Suppression of Invasion of a Hamster Pancreatic Cancer Cell Line by Antisense Oligonucleotides Mutation-matched to K-ras Gene

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Abstract. The anti-invasive activity of antisense oligonucleotides (ASO) specific to the K-ras gene in hamster pancreatic cancer was investigated. HaP-T1, a cell culture derived from BHP-induced hamster pancreatic cancer, was used. After liposome-mediated transfection with mutation-matched and mutation-mismatched ASO in different concentrations, cell proliferation was studied by MTT and MTT-agarose methods. In vitro chemoinvasion assay with the reconstitution of a matrix of a basement membrane onto a filter in a Boyden chamber was performed. Mutation-matched ASO inhibited the tumor growth and invasiveness of HaP-T1 in a dose-dependent manner, while mutation-mismatched ASO were not effective in inhibiting invasion. The present study suggests that antisense oligonucleotides mutation-matched to the K-ras gene may be a new anticancer strategy for pancreatic cancer since they inhibited not only tumor growth but also invasiveness in vitro.

New genes are continually being discovered, some of them related to disease states including pancreatic cancer. Point mutation of the K-ras gene is detected in >80% of human pancreatic cancer (1-3). Moreover, ras activates a multitude of downstream events with roles in cellular processing, including invasion and metastasis. This has provided opportunities for the development of new therapeutics to target a wide range of human diseases. These new drugs are intended to be highly specific: antisense oligonucleotides (ASO) are one of this class of new drugs (4-6). The phosphorotiate antisense oligonucleotides are the current choice for antisense therapy (5, 7, 8). Hamster pancreatic experimental cancer resembles that of humans immunologically, anatomically and genetically (9, 10). We have previously reported on the incidence of the K-ras point mutation in this model achieving a 100% rate (11-13). Thus, this model may be useful for the study of treatment strategies against this type of cancer. Therefore, our aim was to investigate the anti-invasive activity of ASO specific to the K-ras gene in hamster pancreatic cancer.

Materials and Methods

Cells. HaP-T1, a cell culture derived from BHP-induced hamster pancreatic cancer, was used for these experiments (14). Tissue culture was maintained through serial passages with Eagle’s minimal essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (M.A. Bioproducts, Walkersville, MA, USA), non-essential amino acids for MEM (10 ml/l, Dainippon Pharmaceutical Co., Tokyo, Japan) and glutamine (0.3 g/l, Nissui Pharmaceutical Co.), in a 5% CO2 environment. This cell line shows a mutation from GGT to GAT at codon 12 of the K-ras gene (11).

Antisense oligonucleotides design. Mutation-specific antisense oligonucleotide specific to the K-ras gene was designed as follows: antisense (AS-GAT) 5’-CTACGCCATCAGCTCCA-3’ and sense (S-GAT) 5’-TGGAGCTGATGGCGTAG-3’. Mutation-mismatched ASO was designed as follows: antisense (AS-GGT) 5’-CTACGCCACAGCTCCA-3’ and sense (S-GGT) 5’-TGGAGCTGACTGGGCTAG-3’ (15, 16). All oligonucleotides were purified by the HPLC method (Nippon Gene, Toyama, Japan).

Transfection procedure. Oligonucleotides were transfected to the cells by a liposome-mediated method. Briefly, oligonucleotides and liposomes were diluted separately 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. After exposure to the complexes, the medium containing FBS 20% was changed to recover the growth (17). The growth inhibition was determined by two methods: MTT and MTT-agarose assays.

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Key Words: Pancreatic cancer, Syrian golden hamster, K-ras point mutation, invasiveness, antisense oligonucleotides.
Figure 1. Growth inhibition of antisense oligonucleotides by MTT-agarose. C: control, no treatment. Lipof. only: lipofectamine only; S-GAT: sense oligonucleotide targeting GAT mutation; AS-GAT: antisense oligonucleotide targeting GAT mutation; S-GGT: sense targeting GGT mutation; AS-GGT: antisense targeting GGT mutation. Antisense oligonucleotide (AS-GAT) inhibited the tumor growth in a dose-dependent manner.

