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Differential display of expressed genes in pancreatic cancer cells

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Abstract

Activating K-*ras* mutations occur in 80–95% of pancreatic cancers. The purpose of this study was to conduct an open, panoramic survey of gene expression, using K-*ras* status as the axis over which to sub-classify pancreatic cancers. Differential display was used to contrast mRNA purified from exponentially growing PANC-1 and Capan-2 cells (mutated K-*ras*) with Hs766T and BxPC-3 cells (wild-type). Differences were confirmed by Northern analysis. Twenty-five transcripts were differentially expressed by a factor of two or more. Four transcripts were over-expressed and twelve were under-expressed in the mutants relative to the wild-types. The transcripts most strikingly over-expressed by the mutant cell lines were MARCKS, DKFZp547C244, and RPLP2. The transcripts over-expressed by the wild-types were CEACAM6, cDNA AK026924, and myosin light chain-6. Profiling of gene expression with respect to K-*ras* mutation status may lead to new insights into pancreatic cancer pathogenesis, as well as to the identification of novel therapeutic targets.

Keywords: Pancreatic; Adenocarcinoma; Cancer; Differential display; K-*ras*; MARCKS; RPLP2; CEACAM6; Myosin

Progress in adjuvant treatment for pancreatic adenocarcinoma may depend upon grouping individual cases into subtypes according to gene expression patterns [1,2]. Each group may require treatment directed against a different molecular target, irrespective of whether there is a measurable difference in clinical behavior. Some cases could be relatively easy to treat once the mechanisms underlying their malignant behavior are better understood.

At present, there is no well-accepted basis for subdivision for pancreatic cancer derived from gene expression data. However, an activating mutation in K-*ras* is seen in 80–95% of pancreatic cancers [3,4]. Mutated K-*ras* DNA has been detected in pre-invasive ductal lesions and intraductal papillary mucinous tumors [5]. This suggests that K-*ras* gene mutation occurs early in those cancers which have it. The remaining 5–20% of cancers bearing the wild-type (WT) K-*ras* genotype may be assumed to have followed a different pathway to malignancy from an early stage. These two groups probably depend upon different sets of proteins for their phenotypical characteristics. This hypothesis is supported by the work of Aoki et al. [6] who showed that antisense directed against p21 slows the growth of K-*ras* mutant pancreatic cancer cell lines but not the cells with wild-type K-*ras*. In addition, vascular endothelial growth factor expression is associated with K-*ras* status [7].

The hypothesis of this gene expression study was that pancreatic cancers bearing a WT K-*ras* gene have in common many differentially expressed genes compared to cancers with mutated K-*ras*. Consistent expression of an mRNA by a subtype may signify a factor which is mechanistically important in the malignant phenotype.

Cancer cell lines with a mutated K-*ras* were compared to cancer cell lines with the WT gene. There is no 'normal' control because human pancreatic duct cells in culture tend to grow in an unhealthy manner. Comparison to tissues not maintained in culture would likewise be difficult to interpret. The cell lines were selected so that factors other than K-*ras* status were as evenly distributed as possible (Table 1).

Differential display is a method used to compare gene expression patterns irrespective of whether the transcripts have been previously identified. This methodology was chosen to allow a simultaneous four-way comparison.

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Table 1
Comparison of pancreatic cancer cell lines

Cell line	K-*ras* mutation [15,16]	p53 Mutation [15,16]	Differentiation status[a]	Sex[b]	Race[b]	Medium
PANC-1	Mutant	Mutant	Poor [17]	Male	Caucasian	DMEM[c]
Hs766T	WT	Mutant	Moderate [18]	Male	Caucasian	DMEM
Capan-2	Mutant	Mutant	Well differentiated [18,19]	Male	Caucasian	McCoy's 5a
BxPC-3	WT	Mutant	Moderate [18]	Female	Caucasian	RPMI 1640
MIAPaCa2	Mutant	Mutant	Poor [18,20]	Male	Caucasian	DMEM

[a] The differentiation status of the tumor of origin of the cell lines.
[b] The race and sex of the patient from which the cell line was originally cultured.
[c] Dulbecco's Modified Eagle's Medium.

