Molecular Consequences of Silencing Mutant K-ras in Pancreatic Cancer Cells: Justification for K-ras–Directed Therapy

Jason B. Fleming,1 Guo-Liang Shen,1 Shane E. Holloway,1 Mishel Davis,1 and Rolf A. Brekken1,2

1Division of Surgical Oncology, Department of Surgery, and Hamon Center for Therapeutic Oncology Research and 2Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas

Abstract

Mutation of the K-ras gene is an early event in the development of pancreatic adenocarcinoma and, therefore, RNA interference (RNAi) directed toward mutant K-ras could represent a novel therapy. In this study, we examine the phenotypic and molecular consequences of exposure of pancreatic tumor cells to mutant-specific K-ras small interfering RNA. Specific reduction of activated K-ras via RNAi in Panc-1 and MiaPaca-2 cells resulted in cellular changes consistent with a reduced capacity to form malignant tumors. These changes occur through distinct mechanisms but likely reflect an addiction of each cell line to oncogene stimulation. Both cell lines show reduced proliferation after K-ras RNAi, but only MiaPaca-2 cells showed increased apoptosis. Both cell lines showed reduced migration after K-ras knockdown, but changes in integrin levels were not consistent between the cell lines. Both cell lines showed alteration of the level of GLUT-1, a metabolism-associated gene that is downstream of c-myc, with Panc-1 cells demonstrating decreased GLUT-1 levels, whereas MiaPaca-2 cells showed increased levels of expression after K-ras knockdown. Furthermore, after K-ras RNAi, there was a reduction in angiogenic potential of both Panc-1 and MiaPaca-2 cells. Panc-1 cells increased the level of expression of thrombospondin-1, an endogenous inhibitor of angiogenesis, whereas MiaPaca-2 cells decreased the production of vascular endothelial growth factor, a primary stimulant of angiogenesis in pancreatic tumors. We have found that silencing mutant K-ras through RNAi results in alteration of tumor cell behavior in vitro and suggests that targeting mutant K-ras specifically might be effective against pancreatic cancer in vivo. (Mol Cancer Res 2005; 3(7):413-23)

Introduction

Pancreatic cancer is now the fourth leading cause of cancer-related death in the United States, and nearly all patients diagnosed with pancreatic adenocarcinoma die a cancer-related death (1, 2). Novel therapy is needed. Molecular genetic analysis has identified mutations in the p53, p16, Smad4 tumor suppressor genes and the ras proto-oncogene in >80% of pancreatic cancers (3). Sequence analysis identified activating ras mutations as the earliest and most common genetic mutation in pancreas cell transformation and tumor progression (4). K-ras, H-ras, and N-ras mutations are involved in ~25% to 30% of all human cancers (5); however, activating K-ras mutations (in codons 12, 13, and 16) are present in nearly all pancreatic adenocarcinomas (6, 7). These facts, in association with the central importance of ras signaling for cell function and survival, highlight that mutant K-ras is an attractive target for the treatment of pancreatic cancer.

The most developed efforts to target ras for solid tumor therapy are inhibitors of posttranslational farnesylation (FTI) of the ras protein, which blocks membrane attachment and effector pathway signaling; however, studies to date have failed to show a correlation between enzyme inhibitory activity and clinical activity, which may explain the limited therapeutic effect of FTIs in pancreatic cancer trials (8, 9). The specific nature of the activating K-ras mutations offers hope of an equally specific therapy against mutant K-ras. Despite encouraging results in preventing tumor growth in animals (10-12), clinical studies using antisense DNA oligonucleotides against ras have not entered large clinical trials (13, 14). One major problem in designing a sequence-specific anti-ras therapy lies in the fact that wild-type and mutant ras differ only in a single codon. The extraordinary sequence specificity of RNA interference (RNAi) makes it an attractive tool for cancer therapy that may capitalize on the small difference between mutant and normal K-ras genes. Brummelkamp et al. (15) used a retroviral RNAi system to inhibit the expression of mutated K-rasV12 while leaving other ras isoforms unaffected. These studies showed that small interfering RNA (siRNA) for K-ras decreases anchorage-independent growth in a pancreatic cancer cell line (Capan-1).
However, the molecular consequences of specific knockdown of mutant K-ras in human pancreatic cancer cells should be explored to determine the impact of siRNA on cancer cell survival and functions critical for tumor growth and metastasis, such as angiogenesis, metabolism, adhesion, and motility. The overall hypothesis driving the present work is that RNAi therapy directed at specific K-ras mutations identified in pancreatic cancer cells will induce cell death or reverse the neoplastic cellular phenotype.