Figure 2. Inhibitory effect of antisense oligonucleotides on the invasiveness. C: control, no treatment. Lipof. only: lipofectamine only; S-GAT: sense oligonucleotide targeting GAT mutation; AS-GAT: antisense oligonucleotide targeting GAT mutation; S-GGT: sense targeting GGT mutation; AS-GGT: antisense targeting GGT mutation. Antisense oligonucleotide (AS-GAT) effectively inhibited the invasiveness in a dose-dependent manner.
**MTT assay.** Cells were seeded in 96-well plates (3,000 cells/well). The cells became subconfluent in about 48 hours. Then, oligonucleotides were added at different concentrations. DNA complexes were incubated for 4 hours, with recovery of growth for 24 hours. Then, the MTT solution was added and incubated for 4 hours. The solution was aspirated and dimethylsulfoxide (DMSO) added. The absorbance was measured at 570 and 630 nm (15-17).

**MTT agarose.** 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 an upper layer. The plates were incubated for 120 hours. Transfection was performed for 24 hours. MTT was added and incubated for 8 hours. Then, sodium dodecyl sulfate (SDS) 10% was added and left for 12 hours. The absorbance was measured at 570 nm and 630 nm (17).

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

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

All experiments were performed at least in triplicate.

In vitro chemoinvasion assay. In vitro chemoinvasion assay with the reconstitution of a matrix of a basement membrane onto a filter in a Boyden chamber was used. After the transfection in various concentrations, cell suspensions (50,000 cells) were seeded onto 10 μg Matrigel®-coated filters containing 8 μm pore size PET membrane (Becton Dickinson Labware, Franklin Lake, NJ, USA) in cell culture inserts in a haptotatic assay by using fibronectin (20 μg/ml). In the lower chamber, media and FBS 1% were added. Each assay was incubated for 24 hours. The numbers of untreated (positive control), sense-treated and antisense-treated cells which invaded filters were counted using a microscope at a 200-fold magnification (15, 18).

**Results**

Mutation-matched ASO inhibited tumor growth in a dose-dependent manner. It also inhibited the invasiveness of HaP-T1 in a dose-dependent manner. Mutation-mismatched ASO was not effective in inhibiting either the tumor growth or invasion (Figures 1, 2).

**Discussion**

In the progression of cancer, the selective invasive properties of malignant cells are potentially another step in the metastatic process. The invasive behavior of malignant cells involves a number of factors, such as cell adhesion, motility, destruction of host tissue and growth. The motility of tumor cells involves the invasive ability of these to metastasize. Therefore, in treatment strategies against cancer, a drug that may also inhibit invasiveness is suitable.

The present study showed the usefulness of mutation-specific antisense oligonucleotides in inhibiting invasiveness. On the contrary, mutation-mismatched antisense oligonucleotides, represented by a wild-type allele, did not show positive results. We have previously reported similar results when human pancreatic cancer cell lines were used (15, 16).

Schramm et al. (19) showed the cell surface expression of integrins in the K-ras antisense transfected clones of a colon cancer cell line, in which K-ras activation leads to changes in integrin expression. In fact, integrins play an important role in tumor cell attachment to the basement membrane (20). Moreover, Arao et al. (21) reported on the relationship between integrins and invasive activity using pancreatic cancer cell lines. Therefore, in the present study we speculate that antisense oligonucleotides targeting the K-ras gene could have inhibited the integrin expression, consequently inhibiting the invasiveness.

In conclusion, the present study suggests that antisense oligonucleotides mutation-matched to the K-ras gene may be an additional anticancer strategy for pancreatic cancer, inhibiting both tumor growth and invasiveness. However, further studies will be necessary to clarify the mechanisms.

**Acknowledgements**

We thank Dr. Keiichiro Kita, Dr. Yuji Nakada, Dr. Dilson Pereira da Silva Filho, Dr. Hideki Arai, Dr. Takashi Sakamoto, Mr. Huang Cheng Ching and Prof. Keiichi Yamamoto for their helpful comments.

**References**


Received January 4, 2005
Accepted March 3, 2005

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