Materials and methods

Cell culture. The human pancreatic cancer cell lines PANC-1, Capan-2, Hs766T, BxPC-3, and MIA PaCa-2 were obtained from the American Type Culture Collection (Manassas, VA). They were maintained in the media recommended by the ATCC (Table 1) with 10% FBS at 37 °C and 5% CO_2 until they were 70% confluent.

RNA isolation. For differential display, total RNA from PANC-1, Capan-2, BxPC-3, and Hs766T was isolated and DNase-treated using the SV Total RNA isolation kit (Promega, Madison, WI). For Northern analysis RNA was extracted from all five cell lines using Trizol reagent (Gibco BRI, Rockville, MD). RNA was quantified by UV spectrophotometry and SYBR II fluorometry (Sigma, Saint Louis, MI).

First strand synthesis, PCR, and Differential Display. Two micrograms total RNA was primed with 1 μM oligo-dT (70 °C for 3 min), then cDNA was synthesized with 200 units of MMLV reverse transcriptase in a volume of 10 μL at 42 °C for 1 h. DNA was amplified by PCR using 60 combinations of primers from the Delta Differential Display kit (Clontech, Palo Alto, CA). One microliter of DNA was combined with 1 μL of each primer (20 μM), 13.7 μL water, 2 μL 5× Klentaq buffer, 0.3 μL dNTP (5 mM), and 1.0 μL Klentaq polymerase (Clontech, Palo Alto, CA). The PCR program was: 94 °C 5 min, 40 °C 5 min, 68 °C 5 min – one cycle; 94 °C 2 min, 40 °C 5 min, 68 °C 5 min – two cycles; 94 °C 1 min, 60 °C 1 min, 68 °C 2 min – 27 cycles; 68 °C 7 min. For each primer combination, the products from the four cell lines were electrophoresed over 40 cm of 6% polyacrylamide and 7 M urea gel at 1800 V. The gels were silver stained using the method described by Mitchell et al. [8]. Bands which were very dense, but only in both the K-*ras* mutant cell lines, or only both the wild-type lines were cut from the gel. DNA was recovered by boiling in water for 25 min.

Reamplification and identification. Five microliters of eluted DNA was added to 2.5 μL of each of the original primers (20 μM), 31.25 μL water, 5 μL Klentaq buffer, 1.25 μL dNTP (5 mM), and 2.5 μL Klentaq polymerase. The PCR program was stringent: 95 °C for 1 min 15 s, 69 °C 2 min – three cycles; 95 °C 1 min, 68 °C 1 min – three cycles; 95 °C 1 min, 67 °C 1 min, 68 °C 30 s – three cycles; 95 °C 1 min, 66 °C 1 min, 68 °C 30 s – three cycles; 95 °C 1 min, 65 °C 1 min, 68 °C 30 s – 20 cycles. Several bands required two rounds of PCR to yield a product visible on ethidium bromide-stained agarose gels. The products were purified by electrophoresis through 2% agarose and recovered with QIAquick spin columns (Qiagen, Valencia, CA). The products were directly sequenced by the dideoxy fluorescent dye chain termination method, and the transcripts were identified with a BLAST search of the NCBI databases.

Northern analysis. Oligonucleotide probes (Table 2) were synthesized and 5′-labeled with biotin (Sigma-Genosys, TX). Total RNA was electrophoresed through a denaturing gel (1.2% agarose, 2.2 M formaldehyde) and blotted onto positively charged nylon. After cross-linking with 120 mJ/cm^2 UV irradiation, the filter was prehybridized for 4 h at 42° C in 5× SSPE, 5× Denhardt's solution, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA. Hybridization was carried out with 50 ng probe at 42 °C for 16 h. The filter was washed four times in 5× SSPE, 0.1% SDS for 10 min. The filter was processed with the Brightstar BioDetect non-isotopic detection kit (Ambion, Austin, TX) and exposed to XAR film. After detection, the filter was autoclaved at 105 °C for 15 min in 0.1% SDS and re-probed for β-actin. The signal was quantified using ImagePro Plus software (Media Cybernetic, Silver Spring, MD) and normalized to β-actin.

