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Characterization of gene expression in mucinous cystic neoplasms of the pancreas using oligonucleotide microarrays

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Mucinous cystic neoplasms (MCNs) of the pancreas are uncommon neoplasms usually located in the body or tail of the pancreas and usually in females (> 90% of cases). Clinically, they are often misdiagnosed as non-neoplastic pseudocysts leading to failed opportunities for curative resection. To better understand the biology of MCNs and to identify markers of the disease, we performed global gene expression profiling of MCNs using oligonucleotide microarrays. Using laser capture microdissection applied to frozen sections, RNA was extracted from the neoplastic epithelium of MCNs, from the adjacent 'ovarian-type' stroma of MCNs, from histologically normal pancreatic ductal epithelium, from pancreatic acinar tissue and from fibrous stroma in pancreata affected by chronic pancreatitis. Each RNA sample was subjected to two rounds of linear amplification followed by hybridization with U133A gene chips (Affymetrix). The expression patterns of selected genes were confirmed by quantitative RT–PCR and by immunohistochemistry using tissue microarrays containing 19 resected MCNs. A total of 114 known genes were overexpressed in the neoplastic epithelium compared to normal pancreatic ductal epithelium (> 3-fold) including *S100P, PSCA*, c-*myc*, STK6/STK15, *cathepsin E* and *pepsinogen C*. Activation of the Notch pathway in the epithelial component of MCNs was evident by the demonstration of overexpression of Jagged1 and the downstream Notch pathway member Hes1. In the 'ovarian-type' stroma, several genes involved in estrogen metabolism were overexpressed including *STAR* and *ESR1* genes. Some of the genes identified as overexpressed in these neoplasms may be useful as markers that can distinguish MCNs from non-neoplastic pancreatic cystic lesions.

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Introduction

Mucinous cystic neoplasms (MCNs) of the pancreas are relatively rare cystic neoplasms that usually arise in the body or tail of the pancreas. More than 90% arise in females (Thompson *et al*., 1999; Zamboni *et al*., 1999). Pancreatic MCNs were first distinguished from serous cystic neoplasms by Compagno and Oertel (1978). Subsequently, all mucin-producing cystic neoplasms were considered to be MCNs until 1982 when Ohhashi *et al*. (1982) recognized the unique features of intraductal papillary-mucinous neoplasms (IPMNs). MCNs consist of a neoplastic mucin-secreting columnar epithelium and are usually accompanied by a characteristic highly cellular stroma with spindle cells resembling ovarian stroma (Fukushima and Mukai, 1997; Zamboni *et al*., 1999). The distinctive nature of 'ovarian-type' stroma is valuable for diagnosing MCNs especially in cases in which the neoplastic epithelium is denuded from the cyst. Without the presence of an 'ovarian-type' stroma, MCNs can be difficult to distinguish from IPMNs, which are also mucin-producing epithelial neoplasms but which lack a distinct stroma.

Cystic neoplasms represent only a small minority of pancreatic cyst lesions (Solcia *et al*., 1997; Fukushima and Mukai, 1999; Demos *et al*., 2002). Most pancreatic cysts are pseudocysts, which contain an inflammatory and fibrotic tissue wall without an epithelial lining. With improvements in diagnostic imaging, cystic pancreatic lesions are becoming increasingly recognized. The distinction between non-neoplastic pseudocysts and cystic neoplasms is critical, because some cystic neoplasms progress to invasive cancer. Unfortunately, cystic neoplasms are clinically often misdiagnosed as pseudocysts, which leads to delayed diagnosis and failed opportunities for curative resection (Warshaw *et al*., 1990; Demos *et al*., 2002). Molecular markers that could accurately identify neoplastic cystic lesions could help improve the management of patients with cystic lesions. In addition, better knowledge of the molecular alterations that give rise to MCNs could help in the management of patients with these neoplasms. Because MCNs are uncommon and usually only contain a small number of neoplastic cells, molecular studies are difficult and as a result information about these neoplasms has

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largely been derived from immunohistochemical studies of proteins such as p53 (Thompson *et al*., 1999; Zamboni *et al*., 1999; Yoshizawa *et al*., 2002), mucin proteins (Luttges *et al*., 2002), markers of gastric or intestinal differentiation (Zamboni *et al*., 1999), as well as protein expression patterns within the 'ovarian-type' stroma (Fukushima and Mukai, 1997; Izumo *et al*., 2003). In contrast, much more is known about the genetic and epigenetic alterations involved in the development of pancreatic ductal adenocarcinomas, IPMNs and pancreatic intraepithelial neoplasms (PanINs). For example, pancreatic ductal adenocarcinomas can harbor genetic alterations of a large number of genes including *K-ras, p53, p16, SMAD4, BRCA2, MKK4, hMLH1, TGFBR2, TGFBR1, STK11, FANCC, FANCG, ALK4* and *BRAF* genes (Hruban *et al*., 2001; Klein *et al*., 2001; Su *et al*., 2001; Kern *et al*., 2002; van der Heijden *et al*., 2003), as well as numerous alterations in DNA methylation (Ueki *et al*., 2000, 2001; Sato *et al*., 2003a, b). Several similar genetic and/or epigenetic abnormalities have also been described in IPMNs including alteration of the *K-ras, p53* and *STK11/LKB1* genes (Sessa *et al*., 1994; Sato *et al*., 2001, 2002). Global gene expression profiles of pancreatic ductal adenocarcinomas and of IPMNs have provided information about candidate molecular markers of these neoplasms as well as providing information as to their tumor biology (Ryu *et al*., 2001, 2002; Iacobuzio-Donahue *et al*., 2002, 2003a, b; Terris *et al*., 2002; Logsdon *et al*., 2003; Sato *et al*., 2004).

To better understand the biology of MCNs and to identify candidate diagnostic markers, we performed global gene expression profiling of neoplastic epithelial and 'ovarian-type' stromal cell components of MCNs using high-density oligomicroarrays and laser capture microdissection (LCM).

Results

Identification of genes overexpressed in the neoplastic epithelium of MCNs

Sufficient neoplastic epithelial cells for microarray analysis were obtained from six of the eight MCNs, including three adenomas (two flat and one low papillary lesion), two borderline (a flat and a papillary lesion) and one non-invasive mucinous cystic adenocarcinoma (a papillary lesion) (Table 1).

