In vitro Modeling of Human Pancreatic Duct Epithelial Cell Transformation Defines Gene Expression Changes Induced by K-*ras* Oncogenic Activation in Pancreatic Carcinogenesis

Jiaying Qian,[1,2] Jiangong Niu,[4] Ming Li,[1] Paul J. Chiao,[4] and Ming-Sound Tsao[1,2,3]

[1]Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, and Departments of [2]Medical Biophysics and [3]Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada and [4]Department of Surgical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

Genetic analysis of pancreatic ductal adenocarcinomas and their putative precursor lesions, pancreatic intraepithelial neoplasias (PanIN), has shown a multistep molecular paradigm for duct cell carcinogenesis. Mutational activation or inactivation of the K-*ras*, *p16*^{INK4A}, *Smad4*, and *p53* genes occur at progressive and high frequencies in these lesions. Oncogenic activation of the K-*ras* gene occurs in >90% of pancreatic ductal carcinoma and is found early in the PanIN-carcinoma sequence, but its functional roles remain poorly understood. We show here that the expression of K-*ras*^{G12V} oncogene in a near diploid HPV16-E6E7 gene immortalized human pancreatic duct epithelial cell line originally derived from normal pancreas induced the formation of carcinoma in 50% of severe combined immunodeficient mice implanted with these cells. A tumor cell line established from one of these tumors formed ductal cancer when implanted orthotopically. These cells also showed increased activation of the mitogen-activated protein kinase, AKT, and nuclear factor-κB pathways. Microarray expression profiling studies identified 584 genes whose expression seemed specifically up-regulated by the K-*ras* oncogene expression. Forty-two of these genes have been reported previously as differentially overexpressed in pancreatic cancer cell lines or primary tumors. Real-time PCR confirmed the overexpression of a large number of these genes. Immunohistochemistry done on tissue microarrays constructed from PanIN and pancreatic cancer samples showed laminin β3 overexpression starting in high-grade PanINs and occurring in >90% of pancreatic ductal carcinoma. The *in vitro* modeling of human pancreatic duct epithelial cell transformation may provide mechanistic insights on gene expression changes that occur during multistage pancreatic duct cell carcinogenesis. (Cancer Res 2005; 65(12): 5045-53)

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in North America. With an overall 5-year survival rate of 3% (1), pancreatic cancer has one of the poorest prognoses among all cancers (2). Aside from its silent nature and tendency for late discovery, pancreatic cancer also shows unusual resistance to chemotherapy and radiation therapy. Only 20% of pancreatic cancer patients are eligible for surgical resection, which currently remains the only potentially curative therapy (3). To increase survival rate, genes that could be targeted as biomarkers for early detection strategy and for the development of chemopreventive agents need to be identified. Recent genome-wide expression studies have revealed hundreds of putative genes that are differentially expressed in pancreatic cancer tissues/cells compared with the normal pancreas (4–13). Although some of these genes represent promising cancer detection biomarker genes, very few have been validated or studied for their biological roles during the developmental stages of pancreatic ductal cancers (4–13).

Ductal cancer of the pancreas putatively evolves through multistage neoplastic transformation process that are reflected in a series of histologically well-defined precursor lesions termed pancreatic intraepithelial neoplasias (PanIN; ref. 14). PanIN progresses from flat and papillary hyperplastic lesions without dysplasia to papillary lesions with dysplasia and carcinoma *in situ* (15). Molecular analyses in PanIN lesions and cancers have revealed progressively accumulating genetic abnormalities involving several oncogenes and tumor suppressor genes (16). Mutations in the K-*ras* gene seem to occur early, the inactivation of the *p16*^{INK4A} gene at intermediate stages and the inactivation of *p53* and *DPC4/Smad4* at relatively late stage (17, 18). The almost ubiquitous occurrence of K-*ras* oncogenic mutations in pancreatic ductal cancers implicates a critical role for this gene in the pathogenesis and biology of this malignancy.

We report here a dynamic modeling of pancreatic ductal carcinogenesis using a near diploid immortalized duct epithelial cell line established previously from normal human pancreas (19). We show that the HPV16-E6E7 immortalized human pancreatic duct epithelial (HPDE) cells expressing K-*ras*^{G12V} oncogene led to tumorigenic transformation. Gene expression profiling using the high-density oligonucleotide microarrays reveals gene expression changes that are putatively downstream of K-*ras*^{G12V} mutations in the ductal cells and thus are candidate biomarkers for early stages of pancreatic carcinogenesis and early detection of pancreatic cancer patients.

Materials and Methods

Cell culture. The immortal HPDE cell line HPDE6-E6E76c7 (H6c7) and its derivatives were cultured as reported previously (19). Phoenix ecotropic packaging cell line obtained from the American Type Culture Collection (Manassas, VA) was maintained in DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT).

Retroviral infection. Retroviruses were generated by transfecting Phoenix ecotropic packaging cells with the retroviral vector pBabepuro-K-*ras*4B^{G12V} (20) and the corresponding empty vector pBabepuro using

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ming-Sound Tsao, Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9. Phone: 416-946-4426; Fax: 416-946-6579; E-mail: ming.tsao@uhn.on.ca.

LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA). Retroviral supernatants were collected, filtered, and incubated with the target cells in the presence of 4 μg/mL polybrene (Sigma, St. Louis, MO). After 48 hours, cells were subjected to 0.5 μg/mL puromycin (ICN Biomedicals, Irvine, CA) selection until all the untransduced cells have died. To satisfy the institutional biohazard requirement, we first infected H6c7 cells with amphotropic retrovirus produced from plasmid pBMN-IRES-Lyt2-ecoR that carried the murine ecotropic retroviral receptor (eR) gene. H6c7 cells with stable expression of eR was flow sorted and isolated by a dual-laser FACSCalibur using R-phycoerythrin-conjugated rat anti-mouse CD8a (Lyt2) monoclonal antibody (BD Biosciences PharMingen, San Diego, CA).

Anchorage-dependent and anchorage-independent growth assays. Growth curves of cell lines were constructed as described previously (19). Briefly, 10,000 cells were seeded into replicate wells of six-well tissue culture plates. During the next 5 days, replicate wells of trypsin-dissociated cells were counted using the ZM particle counter (Coulter Electronics, Luton, United Kingdom). To determine the proliferative effect of transforming growth factor-β1 (TGF-β1), cells were treated with 10 ng/mL TGF-β1 starting from the second day after seeding (19). The anchorage-independent growth of cells in soft agar medium was evaluated as described previously (19).

Tumorigenic assay. All animal studies were carried out using protocols that have been approved by the Institutional Animal Care Committee. Tumorigenicity in severe combined immunodeficient (SCID) mice was assayed using s.c. and orthotopic implantation. One million cells were used for s.c. injection and 2 million cells for orthotopic model. Cells were suspended in a 50 μL fresh medium that contained 10% Matrigel (BD Biosciences Discovery Labware, Bedford, MA). For s.c. implantation, cells were injected into the abdominal s.c. tissue of SCID mice. The mice were kept for up to 6 months and monitored once weekly. When the tumor was 1 cm in diameter or became ulcerated, the mouse was sacrificed and the tumor was removed, fixed in 10% buffered formalin, and processed for paraffin embedding and histology. For orthotopic implantation, cells were injected directly into the mouse pancreas through a small incision in the abdominal wall. The condition of mice was monitored twice weekly over a period of 6 months, at which time the mice were sacrificed. The pancreas, liver, and spleen were removed and fixed in formalin for routine histologic processing. All the mice used were male and 6 weeks old at the time of implantation.

