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An anti-K-ras ribozyme suppresses oncogene expression and cell growth of human pancreatic cancer

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Hammerhead ribozymes are effective modulators of gene expression due to their simple structure, site-specific cleavage activity and catalytic potential. The K-ras oncogene is thought to play an important role in the growth of pancreatic cancer, because an activated (mutated) ras gene is found in approximately 90 % of human pancreatic cancers. In this study, we designed a hammerhead ribozyme directed against K-ras mRNA at codon 25 [K-ras Rz (25)], and investigated its efficacy in a cultured human pancreatic carcinoma cell line, MIA PaCa-2. K-ras Rz (25) significantly reduced the cellular K-ras mRNA level when introduced into the MIA PaCa-2 cells. The ribozyme suppressed cell growth. K-ras Rz (25) appears capable of reversing the malignant phenotype in human pancreatic carcinoma cells.

Key words : Pancreatic cancer, Ribozyme, Ras, Oncogene, Gene expression

INTRODUCTION

Pancreatic cancer is a devastating disease with an extremely poor prognosis, whose most common form of progression is hepatic metastasis [40, 41]. The reasons for the aggressive growth and metastatic behavior of pancreatic cancer are not entirely understood. In addition, treatment poses several clinical problems, i.e. difficulty in early diagnosis due to the anatomical location and a lack of early symptoms, and limitations of conventional cancer therapy, including surgery, chemotherapy and radiation therapy. Therefore, the development of a new therapeutic strategy for pancreatic cancer, i.e. cancer gene therapy, is one of the most important issues in current medicine [18].

Methods of specific gene modulation to reverse the malignant phenotypes include inhibition of the activated or altered oncogene expression [6, 8, 14, 24, 28, 35, 38]. Hammerhead ribozymes (catalytic RNAs) are derived from satellite RNA of tobacco ringspot virus, and have been developed using a trans-acting RNA enzyme [15, 20]. The hammerhead ribozymes were shown to be effective modulators of gene expression due to their simple structure, site-specific cleavage activity and catalytic potential [2, 5, 10, 13, 17, 34, 36, 39]. The specific cleavage reaction is achieved by complementary binding between the ribozyme's flanking sequence and the target RNA. The transacting ribozymes have the ability to cleave any triplet of NUX (N=any nucleotide, X=A, C or U) as the target RNA sequence. Previously we constructed various ribozymes, and demonstrated ribozyme-mediated specific gene modulation and reversal of the malignant phenotype in cultured cancer cells [11, 16, 19, 25-27, 29-31, 42-45].

The ras oncogene products are located within signal transduction pathways in the cells, and are thought to play an important role in the growth of pancreatic cancer, because an activated (mutated) ras gene is found in approximately 90 % of human pancreatic cancers [1, 3, 4]. In this study, we designed a hammerhead ribozyme directed

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against K-ras mRNA at codon 25, and investigated the ribozyme-mediated reversal of the malignant phenotype of human pancreatic carcinoma cells.

MATERIALS AND METHODS

Cell culture. The human pancreatic carcinoma cell line MIA PaCa-2, composed of undifferentiated carcinoma cells, was obtained from American Tissue Culture Collection (#ATCC CRL-1420; Rockville, MD). The cell line was maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10 % fetal bovine serum (FBS), in addition to 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Gibco BRL, Gaithersburg, MD). The cells were grown in monolayers and passaged every week. They were found to be free of mycoplasma contamination when tested with a Mycoplasma Rapid Detection System (GEN-PROBE, San Diego, CA) every three months. Genomic DNA of the MIA PaCa-2 cells was isolated according to the protocol of Maniatis *et al.* [33]. To detect mutations of the K-ras oncogene in the cell line, DNA sequencing using a kit (dsDNA Cycle Sequencing System, Gibco BRL) was performed. Total RNA from the MIA PaCa-2 cell line and its transfectants was extracted by a single-step procedure using guanidium isothiocyanate and phenol/chloroform.