The cell that gives rise to MCNs has not been established. Therefore, in order to identify genes overexpressed in MCNs relative to their normal pancreatic cell counterpart, we first analysed the gene expression profiles of MCNs to determine if they could indicate its cell of origin. We probed the Affymetrix gene expression profiles of normal and neoplastic pancreatic tissues for a list of genes that are known for their pancreatic ductal or acinar expression (Table 2). Genes expressed in normal pancreatic ductal epithelium with little expression in pancreatic acini, such as Keratin 19, Trefoil factor 1 (TFF1) and Trefoil factor 2 (TFF2), were also highly expressed in MCNs (Table 3). In contrast, acinar-specific genes such as elastase and trypsin were absent or underexpressed in MCNs. Several acinar-specific genes were also detected in the microdissected pancreatic duct samples, suggesting some acinar cell contamination in these microdissections. Indeed, despite the use of the laser capture microscope for microdissections, small amounts of acinar tissue were lifted onto the cap during dissection (Table 3). Overall, these data suggested that the neoplastic epithelium of MCNs is of ductal origin (Figure 1).

We therefore compared the gene expression profiles of MCNs to that of normal-appearing pancreatic ductal epithelium. A total of 206 gene fragments were identified as overexpressed at least threefold in the epithelial neoplastic component of the MCNs ($n = 6$) compared to normal pancreatic ductal epithelium ($n = 5$). Of these

Table 1 Case summary

	Age	*Gender*	*Tumor size (cm)*	*Dissected tissue*	*Histological grade of EC*
1	53	F	1.5	EC	Adenoma, flat
2	57	F	4	OS	Adenoma[a]
3	23	F	13.5	OS	Adenoma[a]
4	49	F	4.5	EC	Adenoma, almost flat
5	31	F	5	EC and OS	Adenoma, low papillary
6	47	F	7.5	EC	Borderline, almost flat
7	38	F	10	EC and OS	Borderline, papillary
8	48	F	9	EC	Carcinoma *in situ*, papillary

EC, epithelial component; OS, 'ovarian-type' stroma. [a]According to the Surgical Pathology Report

Table 2 Primers for quantitative real time RT–PCR

Gene	*Primers*
S100P	
Sense	5′-AGC CAT GGG CAT GAT CAT AG-3′
Antisense	5′-AAC ACG ATG AAC TCA CTG AAG-3′
PSCA	
Sense	5′-CCA CCC TTA ACC CTG TGT TC-3′
Antisense	5′-AAA CTC CCA GGA ACT CAC GTC-3′
HJ1	
Sense	5′-GCG TGG GAT TCC AGT AAT G-3′
Antisense	5′-TTA CAG CCA AAG CCA TAG TAG-3′
HEC	
Sense	5′-TAT TTT CCA GTT CTG AGA AAA TC-3′
Antisense	5′-GGA GCT TGT AGA GAT TTC ATG-3′
c-MYC	
Sense	5′-GAT TCA GAT GAT GAA GAT GAT G-3′
Antisense	5′-GGC GGC ATA GTT GTG CTG-3′
STAR	
Sense	5′-CCCCTAGCACGTGGATTAAC-3′
Antisense	5′-CACATCTGGGACCACTTTAC-3′
ESR1	
Sense	5′-TCA AGG ACA TAA CGA CTA TAT G-3′
Antisense	5′-TGC CCT CCC CAT CAT CTC-3′
L1CAM	
Sense	5′-AAA TGG CTG TGA AGA CCA ATG-3′
Antisense	5′-CCT GGG TGT CCT CCT TAT C-3′
WNT2B	
Sense	5′-GGA GAA GAG GCT TAA GGA TG-3′
Antisense	5′-GTC GCC GCA GGT AAT CAC-3′
SFRP1	
Sense	5′-GGC CCG AGA TGC TTA AGT G-3′
Antisense	5′-TTT TTT CAC TTC TTT TAT TTT CAT C-3′

Table 3 Signal intensity of ductal and acinar markers in microdissected tissues

	MCNs[a]	*Ducts*	*Acini*
Elastase	0.5[b]	8.4	100.0
Trypsin1	0.5	21.9	100.0
Trypsin2	1.9	25.3	100.0
Trypsin3	8.7	30.7	100.0
Keratin19	65.4	100.0	11.4
Trefoil factor 1	125.0	100.0	8.8
Trefoil factor 2	96.2	100.0	3.4

[a]Epithelial component. [b]Relative value compared to the signal intensity of normal appearing ducts or acini

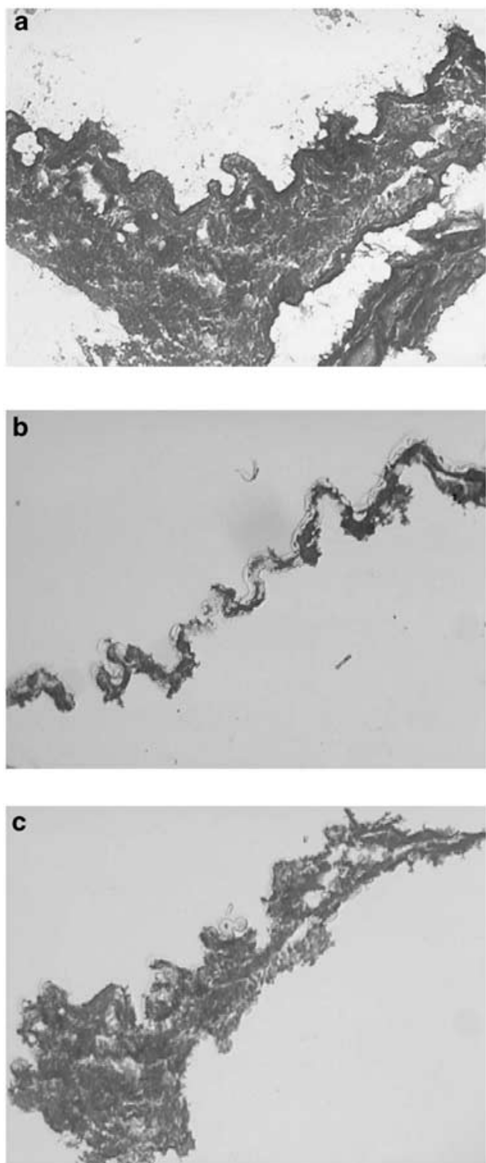

Figure 1 An example of a microdissection obtained using LCM. (**a**) Cystic wall of MCN is composed of lining neoplastic epithelium and 'ovarian-type' stroma. They are separately microdissected. (**b**) Microdissected and captured only lining epithelium. (**c**) 'Ovarian-type' stroma is also microdissected and captured using LCM

