**The Mutant K-ras Oncogene Causes Pancreatic Periductal Lymphocytic Infiltration and Gastric Mucous Neck Cell Hyperplasia in Transgenic Mice**

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**Abstract**

A frequent genetic alteration found in premalignant stages of pancreatic adenocarcinoma is K-ras oncogene point mutation. The mechanistic basis for the inability of K-ras mutation to transform pancreatic ductal cells is unclear, although cooperating events with p16 inactivation, p53 mutation, and SMAD 4 mutation are recognized to be necessary. We have generated a novel mouse model in which the cytokeratin 19 promoter, specifically active in pancreatic ductal cells but not other cell types of the pancreas, is fused to mutant K-ras. This is of direct relevance to human pancreatic cancer because premalignant lesions are found specifically in ductal cells. There is dramatic periductal lymphocytic infiltration in the pancreata of transgenic mice, predominantly CD4 + T lymphocytes, which may act as an adaptive immune response to activated ras-mediated signaling. In addition, gene array analysis reveals an induction of N-cadherin in transgenic mice pancreatic ductal cells, the significance of which relates to promotion of cell adhesion and deterrence of cell migration. Apart from these important biological considerations, there is parallel activity of the cytokeratin 19 promoter in the stem cell region of the gastric epithelium, namely in mucous neck cells. Activated K-ras in this context causes mucous neck cell hyperplasia, a precursor to gastric adenocarcinoma. There is concomitant parietal cell decrease, which is a key step toward gastric adenocarcinoma. Taken together, we have defined how mutant K-ras signaling modulates important molecular events in the initiating events of pancreatic and gastric carcinogenesis.

**Introduction**

Pancreatic adenocarcinoma is a common gastrointestinal malignancy with ~25,000 new cases annually in the United States. Unfortunately, there are ~28,000 deaths related to the disease and its metastatic complications attributable to presentation of patients at advanced stages. Pancreatic adenocarcinomas arise in ductal epithelial cells and undergo a carefully orchestrated program of genetic alterations that arise in premalignant lesions. These eventually culminate in the development of cancer. K-ras oncogene point mutations are found in ~90–95% of all pancreatic ductal adenocarcinomas (1, 2) and, as such, also represent the earliest known genetic alteration in premalignant or precursor lesions. Although K-ras oncogene point mutations are important initiating events, it is clear that inactivation of the p16 and p53 tumor suppressor genes, as well as the SMAD genes, are also important for tumor development and progression (3–7).

The ability to model pancreatic ductal adenocarcinoma and its precursor stages has been hampered by the inability to target mutant Ki-ras in a direct fashion to ductal cells. The elastase promoter is active in acinar cells and has been used to target Ha-ras to the pancreas (8). These mice develop acinar cell tumors. To that end, we have targeted mutant Ki-ras with the cytokeratin 19 promoter to ductal cells (9, 10) to recapitulate the premalignant stages of pancreatic adenocarcinoma. Because this promoter is also active in the gastric isthmus region, we also observe evidence of mucous neck hyperplasia.

**Materials and Methods**

**K19-K-ras V12 Transgene.** For construction of the K19-K-rasV12 Neo-Bam transgene vector, the pCMV-NeoBam vector (gift of B. Vogelstein) was used. The cytomegalovirus promoter in this vector was replaced with the 2.1-kb K19 5′ flanking region plus promoter fragment after KpnI and SalI digestion. The 2.1-kb K19 fragment was generated by PCR using a 5′-CTAGTCTAGACGTCTCAAGTCTCTTTCTAAGACCCAC primer to amplify at ~1970 bp and introduce a 5′ KpnI and BamHI restriction site and a 5′-T ATGGCCGACGTCG ACGGAAAAAGGGACGCAGGTCTGA primer to amplify up to ~46 bp of the K19 promoter linked to a SalI restriction enzyme site. PCR was performed using 20 ng of template DNA, 200 μM each deoxynucleotide triphosphate, 0.5 μM each primer, and 2.5 units of plaqueforming unit Turbo DNA polymerase (Stratagene) in a × 1 reaction buffer provided by the manufacturer. After an initial 60 s denaturation step at 94°C, 30 cycles of amplification were done as follows: denaturation 60 s/94°C, annealing 60 s/60°C, and extension 2 min/72°C, and a final extension at 72°C for 10 min. The K19 promoter PCR product was KpnI/SalI digested and subcloned into the NeoBam vector. The K-ras-V12 cDNA, with a substitution of Gly→Val at codon 12, was obtained by a BamHI digest of the pZIP-K-rasV12 vector (gift of C. Der) and subcloned in the BamHI restriction site of the K19-NeoBam vector between the rabbit β-globin intron and polyadenylation sequence. The construct was verified by DNA sequencing by the dye terminator cycle sequencing method (ABI) in the automated DNA sequencing facility of the University of Pennsylvania.