Western blot and *ras* activity assays. Western blot was done as described previously (21). The primary antibodies used include C-K-*ras* monoclonal antibody (Oncogene, San Diego, CA), AKT antibody (Cell Signaling, Beverly, MA), phospho-AKT (Thr^{308}) antibody (Cell Signaling), p44/42 mitogen-activated protein kinase (MAPK) antibody (New England Biolabs, Beverly, MA), phospho-p44/42 MAPK antibody (Cell Signaling), $p16^{INK4a}$ antibody (Lab Vision NeoMarkers, Fremont, CA), and Smad4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Visualization was accomplished using the horseradish peroxidase–linked anti-rabbit and anti-mouse secondary antibodies (Cell Signaling) and BM chemiluminescence blotting substrate detection (Roche, Indianapolis, IN). The activity of RAS protein was assayed using the ras activation assay kit (Upstate, Charlottesville, VA). Cells were lysed with Mg^{2+} lysis/wash buffer. Active RAS·GTP protein in the cell lysate was precipitated by Raf-1 RBD agarose. The agarose beads were resuspended in 5× sampling buffer and boiled for 5 minutes. The supernatant containing active RAS protein was detected by Western blot as described previously (21).

Electrophoretic mobility shift assay of nuclear factor-κB activity. The nuclear extracts from H6c7 and its derivatives were prepared according to the method of Andrews and Faller (22). DNA-binding assays for nuclear factor-κB (NF-κB) proteins were done with 10 μg nuclear extracts as described by Chiao et al. (23). ^{32}P-labeled double-stranded oligonucleotides (5′-CTCAACAGAGGGGACTTTCCGAGAGGCCAT-3′) containing the κB site found in the HIV long terminal repeat were used as probes. The supershift experiments were done using anti-p65 and anti-p50 antibodies (Santa Cruz Biotechnology). The reactions were analyzed on 4% polyacrylamide gels containing 0.25× Tris-borate EDTA buffer.

RNA isolation and preparation. Total RNA was isolated from cultured cells by guanidinium-phenol extraction (24). RNA was cleaned up using RNeasy Mini kit (Qiagen, Mississauga, Ontario, Canada). The quality of RNA was checked using agarose gel and the Agilent bioanalyzer (Agilent Technologies, Palo Alto, CA).

Affymetrix GeneChip microarray and data analysis. Total RNA (20 μg) was converted to double-stranded cDNA. Biotin-labeled cRNA was generated after an *in vitro* transcription reaction. The cRNA was fragmented and then hybridized to the array HG-U133A (Affymetrix, Santa Clara, CA). Immediately following hybridization, the array underwent an automated washing and staining. Finally, the array was scanned. The software computed intensity for each cell. Sample preparation and hybridization were done at the Microarray Facility of the Hospital for Sick Children (Toronto, Ontario, Canada; http://tcag.bioinfo.sickkids.on.ca/index.php?pagename=microarray.php) using a standard protocol provided by Affymetrix.

Microarray data were analyzed using Affymetrix Microarray Suite 5.0 software. Comparative analysis was done. Data from H6c7 were set as baseline and data from H6c7eR-pBp and H6c7eR-Kr as experiment. Experiment data were compared with baseline data. Genes were considered to be differentially expressed if (*a*) expression changed by at least 2-fold (or signal log ratio ≥1, fold change = $2^{\text{Signal Log Ratio}}$); (*b*) probe sets have a change call of "increase" (I) or "decrease" (D); and (*c*) for increased expression, probe sets must have detection call of "present" (P) in experiment file; for decreased expression, probe sets must have detection call of "present" (P) in baseline file. Other data analysis was done in Excel spreadsheet. Genes were grouped using Gene Ontology tool provided by the NetAffx analysis center (http://www.affymetrix.com/index.affx).

Reverse transcription and quantitative real-time PCR validation. Reverse transcription was completed at 42°C with SuperScript II RNase H^- reverse transcriptase kit (Invitrogen). Real-time PCR was done in a total volume of 25 μL using 10 ng of the first-strand cDNA synthesis mixture as a template. Primers (Supplementary Table S1) were designed by the Primer Express Software (Applied Biosystems, Foster City, CA). The assays were done using Stratagene MX3000P (La Jolla, CA). The relative quantification of gene expression was determined using the comparative C_T method as described previously (25–27). The values of 18S rRNA were used to normalize the expression data. The gene expression level in H6c7eR-Kr cells relative to the H6c7 cells was calculated using the following formulas: $\Delta\Delta C_T = \Delta C_{T\ \text{H6c7eR-Kr}} - \Delta C_{T\ \text{H6c7}}$, fold change = $2^{-\Delta\Delta CT}$.

Immunohistochemistry and tissue microarray. The construction of our pancreatic neoplasia tissue microarrays have been reported previously (27). Immunohistochemistry was done using the peroxidase anti-peroxidase technique following a microwave antigen retrieval procedure (27). Laminin β3 (LAMB3; H-300) antibody (Santa Cruz Biotechnology) was used at 1:500 dilution. The relative staining pattern and intensity were scored using the 0 to 3 scales, which progressed from negative to strong staining. The slides were scored independently (by J.Y.Q. and M.S.T.), and inconsistencies were reconciled with multiheaded microscope. Antibodies for *ADAM8* (H-50), *uPAR* (10G7), *syndecan-1* (DL-101), *lipocalin 2* (F-19), *inhibin βA* (C-18), *DAF* (H-319), and *PIM1* (E-16) were purchased from Santa Cruz Biotechnology. Antibodies for *TIMP1* (clone 102D1) and *FYN* (clone 1S) were purchased from Lab Vision NeoMarkers.

Results

Expression of oncogenic K-*ras* in H6c7 cells. Stable transduction of K-*ras* oncogene into the ecotropic receptor expressing HPDE6-E6E76c7-eR (H6c7eR) cells gave rise to a puromycin-resistant cell population (H6c7eR-Kr), which we confirmed to express the K-RAS protein at significantly higher levels than the control H6c7eR-pBp cells transduced with the viruses containing vector alone (Fig. 1). The mRNA expression level of K-*ras* was also elevated >10-fold in H6c7eR-Kr cells compared with control cells (data not shown). Functional assay using the RAS·GTP-Raf affinity precipitation further confirmed the up-regulated RAS·GTP activities in H6c7eR-Kr cells (Fig. 1).

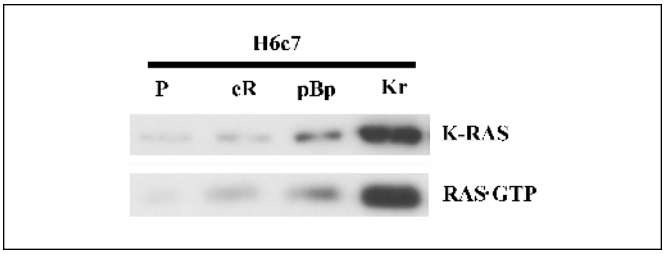

Figure 1. Confirmation of K-*ras* oncogene expression in H6c7eR-Kr cells. *Top,* basal total K-RAS protein levels in H6c7eR-Kr, control H6c7eR, H6c7eR-pBp, and parental H6c7 cells. *Bottom,* expression level of active RAS·GTP, which was detected by Raf affinity precipitation. *P,* parental H6c7 cells; *eR,* H6c7-eR cells expressing ecotropic retroviral receptors; *pBp,* H6c7eR-pBp cells established by infecting H6c7-eR cells with empty vector pBabepuro; *Kr,* H6c7eR-Kr cells created by infecting H6c7-eR cells with pBabepuro-K-*ras*^{G12V}.

