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

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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, p16INK4A, 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-rasG12V 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-kB 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 33 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 p16INK4A 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-rasG12V oncogene led to tumorigenic transformation. Gene expression profiling using the high-density oligonucleotide microarrays reveals gene expression changes that are putatively downstream of K-rasG12V 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-E6E7c7 (Hec7) 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 pBabeuro-K-ras4B12V (20) and the corresponding empty vector pBabeuro using
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-phycocerythrin-conjugated rat anti-mouse CD8α (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 assessed using i.v. 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 (Thr308) antibody (Cell Signaling), p44/42 mitogen-activated protein kinase (MAPK) antibody (New England Biolabs, Beverly, MA), phospho-p44/42 MAPK antibody (Cell Signaling), p16INK4a antibody (BD Biosciences Discovery Labware, Bedford, MA), 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). Active RAS protein was assayed using the ras activation assay kit (Upstate, Charlotteville, VA). Cells were lysed with Mg2+ 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× sample 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 Chio et al. (23).32P-labeled double-stranded oligonucleotides (5′-CTCAACAGGGGGAACGTTCGGAGGAGCAATG-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.

DNA 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 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 datasets and Affymetrix 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 Ct 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: ΔΔCt = ΔCt H6c7eR-Kr − ΔCt H6c7, fold change = 2−ΔΔCt.

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 (13 (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 ADAM5 (H-50), aPAR (10G7), syndecan-1 (DL-101), lipocarnin 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 ectopic receptor expressing HPDE6-E6E7c7-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).
latency of 2 months (Fig. 3). Yielded s.c. tumors in nearly 50% of the SCID mice with an average of mice. The results were summarized in Table 1. H6c7eR-Kr cells revealed poorly differentiated carcinoma with focal glandular and epidermoid differentiation (Fig. 3B). 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. 3C) 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. 3A, 3F). 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. 3A). 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. 3D), the orthotopically growing tumors showed distinct glandular differentiation that closely resembled the histopathology of human pancreatic ductal adenocarcinoma (Fig. 3E).

**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. 4A), 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. 4A) but were higher than that of its pretumorigenic H6c7er-Kr cells. Similar to

**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. 2A and B). The H6c7eR-Kr cells also showed similar growth rate compared with the control H6c7eR cells (Fig. 2C). 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. 2D), 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. 3A), 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 epithelial characteristics (Fig. 3B). 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. 3C) 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.

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that putatively resulted from expression of K-ras. We identified 584 up-regulated genes and 465 down-regulated genes expression between H6c7eR-Kr and H6c7. Using this strategy, we excluded from the gene list that represents the differential differentially expressed between H6c7eR-pBp and H6c7 were line with that of parental H6c7 cell line. Genes showing we then compared the gene expression of control H6c7eR-pBp cell line with that of the parental H6c7 cell line. We selected the genes that occur during multistage carcinogenesis. The expression of the K-RAS protein in H6c7eR-Kr cells did not ras oncoprotein products and additional genes that have been associated with carcinogenesis, such as BGN, PRRS2, 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, 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. 4A). 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. 4B). However, H6c7eR-KrT tumor cell line, established from the H6c7eR-Kr tumor tissues, had significantly higher NF-κB activity (Fig. 4B). Oct-1 probe was used as a loading control (Fig. 4B). The supershift assay confirmed the specificity and identity of the κB DNA-binding activity as p65(ReLa)/p50 heterodimers (Fig. 4C). 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 oncoprotein 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 oncoprotein 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 underegulated 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, p21CIP1 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, PRRS2, 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,
the magnitude of expression changes detected by PCR was comparable with the fold changes detected by the microarray platform.