206 fragments, 114 corresponded to known genes (Table 4), 20 to 'human hypothetical protein' mRNA fragments and 72 to 'expressed sequence tags (ESTs)'. Interestingly, many of the genes identified as overexpressed in the neoplastic epithelial component have been identified as overexpressed in pancreatic ductal adenocarcinomas or in IPMNs including *S100P, prostate stem cell antigen (PSCA), cathepsin E, highly expressed in cancer (HEC),* c-*myc, STK6/STK15* and *pepsinogen C*. We also identified overexpression of Notch signaling pathway-related genes such as *transmembrane protein Jagged1* (increased 4.8-fold) and *dlk* (increased 4.5-fold).

Identification of genes overexpressed in the 'ovarian-type' stroma of MCNs

Since 'ovarian-type' stroma is characteristic of MCNs and is thought to be of fibroblast origin, we compared its gene expression profile to that of fibroblast stromal cells found in the setting of chronic pancreatitis cases. We first identified transcripts expressed at significantly higher levels (>5-fold) in 'ovarian-type' stroma ($n=4$) compared to the stromal cells in chronic pancreatitis ($n=3$). We then eliminated transcripts whose expression call by the Affymetrix Data Mining Tool was absent in two or more of the four 'ovarian-type' stroma samples. By these criteria, 181 gene fragments, including 70 ESTs and 15 hypothetical protein's gene fragments, were found to be overexpressed. A subset of interesting genes from this list is shown in Table 5. Some notable genes in this list included sex hormone-related genes (*steroidogenic acute regulatory protein, STAR*, increased 71.1-fold; *estrogen receptor 1*, *ESR1*, increased 7.3-fold), genes involved in cell proliferation (wingless-type MMTV integration site family, member 2B, WNT2B, increased 8.0-fold; *sFRP 1*, increased 7.1-fold; *fibroblast growth factor 2*, increased 6.6-fold), smooth muscle-related genes (*actin binding LIM protein 1*, increased 5.9-fold; actin alpha 2, increased 5.4-fold; myosin, heavy polypeptide 11, smooth muscle, increased 5.6-fold), genes expressed in the extracellular matrix (*osteoglycin*, increased 12.1-fold) and several neuronal cell-related genes (cell adhesion molecule L1CAM, increased 15.1-fold; neuroepithelial cell transforming gene 1, increased 7.2-fold; brain-derived neurotrophic factor, increased 5.8-fold; neuronal cell adhesion molecule NRCAM, increased 5.0-fold).

Confirmation of expression of selected genes using quantitative RT–PCR

To confirm the expression patterns of genes identified as overexpressed by microarray, we employed quantitative RT–PCR. We examined the expression levels of five genes identified as overexpressed in the neoplastic epithelium of MCNs compared to normal pancreatic duct by microarray (*S100P, PSCA, Jagged1, HEC* and c-*myc*), and five genes (*STAR, ESR1, L1CAM, WNT2B* and *SFRP1*) identified as overexpressed in the ovarian-type stroma compared to microdissected fibrous stroma

Table 4 Selected overexpressed genes in neoplastic epithelium of the MCNs

	GenBank	*Gene name*	*Fold change*	P-*value*[a]
1	NM_005980.1	S100 calcium-binding protein P (S100P)	105.2	0.01
2	AF196478.1	Annexin 14 (ANX14)	34.8	0.08
3	NM_005672.1	Prostate stem cell antigen (PSCA)	7.7	0.004
4	NM_005369.1	MCF.2 cell line-derived transforming sequence (MCF2)	7.1	0.044
5	NM_005904.1	MAD (mothers against decapentaplegic, *Drosophila*) homolog 7 (MADH7)	6.6	0.024
6	NM_001910.1	Cathepsin E (CTSE)	6.6	0.004
7	NM_003158.1	Serine threonine kinase 6 (STK6)	6.5	0.024
8	NM_002570.1	Paired basic amino-acid cleaving system 4 (PACE4)	5.6	0.024
9	U61276.1	Transmembrane protein Jagged 1 (HJ1)	4.8	0.266
10	NM_000860.1	Hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD)	4.8	0.024
11	AB028641.1	SOX11, SRY (sex determining region Y)-box 11	4.6	0.044
12	NM_021103.1	Thymosin, beta 10 (TMSB10)	4.5	0.024
13	NM_001311.1	Cysteine-rich protein 1 (intestinal) (CRIP1)	4.5	0.123
14	U15979.1	Human (dlk)	4.5	0.619
15	NM_015965.1	CGI-39 protein; cell death-regulatory protein GRIM19 (LOC51079)	4.4	0.266
16	NM_021249.1	Sorting nexin 6 (SNX6)	4.3	0.187
17	NM_001490.1	Glucosaminyl (*N*-acetyl) transferase 1, core 2 (beta-1,6-*N*-acetylglucosaminyl-transferase) (GCNT1)	4.2	0.012
18	NM_005924.1	Claudin 15 (CLDN15)	3.9	0.365
19	BC004470.1	Clone MGC:10332, apoptosis-associated speck-like protein containing a CARD	3.9	0.024
20	NM_003979.2	Retinoic acid induced 3 (RAI3)	3.7	0.024
21	BC000733.1	Eucaryotic translation initiation factor 3, subunit 4	3.7	0.266
22	NM_006031.1	Pericentrin (PCNT)	3.6	0.266
23	NM_006101.1	Highly expressed in cancer, rich in leucine heptad repeats (HEC)	3.5	0.123
24	NM_021913.1	AXL receptor tyrosine kinase (AXL), transcript variant 1	3.5	0.365
25	L06633.1	Transcription factor, Sec7 and coiled-coil domains, binding protein	3.4	0.044
26	NM_016425.1	Transmembrane protease, serine 4 (TMPRSS4)	3.3	0.222
27	NM_003247.1	Thrombospondin 2 (THBS2)	3.3	0.004
28	J05594.1	NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (PDGH)	3.3	0.024
29	NM_002630.1	Progastricsin (pepsinogen C) (PGC)	3.3	0.024
30	BC002712.1	v-myc avian myelocytomatosis viral-related oncogene, neuroblastoma derived, clone MGC:3962	3.2	0.075
31	NM_003199.1	Transcription factor 4 (TCF4)	3.2	0.266
32	AF342815.1	Colorectal carcinoma-derived galectin-8 variant I	3.2	0.123
33	NM_003206.1	Transcription factor 21 (TCF21)	3.1	0.187