Cell morphology, growth, and transforming growth factor-β1 responsiveness. The H6c7eR-Kr cells maintained an epithelial-like appearance on tissue culture plates but did not show significant morphologic changes compared with the parent cells (Fig. 2*A* and *B*). The H6c7eR-Kr cells also showed similar growth rate compared with the control H6c7eR cells (Fig. 2*C*). To evaluate the anchorage-independent growth capacity, up to 50,000 of H6c7eR-Kr cells per 60 mm plate were seeded in soft agar medium, but no colony formation was observed after 6 weeks. Furthermore, the H6c7eR-Kr cells still exhibited partial growth inhibition to 10 ng/mL TGF-β1 (Fig. 2*D*), a property similarly observed in the parental H6c7 cells (19). Thus, K-*ras* activation in the H6c7eR cells failed to induce *in vitro* phenotypes of malignant transformation.

Tumorigenic ability of H6c7eR-Kr cells. The tumorigenic potential of these cells was assessed by tumor formation in SCID mice. The results were summarized in Table 1. H6c7eR-Kr cells yielded s.c. tumors in nearly 50% of the SCID mice with an average latency of 2 months (Fig. 3*A*), but no tumor formation was observed in mice injected s.c. with the H6c7eR-pBp cells. Histologic analysis of all four s.c. tumors formed by the H6c7eR-Kr cells revealed poorly differentiated carcinoma with focal glandular and epidermoid differentiation (Fig. 3*B*). The H6c7eR-Kr cells were also implanted orthotopically into the pancreas of five SCID mice. Six months after implantation, although the animals appeared well, two mice developed poorly differentiated carcinomas (Fig. 3*C*) that were histologically similar to the s.c. formed tumors. Metastases were not found. The control H6c7eR-pBp cells were nontumorigenic when injected directly into the pancreas.

We successfully generated a tumor cell line H6c7eR-KrT from the s.c. growing tumors (Fig. 3*A, T4*). In tissue culture, the tumor cells retained their epithelial morphology and showed comparable growth rate as the H6c7eR-Kr cells. They retained partial sensitivity to TGF-β1-induced growth inhibition and expression of *Smad4* gene (data not shown). The H6c7eR-KrT formed tumors in all animals that were implanted either s.c. or orthotopically (Table 1), and the latency of tumor development was also markedly reduced (Fig. 3*A*). Whereas it took 17 weeks for the T4 tumor to become palpable and 23 weeks to reach a 0.5 cm diameter, the tumors formed by the H6c7eR-KrT cell line were palpable 2 weeks after implantation and took ~8 weeks to become 0.5 cm in diameter. However, no metastasis was evident in both cases. Although the tumors formed s.c. by the H6c7eR-KrT cells remained poorly differentiated carcinomas (Fig. 3*D*), the orthotopically growing tumors showed distinct glandular differentiation that closely resembled the histopathology of human pancreatic ductal adenocarcinoma (Fig. 3*E*).

Activation of pathways downstream of K-*ras*. The activation of K-RAS protein in H6c7eR-Kr cells led to the activation of its downstream effectors, such as AKT and MAPK. The phospho-AKT and phospho-MAPK levels were enhanced in the H6c7eR-Kr cells compared with the parental or vector transduced control (Fig. 4*A*), as were the levels of K-RAS protein. The tumor cell line H6c7eR-KrT and tumor tissue that it formed showed further increases in the K-RAS protein expression levels as well as in the level of MAPK activation. It is worth noting that the K-RAS protein levels of the H6c7eR-KrT cells and tumor tissue were comparable with that expressed in a pancreatic cancer cell line PK1 (Fig. 4*A*) but were higher than that of its pretumorigenic H6c7er-Kr cells. Similar to

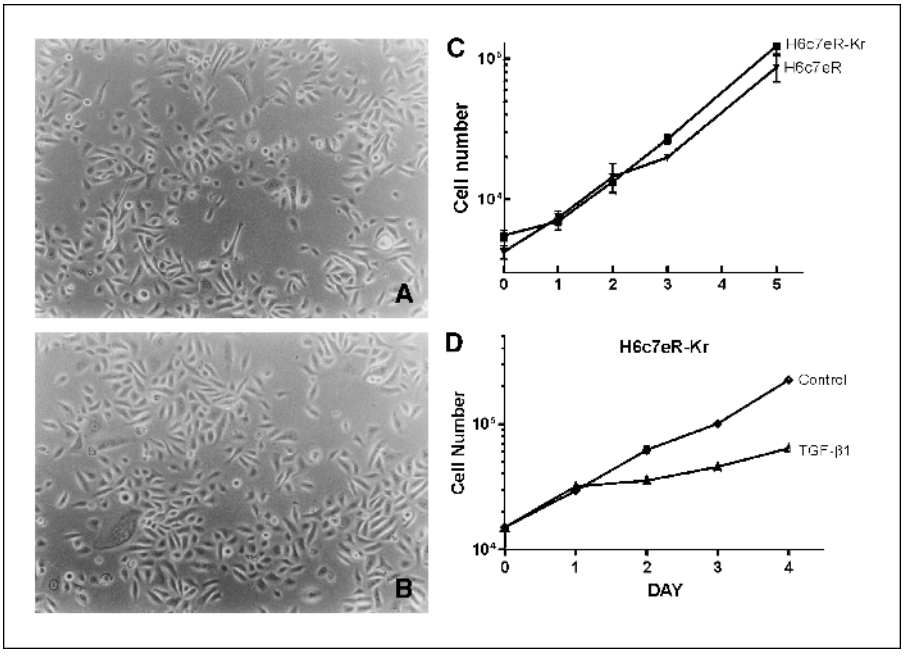

Figure 2. Growth properties of H6c7eR-Kr cells. *A* and *B,* phase-contrast appearances of H6c7eR-Kr and H6c7 cells growing on a plastic surface, respectively. *C,* growth curve of H6c7eR-Kr and control H6c7-eR cells. *D,* inhibitory effect of TGF-β1 on H6c7eR-Kr cells. For the latter experiments, untreated and treated cells were initially seeded in replicates and counted at various points indicated after treatment by 10 ng/mL TGF-β1 or without treatment (*Control*).

Table 1. Tumor formation in SCID mice following implantation of derived cell lines

Cell line	No. mice with tumor/no. mice implanted s.c.	No. mice with tumor/no. mice implanted orthotopically
H6c7eR-pBp	0/7	0/5
H6c7eR-Kr	4/7	2/5
H6c7eR-KrT	5/5	3/3

NOTE: Cells were injected into each animal and animals were sacrificed under one of the following conditions: tumor has formed and reached 1.0 cm in diameter; ulceration started on the surface of the tumor; the mice were morbid; or the mice were kept for as long as 6 months after implantation.

the parental H6c7 cells that express the HPV16-E6E7 gene, the H6c7eR-Kr cells and the tumor cell line/tissue still expressed the normal p16 and SMAD4 proteins (Fig. 4*A*). In contrast, the PK1 pancreatic cancer cell line showed the typical loss of p16 and SMAD4 expression.

