-PaS-2. MMC had to be increased by 10-fold to inhibit MS-PaS-1 and 50-fold to inhibit MS-PaS-2. However, IC_{50} was the same when antisense oligonucleotide was tried in these 3 cell lines. Conclusion: Antisense oligonucleotide-targeted K-ras gene may be a good choice for therapy because it could inhibit the growth in metastatic and remetastatic cells as well as in primary tumor cells.*

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Pancreatic cancer has one of the worst prognoses of all malignancies (1,2). The incidence of the disease is nearly equivalent to the death rate associated with the diagnosis of pancreatic cancer (3). This poor prognosis relates mainly to its late diagnosis, although better imaging techniques and safer surgery have been developed (4). At the time of the diagnosis, metastasis has already spread in most cases.

K-*ras* point mutation at codon 12 has a relationship of about 65 to 90% with pancreatic cancer in humans (5-10) and 90 to 95% with a nitrosamine-induced hamster pancreatic cancer model (11-19). Cancer therapy should also include the treatment of metastatic disease because it is known that the properties of metastatic cells may vary considerably from those of the primary tumor. Additionally, some reports attribute failure of chemotherapy regimens to acquired drug chemoresistance (20,21). However, hypothetically metastases have heterogeneous populations, responsible for their resistant phenotype to the drug.

Considerable efforts have been made to develop drugs that will interfere with the function of Ras proteins, with the hope that the removal of the Ras-dependent growth signal from tumor cells might cause their growth arrest or death. Thus, among the variety of drugs targeting this gene, farnesyl transferase inhibitors and antisense oligonucleotides have been developed. In addition, considering that the goal of gene therapy for cancer is to inhibit the constitutive signals that drive tumor growth, antisense oligonucleotides could be a good choice to deal with this disease.

In previous reports with the K-*ras* point mutation as the target gene, Alemany *et al.* (22) reported that antisense K-*ras* fragment transfected by adenovirus vector inhibited cell growth of lung cancer with a mutant K-*ras*. Aoki *et al.* (23) stated that antisense K-*ras* expression plasmid inhibited K-*ras* p21 protein production and growth in human pancreatic cancer cell lines and suppressed the pancreatic tumor development *in vivo* (24). Kita *et al.* (25) and Nakada *et al.* (26) demonstrated the antiproliferative effect on cultured pancreatic cancer cell lines.

The aim of this study was to clarify if the same drugs, which can inhibit the tumor growth in the parental cell line, can also inhibit pancreatic metastatic cell line growth at the same concentrations and to compare the inhibition with antisense oligonucleotides mismatched to K-*ras* gene. Moreover, inhibition in a remetastatic pancreatic cell line, *i.e.*, established from "return trip" metastases of MS-PaS-1, in a hamster pancreatic cancer model, was studied.

Materials and Methods

Experimental animals. Four male Syrian golden hamsters (GN strain) aged 12 weeks, weighing on average 126 g, purchased from Nippon Institute for Biological Science (Ohme, Tokyo, Japan), were used. The hamsters were fed with clean water and chow *ad libitum* and kept in 12/12 hours day/night cycle.

Cell line. HaP-T1, a BHP-induced hamster pancreatic cancer cell line established by Saito *et al.* (27), was used for the experiments. The tissue culture was maintained through serial passages using Eagle's Minimal Essential Medium (MEM), supplemented with Fetal Bovine Serum (FBS) 10%, in a 5% CO_2 environment. K-*ras* point mutation at codon 12 from GGT to GAT is present in this cell line (17-19).

Experimental design. Liver implantation of pancreatic cancer tissue was performed as previously described (28). The resulting pancreatic metastasis was then resected and a metastatic pancreatic cancer cell line was established. Next, the liver implantation procedure was performed once more and the resulting pancreatic metastasis, *i.e.*, remetastasis because it was derived from a metastatic cell line, MS-PaS-1, was resected in order to establish a remetastatic pancreatic cancer cell line (Figure 1).

Preparation of tumor cell suspensions for in vivo injections. Subconfluent cultures of HaP-T1 were harvested after treatment with 0.25% trypsin and 0.02% EDTA for 10 min. The trypan-blue dye exclusion test was used to determine the concentration of viable cells. Cells with viability over 90% were suspended in serum-free medium for *in vivo* injections.

Subcutaneous implantation. After diethyl-ether inhalation anesthesia and asepsy, a tumor cell suspension (2 x 10^6 cells/ml, 0.1 ml) was inoculated subcutaneously. One month later, the hamsters were reanesthetized, the tumor was resected and minced into pieces of 1 mm^3.