Design of hammerhead ribozymes. We designed an anti-ras ribozyme [K-ras Rz (25)] to specifically cleave c-K-ras oncogene transcripts at codon 25 (Fig. 1). We also prepared a disabled ribozyme [dis-K-ras Rz (25)] containing a single base change (G to A) in the catalytic core, which was used as a control. We cloned K-ras Rz (25) or dis-K-ras Rz (25) into the expression plasmid vector, pCR3.1 (Invitrogen, Carlsbad, CA), containing the cytomegalovirus (CMV) promoter region and the neomycin (G418) resistance gene. For cloning, the following synthetic deoxyoligonucleotides were used as primers:

5'- GG TCG ACG AAT TCT GAT TCT CTG ATG AGT CCG TG -3' (ras Rz 25-S), 5' - AAA GCT TGC TAA TTT TCG TCC TCA CGG ACT CAT -3' (ras Rz 25-A), for K-ras Rz (25); and 5'- GG TCG ACG AAT TCT GAT TCT CTA ATG AGT CCG TG -3' (ras dis-Rz 25-S), 5'- AAA GCT TGC TAA TTT TCG TCC TCA CGG ACT CAT -3' (ras Rz 25-A), for dis-K-ras Rz (25). By 30 cycles of PCR, we synthesized double-stranded DNA fragments of K-ras Rz (25) or dis-K-ras Rz (25). The primers, ras Rz 25-S and ras dis-Rz 25-S have a Sal I site at their 5' ends, while ras Rz 25-A has a Hind III site at its 3' end, to facilitate insertion of K-ras Rz (25) or dis-K-ras Rz (25) into the multiple cloning site of the pCR3.1 plasmid. To confirm that the complete ribozyme was inserted into the vector, we sequenced the plasmid DNA.

MIA PaCa-2 cells transfected with anti-K-ras ribozyme. The transfection of MIA PaCa-2 cells with pCR3.1/K-ras Rz (25) or pCR3.1/dis-K-ras Rz (25) was performed by the lipofectin method (Lipofectin Reagent, Gibco BRL). Plasmid-containing cells were selected in Dulbecco's modified Eagle's medium supplemented with 800 μg/ml of G418 (Gibco BRL) for 4-6 weeks, and subsequently clones of K-ras Rz (25) [MIA PaCa-2/K-ras Rz (25)], dis-K-ras Rz (25) [MIA PaCa-2/dis-K-ras Rz (25)] or pCR3.1 (MIA PaCa-2/pCR3.1, transfected with pCR3.1 plasmid vector only) transfectants were isolated. Ribozyme expression in the cells was confirmed by Real Time PCR (Perkin-Elmer, Norwalk, CT) using the pCR3.1 primer pairs:

5'- CCA AGC TGG CTA GCG TTT AAA -3' (pCR3.1-S), 5'- GGC TTA AGA CGT CTA TAG GTC -3' (pCR3.1-A), and an oligonucleotide probe: 5'- CTC GGA TCC ACT AGT CCA GTG TGG TGG -3'.

K-ras gene expression. Reverse transcription (RT)-PCR was performed according to a commercial protocol using a thermal cycler GeneAmp PCR System 9600 (Perkin-Elmer). SuperScript I (Gibco BRL) and Taq DNA polymerase (Perkin-Elmer) were used for the RT-PCR. K-ras gene expression was semi-quantitatively detected by RT-PCR and Real Time PCR using the primers.

5'-GAC GAA TAT GAT CCA ACA ATA GAG GAT T -3' (K-ras S), 5'-TAC TGG TCC CTC ATT GCA CTG TA -3' (K-ras A), and 5'-TCT CTT GGA TAT TCT CGA CAC AGC AGG TCA T -3' (K-ras P) for K-ras gene expression; and 5'- ATC TTC AAA CCT CCA TGA TG -3' (β2 m S) and 5'- AAC CCC ACT GAA AAA GAT GA -3' (β2 m A) for β 2-microglobulin gene expression.