NF-κB, another transcription factor, which acts downstream of K-*ras*, was reported previously to be constitutively activated in most human pancreatic cancers but not in normal pancreas (28). The expression of the K-RAS protein in H6c7eR-Kr cells did not significantly increase NF-κB activity compared with the control cells (Fig. 4*B*). However, H6c7eR-KrT tumor cell line, established from the H6c7eR-Kr tumor tissues, had significantly higher NF-κB activity (Fig. 4*B*). Oct-1 probe was used as a loading control (Fig. 4*B*). The supershift assay confirmed the specificity and identity of the κB DNA-binding activity as p65(RelA)/p50 heterodimers (Fig. 4*C*). These results suggest that constitutive NF-κB activation may play a critical role in the tumorigenic transformation of pancreatic ductal epithelial cells, and this could be achieved by very high levels of K-*ras* expression or in conjunction with other genetic alterations that occur during multistage carcinogenesis.

Molecular profiling of the H6c7eR-Kr cells. The Affymetrix oligonucleotide microarray platform was used to characterize the transcriptional response of H6c7 cells to K-*ras* oncogene transduction. RNA extracted from H6c7eR-Kr cells, H6c7eR-pBp cells, and parental H6c7 cells was profiled on the Affymetrix HG-U133A chips, which contains ~22,000 probe sets representing >19,000 transcripts derived from 16,000 human genes.

To identify genes that are differentially expressed in H6c7eR-Kr cells, we first compared the gene expression of H6c7eR-Kr cell line with that of the parental H6c7 cell line. We selected the genes that have robust changes as described in Materials and Methods. To eliminate the effects of empty vector on transcriptional regulation, we then compared the gene expression of control H6c7eR-pBp cell line with that of parental H6c7 cell line. Genes showing differentially expressed between H6c7eR-pBp and H6c7 were excluded from the gene list that represents the differential expression between H6c7eR-Kr and H6c7. Using this strategy, we identified 584 up-regulated genes and 465 down-regulated genes that putatively resulted from expression of K-*ras* oncogene in the H6c7 cells. Genes with changes >4-fold are shown in Supplementary Tables S2 and S3. Based on their known biological functions, these differentially expressed genes were mainly involved in transcription, proteolysis, cell proliferation, death, adhesion, cell surface receptor, and intracellular signaling (Table 2).

To further validate the microarray expression profiling results and identify putative pancreatic cancer tumor marker genes, we compiled the publicly available microarray and Serial Analysis of Gene Expression data on genes that are differentially expressed in pancreatic cancer (4–13). We then compared our list of genes with this published list of genes. Among the 584 up-regulated genes, 42 genes were reported previously as overexpressed in primary pancreatic cancer or cancer cell lines (Table 3). We identified only 10 overlapping down-regulated genes, as there were limited published data on underexpressed genes in pancreatic cancers (data not shown).

Validation of microarray data by real-time PCR. To validate the microarray results, we selected 24 among the 42 overexpressed genes and 2 known *ras*-induced genes, $p21^{cip1}$ and *VEGF* for real-time PCR confirmation of differential expression (Fig. 5). The 24 genes included 15 genes for which antibodies were available for the protein products and additional genes that have been associated with carcinogenesis, such as *BGN, PRSS2*, and *PTP4A1* (29–31). Eighteen genes were confirmed to be differentially overexpressed in the H6c7eR-Kr cells compared with the H6c7 cells. For most genes,

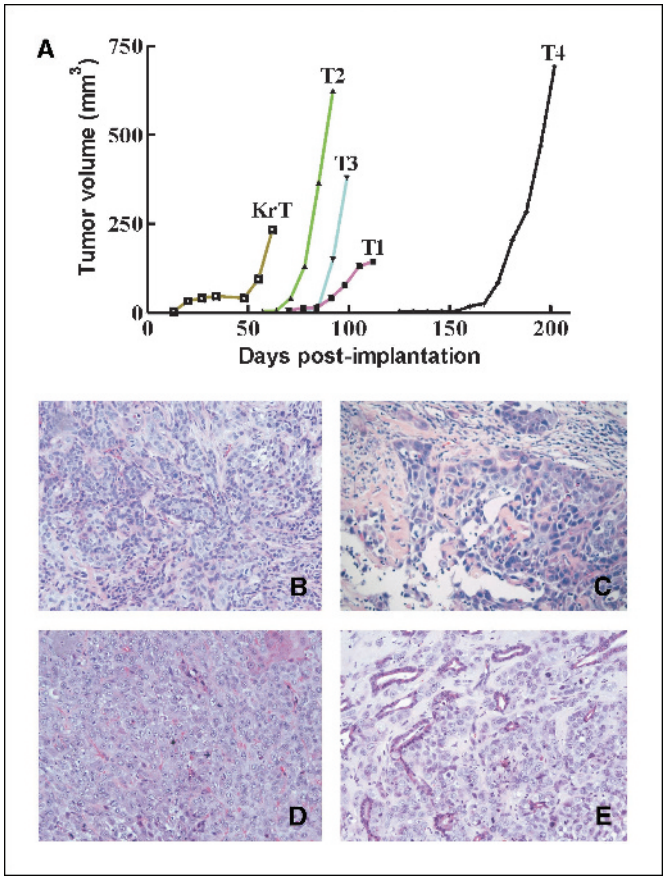

Figure 3. Tumorigenicity of H6c7eR-Kr cells. *A,* growth rates of s.c. tumors formed by H6c7eR-Kr cells injected into four SCID mice (*T1-T4*). The tumor was monitored once weekly. The size of tumor was measured since the onset of the tumor. Volume of the tumor was calculated using the formula: V = length × width^2 × 0.52. A primary tumor cell line H6c7eR-KrT was derived from T4 s.c. tumor. The growth curve of the tumor formed by H6c7eR-KrT cells (*KrT*) is also illustrated. *B* and *C,* histology of s.c. and orthotopic tumors derived from H6c7eR-Kr cells, respectively. *D* and *E,* s.c. and orthotopic tumors formed by H6c7eR-KrT cells, respectively. Magnification, ×100 (H&E).