Results

Specificity of Mutant K-ras siRNA in Human Pancreatic Cancer Cells

Transfection of human pancreatic cancer cell lines, Panc-1 and MiaPaca-2, with duplexed oligonucleotides specific for mutations in the K-ras gene found in either cell line reduced the expression of K-ras protein (Fig. 1A). The reduction of gene/protein expression was specific as oligonucleotides with a single bp alteration (Mismatch; Fig. 1A) or specific for other targets (Lamin; Fig. 1A) were ineffective at reducing the level of K-ras expression. Furthermore, exposure of cells to siRNA constructs encoding other wild-type sequences within the K-ras open reading frame also inhibited K-ras protein expression. A dose-dependent decrease in K-ras protein was observed after exposure of the cells to increasing concentrations of siRNA (25, 50, or 100 nm; Fig. 1B). Additionally, a temporal decrease in protein expression was observed with maximal inhibition of K-ras protein levels in MiaPaca-2 and Panc-1 cells 48 hours after exposure to 100 nm mutant-specific K-ras siRNA (Fig. 1B). Exposure of Panc-1 cells to siRNA specific for lamin A/C selectively reduced lamin protein levels, but exposure of the cells to siRNA specific for wild-type or mutant K-ras did not alter the level of lamin A/C (Fig. 1C). To show sequence specificity of the siRNA, Panc-1 and MiaPaca2 cells were treated with the siRNA oligonucleotides encoding the activating K-ras mutation found in each cell line. The Panc-1–specific siRNA reduced the level of K-ras protein in Panc-1 cells but not MiaPaca2 cells (Fig. 1D). The MiaPaca2-specific siRNA was also specific such that it only reduced the level of K-ras protein in the MiaPaca2 cells (Fig. 1D). Importantly, alterations in protein kinase R expression (Fig. 1E) or phosphor-PKR (data not shown) were not observed after K-ras siRNA exposure; this protein is associated with interferon response to ectopic RNA exposure (16, 17).

Colony Formation, Cell Proliferation, and Apoptosis after K-ras siRNA

Panc-1 or MiaPaca-2 cells exposed to mutant-specific K-ras siRNA showed decreased cell proliferation, colony formation, and alteration in cell cycle proteins (Fig. 2). Both cell lines showed a 75% reduction in colony formation after treatment with mutant-specific K-ras siRNA (Fig. 2B) and both cell lines

FIGURE 1. Reduction of the level of K-ras protein in pancreatic tumor cells with mutant-specific siRNA. A. K-ras protein levels in whole cell lysates prepared from cells pretreated for 48 hours with siRNA (100 nmol/L) specific for lamin A/C (Lamin), wild-type K-ras (WT K-ras), a mismatch version of the RNA oligospecific for the activating mutation in either Panc-1 or MiaPaca-2 cells (Mismatch), mutant K-ras (K-ras), or Oligofectamine alone (Control). B. K-ras protein levels in Panc-1 and MiaPaca-2 cell lysates after titration of the mutant-specific K-ras siRNA or a time course of mutant-specific K-ras siRNA (100 nmol/L). β-actin protein levels were used as a cell lysate loading control. C. The level of lamin in Panc-1 cells after treatment with siRNA specific for lamin A/C, wild-type K-ras, mutant K-ras, and Oligofectamine alone was determined by Western blotting. D. K-ras β-actin protein levels in Panc-1 (P) and MiaPaca2 (M) cells after exposure to mutant-specific K-ras siRNA. The siRNA oligonucleotides used encoded the sequence specific for the mutation found in Panc-1 (K-ras (P)) or MiaPaca2 (K-ras (M)) cells. E. The level of PKR protein assessed by Western blotting in Panc-1 and MiaPaca-2 cell lysates after titration of mutant-specific K-ras siRNA.
had a reduced capacity to proliferate after K-ras knockdown (Fig. 2C and D). To determine the molecular changes associated with decreased cell proliferation, we analyzed the changes in the level expression of cell cycle proteins by Western blot analysis. Panc-1 and MiaPaca-2 cells treated with mutant-specific K-ras siRNA showed a time- and dose-dependent decrease in the expression of cyclin D1 and a concomitant increase in the level of p27cip1/kip 1. Additionally, Panc-1, but not MiaPaca-2 cells, showed a reduction in the level of p21 waf1 (Fig. 2A). p21 waf1 was not detectable in MiaPaca-2 cells (Fig. 2A).