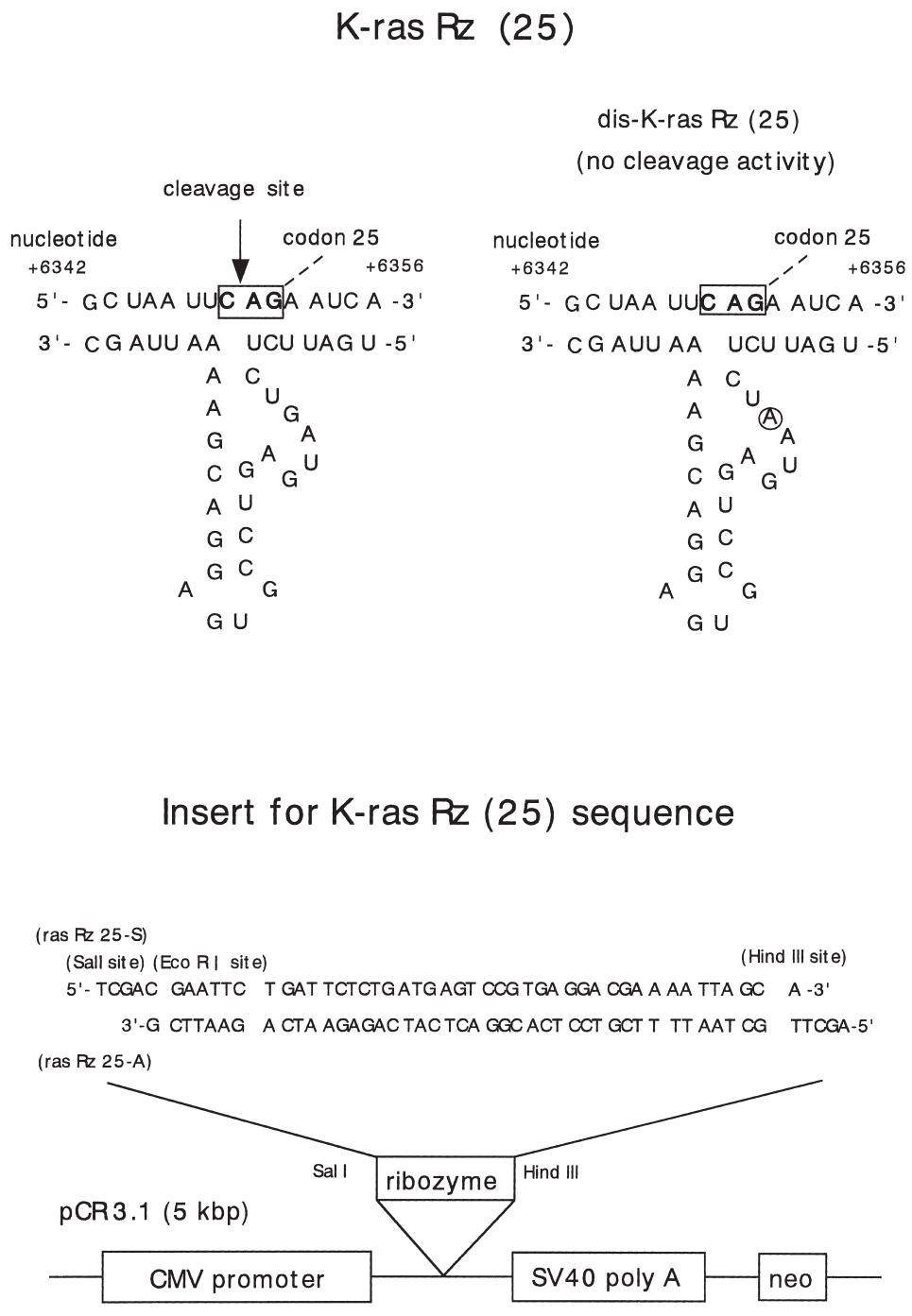

Fig. 1 Design of the anti-K-ras ribozyme [K-ras Rz (25)]. The hammerhead ribozyme targets the UUC sequence of the K-ras oncogene transcript at codon 25 (top). The codon 25 triplet is CAG. The disabled ribozyme contains a single G to A change in the catalytic core of the hammerhead structure. The pCR3.1 plasmid is driven by the cytomegalovirus (CMV) promoter (bottom). The inserted K-ras ribozyme sequence is cloned between the CMV promoter and SV 40 poly A signal.