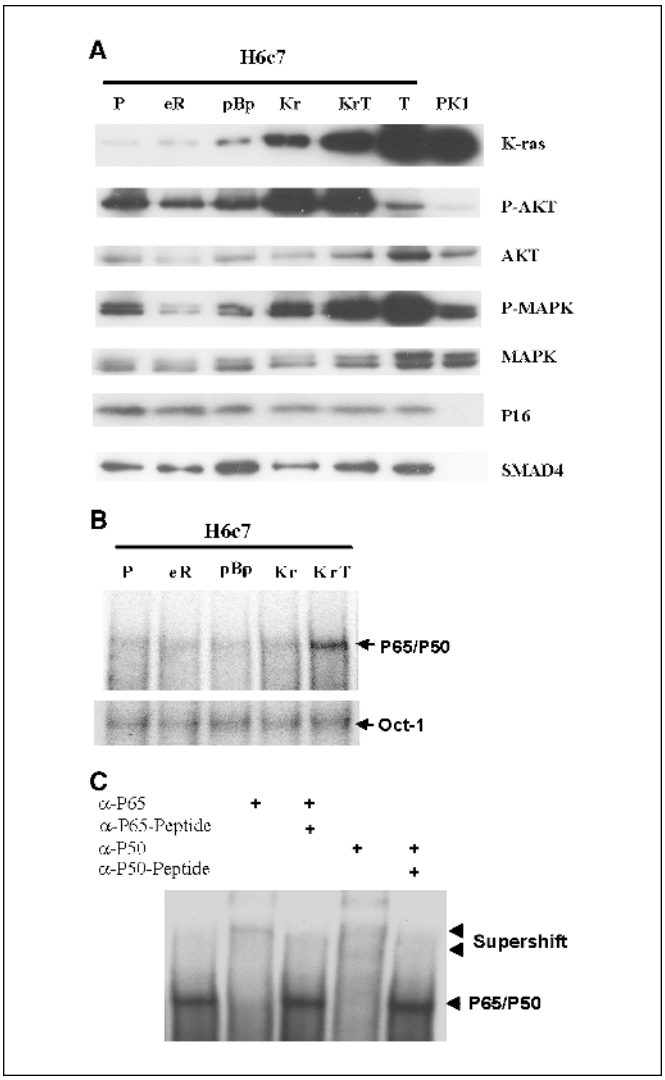

Figure 4. Activation of regulatory and signaling proteins in H6c7eR-Kr cells. *A,* Western blot of K-RAS protein, phospho-AKT (P-AKT), total AKT, phospho-MAPK (P-MAPK), total MAPK, P16, and SMAD4 in a panel of cell lines, including H6c7 parental cells, H6c7-eR, H6c7eR-pBp, H6c7eR-Kr, H6c7eR-KrT, T4 s.c. tumor (*T*), and a pancreatic cancer cell line (*PK1*). *B,* NF-κB activity in H6c7 cells and its derivatives was determined by electrophoretic mobility shift assay. *C,* nuclear extract from H6c7eR-KrT cells was used for the supershift assays to show the specificity of P65 and P50 antibodies.

the magnitude of expression changes detected by PCR was comparable with the fold changes detected by the microarray platform.

Overexpression of laminin β3 in pancreatic intraepithelial neoplasias and primary pancreatic cancers. Pancreatic cancer is believed to evolve from the precursor ductal lesions PanINs. Immunohistochemical analysis of different stages of PanIN led to the understanding of neoplastic progression in pancreatic duct cell carcinogenesis. Among the 42 genes listed in Table 3, we selectively did immunohistochemical evaluation of the highly expressed genes for which commercial antibodies were available. Among all the antibodies we tested, only the antibody for LAMB3 was confidently considered to show specific tissue staining (Fig. 6). There was a distinctly progressive enhancement of LAMB3 expression in the PanIN-cancer sequence (Fig. 6*E*). High-level (grades 2 and 3) cytoplasmic staining of LAMB3 was detected in >85% (34 of 40) pancreatic cancer tissue samples (Fig. 6*D* and *E*). Increased cytoplasmic and membranous staining was also observed in 12 of 37 (32%) of high-grade PanIN (2 and 3) lesions (Fig. 6*B* and *C*). However, no or faint staining was observed in all except one normal pancreatic duct epithelia (Fig. 6*A*) or PanIN-1 lesion. It is worth noting that strong membranous staining was only observed in the high-grade PanIN lesions and invasive cancer cells showed mainly ectopic expression of this protein in the cytoplasm.

Discussion

We have shown that the stable transduction and expression of the K-*ras* oncogene into HPV16-E6E7 immortalized HPDE cells yielded incomplete tumorigenic transformation phenotype, with only 50% of the animals implanted with this cell population forming tumors. This transformation event was not apparent from the *in vitro* properties of this cell line, as the cells maintained a monolayer epithelial-like appearance, failed to grow in soft agar, and remained partially sensitive to TGF-β-elicited growth inhibition. Full malignancy transformation as reflected in a cell line that was established from one of these tumors seems associated with further elevation of K-*ras* oncogene expression levels and activation of the NF-κB pathway, suggesting that the additional genetic or epigenetic changes that led to the latter play essential role in pancreatic carcinogenesis. By comparing the gene expression profiles of K-*ras* expressing and control HPDE cell lines, we have identified genes whose expression levels were putatively modulated by K-*ras* oncogenic activation in pancreatic duct cells. These included 42 genes that were reported previously as overexpressed in pancreatic cancer. Among these, we have further validated using tissue microarrays that the overexpression of LAMB3 occurred during the late stages of PanIN-carcinoma sequence; thus, LAMB3 potentially represents an excellent candidate gene that should be studied further as potential early detection biomarker for pancreatic cancer.

This is the first demonstration that K-*ras* oncogene in conjunction with inactivation of p53 function and deregulated Rb signaling can induce tumorigenic transformation in HPDE cells. Lohr et al. (32) have reported previously that bovine pancreatic duct cells also became tumorigenic after sequential transfection with the SV40 large T antigen and K-*ras* oncogene. As both HPV16-E6E7 and SV40 large T antigen inactivate p53 and Rb pathways, results of these studies suggested a common set of obligate genetic events for the malignant transformation of pancreatic duct cells, similar to that reported previously in other human epithelial cell

Table 2. Number of deregulated genes in H6c7eR-Kr cells according to known biological functions

Annotated functions	No. up-regulated genes	No. down-regulated genes
Transcription	62	44
Proteolysis	20	20
Cell proliferation	36	52
Cell death	16	15
Cell adhesion	16	10
Cell surface receptors	24	14
Intracellular signaling	32	19

Table 3. Genes reported previously as overexpressed in pancreatic cancer and are also identified in this study as putative downstream targets of K-*ras* oncogene activation in pancreatic duct cells

Unigene	Gene symbol	Title
Hs.86947	*ADAM8*	A disintegrin and metalloproteinase domain 8
Hs.821	*BGN*	Biglycan
Hs.7327	*CLDN1*	Claudin 1
Hs.408864	*DAF*	Decay accelerating factor for complement (CD55)
Hs.171596	*EPHA2*	EphA2
Hs.438862	*EPS8L1*	EPS8-like 1
Hs.390567	*FYN*	FYN oncogene related to SRC, FGR, YES
Hs.169946	*GATA3*	GATA-binding protein 3
Hs.74576	*GDI1*	GDP dissociation inhibitor 1
Hs.24049	*GOLGA2*	Golgi autoantigen, golgin subfamily A, 2
Hs.2178	*HIST2H2BE*	Histone 2, H2be
Hs.3618	*HPCAL1*	Hippocalcin-like 1
Hs.40968	*HS3ST1*	Heparan sulfate (glucosamine) 3-*O*-sulfotransferase 1
Hs.727	*INHBA*	Inhibin, βA (activin A, activin AB α polypeptide)
Hs.149846	*ITGB5*	Integrin, β_5
Hs.376874	*KCNK1*	Potassium channel, subfamily K, member 1
Hs.436983	*LAMB3*	Laminin β3
Hs.204238	*LCN2*	Lipocalin 2 (oncogene 24p3)
Hs.79299	*LHFPL2*	Lipoma HMGIC fusion partner–like 2
Hs.3844	*LMO4*	LIM domain only 4
Hs.2399	*MMP14*	Matrix metalloproteinase 14 (membrane inserted)
Hs.364345	*NNMT*	Nicotinamide *N*-methyltransferase
Hs.423	*PAP*	Pancreatitis-associated protein
Hs.79769	*PCDH1*	Protocadherin 1 (cadherin-like 1)
Hs.82101	*PHLDA1*	Pleckstrin homology-like domain, family A, member 1
Hs.81170	*PIM1*	Pim-1 oncogene
Hs.179657	*PLAUR*	Plasminogen activator, urokinase receptor
Hs.96	*PMAIP1*	Phorbol-12-myristate-13-acetate–induced protein 1
Hs.2499	*PRKCL1*	Protein kinase C–like 1
Hs.438582	*PRNP*	Prion protein (p27-30; Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)
Hs.511525	*PRSS2*	Protease, serine, 2 (trypsin 2)
Hs.146688	*PTGES*	Prostaglandin E synthase
Hs.227777	*PTP4A1*	Protein tyrosine phosphatase type IVA, member 1
Hs.194691	*RAI3*	Retinoic acid induced 3
Hs.21858	*SERPINE2*	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
Hs.235782	*SLC21A12*	Solute carrier family 21 (organic anion transporter), member 12
Hs.410977	*TAGLN*	Transgelin
Hs.446641	*TIMP1*	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity)
Hs.83883	*TMEPAI*	Transmembrane, prostate androgen-induced RNA
Hs.278896	*UGT1A10*	UDP glycosyltransferase 1 family, polypeptide A10
Hs.26077	*WFS1*	Wolfram syndrome 1 (wolframin)
Hs.82109	*SDC1*	Syndecan-1