Results

Differential display

Approximately 6000 bands were visualized clearly. The vast majority of the transcripts were identical for all four cell lines: only eight transcripts were present uniquely in a single cell line. Twelve bands were seen which were clearly over-expressed in the WT cell lines exclusively. Four were over-expressed only in the mutant cells. A representative example of a gel with a differentially expressed product is shown in Fig. 1.

Table 2
Sequences of probes used for Northern analysis

Gene	Sequence of biotinylated oligonucleotide probe
RPLP2	5′-AATACCCTGGGCAATGACGTCTTCAATGT-3′
MARCKS	5′-ATAAACCAGCAATAAACAATAAAGCCTATACAACTTGTAG-3′
CEACAM6	5′-TGTAGCCATTTTAACCAAGCAGCACATTTGTTAATT-3′
Myosin light 6	5′-AAAGTCCAGCACCTTCACATTCATCTCATCACTCT-3′
cDNA AK026924	5′-GCTTGTACTTCTCCATACTGAGCACCCCAGCCCATAGG ATG-3′
cDNA DKFZp547C244	5′ GTTTTACTATCTCACCACTCTTCTGGGCATCCCCTGCCCTGCA-3′
β-Actin	5′-ATTTCCCGCTCGGCCGTGGTGGTGAAGCTGTAGC-3′

The sequences were compared to the NCBI databases to confirm specificity for the target sequences.

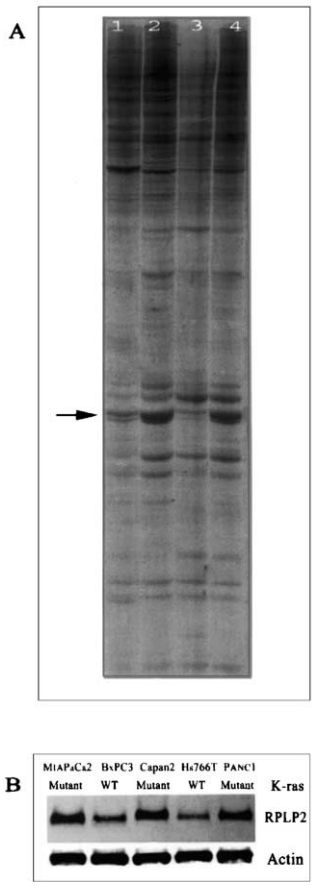

Fig. 1. (A) A representative differential display gel showing the band subsequently identified as RPLP2 (arrow). Lane 1: BxPC-3, Lane 2: Capan-2, Lane 3: Hs766T, and Lane 4: PANC-1. (B) Northern analysis using probe for RPLP2 (430 bp) and β-actin.

Of these differentially displayed bands, the ten with the greatest contrast were selected for PCR amplification. Six yielded sequences which showed great homology with published human DNA sequences. The identities of these genes are shown in Table 3. The remaining four bands gave sequences which included the same primer sequence at each end of the amplicon. This is a known limitation of direct sequencing of differential display products [9], and the sequences of these DNA strands could not be determined.

Northern analysis

Northern analysis (Fig. 1) showed that the differences observed with differential display were generalizable to different passages of the cells and to the additional cell line MIA PaCa-2. The magnitude of the differential expression was 1.5-fold or higher for each of the six transcripts (Table 3).

Discussion

The cell lines are very similar to each other at the gene transcription level: Only 25 out of 6000 bands (0.4%) showed significant differences in density between them. The differences tended to occur in a pattern, specifically, in association with the K-*ras* status. These data support the hypothesis underlying this study which is that pancreatic cancers with a WT K-*ras* can be grouped separately at the molecular level. This conclusion is important because it shows that K-*ras* WT cancer should be considered separately in experiments designed to test potential therapies for pancreatic cancer. Pancreatic cancers need to be broken down into smaller subgroups so that such targets can be found.