Overexpression of laminin B3 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. 6E). High-level (grades 2 and 3) cytoplasmic staining of LAMB3 was detected in >85% (34 of 40) pancreatic cancer tissue samples (Fig. 6D and E). Increased cytoplasmic and membranous staining was also observed in 12 of 37 (32%) of high-grade PanIN (2 and 3) lesions (Fig. 6B and C). However, no or faint staining was observed in all except one normal pancreatic duct epithelia (Fig. 6A) or PanIN-1 lesion. It is worth noting that strong membranous expression 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 tumorogenic 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>
<thead>
<tr>
<th>Annotated functions</th>
<th>No. up-regulated genes</th>
<th>No. down-regulated genes</th>
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<tbody>
<tr>
<td>Transcription</td>
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<tr>
<td>Proteolysis</td>
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Our finding also provides a strong and direct experimental evidence that genetic aberrations commonly found in PanIN lesions (i.e., K-ras mutations) deregulated G1-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 p14arf 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 required for malignant transformation in this model.

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

<table>
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<tr>
<th>Unigene</th>
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<tr>
<td>Hs.86947</td>
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<td>INHBA</td>
<td>Inhibin, βA (activin A, activin AB α polypeptide)</td>
</tr>
<tr>
<td>Hs.149846</td>
<td>ITGB5</td>
<td>Integrin, β3</td>
</tr>
<tr>
<td>Hs.376874</td>
<td>KCNK1</td>
<td>Potassium channel, subfamily K, member 1</td>
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<tr>
<td>Hs.436983</td>
<td>LAMB3</td>
<td>Laminin β3</td>
</tr>
<tr>
<td>Hs.204238</td>
<td>LCN2</td>
<td>Lipocalin 2 (oncogene 24p3)</td>
</tr>
<tr>
<td>Hs.79299</td>
<td>LHFPL2</td>
<td>Lipopma HMGIC fusion partner-like 2</td>
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<td>Hs.3844</td>
<td>LMO4</td>
<td>LIM domain only 4</td>
</tr>
<tr>
<td>Hs.2399</td>
<td>MMP14</td>
<td>Matrix metalloproteinase 14 (membrane inserted)</td>
</tr>
<tr>
<td>Hs.364345</td>
<td>NNMT</td>
<td>Nicotinamide N-methyltransferase</td>
</tr>
<tr>
<td>Hs.423</td>
<td>PAP</td>
<td>Pancreatitis-associated protein</td>
</tr>
<tr>
<td>Hs.79769</td>
<td>PCDH1</td>
<td>Protocadherin 1 (cadherin-like 1)</td>
</tr>
<tr>
<td>Hs.82101</td>
<td>PHLD1A</td>
<td>Pleckstrin homology-like domain, family A, member 1</td>
</tr>
<tr>
<td>Hs.81170</td>
<td>PIN1</td>
<td>Pin-1 oncogene</td>
</tr>
<tr>
<td>Hs.179657</td>
<td>PLAUR</td>
<td>Plasminogen activator, urokinase receptor</td>
</tr>
<tr>
<td>Hs.2499</td>
<td>PRKCL1</td>
<td>Protein kinase C-like 1</td>
</tr>
<tr>
<td>Hs.438582</td>
<td>PRNP</td>
<td>Prion protein (p27-30; Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)</td>
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<tr>
<td>Hs.511525</td>
<td>PRSS2</td>
<td>Protease, serine, 2 (trypsin 2)</td>
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<td>Prostaglandin E synthase</td>
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<td>PTP4A1</td>
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<td>RA1</td>
<td>Retinoic acid induced 3</td>
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<td>Hs.21858</td>
<td>SERPINE2</td>
<td>Serine (or cysteine) proteinase inhibitor, clade E (nexitin, plasminogen activator inhibitor type 1), member 2</td>
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<td>SLC21A12</td>
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<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity)</td>
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<td>TMEPAI</td>
<td>Transmembrane, prostate androgen-induced RNA</td>
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<td>UGT1A10</td>
<td>UDP glycosyltransferase 1 family, polypeptide A10</td>
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<td>WFS1</td>
<td>Wolfram syndrome 1 (wolframin)</td>
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<tr>
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<td>SDC1</td>
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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. A third possibility is that 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 p21cip1, 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 p21cip1 was likely to have occurred (37, 38). Previous reports indicated that the overexpression of p21cip1 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
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 H6c7El-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.

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Jiaying Qian, Jiangong Niu, Ming Li, et al.


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