Apoptosis of both cell lines after exposure to mutant-specific K-ras siRNA was measured by the use of the DNA-binding dye Hoechst 33342 (18, 19), which allows for evaluation of nuclear morphology. Additionally, the level of activation of caspase-3 and caspase-9 was determined by Western blot analysis. These studies identified condensed nuclei in 30% to 40% of MiaPaca-2 cells as well as a coincident increase in the expression of activated caspase-3 and caspase-9 after exposure to mutant-specific K-ras siRNA (25 nm; Fig. 3A and B). Panc-1 cells, on the other hand, did not show an increase in the number of apoptotic cells nor was there any detectable increase in the activation of caspase-3 or caspase-9 (Fig. 3A).

Cell Migration after K-ras siRNA

The effect of siRNA-mediated inhibition of mutant K-ras expression on the migration of MiaPaca-2 and Panc-1 cells through an extracellular matrix was examined using a modified Boyden chamber assay (20-22). We observed an ~70% reduction in cell migration in both cell lines after exposure to mutant-specific K-ras siRNA (100 nmol/L; Table 1, Fig. 4). Pancreatic cancer cell migration through an extracellular matrix requires interaction of cell surface integrins with the extracellular matrix. Therefore, we examined the expression of the fibronectin-binding integrin α5β1 in both cell lines after K-ras RNAi. Both MiaPaca-2 and Panc-1 cells showed a reduction in
the level of α5β1 integrin by immunocytochemistry (Fig. 5A and B). The level of α5 integrin was reduced significantly in Panc-1 cells treated with K-ras siRNA, whereas the level of β1 integrin dropped significantly in the MiaPaca-2 cells treated with K-ras siRNA. Western blot analysis of whole cell lysates confirmed the immunocytochemical staining (data not shown).

Alterations in the Angiogenic Balance after K-ras siRNA

Angiogenesis is critical to the formation of metastatic tumors and increased microvascular activity in primary pancreatic adenocarcinoma tumors is associated with metastasis and decreased patient survival (23, 24). Angiogenesis is regulated tightly by the balance of promoters and inhibitors, such as vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1), a primary stimulant and inhibitor of angiogenesis, respectively (25). The expression of VEGF and TSP-1 by Panc-1 and MiaPaca-2 cells was examined after exposure to mutant-specific K-ras siRNA. The baseline level of VEGF production in Panc-1 and MiaPaca-2 cells in culture was 750 and 550 pg/mL, respectively. After K-ras RNAi treatment, VEGF production was moderately reduced in the Panc-1 cells but dropped substantially to <100 pg/mL (P < 0.001) within 72 hours after MiaPaca-2 cells were exposed to mutant-specific K-ras siRNA (Table 2). Alternatively, Panc-1 cells showed a time- and dose-dependent increase of the level of expression of TSP-1 transcript (Fig. 6C) and protein (Fig. 6A and C) after treatment with K-ras siRNA. TSP-1 expression was absent in the MiaPaca-2 cells at baseline and after siRNA treatment.

A primary mechanism that controls TSP-1 expression is the transcription factor and oncogene c-myc (26). Therefore, we examined the expression of c-myc in Panc-1 and MiaPaca-2 after K-ras siRNA exposure. Protein levels of the c-myc oncogene were present in both Panc-1 and MiaPaca-2 cells at baseline; however, 48 hours after siRNA exposure, c-myc levels were persistently high in surviving MiaPaca-2 cells, but decreased to below baseline in Panc-1 cells (Fig. 6B). In both cell lines, the level of c-myc correlated inversely with TSP-1 expression.