Using both differential display and Northern analysis we have shown that the K-*ras* mutant cell lines consistently over-express RPLP2, MARCKS, and DKF Zp5 47C244; they consistently under-express CEA CAM6, myosin light chain-6, and cDNA AK026924.

Most of these genes have functions which could be important in malignant behavior and they may have potential as targets for therapy. These functions are briefly discussed below.

RPLP2

Ribosomal protein large P2 exists as a dimer with RPLP1 in the large subunit of the ribosome. It plays an

Table 3
Genes identified as differentially expressed between K-*ras* mutant and WT pancreatic cancer cell lines

Gene	Cells showing over-expression	Difference on Northern blot[a]	Base match with published sequence[b]	Accession number	Chromosomal gene location
RPLP2	Mutant	2.3-fold	148/148 (100%)	BC007573	11p15.5
MARCKS	Mutant	1.5-fold	320/327 (97%)	XM_011470	6q22.2
DKFZp547C244	Mutant	1.7-fold	319/319 (100%)	AL442093	12
CEACAM6	WT	60-fold	297/300 (99%)	XM_029919	19q13.2
AK026924	WT	1.5-fold	142/143 (100%)	XM_033132	22
Myosin LC6[c]	WT	2.5-fold	162/180 (90%)	AK026164	12q13.13

[a] All results are normalized to β-actin. The mean of the two wild-type cell lines was compared to the mean of the three K-*ras* mutant cell lines.
[b] The number of base-pair matches between the PCR products (excluding the primers) and the published sequences is given.
[c] Myosin light chain-6.

important role in the elongation step of protein synthesis. Experiments with Saccharomyces bearing genetic modifications to inactivate the P1 and P2 proteins have shown that these proteins are not essential for eukaryotic life. However these cells translate a different set of proteins from the RNA pool [10]. They also show different growth and sporulation behavior, which is reversed upon transfection with the missing gene.

MARCKS

Myristoylated alanine-rich protein kinase C substrate is the most abundant cellular substrate for PKC. The MARCKS protein binds calmodulin, actin, and synapsin. Down-regulation of MARCKS has been described in cells which had reverted from a *ras*-transformed cell line [11]. In that study the investigators attempted to alter the phenotype by transfecting a MARCKS construct to restore the MARCKS protein. *Ras*-transformed cells transfected with MARCKS exhibited unusually poor adherence following treatment with phorbol esters (which activate PKC).

CEACAM6

Carcinoembryonic Antigen Associated Cell Adhesion Molecule-6 (also known as non-specific cross-reacting antigen) is commonly over-expressed in human cancers. CEACAM6 has been shown to be present at very low levels in most pancreatic tissues in a previous study [12]. In this study, levels were virtually undetectable in the K-*ras* mutant cell lines. This gene functions as an intercellular adhesion molecule and may inhibit the apoptosis normally seen when cells detach from the basement membrane [13].

Myosin light chain-6

This gene product is the smooth muscle myosin light chain. Myosin, together with actin and microtubules, is important in cell motility and adhesion. Keneko et al. [14] have shown that myosin light chain kinase inhibitors block invasion and adhesion of some pancreatic cancer cell lines.

cDNA AK026924

This transcript is found in the NCBI databases only in RNA derived from hepatoma and small cell carcinoma of the lung. No function is known.

cDNA FZp547C244

This transcript was first sequenced from a human fetal brain sample. Data mining of the National Institutes of Health SAGE database and NCBI databases shows expression of this transcript at high levels exclusively in malignant tumors and normal ovarian tissue. The functional role of this RNA species is unknown.

The results of this study suggest that design of pancreatic cell line experiments should take into account K-*ras* status. The results of studies aiming to advance the understanding of pancreatic cancer biology or to test the efficacy of therapies may be different depending on the K-*ras* status.

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