lines (20, 33). Our finding also provides a strong and direct experimental evidence that genetic aberrations commonly found in PanIN lesions (i.e., K-*ras* mutations) deregulated G_1-S cell cycle checkpoint exemplified by *p16* mutations, and *p53* mutations indeed play a critical role in the multistage progressive transformation of pancreatic duct epithelial cells in PanIN lesions (14).

Our current knowledge on the putative roles of these genetic mutations in pancreatic carcinogenesis is largely represented by static images of when and how frequent these mutations occur in the PanIN and carcinoma lesions, but we remain ignorant of their biological impacts. It is with this in mind that we established previously the HPDE cell lines from normal human pancreatic duct fragments to establish an *in vitro* dynamic model of human pancreatic duct cell carcinogenesis (34). Nevertheless, because tumors were formed in only 50% of the SCID mice implanted by the H6c7eR-Kr cells and that the tumor cell lines established from one of these tumors showed greatly enhanced tumorigenicity, it suggests that additional genetic or epigenetic changes may be required in addition to K-*ras* oncogene to fully transform these HPV16-E6E7 immortalized HPDE cells into highly malignant cancer cells. A recent mouse model also found that pancreas-specific K-*ras* mutation alone induced focal pancreatic ductal lesions, but it was only when this was complemented by inactivation of the $p14^{arf}$ gene that malignant pancreatic cancer developed (35). Because the H6c7eR-KrT cells still express normal Smad4 protein, our results indicate that *Smad4* inactivation is not

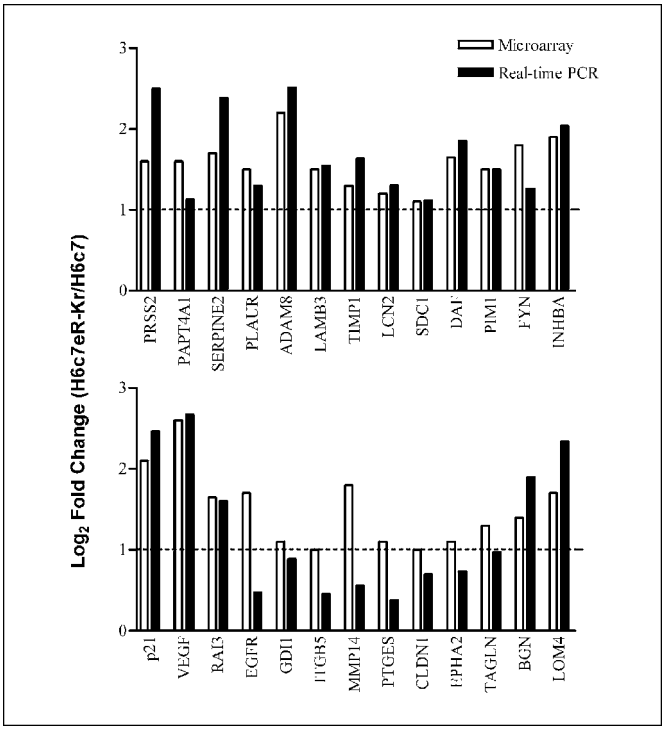

Figure 5. Real-time PCR validation of candidate genes. *Columns,* $\log_2$ fold changes of the mRNA levels of each gene in H6c7eR-Kr cells relative to that expressed in H6c7 cells as obtained from microarrays or real-time PCR studies.

absolutely necessary for the tumorigenic transformation of HPDE cells. Nevertheless, our results also suggest that in HPDE cells abrogation of the TGF-β signaling cannot be adequately achieved by K-*ras* oncogene activation alone and thus will likely require inactivation of the *Smad4* gene. The HPDE cells provide an important dynamic model for future studies on the functional role of *Smad4* gene in pancreatic duct cell carcinogenesis. In addition, introduction of other genetic aberrations implicated in the PanIN-cancer sequence into this cell model could better define their potential role in pancreatic cancer progression. Tumors formed by both H6c7eR-Kr and H6c7eR-KrT cells failed to develop metastasis. These cell lines provide an appropriate model to test other pancreatic cancer–associated genes for their role in metastasis.

Several other possibilities could be investigated to identify the additional malignancy-associated genetic or gene expression changes required for full malignant transformation of the K-*ras* oncogene–expressing HPDE cells. It is possible that there was further selection among the implanted population of H6c7eR-Kr cells for clones that have acquired the additional genetic aberration critical for tumorigenic growth *in vivo*. A second possibility is that K-*ras* oncogene generates genomic instability, which could lead to the acquisition of such genetic changes necessary for tumorigenic growth *in vivo*. The fact that H6c7eR-KrT cells showed much higher expression level of K-RAS protein compared with the H6c7eR-Kr cells and that constitutive activation of NF-κB was detected only in H6c7eR-KrT cells favors the first possibility. Future studies that investigate the tumorigenicity of clones of H6c7eR-Kr cells with varying K-*ras* levels may provide further insights into the importance of expression level of this oncogene in pancreatic carcinogenesis. It is possible that very high levels of K-*ras* oncogene expression is necessary for the activation of NF-κB pathway (36), and the latter is critical for the H6c7eR-Kr cells to fully manifest their *in vivo* tumorigenic potential.

Using the microarray technology, we are able to appreciate global transcriptional changes induced by K-*ras* oncogene in the near normal human pancreatic duct cells. All together, we identified 584 up-regulated genes and 465 down-regulated genes. Several known *ras*-up-regulated genes, including $p21^{cip1}$, *ets1, ets2*, and *VEGF*, were also overexpressed in H6c7eR-Kr cells. As there is no functional p53 in the H6c7 cells due to E6 immortalization (19), a p53-independent activation of $p21^{cip1}$ was likely to have occurred (37, 38). Previous reports indicated that the overexpression of $p21^{cip1}$ protein occurred early and progressively increased during the PanIN-cancer sequence (17, 39). Our data suggest that such up-regulation is likely secondary to a transcriptional induction by K-*ras* mutations and downstream constitutive activation of the RAS-Raf-MAPK pathway.