**Transgenic Mice.** For microinjection, the purified K19-K-rasV12 NeoBam vector was linearized by a BSMBI/EcoQ restriction enzyme digest to release the K19-K-rasV12 transgene, purified using Elutips, and used for microinjection. The DNA was injected into B6 × SJL recipient oocytes and transferred to pseudopregnant females. Genotyping of founders and offspring was performed using PCR with primers specific for the transgene. The sense primer (5′-GGCTGGCGTGAAATATTCT), spanning the β-globin intronic sequence, and the antisense primer (5′-GCTGTATCGTCAAGGCACTC) recognize a single PCR band corresponding to the expected product of 360 bp. Transgene incorporation was confirmed by Southern blot analysis after a digestion. The 2.1-kb K19 fragment was generated by PCR using a 5′-CTAGTCTAGACGTCTCAAGTCTCTTTCTAAGACCCAC primer to amplify at ~1970 bp and introduce a 5′ KpnI and BamHI restriction site and a 5′-T ATGGCCGACGTCG ACGGAAAAAGGGACGCAGGTCTGA primer to amplify up to ~46 bp of the K19 promoter linked to a SalI restriction enzyme site. PCR was performed using 20 ng of template DNA, 200 μM each deoxynucleotide triphosphate, 0.5 μM each primer, and 2.5 units of plaqueforming unit Turbo DNA polymerase (Stratagene) in a × 1 reaction buffer provided by the manufacturer. After an initial 60 s denaturation step at 94°C, 30 cycles of amplification were done as follows: denaturation 60 s/94°C, annealing 60 s/60°C, and extension 2 min/72°C, and a final extension at 72°C for 10 min. The K19 promoter PCR product was KpnI/SalI digested and subcloned into the NeoBam vector. The K-ras-V12 cDNA, with a substitution of Gly→Val at codon 12, was obtained by a BamHI digest of the pZIP-K-rasV12 vector (gift of C. Der) and subcloned in the BamHI restriction site of the K19-NeoBam vector between the rabbit β-globin intron and polyadenylation sequence. The construct was verified by DNA sequencing by the dye terminator cycle sequencing method (ABI) in the automated DNA sequencing facility of the University of Pennsylvania.
Cell Culture and Transient Transfection. The human pancreatic ductal adenocarcinoma cell lines Panc-1 and Capan-2 (American Type Culture Collection) were grown in 5% CO₂ at 37°C as subconfluent monolayers in DMEM supplemented with 10% volume for volume FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1% t-glutamine (Sigma Chemical Co., St. Louis, MO). Experiments were carried out in the log phase of growth after the cells were seeded for 24 h.

Transient transfections of cells with the DNA construct were performed using the liposomal DNA transfer technique. After an attachment period of 12 h, cells were incubated with 800 µl of the transfection mixture containing 1 µg of plasmid DNA and 20 µl of LipofectAMINE reagents in Opti-MEM medium (Life Technologies, Inc., Arlington Heights, IL). Cells were incubated for 5 h, followed by exchange with fresh medium supplemented with FCS. Transfection efficiency was routinely monitored by cotransfection with pGreen Lantern-1 (Life Technologies). After 48 h, cells were washed twice with PBS and resuspended in Mg²⁺/EGTA lysis buffer (Upstate Biotechnology, Inc.) containing 25 mM HEPES (pH 7.5), 125 mM sodium chloride, 5 mM EDTA and 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, and 2% glycerol supplemented with a proteinase inhibitor cocktail (Boehringer Mannheim). Protein lysates were used for Western blotting and ras activity assays.

RT-PCR. Total RNA was isolated from monolayer cultures using TRIzol reagent (Life Technologies). For the semiquantitative analysis of transcripts encoding N-cadherin and glyceraldehyde-3-phosphate dehydrogenase, 200 ng of total RNA were reverse transcribed and amplified according to the protocol of the Titan One tube RT-PCR system (Roche, Mannheim, Germany). PCR primers were designed to anneal to specific sequences of the mouse N-cadherin (forward, 5'-GGCGTCTGTGGAGGCTTCTGGTGAA-3', reverse, 5'-GTGA-H11032/H11032/GGCGTCTGTGGAGGCTTCTGGTGAA-3') and adenocarcinoma (around 1.1 kb) fragment. The sequences of the primers for glyceraldehyde-3-phosphate dehydrogenase were 5'-TGGTAACGCCGTGTGGCTTGGTGAGA-3' and 5'-AACAGCTTGGTGCGAGCA-3', yielding a 440 bp cDNA product. Data shown were obtained with 15 PCR cycles.