[a]Mann–Whitney test

Table 5 Selected overexpressed genes in 'ovarian-type' stroma

	GenBank	*Description*	*Fold change*	P-*value*[a]
1	NM_000349.1	Steroidogenic acute regulatory protein (STAR)	71.1	0.029
2	NM_006614.1	Cell adhesion molecule with homology to L1CAM (close homolog of L1) (CHL1)	15.5	0.029
3	NM_001262.1	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) (CDKN2C)	15.3	0.029
4	NM_014057.1	Osteoglycin (osteoinductive factor, mimecan) (OGN)	12.1	0.029
5	NM_007361.1	Nidogen 2 (NID2)	10.5	0.029
6	NM_000552.2	von Willebrand factor (VWF)	10.1	0.029
7	NM_014060.1	MCT-1 protein (MCT-1)	9.0	0.029
8	NM_024494.1	Wingless-type MMTV integration site family, member 2B (WNT2B), transcript variant WNT-2B2	8.0	0.514
9	NM_000115.1	Endothelin receptor type B (EDNRB), transcript variant 1	7.9	0.514
10	NM_002290.2	Laminin, alpha 4 (LAMA4)	7.3	0.029
11	NM_000125.1	Estrogen receptor 1 (ESR1)	7.3	0.029
12	NM_005863.1	Neuroepithelial cell transforming gene 1 (NET1)	7.2	0.029
13	NM_003012.2	Secreted frizzled-related protein 1 (SFRP1)	7.1	0.086
14	NM_002006.1	Fibroblast growth factor 2 (basic) (FGF2)	6.6	0.029
15	NM_001147.1	Angiopoietin 2 (ANGPT2)	6.6	0.086
16	NM_014799.1	Hephaestin (HEPH)	6.4	0.029
17	NM_005025.1	Protease inhibitor 12 (neuroserpin) (SERPINI1)	6.0	0.314
18	NM_001709.1	Brain-derived neurotrophic factor (BDNF)	5.8	0.029
19	NM_022844.1	Myosin, heavy polypeptide 11, smooth muscle (MYH11), transcript variant SM2	5.6	0.029
20	NM_001613.1	Actin, alpha 2, smooth muscle, aorta (ACTA2)	5.4	0.029
21	NM_012082.2	Friend of GATA2 (FOG2)	5.4	0.029
22	NM_001855.1	Collagen, type XV, alpha 1 (COL15A1)	5.2	0.029
23	NM_005010.1	Neuronal cell adhesion molecule (NRCAM)	5.0	0.314

[a]Mann–Whitney test

obtained from a resected chronic pancreatitis specimen. All 10 genes were overexpressed in the MCN tissues by quantitative RT–PCR when compared to their respective controls (Figures 2 and 3). The mean expression levels of *S100P, PSCA, Jagged1, HEC* and c-*myc* in neoplastic epithelial samples were increased 21.3-, 4.04-, 1.8-, 4.9- and 4.9-fold compared to those in normal ductal epithelial samples, respectively. Generally, gene expression levels were higher in lesions of higher histological grade (borderline and carcinoma *in situ*). The mean expression levels of *STAR, ESR1, L1CAM, WNT2B* and *SFRP1* in 'ovarian-type' stromal samples were increased 65.6-, 26.5-, 16.1-, 1.8- and 5.0-fold compared to the fibrous stromal tissue samples, respectively. Interestingly, the expression levels of four out of the five above-described genes in cultured fibroblast cells were decreased compared to microdissected fibrous tissue samples from surgically resected specimens.

Immunohistochemical analysis

To evaluate whether the transcripts identified by oligonucleotide microarray analysis were overexpressed at the protein level, we performed immunohistochemistry on tissue microarrays (TMAs) using commercially available antibodies for four proteins (Jagged, PSCA,

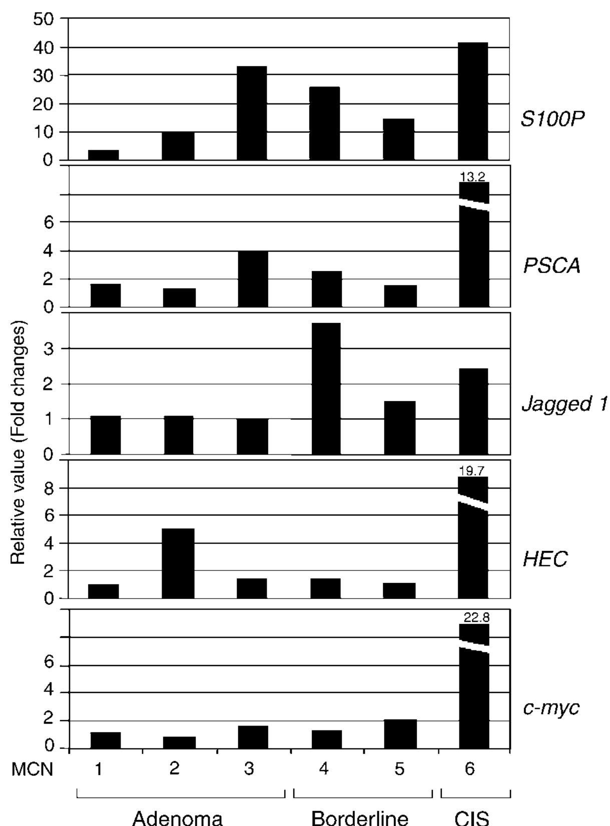

Figure 2 Quantitative RT–PCR analysis of five genes in neoplastic epithelium of the MCNs. The fold change levels on the vertical axis are relative to the expression in normal ductal cell samples ($n = 5$)