Liver implantation of the tumor and establishment of cell lines. Animals were anesthetized with diethyl-ether inhalation and sodium pentobarbital (5mg/kg/body weight) intraperitoneally. As previously described, one piece of tumor was implanted in the frontal lobe of the liver (28). The hole was closed with Vycril® 7-0 suture (Ethicon Co., NJ, USA). The abdominal wall was closed in two layers with 4-0 nylon sutures.

Animals were followed-up and necropsy performed after death. Metastases found in the splenic lobe of the pancreas were resected and surrounding necrotic tissue was removed and cut into fragments of 1 mm^3. Pieces were washed 3 times with MEM with FBS 10%. An explantation technique was employed using MEM supplemented with FBS 20%, as previously described (27). After 3

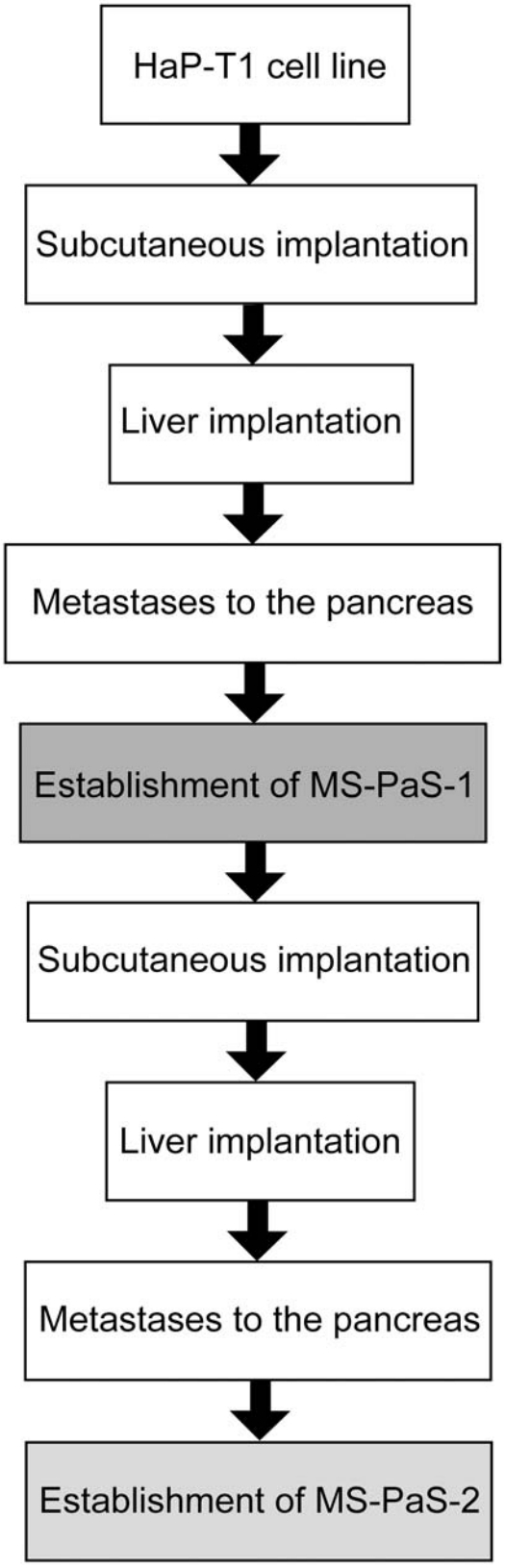

Figure 1. *Experimental protocol. See text for more details.*

days, the tumor pieces were taken out and the cells were covered with media. One week later, fibroblasts were absent and the first passage was performed. After 20 passages, the growth curve was established by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) according to Mosmann's method. The doubling-time was 19 hours. This cell line was named as MS-PaS-1.

Next, a cell suspension of MS-PaS-1 was implanted subcutaneously. After one month, the tumor was resected, minced and one fragment was implanted into the liver, as described above. After follow-up and death of the animals, establishment of the cell line was made as described above. The doubling-time was 15 hours. This remetastatic pancreatic cell line, because it was established from MS-PaS-1, was named as MS-PaS-2.

Sequencing of K-ras exon 1 in metastatic and remetastatic pancreatic cell lines. DNA extraction, amplification by PCR, purification of recovered DNA and direct sequencing were performed as previously described (17). Briefly, electrophoretically segregated, PCR-amplified fragments of K-*ras* exon 1 were purified with Suprec-01 devices (Takara Shuzo, Kyoto, Japan) and then sequenced directly with the Auto Cycle Sequencing kit (Pharmacia, Uppsala, Sweden) using fluorescent primers. Sequencing reactions were analyzed with A.L.F. DNA Sequencer (Pharmacia) according to the manufacturer's instructions.

Direct sequencing detected GGT to GAT transition at codon 12 of K-*ras* exon 1 in MS-PaS-1 and MS-PaS-2, coinciding with parental cell line (HaP-T1) mutation.