Western Blot Analysis and ras Activity Assay. Fresh pancreatic tissues were obtained from transgenic animals and wild-type littermate controls. The entire pancreas was immediately transferred to Mg²⁺/EGTA lysis buffer (Upstate Biotechnology) with protease inhibitors (Boehringer Mannheim), sonicated on ice two times for 45 s to disrupt the tissue, and centrifuged at 4°C for 20 min. Protein concentration of the supernatant was determined by a colorimetric method (Bio-Rad) and adjusted to 1 µg/µl. Lysates (500 µg) were precleared with 10 µg of glutathione S-transferase-agarose (Santa Cruz Biotechnology), followed by incubation with 10 µg of Raf-1 RBD agarose conjugate (Upstate Biotechnology) for 30 min at 4°C. Beads were collected by pulse centrifugation and washed twice in lysis buffer. Raf-1 RBD-precipitated ras-GTP was eluted on a 15% SDS-PAGE. To determine total ras protein, 10 % H11003/H11003/H11003/H11003/H11003/H11003/H11003/H11003/H11003 of the Titan One tube RT-PCR system (Roche, Mannheim, Germany). PCR was used at a concentration of 1:5000 dilution. In addition, blots were probed with anti-MAPK, conjugated sheep antimouse (Amersham Pharmacia Biotech) antibody, was used at a 1:5000 dilution. 3 The abbreviations used are: RT-PCR, reverse transcription-PCR; HRP, horseradish peroxidase; TNB, 2-nitro-5-thiobenzoate; AAA, Anguilla anguilla; MAPK, mitogen-activated protein kinase.

Histology and Immunohistochemistry. Five transgenic mice from each of the three founder lines were sacrificed at 6, 9, 12, 15, and 18 months (yielding 15 transgenic mice at each time point), along with an equivalent number of wild-type littermates. Six hours before euthanasia, animals were labeled with 200 mg of BrdUrd/kg body weight by i.p. injection. Tissues (pancreatic, gastric, liver, esophagus, small intestine, colon, kidney, spleen, and lung) were fixed in 4% paraformaldehyde and paraffin embedded. Protein and tissue sections were mounted on adhesive-coated slides. Consecutive sections were stained with H&E. Sections of wild-type and transgenic animals were screened by independent investigators, and histopathology was confirmed independently by a veteran pathologist.

For immunohistochemistry, slides were dewaxed and rehydrated, immersed in 10 mM citric acid (pH 6.0), and boiled for 6 min to retrieve the antigen before immunostaining. This was followed by pretreatment with 3% H₂O₂ in methanol to quench endogenous peroxidase activity and then blocked for 15 min with a protein blocking reagent (Vector). Primary antibodies were incubated with tissue sections and included mouse anti-BrdU monoclonal antibody (Boehringer Mannheim) at a 1:500 dilution, anti-AAA lectin antibody (San Mateo) at 1:20 dilution, and anti-H+K+ATPase (Medical and Biological Laboratories Co.) at 1:2000 dilution, each followed by incubation with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) in a 1:100 dilution for 30 min. Signals were developed by the avidin-biotin peroxidase method using the 3,3′-diaminobenzidine substrate kit (Vector Laboratories). The specificity of the staining was confirmed for each antibody by control reactions in which the primary antibody was omitted. Sections were counterstained with hematoxylin.

For CD4 and CD8 immunohistochemistry, fresh pancreas tissues were either positioned in Tissue Tek and immediately frozen in liquid N₂ or frozen, then positioned in Tissue Tek, and immediately frozen again. Tissues were sectioned at 6–10 µm in a cryostat at −18°C and, slides were stored at −80°C. Slides were thawed at room temperature for 20–30 min, and all subsequent steps were done at room temperature. Sections were fixed in 2% paraformaldehyde for 5 min and washed three times in 1 × PBS (3–5 min/wash).