Alteration in Cell Metabolism after K-ras siRNA

Tumor cells growing under conditions of normal oxygen tension show elevated glycolytic rates that correlate with increased expression of glycolytic enzymes and glucose transporters, a phenomenon termed the Warburg effect (27, 28). One example of the Warburg effect is the level of expression of glucose transporter-1 (GLUT-1). GLUT-1 expression is regulated by c-myc, is expressed at a high level in many solid tumors, including pancreatic cancer, and is associated with tumor metastasis (29). The expression of GLUT-1 was examined in Panc-1 and MiaPaca-2 cells after exposure to the mutant-specific K-ras siRNA (Fig. 7). Consistent
with the level of c-myc expression in each cell line, MiaPaca-2 cells showed an increase in GLUT-1 protein expression within 48 hours of exposure to 100 nmol/L of mutant-specific K-ras siRNA; however, transcription and protein expression of GLUT-1 decreased in Panc-1 cells after exposure to K-ras siRNA (Fig. 7). The increase in GLUT-1 protein levels in MiaPaca-2 cells exposed to mutant-specific K-ras siRNA seems to occur at a posttranscriptional level because the amount of GLUT-1 transcript does not change after siRNA treatment.

Discussion

The present study was undertaken to determine the molecular consequences of silencing mutant K-ras production in human pancreatic adenocarcinoma cells. The major finding to result is that specific knockdown of mutant K-ras through RNAi disrupts the malignant phenotype of pancreatic adenocarcinoma cells by interfering with multiple characteristics critical to the metastatic program.

Hanahan and Weinberg (30) described the principle characteristics of neoplastic cells to include unregulated growth,
levels of c-myc expression, and Panc-1 and MiaPaca-2 cells fall (41). Seventy percent of malignant tumors show increased growth, protein synthesis, and mitochondrial function (38), which is a normal consequence of c-myc accumulation (40). Increased levels of c-myc activity promote an angiogenic switch (38), which is a normal consequence of c-myc accumulation (40).

Stabilization and accumulation of the normally short-lived c-myc, which promotes transformation and survival (36-39). It results in toto (31-33). Recently, c-myc–induced mammary transformation of epithelial cells has been noted to be a potentially broad-based therapy for this disease. The genetic profile of Panc-1 and MiaPaca-2 cells represent >90% of the mutations found in human pancreatic adenocarcinoma tumors. In addition to mutant K-ras, both cell lines possess p53 mutations and homozygous deletion of p16 Ink4a. Fifty percent of human pancreatic tumors possess an interrupted K-ras in pancreatic cancer cells results in decreased angiogenic potential. The synergy between the c-myc and ras pathways in malignant transformation of epithelial cells has been noted previously (31-33). Recently, c-myc–induced mammary tumorigenesis was observed to proceed through a preferred secondary oncogenic pathway involving K-ras2 over other ras species (34). Furthermore, Yeh et al. (35) described that the molecular interaction between ras and c-myc results in the stabilization and accumulation of the normally short-lived c-myc, which promotes transformation and survival (36-39). It is also known that activated ras protects cells from apoptosis, which is a normal consequence of c-myc accumulation (40). Increased levels of c-myc activity promote an angiogenic switch (38), growth, protein synthesis, and mitochondrial function (41). Seventy percent of malignant tumors show increased levels of c-myc expression, and Panc-1 and MiaPaca-2 cells fall.

Within this group. When K-ras was silenced in Panc-1 and MiaPaca-2 cells, the observed cellular phenotypic changes in proliferation and apoptosis, cell adhesion and migration, angiogenic profile, and metabolism can potentially be explained by changes in c-myc–controlled genes.

The genetic profile of Panc-1 and MiaPaca-2 cells represent >90% of the mutations found in human pancreatic adenocarcinoma tumors. In addition to mutant K-ras, both cell lines possess p53 mutations and homozygous deletion of p16 Ink4a. Fifty percent of human pancreatic tumors possess an interrupted Smad/transforming growth factor-β signaling pathway (3). In our study, Panc-1 cells retain transforming growth factor-β signaling via the Smad4 mechanism, but MiaPaca-2 cells lack a functional transforming growth factor-β receptor 2. Transforming growth factor-β signaling is known to promote degradation of c-myc protein levels, which protects cells from the deleterious effects of c-myc overexpression and offers a potential explanation for the different responses of Panc-1 and MiaPaca-2 cells to K-ras siRNA (42, 43).