Chemotherapy drugs. The chemotherapy drugs used were: 5-Fluorouracil (5-FU), (Sigma Chemicals, Co., Japan) and Mitomycin C (MMC), (Sigma Chemicals, Co.).

Design of antisense oligonucleotides. A mutation-specific antisense oligonucleotide specific to K-*ras* gene was designed as follows: antisense 5'-CTACGCATCAGCTCCA-3' and sense 5'-TGGAGC TGATGGCGTAG-3'. The underlined bases represent the target sites. The definition for the length of oligonucleotides was based on previous reports (24-26). Oligonucleotides purified by HPLC method were purchased from Nippon Gene, Toyama, Japan.

Transfection procedure. Oligonucleotides were transfected to the cells by the liposome-mediated method (24,25). Briefly, oligonucleotides and liposomes were diluted separately. The mixtures were diluted to different concentrations and were incubated for 30 minutes at room temperature to form complexes. Next, they were added to subconfluent cultures and incubated for 4 hours or 24 hours. After exposure to complexes, the medium containing FBS 20% was changed to recover the growth. The growth inhibition was determined by two methods: MTT and MTT-agarose assays.

MTT assay. Parental, metastatic and remetastatic pancreatic cells were seeded (3,000 cells/well). The cells became subconfluent in about 48 hours. Then, the chemotherapy drugs or oligonucleotides were added at different concentrations. 5-FU or MMC were incubated for 4 hours. DNA complexes were incubated for 4 hours, with recovery of growth for 24 hours. Then, MTT solution was added and incubated for 4 hours. The solution was aspirated. Dimethylsulfoxide (DMSO) was added. The absorbance was measured at 560 and 630 nm (25).

MTT agarose assay. Agarose 0.5% was prepared using MEM, supplemented with FBS 20% and added as a lower layer to 96-well plates. After 4 hours in the refrigerator and 30 minutes in the incubator, agarose 0.3% containing 3,300 cells/well was added as the upper layer. The plates were incubated for 120 hours. Drugs were added and left for 12 hours. Oligonucleotide complexes were left for 24 hours. MTT solution was added and the plates were incubated for 8 hours. Then, to solubilize the agarose and formazan products, SDS 10% was added and left for 12 hours in the incubator (29). The absorbance was measured in the same manner as used in the MTT assay.

Absorbance index. The absorbance index of both MTT and MTT-agarose was calculated as follows:

$$\% \text{ inhibition index} = \frac{\text{negative control} - \text{treated cells}}{\text{negative control}} \times 100$$

In the case of oligonucleotides, the negative control were the "sense treated cells". Therefore, the index was calculated as follows:

$$\% \text{ inhibition index} = \frac{\text{"sense treated cells"} - \text{"antisense treated cells"}}{\text{"sense treated cells"}} \times 100$$

IC_{50} (inhibitory concentration of 50% of the cells) was adjusted to observe the response in the 3 cell lines. All experiments were performed at least in triplicate.

Results

The inhibitory concentration (IC_{50}) of 5-FU, which inhibited the HaP-T1, had to be increased by 50-fold to inhibit MS-PaS-1 and 100-fold to inhibit MS-PaS-2. MMC had to be increased by 10-fold to inhibit MS-PaS-1 and 50-fold to inhibit MS-PaS-2. However, IC_{50} was the same when antisense oligonucleotide was tried in these 3 cell lines (Table I).

Discussion

Pancreatic cancer is still a medical challenge because the mortality rate corresponds to the number of new cases diagnosed every year (3). The curative resection rate is on average 20% (4,30,31). To improve the death rate due to this disease, adjuvant or neoadjuvant therapies have to be effective enough to inhibit metastatic growth. Knowing that K-*ras* point mutation has a relationship of about 90% with pancreatic cancer (5-19), gene therapy against this gene could be a good choice. For this purpose, antisense oligonucleotides could be used to deal with this disease.

Even with the development of new drugs, the effectiveness of chemotherapeutic protocols is low (32,33). Failure rates are due to metastases or local recurrence. To confront possible micrometastases, it might be necessary to increase the drug concentration. However, by increasing this concentration, systemic side-effects could also be increased. Therefore, a therapy the dosage of which could be maintained and which had low side-effect rates seems to be the ideal approach.

Table I. *Inhibitory concentration (IC_{50}) of the cell growth.*

	HaP-T1 (μg/ml)	MS-PaS-1 (μg/ml)	MS-PaS-2 (μg/ml)
5-FU			
MTT	0.3	15[a]	30[b]
MTT-agarose	0.2	4[c]	10[d]
MMC			
MTT	0.005	0.05[e]	0.25[f]
MTT-agarose	0.03	0.3[g]	0.9[h]
ASO			
MTT	0.4	0.4	0.4
MTT-agarose	0.3	0.3	0.3

5-FU: 5 Fluorouracil; MMC: Mytomycin C; ASO: Antisense oligonucleotides. Concentrations to inhibit the tumor growth [a]increased 50-fold, [b]increased 100-fold, [c]increased 20-fold, [d]increased 50-fold, [e]increased 10-fold, [f]increased 50-fold, [g]increased 10-fold and [h]increased 30-fold, when compared with parental cell line HaP-T1.