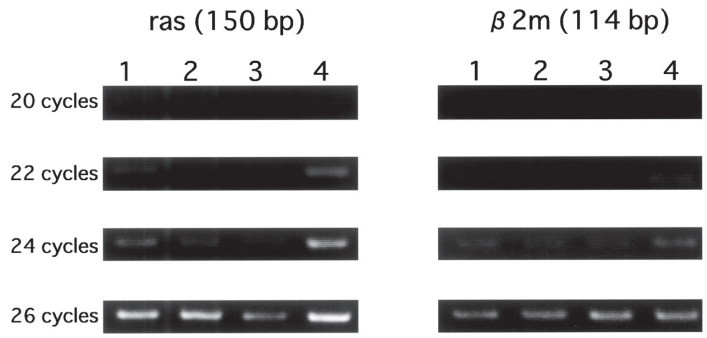

Fig. 2 K-ras (150-bp PCR products) expression in MIA PaCa-2 pancreatic cancer cells detected by RT-PCR (20, 22, 24 and 26 cycles of PCR).
Lane 1, MIA PaCa-2 parental cells; Lane 2, MIA PaCa-2 transfected with pCR3.1 vector only; Lane 3, MIA PaCa-2 transfected with K-ras Rz (25); and Lane 4: MIA PaCa-2 transfected with dis-K-ras Rz (25). K-ras Rz (25) down-regulated expression of K-ras mRNA. The K-ras mRNA level is significantly decreased in the MIA PaCa-2/K-ras Rz (25) cells. Beta-2 microglobulin (β 2 m, 114-bp PCR products) is an internal control of gene expression.

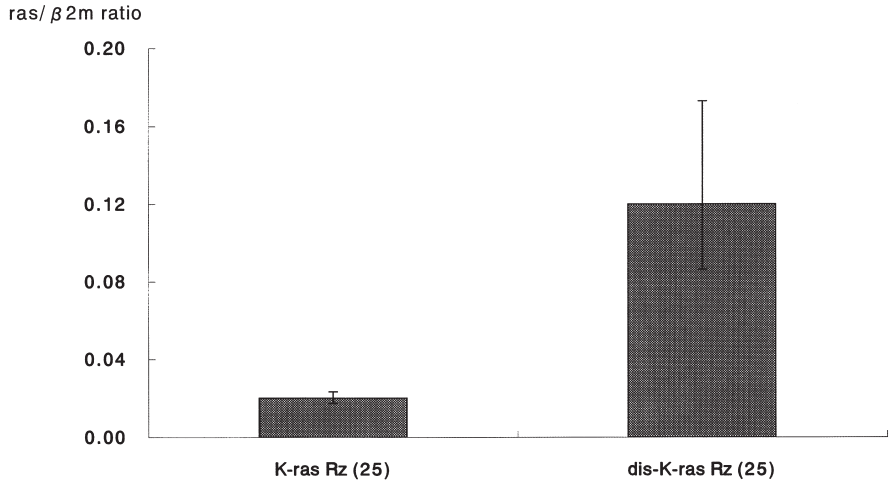

Fig. 3 K-ras expression in MIA PaCa-2 pancreatic cancer cells detected by Real Time PCR. The expression of K-ras mRNA is evaluated as a ratio of ras/beta-2 microglobulin (β 2 m). The K-ras mRNA level in MIA PaCa-2/K-ras Rz (25) cells is 16.7 % of that in MIA PaCa-2/dis-K-ras Rz (25) cells.

Generation time assay of MIA PaCa-2 and transfectant cells. To determine the efficacy of K-ras Rz (25), we enumerated the parental MIA-PaCa2 cells and transfectants [MIA PaCa-2/K-ras Rz (25), MIA PaCa-2/dis-K-ras Rz (25) and MIA PaCa-2/pCR 3.1]. These cell lines were plated at a density of 100 cells/dish on day 0. We counted cell numbers in all lines with a Coulter Counter (Beckman Coulter, Fullerton, CA). The generation time (doubling time) was evaluated using the formula: (t - t0) × log 2 / (log N - log N0), where N and N0 are cell numbers at t and t0 (during logarithmic growth phase) [18]. These experiments were performed twice in duplicate.