The balance between cancer cell production and excretion of proangiogenic (e.g., VEGF) and antiangiogenic (e.g., TSP-1) factors defines the ability of neoplastic cells to recruit new vasculature and progress to metastasis (26). Oncogenic ras has been shown repeatedly to promote VEGF secretion from neoplastic cells (44) and both Panc-1 and MiaPaca-2 produce high levels of VEGF in culture. Ablation of K-ras activity resulted in marked reduction in VEGF production from MiaPaca-2 cells and a more modest reduction from Panc-1 cells. These results confirm that there are multiple oncogenic pathways capable of regulating VEGF levels and suggest that c-myc does not directly control VEGF production in MiaPaca-2 cells. However, it is conceivable that c-myc is an important component driving VEGF production in the Panc-1 cells because
the level of VEGF does drop after c-myc levels are reduced by K-ras RNAi (45). Previous work suggests that c-myc represses the expression of TSP-1 (46). Furthermore, H-ras has been shown to repress TSP-1 expression and increase tumor angiogenesis (26). K-ras might serve a similar function in Panc-1 cells as loss of K-ras results in increased TSP-1 levels coincident with the reduction of c-myc protein levels. In MiaPaca-2 cells, c-myc levels were persistent after K-ras reduction. We were unable to detect TSP-1 at the mRNA level or at the protein in whole cell lysates or in ethanol-precipitated tumor cell–conditioned media (data not shown). If the changes in VEGF and TSP-1 levels after K-ras knockdown in Panc-1 and MiaPaca-2 cells are taken together, they predict that the molecular changes after specific knockdown of mutant K-ras will potentially result in an antiangiogenic phenotype.

Differences in the level and activity of c-myc after knockdown of K-ras in Panc-1 and MiaPaca-2 cells potentially explains the alterations in cell survival and integrin expression we observed between the two cell lines. c-myc promotes cell cycle progression through sequestration of p27 and repression of p21 and p15 (40, 47). Accordingly, an increase in p27 and p21 was observed in Panc-1 cells 24 hours after reduction in K-ras.
and c-myc, whereas no such changes are observed in MiaPaca-2 cells after K-ras siRNA. Cell proliferative and apoptotic pathways are coupled because induction of the cell cycle sensitizes the cell to apoptosis. The apoptotic pathway is suppressed in the presence of appropriate survival factors. Oncogenic K-ras acts as a survival signal and suppresses c-myc–induced apoptosis (48). However, inhibition of K-ras allows c-myc to trigger caspase-related apoptosis as was observed in the MiaPaca-2 cells. Therefore, Panc-1 cells, with decreased c-myc levels, showed no evidence of increased apoptosis after siRNA-mediated reduction of K-ras.

Tumor cell migration and interaction with the extracellular matrix is critical for metastasis. Reduction of mutant K-ras levels resulted in decreased migration of both Panc-1 and MiaPaca-2 cells consistent with the results of Yan et al. (49, 50). Expression of β1 integrin promotes metastasis of ras–myc–transformed fibroblastoid cell tumors in animal studies (51), and the interaction between integrin α5β1 and fibronectin promotes the survival of growth-arrested cancer cells at sites of distant metastasis (52). The integrin α5 promoter contains a CCAAT/enhancer binding protein binding site and CCAAT/enhancer binding protein overexpression represses integrin α5 expression (53). Furthermore, ras activity can regulate the expression of CCAAT/enhancer binding proteins (54, 55), which might explain, at least in part, the reduction of integrin α5 levels in Panc-1 cells after K-ras siRNA (Fig. 5). This is supported by proteomic analysis of myc-regulated proteins that identified integrin β1 and other adhesion molecules as targets of c-myc regulation (56). Another study showed that N-myc overexpression results in decreased β1 levels and increased apoptosis (57), which is similar to the results we obtained in MiaPaca-2 cells after reduction of K-ras levels.