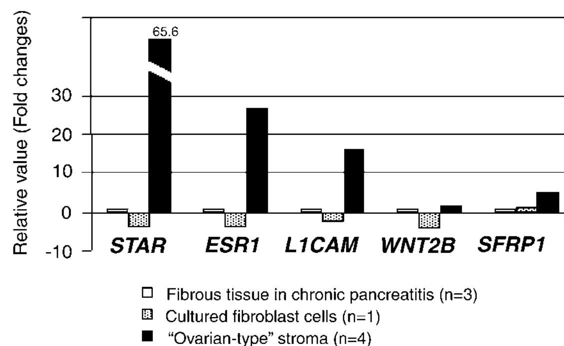

Figure 3 Quantitative RT–PCR analysis of five genes in 'ovarian-type' stroma of the MCNs. Solid bars and dot-blot bars represent a fold change levels of gene expression in 'ovarian-type' stroma ($n = 4$) and in cultured fibroblastic cells ($n = 1$) compared to that of microdissected fibrous stromal cells in chronic pancreatitis ($n = 3$)

Frizzled and L1CAM), whose transcripts were overexpressed in either the epithelium or stroma of MCNs by oligonucleotide microarray analysis. We also evaluated the expression of HES1, a downstream member of the Notch pathway, on frozen section slides of six MCNs to further evaluate the significance of overexpression of Jagged1 and Dlk in the epithelia of MCNs. In all, 57 of the 76 TMA cores of MCNs were evaluable by immunohistochemistry. We evaluated cores from 23 epithelial lesions (13 adenomas, five borderline MCNs and five mucinous cystic adenocarcinomas) and cores from 14 areas of 'ovarian-type' stroma (cores from the same neoplasm of the same grade were counted as one lesion). Five of the 19 MCN neoplasms contained regions of tumor of more than one grade. Jagged1 protein expression was evident in both the cytoplasm and nuclei of the neoplastic cells with less intense expression in normal-appearing pancreatic ductal epithelium, in acinar cells and in islet cells. Seven of 15 cores of ovarian-type stroma had weak nuclear expression of Jagged1. Overall, 30.4% (7/23) of all the MCNs expressed Jagged by immunohistochemistry. Intense and prevalent nuclear staining of HES1 protein was observed in both borderline and carcinoma *in situ* neoplasms (MCN-5 and -6) (Figure 4c). Those two neoplasms showed marked papillary growth. No staining in the stromal cells was observed. PSCA protein expression was seen in 34.8% (8/23) of MCNs with expression localized to the cytoplasm of the neoplastic cells (Figure 4b and d). Expression was more likely in high-grade MCNs than in low-grade MCNs, although this difference did not reach statistical significance; Jagged1 expression was seen in 50% of high-grade and 15.4% of low-grade lesions. PSCA was expressed in 50% of high-grade and 23.1% of low-grade lesions.

Four of the 14 'ovarian-type' stroma tissues labeled intensely with the anti-Frizzled antibody, and four other lesions were weakly positive for Frizzled. L1CAM protein was strongly expressed in peripheral nerve tissues. Two of 14 'ovarian-type' stroma showed cytoplasmic and fine meshes of net-like membranous staining (Figure 4f). In contrast to the labeling

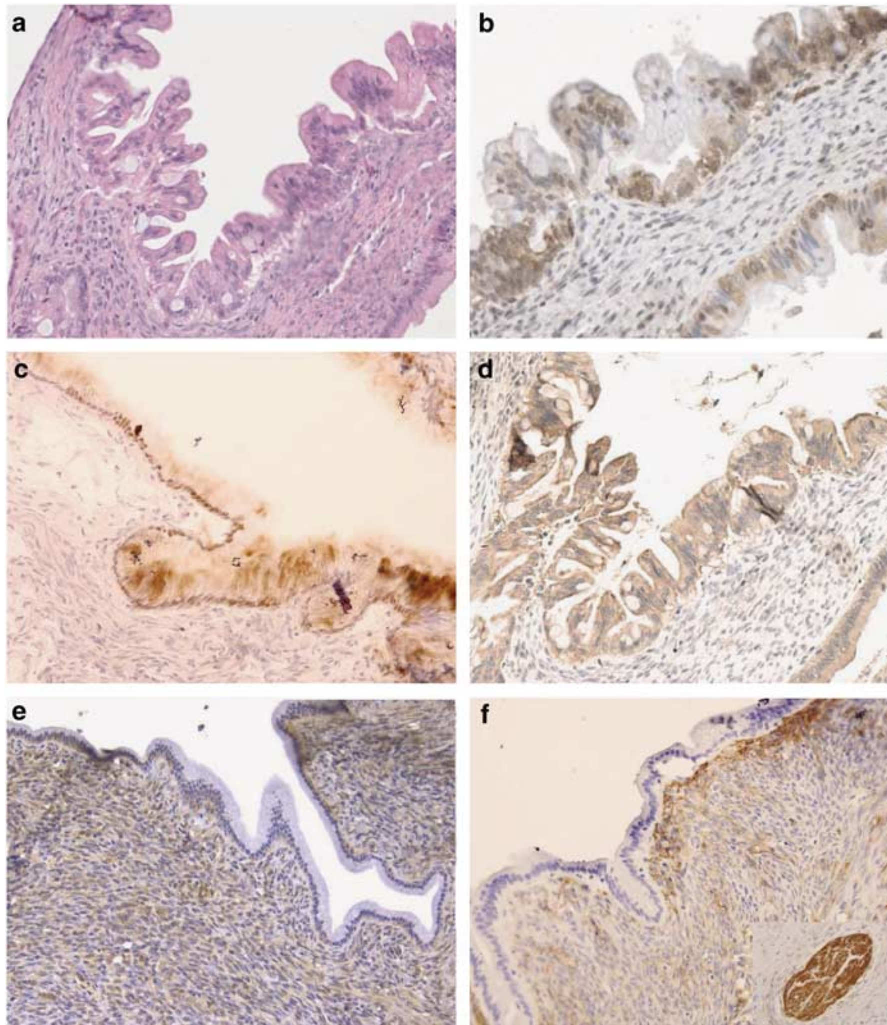

Figure 4 Immunohistochemical validation of five genes overexpressed in MCNs. Shown are representative tissue sections including H&E labeled in (**a**) and positive expression for PSCA (**b**), Jagged (**c**), HES1 (**d**), Frizzled (**e**) and L1CAM (**f**). HES1 labeling was performed on frozen sections of pancreatic tissue. Immunohistochemistry of the remaining genes was carried out on sections of formalin-fixed paraffin-embedded tissue microarrays. The inset of (**f**) shows strong positive staining of a peripheral nerve tissue as a control

of stroma fibroblasts, the lining neoplastic epithelium did not label.