Genetic alterations were identified using Southern blot analysis, using the liposomal DNA transfer technique. After an attachment period of 20 min, the secondary antibody, HRP-conjugated sheep antimouse (Amersham Pharmacia Biotech) antibody, was used at a 1:2000 dilution. Avidin block for 10 min, and biotin block for 10 min (both from Vector Laboratories). After a single wash with 1 × PBS, tissue sections were incubated with primary antibody for 1 h. After three washes in 1 × PBS, sections were incubated with a 1:100 dilution of streptavidin–horse radish peroxidase (NEN Life Sciences) in TBN in 30 min, followed by another set of 1 × PBS washes, and incubated for 5 min in cynamine 3 substrate diluted 1:100 in amplification diluent (NEN Life Sciences). After another set of 1 × PBS washes, sections were counterstained with 20 µg/ml 4′,6-diamidino-2-phenylindole (Sigma) for 5 min, rinsed in 1 × PBS, dried, and mounted with Vectashield Mounting Medium (Vector Laboratories) and a cover slip. Sections were viewed never >16 h after the staining using a fluorescent microscope. Images were captured using Magnafire 2.0 software. The primary antibodies against CD4 and CD8 (0.5 mg/ml; PharMingen) were all diluted 1:200 in TBN.

Results

Generation of the K19-K-ras V12 Transgenic Mice and Increased ras Activity in Pancreatic Tissues. The K19–K-ras-V12 transgene was constructed from a 2.1-kb genomic fragment of the 5′ flanking region/promoter of the mouse K19 gene (8, 9) and linked to the full-length human K-ras gene with a point mutation in codon 12 (Gly→Val), which represents one of the most common K-ras mutations in human pancreatic adenocarcinoma. An intronic fragment from the rabbit β-globin was inserted between the K19 promoter and K-ras gene to enhance gene expression. This construct was tested initially by overexpression in human pancreatic ductal cell lines (Fig. 1A). By Western blotting, there is increased K-ras expression and increased ras activity from the transcribed transgene construct compared with mock-transfected cells.

Three independent founder lines (A, B, and C) were generated. Genotyping of founders and F1 and F2 offspring was performed by PCR with primers specific for the transgene and by Southern blot analysis demonstrating integration of the transgene (data not shown). To test the functional activity of the transgene in vivo, ras-activity and expression of downstream effectors were tested in fresh pancreatic
tissues from transgenic animals and wild-type littermate controls (Fig. 1B). The total level of ras protein in whole pancreatic extracts from transgenic and wild-type mice was equal. However, Western blot analysis demonstrated activated K-ras was increased in all three founder lines with concomitant increase in cyclin D1. Although phosphorylated MAPK was increased in only one founder line, it should be noted that the functional effects of activated ras may be independent of phosphorylated MAPK in human pancreatic cancer (11). Furthermore, BrdU labeling of mice revealed a 2-fold increase in BrdU immunostaining within pancreatic ductal epithelia of transgenic mice in all founder lines compared with wild-type littermates starting at 6 months of age (data not shown).

**Overexpression of Activated ras Leads to Lymphocytic Infiltration Around Pancreatic Ducts.** Age-matched transgenic and wild-type mice littermates were sacrificed between ages 6 and 18 months for histological analysis. Starting at 6 months of age, there was dramatic evidence of lymphocytic infiltration around ducts, both interlobular and intralobular, in all three founder lines (Figs. 2, A and B). To phenotype the lymphocytes, immunostaining revealed the presence of CD4+ predominant T lymphocytes and occasional CD8+ T lymphocytes (Fig. 2C). In particular, CD4 was most prevalent because many ducts had pockets of positively stained cells around them. CD8 could be found in smaller groupings near and almost within a few ducts, although there were more ducts with pockets of CD4-positive cells than those with CD8-positive cells. In nontransgenic pancreas, there was no staining for CD4, CD8, with an irrelevant isotype antibody (data not shown).

There was no evidence of architectural or morphological changes in acinar or islet cells. Specific examination of pancreatic ductal epithelial cells showed occasional changes of hyperplasia (Fig. 2D), which is observed as an initial step of pancreatic cancer in the pancreatic intraepithelial neoplasm classification of human pancreatic ductal adenocarcinomas and associated with K-ras oncogene point mutations (12). These changes were not seen in any wild-type littermates followed ≤18 months.

Microarray analysis (Affymetrix Microarray Suite 5.0, U74Av2 GeneChips) was performed on pancreatic ductal cells isolated from K19-K-ras-V12 mice compared with wild-type mice. This revealed an increase in N-cadherin expression (3.8 × increase in microarray) that was confirmed both at the RNA (4.5 × increase) and protein...
increase) levels in transgenic mouse pancreatic ductal cells compared with those from wild-type mice (Fig. 3). It is known that N-cadherin-mediated adhesion is critical for maintaining cell–cell interactions in tissues undergoing cellular rearrangements and increased mechanical stress associated with morphogenesis.