Normal nontransformed mammalian cells use oxygen to generate energy, whereas cancer cells rely on glycolysis for energy and are, therefore, less dependent on oxygen. This results in increased survival of tumor cells in the hypoxic microenvironment of the tumor (28). Increased reliance on glycolysis is often accompanied by an increase in the rate of glucose transport (58). A significant increase in GLUT-1 mRNA has been described in tumors of the esophagus, colon, pancreas, and lung (59, 60), whereas GLUT-1 protein expression in gastric adenocarcinoma is associated with metastasis and decreased patient survival (61). Studies in fibroblasts overexpressing c-myc have shown that c-myc regulates the expression of GLUT-1 and other genes critical to glucose metabolism (29). Our results suggest that the level of GLUT-1 is differentially regulated in Panc-1 and MiaPaca-2 cells after reduction of K-ras, which likely reflects the alternate regulation of c-myc in these cells.

Our findings are another example of the complexity of multistage process of carcinogenesis and the “bizarre” molecular circuitry of cancer cells (62). The results, taken together, show that in cells addicted to mutant K-ras, in this case Panc-1 and MiaPaca-2 cells, the maintenance of the malignant phenotype is dependent on the activity of an oncogene that is also crucial for the development of the initial tumor, as suggested by Weinstein (62). We conclude that the development of mutant-specific K-ras siRNA as a potential therapeutic is justified.

Materials and Methods

Cell Culture

The human pancreatic cancer cells lines, Panc-1 and MiaPaca-2 (CRL-1469 and CRL-1420, American Type Culture Collection, Manassas, VA), were maintained in culture with DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Gemini Bio-Products, Woodland, CA) at 37°C in a humidified atmosphere of 5% CO₂.

K-ras RNAi

Using the described mutations identified in the MiaPaca-2 (G12C) and Panc-1 (G12D) cell lines, RNA oligonucleotides were synthesized by the Center for Biomedical Inventions (University of Texas Southwestern, Dallas, TX). The following oligonucleotide sequences (sense/antisense) were used: for wild-type K-ras, 5′-GTGGAGCTGGCGTAG-3′/5′-CAACCTGACCAGC-3′; for MiaPaca-2 mutant K-ras, 5′-GTGGAGCTGGCGTAG-3′/5′-CCACCTCTGACCAGCGATC-3′; and for Panc-1 mutant K-ras, 5′-GTGGAGCTGGCGTAG-3′/5′-CAACCTCGAC-TACCAGCATC-3′; scrambled 5′-CGAAGUGUGUGUGUGUGUGCC-3′/5′-GCCACACACACACACCUUCG-3′. Sense and antisense oligonucleotides were resuspended to 50 μM/L in diethyl pyrocarbonate–treated water, mixed, heated for 2 minutes at 90°C, and annealed for 1 hour at 37°C. Annealed oligos (20 μM/L) were aliquoted and stored at −80°C until use.

For RNAi Panc-1 (3.5 × 10⁴) and MiaPaca-2 (4.5 × 10⁴), cells were incubated in a 12-well plate (BD Falcon, Bedford, MA) overnight (14–16 hours), washed, and incubated in Opti-MEM (Invitrogen), and exposed to duplexed siRNA oligonucleotides in the presence of Oligofectamine (Invitrogen) for 6 hours. Cells were incubated with duplexed siRNA oligonucleotides under two different conditions: increasing siRNA concentrations (25, 50, or 100 nmol/L) for 24, 48, and 72 hours each or 100 nmol/L for 48, 72, or 96 hours. Six hours posttransfection, the media was changed to DMEM with 10% FCS. At the indicated time point, the supernant or cells from three identical wells were harvested and pooled for analysis.

Cell Proliferation, Colony Formation, and Apoptosis Analysis

Cell proliferation was assessed by seeding Panc-1 or MiaPaca-1 cells into 12- or 24-well plates on day −1 and exposing to K-ras or control siRNA (100 nmol/L) for 6 hours on days 0 and 2. Representative wells were counted on the days indicated. Cell proliferation was also assessed using a MTS assay (data not shown). Briefly, cells were plated into 12-well plates (3.5 × 10⁴–4.0 × 10⁴ per well) and exposed to siRNA (25, 50, or 100 nmol/L) for 6 hours on days 1 and 2. Cells were harvested on day 3 and reseeded (2 × 10⁴/well) in a 96-well plate for 48 hours before the MTS assay, which was done according to the instructions of the manufacturer (Promega, Madison, WI). Colony formation was assessed as follows: enhanced green fluorescent protein–transfected Panc-1 and MiaPaCa-2 cells were treated with Oligofectamine or 100 nmol/L mutant-specific K-ras siRNA for 6 hours on days 1 and 2. Cells were harvested on day 3 and reseeded in
triplicate at a concentration of 2,500 cells per 35 × 10 mm dish (Nalge Nunc International, Rochester, NY) in 0.33% top agarose (SeaKem Agarose, FMC BioProducts, Rockland, ME). Dishes were incubated at 37°C for 12 days. Colonies were identified microscopically (Nikon SMZ-1500, Nikon, Lewisville, TX). Colonies of ≥0.1 mm in diameter were counted and expressed as mean number of colonies/well ± SD.