Discussion

In this study, we used high-density oligonucleotide microarrays, LCM and liner RNA amplification to identify genes overexpressed in MCNs. Several of the genes identified as overexpressed are likely to contribute to the biological features of MCNs and may also have diagnostic potential as molecular markers. MCNs are a relatively uncommon neoplasm compared to usual pancreatic cancers and relatively few frozen specimens of MCNs of the pancreas are available for research studies. For this reason and to confirm our gene expression results, we performed immunohistochemical analysis of candidate overexpressed genes in a larger independent series of MCNs.

Among the genes we identified as overexpressed in the neoplastic epithelium of MCNs were several members of the Notch pathway including *Jagged1* and *dlk*. In addition, since the *HES1* gene fragment was not contained on the U133A gene chip, we performed Hes1 immunohistochemistry, which demonstrated intense nuclear expression of HES1 protein in high-grade MCNs. The Notch signaling pathway has been reported to play an essential role in cellular specification, proliferation and differentiation in a wide variety of developmental processes including pancreatic differentiation (Apelqvist *et al*., 1999; Maillard and Pear, 2003). Aberrant Notch signaling has also been observed in several human cancers, including pancreatic ductal adenocarcinoma (Miyamoto *et al*., 2003), mammary cancers in mice (Jhappan *et al*., 1992), acute T-cell lymphoblastic leukemia (Ellisen *et al*., 1991), Hodgkin's and anaplastic large cell lymphoma (Jundt *et al*., 2002) as well as cervical cancer (Zagouras *et al*., 1995). These

studies implicate Notch pathway activation as oncogenic. However, in certain cell types, notch activation is tumor suppressive. For example, loss of Notch leads to sustained overexpression of Gli2 in the skin, promoting tumor development (Nicolas *et al*., 2003). Hes1 is also overexpressed in early PanINs, suggesting that it may contribute to early pancreatic neoplastic development (Miyamoto *et al*., 2003). Other investigators have observed overexpression of the Notch4 ligand in pancreatic ductal adenocarcinomas (Crnogorac-Jurcevic *et al*., 2002). The finding of overexpression of members of the Notch pathway in MCNs suggests that this pathway is important in MCN development.

STK15 (STK6/aurora-A) is a novel serine threonine kinase that is overexpressed in multiple tumor types. Stk6 is located on an amplicon frequently amplified and overexpressed in breast (Hodgson *et al*., 2003), pancreatic (Mahlamaki *et al*., 2002; Li *et al*., 2003) and other cancers. A polymorphism in STK6 identified as a modifier of cancer risk in mice led to investigations demonstrating that the same 91A variant was associated with an increased risk of developing colon cancer and tumors were much more likely to amplify the 91A variant than the normal allele (Ewart-Toland *et al*., 2003). The 91A variant has also been shown to increase the risk of developing esophageal cancer (Miao *et al*., 2004). Overexpression of STK15 occurs in many tumors without evidence of gene amplification (Hodgson *et al*., 2003). Importantly, STK15 inhibitors show considerable promise as anticancer agents in preclinical models (Sausville, 2004).

The ‘ovarian-type’ stroma of MCNs is composed of densely packed spindle cells expressing a smooth muscle phenotype, along with estrogen and/or progesterone receptors (Fukushima and Mukai, 1997; Zamboni *et al*., 1999). This stroma exhibits a variable degree of luteinization characterized by presence of epithelioid cells with round to oval nuclei and abundant clear or eosinophilic cytoplasm, and these cells are known to express calretinin and alpha-inhibin (Zamboni *et al*., 1999). The genes overexpressed in ‘ovarian-type’ stroma included sex hormone-related genes such as *STAR* and *ESR1*. The STAR protein, by enhancing the metabolism of cholesterol into pregnenolone, has a key role in steroid hormone synthesis, and is responsible for the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where it is cleaved to pregnenolone (Sorianello *et al*., 2002). In the normal ovary, STAR is expressed in luteinized follicles and in theca cells (Sorianello *et al*., 2002). We observed overexpression of STAR transcript in ‘ovarian-type’ stroma, although other genes involved in progesterone and androgen biosynthesis were expressed at low levels. This pattern is similar to that seen in the postmenopausal ovary (Jabara *et al*., 2003). Another gene identified as overexpressed in ovarian stroma was the secreted Fizzled-related protein (sFRP) known for its function as a modulator of Wnt signaling. The Wnt pathway influences many biological processes such as cell polarity, adhesion, tumorigenesis and apoptosis (Jones and Jomary, 2002). Several reports have described the relationship between sFRP expression and high estrogen status; sFRP expression in human uterine leiomyomas correlates with estrogen level (Fukuhara *et al*., 2002), and sFRP and estrogen receptor-alpha transcripts colocalize in decidual cells during pregnancy (Ernst *et al*., 2002). Other genes known to be estrogen induced in the setting of breast cancer or in normal breast epithelium were not overexpressed in MCNs (data not shown) (Seth *et al*., 2002; Frasor *et al*., 2003). The availability of frozen ovarian stroma tissue from additional patients with MCNs would permit comparison of this tissue with normal ovary to identify further similarities as well as differences between these tissue types. Such a comparison could identify genes mediating the growth and maintenance of ovarian stroma in MCNs. Overall, the finding of genes implicated in female genital organ function suggests that the almost exclusive female predominance of MCNs could result from the sensitivity of MCNs to female hormones.