Genome-wide expression profiling studies (4–13) have identified ~860 genes differentially expressed in pancreatic cancer cell lines or tumors. These genes are potential diagnostic biomarker genes or target genes for the development of novel therapeutics. Only a few of these genes were confirmed in multiple studies, thus creating a dilemma as to which of the genes should be pursued first in validation studies. The same issue confronted us with the list of genes that we found to be differentially expressed following K-*ras* oncogene activation in the H6c7 cells. We therefore cross-referenced our list of *ras*-induced genes and the compiled published gene list. This comparison yielded 42 genes that were

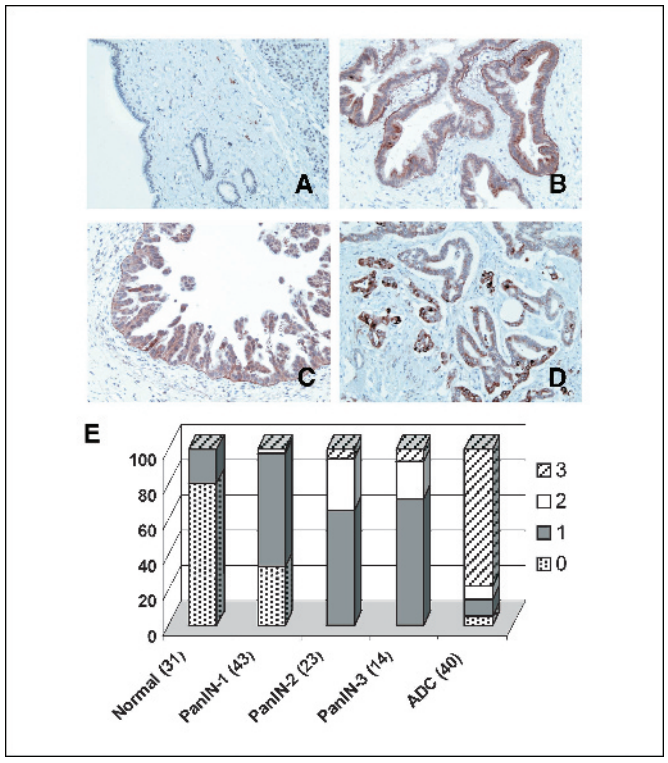

Figure 6. Immunohistochemical staining of LAMB3 in pancreatic ductal lesions. *A,* normal pancreatic duct epithelium shows no staining in duct cells. *B* and *C,* increased cytoplasmic and membranous staining was observed in PanIN-2 and PanIN-3 lesions, respectively. *D,* heterogeneous but strong cytoplasmic staining was detected in invasive pancreatic ductal adenocarcinoma cells. *E,* frequency of PanIN lesions and adenocarcinoma (*ADC*) showing various levels of LAMB3 immunostaining. The staining intensity was scored on a 0 to 3 scale from negative to strong immunoreactivity. Magnification, ×100 (*A-D*).

reported previously as overexpressed in pancreatic cancer and were also identified in the current study. Because these genes are up-regulated by K-*ras* oncogene in HPDE cells derived from normal pancreas and their overexpression persisted in invasive pancreatic cancer, we postulate that they would be candidate tumor markers for early detection. Among these 42 genes, *PLAUR* was reported to be up-regulated by activated extracellular signal-regulated kinase-1 pathway in colon cancer (40). Its transcription was shown to be stimulated by constitutively active H-*ras* (41), and it has been implicated in the invasiveness of pancreatic cancer cells (42). *BGN* and *TIMP1* were also found to be overexpressed in pancreatic cancer (30, 43). Among the 26 genes we chose to validate by quantitative real-time PCR, the overexpression of 18 genes in H6c7eR-Kr cells were confirmed. Due to the limited availability of commercial antibodies against these gene products and high rate of unsatisfactory quality of these antibodies for immunohistochemistry on formalin-fixed and paraffin-embedded tissue sections, we were only able to confirm the overexpression of LAMB3 in pancreatic cancer and PanIN tissue samples. LAMB3 encodes the β3 chain of laminin-5, which is composed of α3, β3, and γ2 chains (44). Laminin, the key component of basement membrane, is involved in cell growth, migration, adhesion, and angiogenesis (45). Pancreatic cancer was shown to synthesize and deposit laminin-5 in the basement membrane (46), and the γ2 chain was reported to be a new prognostic marker for invasive pancreatic cancer (47). However, the function of β3 subunit in pancreatic cancer is not well understood. Ohnami et al. (48) reported previously that transduction of antisense K-*ras* in a pancreatic cancer cell line down-regulated the expression of LAMB3. This result and ours indicate that LAMB3 is very likely a downstream target of oncogenic K-*ras* activation in pancreatic duct cells. Our finding that LAMB3 is overexpressed at high frequency in pancreatic cancers and parallel to the PanIN-adenocarcinoma progression suggests that LAMB3 could potentially play an important mechanistic role in this process. Further studies on the potential utility of LAMB3 as a cancer biomarker are warranted.

In conclusion, we have shown that HPDE cell line is a powerful model to dissect the mechanistic roles of various genetic aberrations identified previously in pancreatic cancer and its precursor PanIN lesions. Such studies may also provide a more efficient strategy to identify relevant genes that should be investigated further as candidate biomarkers for early detection of pancreatic cancer patients.

Acknowledgments

Received 9/8/2004; revised 1/11/2005; accepted 3/28/2005.

Grant support: Canadian Institutes of Health Research grant MOP49585 (M-S. Tsao), USPHS grant R01CA097159, Lockton Fund for Pancreatic Cancer Research, and Topfer Fund for Pancreatic Cancer Research (P.J. Chiao).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank James Ho for immunohistochemistry work, Wendy Zhang for assistance in microarray data analysis, Dr. William Hahn for the contribution of pBabepuro-K-*ras* plasmid, Dr. Linda Penn for providing the retrovirus for ecotropic receptors, and Dr. David Hedley for advice in flow cytometry analyses.