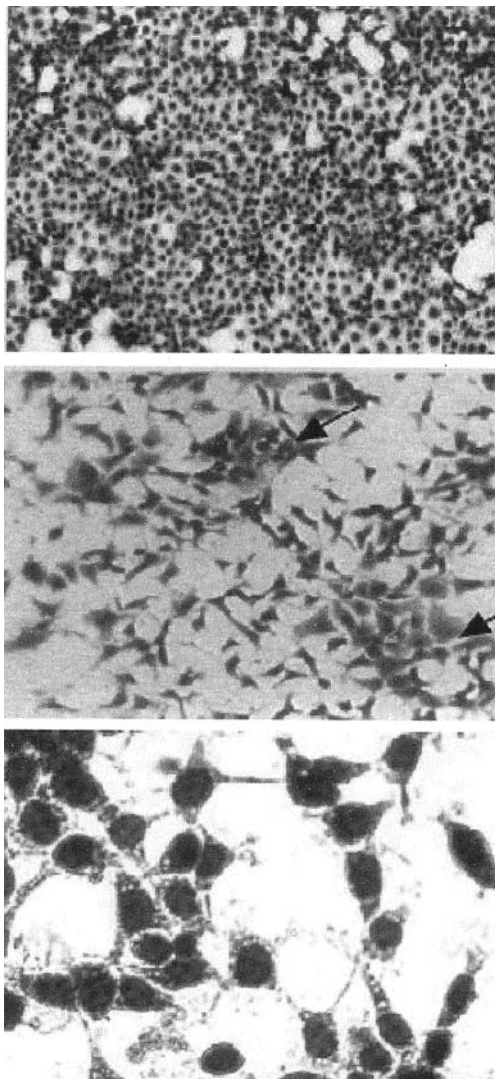

Figure 2. *General appearance of cell cultures. Subconfluent cell culture dishes were fixed in methanol and stained with Giemsa. A. HaP-T1 (parental cell line) (40X). B. MS-PaS-1 (40X). C. MS-PaS-2 (100X). Note the difference between the spatial distribution of the cells and the gross appearance of the cytoplasm. MS-PaS-1 (B) grows in colonies (black arrows) and has fibroblast-like appearance. MS-PaS-2 (C) is similar to MS-PaS-1.*

On the other hand, it has been hypothesized that the growth rate of metastasis may be higher than that of the primary tumor, which could also explain the failure of chemotherapy regimens. Moreover, metastatic recurrence even after metastasis eradication may be a consequence of remaining micrometastatic cell growth or the appearance of remetastatic lesions. These metastatic or remetastatic cells could have had an acquired drug resistance or a clonal selection could have happened. Nevertheless, a therapy targeting these cells at a molecular level could be suitable.

In the present experiments, although the three cell lines were of pancreatic origin, the doubling-time was different in each of them. The increasing concentration of 5-FU and MMC that was applied in metastatic and remetastatic cell lines showed that standard doses for the primary tumor, *i.e.*, parental cell line, may not be effective in the treatment of possible metastatic lesions *in vitro*. On the contrary, antisense oligonucleotides targeting K-*ras* point mutation were effective at the same doses in all three cell lines. In fact, this strategy uses a nucleotide specific sequence ("antisense") designed to hybridize to a target messenger RNA (mRNA) transcript ("sense") *via* Watson-Crick base pairing. Thus, the formation of this DNA:RNA heteroduplex results in mRNA inactivation and consequent inhibition of synthesis of protein product.

Since the same doses of antisense oligonucleotides could be confirmed by MTT-agarose assay, which may mimic chemosensivity tests *in vivo* (29), these results encourage us to try this dosage *in vivo*. In addition, if the concentration of antisense oligonucleotides for gene therapy of primary as well as metastatic and remetastatic cells were the same, we hypothesize that side-effects could be minimized when compared to treatments with 5-FU and MMC.

In conclusion, this study suggests that the absence or failure of response to chemotherapy may be a consequence of a clonal selection through the metastatic process and not due to acquired drug resistance. Moreover, considering that the goal of gene therapy for cancer is to inhibit the constitutive signals that drive tumor growth, antisense oligonucleotide-targeted K-*ras* gene may be a good choice of therapy because it can inhibit the growth of metastatic and remetastatic cells as well as of primary tumor cells.

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