The L1CAM is a membrane glycoprotein of the immunoglobulin family and has an important function in development of the nervous system by regulating cell adhesion and migration (Schachner, 1997; Kamiguchi *et al*., 1998). However, L1CAM expression is seen not only in nervous tissues but has also been detected in lymphocytes, granulocytes, epithelial cells of the intestinal and urogenital tract, and in the epidermis (Thor *et al*., 1987; Kowitz *et al*., 1992; Kalus *et al*., 2003). L1CAM expression has also been detected in several highly malignant tumors (Deichmann *et al*., 2003; Fogel *et al*., 2003a, b). We confirmed the characteristic membranous staining pattern in ovarian-type stroma in two of 14 cases by immunohistochemistry.

In the clinical setting, it remains difficult to distinguish neoplastic from non-neoplastic pancreatic cysts either by radiological, endoscopic or biochemical means. Previous attempts to differentiate neoplastic from non-neoplastic cysts include analysis of aspirated cyst fluid for cytology, enzymes and tumor markers (Rubin *et al*., 1994; Hammel *et al*., 1995; Sand *et al*., 1996; Sperti *et al*., 1996, 1997). Thus, there is a need for a panel of markers that can accurately differentiate neoplastic from non-neoplastic pancreatic cysts. Our list of overexpressed genes in MCNs can be used in future studies whose aim is to determine the diagnostic utility of markers detected at the RNA or protein level in needle aspirates of pancreatic cysts.

In conclusion, we have identified several genes that are overexpressed in MCNs that indicate alterations in several important biochemical pathways. Knowledge of the gene expression profiles of MCNs may be useful in the differential diagnosis of these lesions in the clinical setting.

Materials and methods

Patients and specimens

Fresh frozen tissues from pancreatic resections containing MCNs were obtained from the tumor bank of the Department

of Pathology from eight patients (all women, 23–57 years, mean 43.3 years) who underwent distal pancreatectomy at the Johns Hopkins Hospital between the years 1989 and 2000 (Table 1). The MCNs had been stored at −70°C since their resection with the approval of the Johns Hopkins Committee for Clinical Investigation. The diagnosis of MCN was based on WHO criteria: a cystic epithelial neoplasm that lacked communication with the pancreatic duct system and that is composed of columnar, mucin-producing epithelium, supported by ovarian-type stroma (Zamboni *et al*., 2000). The eight MCNs included five adenomas, two borderline neoplasms and one *in situ* adenocarcinoma.

To obtain histologically normal pancreatic ductal epithelium and acinar cells for comparison, eight normal pancreatic frozen tissues were collected from histologically normal-appearing pancreata. The pancreatic ductal epithelia were dissected from larger interlobular ducts. Five pancreata were resected from patients with pancreatic ductal adenocarcinoma, two were from patients with chronic pancreatitis and one was from a patient with an MCN (49–72 years, mean 62.4 years). As a control for the analysis in 'ovarian-type' stroma, fibrous stromal cells without marked inflammatory cell aggregate were microdissected from the surgical resection specimens of three patients with chronic pancreatitis (19–72 years, mean 49 years). Fibroblast cells were also obtained from the surgical resection specimens of a 33-year-old male patient with chronic pancreatitis and grown *in vitro* at low passage (a gift from Dr Masao Tanaka and Dr Kazuhiro Mizumoto, Kyushu University, Fukuoka, Japan). These culture cells were used as a control for the quantitative RT–PCR analysis.

Laser capture microdissection

Frozen tissue sections (8 μm) were cut and the slides were stored immediately at −70°C until subsequent fixation. Every second section was labeled with hematoxylin and eosin (H&E) and covered with a glass coverslip for diagnostic purposes. Tissue sections that underwent LCM were defrosted, fixed (70% ethanol for 3 min) and dehydrated (100% ethanol for 3 min, xylene for 5 min). Sections were dissected using a Pixcell II LCM system (Arcturus Engineering Inc., Mountain View, CA, USA) with a 7.5 μm diameter laser beam (Figure 1). Generally, cells were obtained from 10–20 sections by LCM in order to have sufficient numbers of cells for subsequent RNA amplification.

RNA extraction and amplification

Total RNA was extracted from microdissected tissue using the Picopure RNA isolation kit (Arcturus) according to the manufacturer's instructions. After determining RNA quality using RT–PCR amplification of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, the extracted RNA was subjected to two rounds of linear amplification using the RiboAmp RNA amplification kit (Arcturus). During the second round of amplification, the Enzo® BioArray (tm) HighYield (tm) RNA Transcript Labeling Kit was used to label the RNA during the *in vitro* transcription (IVT) step (Enzo Diagnostics Inc., Farmingdale, NY, USA). If after IVT the RNA yield was not sufficient for hybridization (>15 μg), additional cells were laser capture microdissected from the same neoplasm and the RNA extraction and two rounds of amplification process were repeated until sufficient RNA was obtained for oligonucleotide microarray analysis.

Oligonucleotide array hybridization and data analysis

A 15 μg of total RNA was used for array hybridization as per the manufacturer's protocol. The fragmented cRNA was hybridized to the Human Genome U133A chips (Affymetrix, Santa Clara, CA, USA) with 18 462 genes/EST transcripts at 45°C for 16 h. The washing and labeling procedures were performed using the Affymetrix Fluidics Station according to the manufacturer's instructions. The arrays were then scanned using a Gene Array Scanner (Agilent, Palo Alto, CA, USA), and signal intensity for each transcript (background-subtracted and adjusted for noise) was determined using Microarray Suite Software 5.0 (Affymetrix).

One-step SYBR Green -based quantitative RT–PCR

To confirm gene expression differences identified by microarray data, 10 genes were selected from the GeneChip list of overexpressed genes. One-step SYBR Green-based RT–PCR amplification was performed using an ABI7700 thermocycler (Applied Biosystems, Foster City, CA, USA). After optimization of each of the primer pairs, samples were assayed in a 50-μl reaction mixture containing 2 μl of sample RNA and optimal concentration of each of the primers by using the QuantiTect SYBR Green RT–PCR kit (Qiagen, Valencia, CA, USA). The primer sequences are shown in Table 2. The thermal profile for one-step SYBR Green-based RT–PCR consisted of a 30-min RT step at 50°C and 15 min of *Taq* polymerase activation at 95°C, followed by 43 cycles of PCR at 95°C for 20 s (denaturation), 58°C for 30 s (annealing) and 72°C for 30 s (extension).