References

1. Warshaw AL, Fernandez-Del Castillo C. Pancreatic carcinoma. N Engl J Med 1992;326:455–65.
2. Magee CJ, Ghaneh P, Neoptolemos JP. Surgical and medical therapy for pancreatic carcinoma. Best Pract Res Clin Gastroenterol 2002;16:435–55.
3. Yeo TP, Hruban RH, Leach SD, et al. Pancreatic cancer. Curr Probl Cancer 2002;26:176–275.
4. Friess H, Ding J, Kleeff J, et al. Microarray-based identification of differentially expressed growth- and metastasis-associated genes in pancreatic cancer. Cell Mol Life Sci 2003;60:1180–99.
5. Nakamura T, Furukawa Y, Nakagawa H, et al. Genome-wide cDNA microarray analysis of gene expression profiles in pancreatic cancers using populations of tumor cells and normal ductal epithelial cells selected for purity by laser microdissection. Oncogene 2004;23:2385–400.
6. Logsdon CD, Simeone DM, Binkley C, et al. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. Cancer Res 2003;63:2649–57.
7. Crnogorac-Jurcevic T, Efthimiou E, Nielsen T, et al. Expression profiling of microdissected pancreatic adenocarcinomas. Oncogene 2002;21:4587–94.
8. Grutzmann R, Foerder M, Alldinger I, et al. Gene expression profiles of microdissected pancreatic ductal adenocarcinoma. Virchows Arch 2003;443:508–17.
9. Iacobuzio-Donahue CA, Ashfaq R, Maitra A, et al. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. Cancer Res 2003;63:8614–22.
10. Iacobuzio-Donahue CA, Maitra A, Olsen M, et al. Exploration of global gene expression patterns in pancreatic adenocarcinoma using cDNA microarrays. Am J Pathol 2003;162:1151–62.
11. Iacobuzio-Donahue CA, Maitra A, Shen-Ong GL, et al. Discovery of novel tumor markers of pancreatic cancer using global gene expression technology. Am J Pathol 2002;160:1239–49.
12. Tan ZJ, Hu XG, Cao GS, Tang Y. Analysis of gene expression profile of pancreatic carcinoma using cDNA microarray. World J Gastroenterol 2003;9:818–23.
13. Han H, Bearss DJ, Browne LW, Calaluce R, Nagle RB, Von Hoff DD. Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray. Cancer Res 2002;62:2890–6.
14. Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. Clin Cancer Res 2000;6:2969–72.
15. Hruban RH, Adsay NV, Albores-Saavedra J, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am J Surg Pathol 2001;25:579–86.
16. Hruban RH, Wilentz RE, Kern SE. Genetic progression in the pancreatic ducts. Am J Pathol 2000;156:1821–5.
17. Bardeesy N, Depinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer 2002;2:897–909.
18. Hilgers W, Kern SE. Molecular genetic basis of pancreatic adenocarcinoma. Genes Chromosomes Cancer 1999;26:1–12.
19. Ouyang H, Mou L, Luk C, Liu N, Karaskova J, Squire J, Tsao MS. Immortal human pancreatic duct epithelial cell lines with near normal genotype and phenotype. Am J Pathol 2000;157:1623–31.
20. Lundberg AS, Randell SH, Stewart SA, et al. Immortalization and transformation of primary human airway epithelial cells by gene transfer. Oncogene 2002;21:4577–86.
21. Liu N, Furukawa T, Kobari M, Tsao MS. Comparative phenotypic studies of duct epithelial cell lines derived from normal human pancreas and pancreatic carcinoma. Am J Pathol 1998;153:263–9.
22. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 1991;19:2499.
23. Chiao PJ, Miyamoto S, Verma IM. Autoregulation of IκBα activity. Proc Natl Acad Sci U S A 1994;91:28–32.
24. Tsao MS, Zhu H, Viallet J. Autocrine growth loop of the epidermal growth factor receptor in normal and immortalized human bronchial epithelial cells. Exp Cell Res 1996;223:268–73.
25. Zhu CQ, Blackhall FH, Pintilie M, et al. Skp2 gene copy number aberrations are common in non-small cell lung carcinoma, and its overexpression in tumors with ras mutation is a poor prognostic marker. Clin Cancer Res 2004;10:1984–91.
26. Wang KK, Liu N, Radulovich N, et al. Novel candidate tumor marker genes for lung adenocarcinoma. Oncogene 2002;21:7598–604.
27. Al Aynati MM, Radulovich N, Riddell RH, Tsao MS. Epithelial-cadherin and β-catenin expression changes in pancreatic intraepithelial neoplasia. Clin Cancer Res 2004;10:1235–40.
28. Wang W, Abbruzzese JL, Evans DB, Larry L, Cleary KR, Chiao PJ. The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin Cancer Res 1999;5:119–27.
29. Zeng Q, Dong JM, Guo K, et al. PRL-3 and PRL-1 promote cell migration, invasion, and metastasis. Cancer Res 2003;63:2716–22.
30. Weber CK, Sommer G, Michl P, et al. Biglycan is overexpressed in pancreatic cancer and induces G_1-arrest in pancreatic cancer cell lines. Gastroenterology 2001;121:657–67.
31. Moilanen M, Sorsa T, Stenman M, et al. Tumor-associated trypsinogen-2 (trypsinogen-2) activates procollagenases (MMP-1, -8, -13) and stromelysin-1 (MMP-3) and degrades type I collagen. Biochemistry 2003;42:5414–20.
32. Lohr M, Muller P, Zauner I, et al. Immortalized bovine pancreatic duct cells become tumorigenic after transfection with mutant k-*ras*. Virchows Arch 2001;438:581–90.
33. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA. Creation of human tumour cells with defined genetic elements. Nature 1999;400:464–8.
34. Furukawa T, Duguid WP, Rosenberg L, Viallet J, Galloway DA, Tsao MS. Long-term culture and immortalization of epithelial cells from normal adult

human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. Am J Pathol 1996; 148:1763–70.

35. Aguirre AJ, Bardeesy N, Sinha M, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. Genes Dev 2003;17:3112–26.

36. Mayo MW, Norris JL, Baldwin AS. Ras regulation of NF-κB and apoptosis. Methods Enzymol 2001;333: 73–87.

37. Hirose T, Sowa Y, Takahashi S, et al. P53-independent induction of gadd45 by histone deacetylase inhibitor: coordinate regulation by transcription factors Oct-1 and NF-Y. Oncogene 2003;22:7762–73.

38. De Siervi A, Marinissen M, Diggs J, Wang XF, Pages G, Senderowicz A. Transcriptional activation of p21(waf1/cip1) by alkylphospholipids: role of the mitogen-activated protein kinase pathway in the transactivation of the human p21(waf1/cip1) promoter by Sp1. Cancer Res 2004;64:743–50.

39. Biankin AV, Kench JG, Morey AL, et al. Overexpression of p21(WAF1/CIP1) is an early event in the development of pancreatic intraepithelial neoplasia. Cancer Res 2001;61:8830–7.

40. Lengyel E, Wang H, Gum R, Simon C, Wang Y, Boyd D. Elevated urokinase-type plasminogen activator receptor expression in a colon cancer cell line is due to a constitutively activated extracellular signal-regulated kinase-1-dependent signaling cascade. Oncogene 1997;14:2563–73.

41. Muller SM, Okan E, Jones P. Regulation of urokinase receptor transcription by Ras- and Rho-family GTPases. Biochem Biophys Res Commun 2000;270:892–8.

42. Paciucci R, Tora M, Diaz VM, Real FX. The plasminogen activator system in pancreas cancer: role of t-PA in the invasive potential *in vitro*. Oncogene 1998;16:625–33.

43. Bramhall SR, Stamp GW, Dunn J, Lemoine NR, Neoptolemos JP. Expression of collagenase (MMP2), stromelysin (MMP3) and tissue inhibitor of the metalloproteinases (TIMP1) in pancreatic and ampullary disease. Br J Cancer 1996;73:972–8.

44. Patarroyo M, Tryggvason K, Virtanen I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. Semin Cancer Biol 2002;12:197–207.

45. Engbring JA, Kleinman HK. The basement membrane matrix in malignancy. J Pathol 2003;200:465–70.

46. Tani T, Lumme A, Linnala A, et al. Pancreatic carcinomas deposit laminin-5, preferably adhere to laminin-5, and migrate on the newly deposited basement membrane. Am J Pathol 1997;151:1289–302.

47. Katayama M, Sanzen N, Funakoshi A, Sekiguchi K. Laminin γ2-chain fragment in the circulation: a prognostic indicator of epithelial tumor invasion. Cancer Res 2003;63:222–9.

48. Ohnami S, Matsumoto N, Nakano M, et al. Identification of genes showing differential expression in antisense K-*ras*-transduced pancreatic cancer cells with suppressed tumorigenicity. Cancer Res 1999;59: 5565–71.