RESULTS

Ribozyme-mediated down-regulation of K-ras m-RNA expression in MIA PaCa-2 cells.

The human pancreatic carcinoma cell line MIA PaCa-2 had a TTC triplet at codon 24-25 of the K-ras oncogene, as well as a homozygous TGT mutation at codon 12. K-ras transcripts of the MIA PaCa-2 cells had

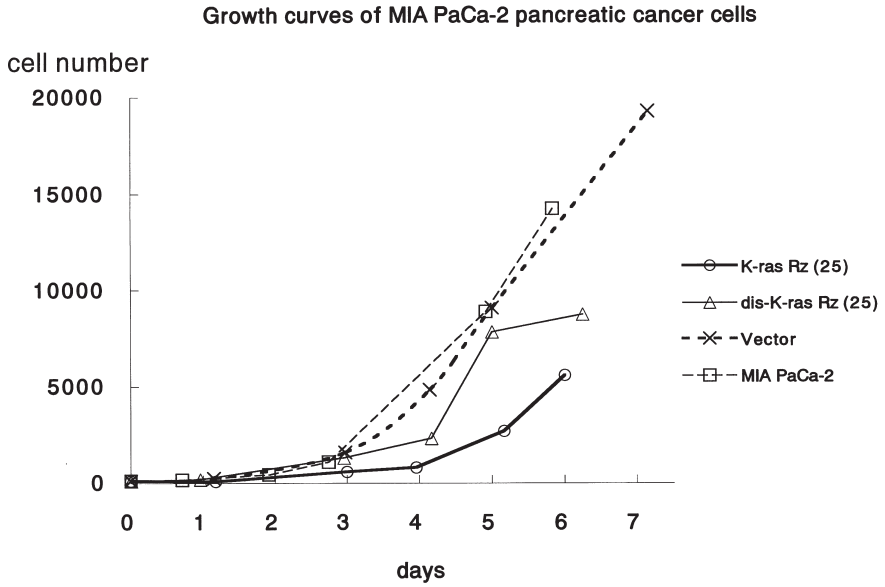

Fig. 4 Growth curves of MIA PaCa-2 pancreatic cancer cell lines. ○ K-ras Rz (25), MIA PaCa-2 cells transfected with K-ras Rz (25); △ dis-K-ras Rz (25), MIA PaCa-2 cells transfected with dis-K-ras Rz (25); × Vector, MIA PaCa-2 cells transfected with pCR3.1 vector only; and □ MIA PaCa-2 parental cells. The MIA PaCa-2 cells transfected with K-ras Rz (25) exhibit a longer generation time (19.6 hours, 1.2-fold) than the parental cells. Plotted data are averages of results performed twice in duplicate.

Table 1 Generation time of MIA PaCa-2 cells

Cells	Generation time	
MIA PaCa-2 (parental cells)	17.1 hours	100 %
MIA PaCa-2/pCR3.1 (vector)	17.2 hours	101 %
MIA PaCa-2/K-ras Rz (25)	19.6 hours	115 %
MIA PaCa-2/dis-K-ras Rz (25)	18.9 hours	111 %

Generation time is calculated on day 3 and day 5.

a UUC sequence at codon 25, which is cleavable by K-ras Rz (25). A significant decrease in the K-ras mRNA level was detected in the MIA PaCa-2/K-ras Rz (25) cells (Fig. 2). In contrast, the mRNA level was not significantly altered in the MIA PaCa-2/dis-K-ras Rz (25) cells. Then the Real Time PCR was performed, and demonstrated that the K-ras mRNA level in the MIA PaCa-2/K-ras Rz (25) cells was 16.7 % of that in the MIA PaCa-2/dis-K-ras Rz (25) cells (Fig. 3).