Apoptosis was judged by nuclear condensation. Briefly, Panc-1 (3.5 × 10^4) and MiaPaca-1 (4.5 × 10^4) cells per well were seeded into 12-well plates and exposed to siRNA (25, 50, or 100 nmol/L) for 6 hours on days 1 and 2. On day 4 (72 hours after siRNA treatment), 0.1 mL of Hoechst 33342 at a concentration of 2 μg/mL was added to each well and incubated at room temperature for 15 minutes. The cells were fixed and then examined using a Nikon Eclipse TE2000 microscope, photographed, and analyzed as above.

**Cell Migration Assay**

The migration of human pancreatic cancer cells through an artificial extracellular matrix (Matrigel, BD Biosciences, Bedford, MA) was assessed using a modified Boyden chamber assay. Briefly, Panc-1 (3.0 × 10^5) or MiaPaca-2 (4 × 10^5) cells were exposed to 100 nmol/L siRNA for 48 hours, harvested, counted, and ~1 × 10^4 cells placed on Matrigel-coated 8 μm pore polycarbonate inserts (BD Biosciences) in a 24-well tissue culture plate. The cells were incubated for 48 hours at 37°C, with serum-free media in the upper chamber and media containing serum (5%) in the lower chamber. Cell migration was determined by scraping the top of the insert with a Q-Tip and fixing and staining the bottom/under side of the insert with hematoxylin (Diff-Quick, Dade Berhring, Inc., New York). The porous membrane was removed from the insert and placed on a glass slide under a cover slip. The number of migrated cell per 10 high-powered fields was quantified for each condition in triplicate and expressed as a mean number of cells per high-powered field.

**Reverse Transcription-PCR**

Total cellular RNA was harvested from human Panc-1 and MiaPaca-2 cells using Trizol (Invitrogen) after exposure of the cells to control or mutant-specific K-ras siRNA. RNA was also harvested from endothelial cells (human umbilical vascular endothelial cell, NEC Technologies, Rensselaer, NY) in a similar manner. After DNase I treatment (Ambion, Austin, TX) and quantification, cDNA was synthesized using reverse transcriptase AMV, M-MuLV, or Tth DNA polymerase (Roche Diagnostics Corp., Indianapolis, IN). PCR was then done using the following oligonucleotide sequences (sense/antisense; IDT, Coralville, IA): GLUT-1, 5’-CAAATGACCTCAATTTCTAATCCTACCTCCTGG-3’/5’-CGGGTGCTTATCATACCTTGGTGCGT-3’; TSP-1, 5’-GAGGACGCAAAACGACTAAGG-3’/5’-TGAGGATGGGAGATCG-3’; glyceraldehyde-3-phosphate dehydrogenase, 5’-TCCACCAACCTGTTGCTGTAG-3’/5’-GACCACAGTCATGACACT-3’.