**Mutant K-ras Signaling Leads to Mucous Neck Cell Hyperplasia in the Stomach.** We have shown previously that the K19 promoter is highly active in the mucous neck cell compartment of the stomach, a site where stem cells of this organ are believed to reside. In a striking fashion, we found in all transgenic mice evidence of mucous neck cell hyperplasia as early as 3–6 months with persistence ≥18 months with confirmation by immunohistochemistry (Fig. 4). This finding was not apparent in any wild-type mice. In addition, there was a decrease in parietal cell mass in transgenic mice but not in any wild-type mice (Fig. 4).

**Discussion**

Using the K19 promoter as a platform for generation and characterization of transgenic mice that might potentially recapitulate premalignant stages of pancreatic ductal and gastric cancer, we targeted mutant K-ras with the K19 promoter. This resulted in prominent

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**Fig. 3. Increased N-cadherin expression in pancreatic ductal cells from K19-K-ras-V12 versus wild-type mice.** A, RT-PCR. B, Western blot analysis.

phenotypes, namely a periductal lymphocytic infiltration in the pancreas and hyperplasia of gastric mucous neck cells.

We observed occasional evidence of pancreatic ductal hyperplasia in transgenic mice, consistent with the human counterpart in the pancreatic intraepithelial neoplasm classification. It is conceivable that the frequency of hyperplasia and even dysplasia was impeded by the recruitment of CD4+ T cells and, to a lesser extent, CD8+ T cells, which are known to play a central role in the host adaptive immune response. This can be consistent with observations that overlapping epitopes in mutant ras oncogene peptides activate CD4+ and CD8+ T-cell responses (13, 14). Furthermore, mutant ras has been noted to increase recruitment of natural killer cells and natural killer cell-mediated lysis (15). Thus, the recruitment and infiltration of CD4+ T cells may modulate the host immune surveillance against early neoplastic changes in the pancreas.

The consistent induction of N-cadherin in the pancreatic ductal cells of K19-K-ras-V12 transgenic mice may serve as a checkpoint to the proliferation-mediated and growth-promoting effects of activated ras given N-cadherin’s role in adhesion and motility (16). The fundamental importance of N-cadherin in the pancreas is underscored by the finding that deficiency of N-cadherin causes agenesis of the dorsal pancreas (17). Furthermore, a dominant-negative version of N-cadherin, when expressed in mouse embryonic stem cells under the control of promoters active in small intestinal epithelial cells and resulting in adult chimeric mice, leads to disturbances in cell migration (18), mucosal inflammation (consistent with Crohn’s disease), and adenomas. Thus, we postulate that although K-ras mutations may be crucial initiating events in pancreatic carcinogenesis, there are counterbalances with induction of N-cadherin and periductal lymphocytic infiltration. To overcome these effects, inactivation of the p16INK4a, SMAD4, and p53 genes is necessary (19–22). To that end, mutation in oncogenic Ki-ras may require combination with inactivation with one or more of these genes to induce cancer, which is the basis for current investigation by our group. Another advantage of our approach is that we are using activated Ki-ras in contrast to activated Ha-ras, which has been classically used in the pancreas and other organs.

There was mucous neck cell hyperplasia in the gastric epithelium of transgenic mice, which is known to be a precursor of gastric adenocarcinoma. Concurrent with this hyperplasia was a decreased number of parietal cells. These changes were present in all founder lines and evident up to 18 months of age. Multilobed stem cells in the isthmus of the gastric epithelium give rise to different cell lineages: (a) acid-producing parietal cells; (b) mucus-producing pit cells; and (c) pepsinogen and intrinsic factor-producing zymogenic cells. Members of the parietal cell lineage are believed to instruct the mucous neck-zymogenic cell lineage, even before maturation of the complete gastric epithelium subunits (23, 24). Our findings support interplay between different cell lineages within the gastric epithelium and that activation of ras signaling in mucous neck cells may instruct a program to diminish parietal cell differentiation and promote mucous neck cell hyperplasia. Indeed, parietal cell loss, atrophic gastritis, and achlorhydria are hallmarks of precursor stages of gastric adenocarcinoma (25).

In aggregate, our findings support an important role for mutant K-ras in the development of premalignant stages in the pancreas and stomach and establish an important mechanistic-based model for translation into chemopreventive and therapeutic strategies, as well as genome-wide analysis.

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References

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