Ribozyme-mediated growth suppression of MIA PaCa-2 cells.

The transfectants showed similar morphological features to the parental MIA PaCa-2 cells under our cell culture conditions. The generation time of the MIA PaCa-2 parental cells was 17.1 hours (Fig. 4, Table 1). The MIA PaCa-2/K-ras Rz (25) cells exhibited longer generation time, 19.6 hours (1.2-fold longer).

DISCUSSION

In this study, we constructed a hammerhead ribozyme to cleave the K-ras mRNA transcript at codon 25, and examined the biological effects in stable transfectants of the human pancreatic carcinoma cell line MIA PaCa-2. The anti-K-ras ribozyme, K-ras Rz (25), down-regulated K-ras gene transcription, and suppressed the growth of MIA PaCa-2 cells.

Cancer is a genetic disease, and alterations to oncogenes/tumor suppressor genes, such as mutation, amplification, overexpression and deletion, are found in various cancer cells [7, 9, 23]. A point mutation of the ras oncogene family activates its p21 gene products, and affects cancer cell growth and the malignant phenotype [1]. The activated p21 ras oncogene products were suggested to alter the cellular signal transduction pathways and to affect the neoplastic growth of pancreatic cancers [3]. Therefore, activated (mutant) K-ras gene transcripts can be used as potential targets of specific gene modulation for suppressing the growth of pancreatic cancers.

Mutations in the ras oncogene have been found in approximately 90 % of human pancreatic ductal adenocarcinomas [1]. Most of these changes, such as GGT (wild type) to GAT, GTT, and TGT mutations, have been located at codon 12 of the K-ras gene [21]. Previously, we designed a ribozyme targeting activated K-ras mRNA (GTT gene mutation at codon 12), and demonstrated a ribozyme-mediated reversal of the malignant phenotype of pancreatic carcinoma cells [43]. The ribozyme directed against activated K-ras mRNA specifically targeted a single-base mutation of the K-ras mRNA in a cell-free system, as well as in a cell culture system [22, 44]. The anti-K-ras ribozyme specifically cleaved the sequence GUU, but did not affect the other sequences of K-ras mRNA at codon 12; i.e. the anti-K-ras ribozyme we designed previously is thought to have no effect on pancreatic carcinomas with a GAT or TGT mutation of K-ras codon 12. In the present study, therefore, we constructed a new ribozyme, K-ras Rz (25), to cleave the K-ras mRNA transcripts at codon 25, because the UUC triplet in this region is one of the most cleavable sequences (GCU, UUC, CUC, GUA and GUU) of hammerhead ribozymes [32]. Mutations of the K-ras gene at codon 25 have not been found in ordinary human cancers, including human pancreatic ductal adenocarcinomas. Therefore, K-ras Rz (25) is able to down-regulate any kinds of K-ras gene transcripts, such as K-ras mRNA with a UGU or GAU mutation at codon 12, and could be capable of reversing ras-associated malignant phenotypes of human cancers. K-ras Rz (25) was shown to down-regulate K-ras mRNA, which are similar to knockout of the K-ras gene. We speculate that K-ras Rz (25) could suppress K-ras transcripts of any cancer cells, as well as normal cells in general. In this study, K-ras Rz (25) efficiently suppressed K-ras gene transcription as well as the growth of MIA PaCa-2 cells which expressed K-ras mRNA. In addition, dis-K-ras Rz (25) mildly suppressed growth of MIA PaCa-2 cells. The mild growth suppression is thought to be antisense effects of disabled ribozyme without cleavage reaction [20]. The pCR3.1 plasmid used in this study was driven by the CMV promoter, and not by a tissue-specific promoter. For the application of ribozyme-mediated gene modulation, it is necessary to investigate the use of a tissue-specific promoter. One potential candidate is the carcinoembryonic antigen (CEA) promoter because pancreatic carcinomas frequently produce large amounts of CEA [12, 37]. When targeted at a tumor-selective gene and tissue-specific promoter, ribozymes is thought to offer minimal toxicity for *in vivo* applications in cancer therapy.

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