**Western Blot**

Cells were harvested as described above and were lysed in 50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 1 mmol/L sodium orthovanadate, 5 mmol/L NaF, 20 mmol/L β-glycerophosphate, and protease inhibitors (Complete, Roche Diagnostics). Fifty micrograms of protein, as determined by a modified Bradford protein assay (Bio-Rad, Hercules, CA), were loaded per well onto a precast 4% to 12% polyacrylamide gradient gel (Invitrogen). Proteins were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were blocked in 5% nonfat milk and incubated in primary antibody. Antibodies against the following proteins were used: K-ras (sc-30), PKR (sc-6282), p21 (sc-756), ERK1 (sc-94), and MKP2 (sc-17821) from Santa Cruz Biotechnology (Santa Cruz, CA); p27 (RB-9019), PARP (Ab-2, RB-1516), p53 (Ab-6, MS-187), GLUT-1 (Ab-1, RB-078), and TSP-1 (Ab11, MS-1066 and Ab-2, MS-419) from Lab Vision (Fremont, CA); β-actin (clone AC-40) from Sigma-Aldrich, Inc. (St. Louis, MO); c-myc (9E10, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa), integrins α2, α5, β1, and β5 (AB1944, AB1928, AB1952, and AB1926, respectively), and activated caspase-3 (AB3623) from Chemicon International (Temecula, CA); phospho-ERK1/2, phospho-Raf, phospho-AKT, cleaved caspase-9, and phospho-PKR from Cell Signaling Technology (Beverly, MA); and cyclin D1 (SA-260) from BIOMOL (Plymouth Meeting, PA). Mab 133 specific for human TSP-1 was provided generously by Dr. Joanne Murphy-Ullrich (University of Alabama Birmingham, Birmingham, AL). After incubation with primary antibody, the membranes were washed in PBS + 0.1% Tween 20 and incubated with the appropriate peroxidase–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by development with chemiluminescence substrate (Pierce Biotechnology, Inc., Rockford, IL) and exposure to X-ray film (Kodak, Rochester, NY). TSP-1 protein used as control was obtained from human umbilical vascular endothelial cell lysate (Lab Vision), Dr. Joanne Murphy-Ullrich, and purchased from Protein Sciences (Meriden, CT).

**Immunocytochemistry**

Cells were seeded in 12-well plates and treated with control or K-ras siRNA as described. Forty-eight hours later, cells (2 × 10^5-3 × 10^5) were plated onto LabTek II chamber slides (Nalge Nunc) and incubated at 37°C overnight, washed with PBS, and fixed in 4% paraformaldehyde (cells were fixed 72 hours after siRNA treatment). The slides were blocked with 5% FCS/TBST and exposed to primary antibodies at a concentration of 5 μg/mL. Antibodies against the following proteins were used: GLUT-1, integrins α5, β1, and β5. The cells were counterstained with Hoechst 33342 dye (Molecular Probes, Eugene, OR) and exposed to 1:500 Cy-3 or FITC-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Cells were examined using an Nikon E600 upright microscope (Nikon) fitted with a CoolSNAP HQ camera (Photometrics, Tucson, AZ) and signal quantified using Meta Morph software. Images to be compared were captured under identical conditions (e.g., exposure time and image scaling) and a threshold intensity level was set by examination of sections stained with secondary alone. The percentage of pixels reaching the threshold level was
determined for each target and for 4′,6-diamidino-2-phenylindole in each image. The signal intensity/pixel number for 4′,6-diamidino-2-phenylindole was used to normalize the specific signal for each target antibody.

**VEGF Production**

After exposure of Panc-1 (3 × 10^5) or MiaPaca-2 (5 × 10^5) cells to 100 nmol/L mutant-specific K-ras siRNA or Oligofectamine alone (for 24, 48, and 72 hours), the cells were harvested, counted, resedeed, and cultured in DMEM with 10% FCS without siRNA for 24 hours for a total of 48, 72, and 96 hours after initial siRNA exposure. The supernatants were harvested and stored at −80°C. The concentration of human VEGF was determined using the human VEGF immunoassay kit (R&D Systems, Minneapolis, MN).

**Acknowledgments**

We thank Dr. Latha Shivakumar for technical assistance; Drs. John Minna and Helene Sage, members of our laboratory, and David Shames for helpful discussions and support; and Dr. Joanne Murphy-Ullrich for providing TSP-1 and anti–TSP-1 antibodies for confirmation of our results.

**References**


Molecular Consequences of Silencing Mutant K-ras in Pancreatic Cancer Cells: Justification for K-ras–Directed Therapy


Updated version
Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/3/7/413

Cited articles
This article cites 61 articles, 18 of which you can access for free at:
http://mcr.aacrjournals.org/content/3/7/413.full.html#ref-list-1

Citing articles
This article has been cited by 23 HighWire-hosted articles. Access the articles at:
/content/3/7/413.full.html#related-urls

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.