Tissue microarrays

To further confirm the gene expression patterns identified by Affymetrix gene expression profiling, we determined the tissue protein expression patterns overexpressed in genes in an independent series of MCNs. Formalin-fixed paraffin-embedded blocks of a series of MCNs were obtained from the surgical pathology archives of the Department of Pathology at the Johns Hopkins Hospital. The MCN TMAs contained 76 microcores from 19 MCN resection specimens. For the TMA construction, representative areas containing morphologically defined MCNs were circled on the glass slides and used as a template. TMAs were constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD, USA). The diameter of the punched core was 1.4 mm. Serial sections were cut from these TMAs, of which one was labeled with H&E as a reference.

Immunohistochemistry

TMA sections (4 μm) mounted on positively charged slides were incubated for 30 min and deparaffinized by standardized methods. Antigen retrieval was performed for 20 min, in 10 mM sodium citrate buffer (pH 6.0) heated at 95°C in a steamer, followed by cooling off for 20 min. After blocking endogenous peroxidase activity with a 3% aqueous H_2O_2 solution for 5 min, the primary antibodies were incubated with the sections at each final concentration for 60 min in a Dako automatic immunostainer (Dako, Carpinteria, CA, USA). The same 8 μm frozen sections that were used to microdissect the MCNs were stained with the HES1 antibody using similar methods outlined above with the exception that the Hes1 antibody did not require an antigen retrieval step. The primary antibodies used for immunohistochemistry were a rabbit polyclonal antibody against fizzled-related protein (H-300, Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution

1 : 25), Jagged (H-66, Santa Cruz, 1 : 100) and a mouse monoclonal antibody against the L1 cell adhesion molecule (L1CAM, UJ127, Lab Vision, Fremont, CA, USA, 1 : 100). Antibodies against prostate stem cell antigen (PSCA, clone 1G8, 1 : 200) and HES1 (1 : 100) were kindly provided by Dr Robert E Reiter, Department of Urology, University of California and Dr Tetsuo Sudo, Toray Industries Inc., respectively. For every TMA slide labeled, a control slide was incubated, with Tris-buffered saline buffer substituted for the primary antibody. The EnVision + DAB + detection kit (Dako, Carpinteria, CA, USA) was used for the detection of immunolabeling. Sections were counterstained with hematoxylin. Before immunohistochemical evaluation, each MCN core arrayed on the TMA was graded into adenoma, borderline and carcinoma lesions using the H&E-stained slide. Immunolabeling patterns were scored as either positive (positive labeling in >10% of neoplastic cells) or negative. Immunohistochemical labeling was considered positive if at least one of the tissue cores from the case showed positive immunostaining.

Data analysis

The Affymetrix Data Mining Tool software was used to compare the gene expression profiles of signal intensity of each group. Gene expression differences of at least threefold (fold change) between neoplastic epithelium and normal ductal epithelium were considered significant. For the comparison of ovarian-type stroma with the fibrous stroma of chronic pancreatitis, differences of at least fivefold were considered significant. Gene expression analysis using real-time, quantitative PCR data utilized the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

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References

Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl M and Edlund H. (1999). *Nature*, **400,** 877–881.
Compagno J and Oertel JE. (1978). *Am. J. Clin. Pathol.*, **69,** 573–580.
Crnogorac-Jurcevic T, Efthimiou E, Nielsen T, Loader J, Terris B, Stamp G, Baron A, Scarpa A and Lemoine NR. (2002). *Oncogene*, **21,** 4587–4594.
Deichmann M, Kurzen H, Egner U, Altevogt P and Hartschuh W. (2003). *J. Cutan. Pathol.*, **30,** 363–368.
Demos TC, Posniak HV, Harmath C, Olson MC and Aranha G. (2002). *AJR Am. J. Roentgenol.*, **179,** 1375–1388.
Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD and Sklar J. (1991). *Cell*, **66,** 649–661.
Ernst T, Hergenhahn M, Kenzelmann M, Cohen CD, Bonrouhi M, Weninger A, Klaren R, Grone EF, Wiesel M, Gudemann C, Kuster J, Schott W, Staehler G, Kretzler M, Hollstein M and Grone HJ. (2002). *Am. J. Pathol.*, **160,** 2169–2180.
Ewart-Toland A, Briassouli P, de Koning JP, Mao JH, Yuan J, Chan F, MacCarthy-Morrogh L, Ponder BA, Nagase H, Burn J, Ball S, Almeida M, Linardopoulos S and Balmain A. (2003). *Nat. Genet.*, **34,** 403–412.
Fogel M, Gutwein P, Mechtersheimer S, Riedle S, Stoeck A, Smirnov A, Edler L, Ben-Arie A, Huszar M and Altevogt P. (2003a). *Lancet*, **362,** 869–875.
Fogel M, Mechtersheimer S, Huszar M, Smirnov A, Abu-Dahi A, Tilgen W, Reichrath J, Georg T, Altevogt P and Gutwein P. (2003b). *Cancer Lett.*, **189,** 237–247.
Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR and Katzenellenbogen BS. (2003). *Endocrinology*, **144,** 4562–4574 Epub 2003 Jul 4510.
Fukuhara K, Kariya M, Kita M, Shime H, Kanamori T, Kosaka C, Orii A, Fujita J and Fujii S. (2002). *J. Clin. Endocrinol. Metab.*, **87,** 1729–1736.
Fukushima N and Mukai K. (1997). *Pathol. Int.*, **47,** 806–808.
Fukushima N and Mukai K. (1999). *Adv. Anat. Pathol.*, **6,** 65–77.
Hammel P, Levy P, Voitot H, Levy M, Vilgrain V, Zins M, Flejou JF, Molas G, Ruszniewski P and Bernades P. (1995). *Gastroenterology*, **108,** 1230–1235.
Hodgson JG, Chin K, Collins C and Gray JW. (2003). *Breast Cancer Res. Treat.*, **78,** 337–345.
Hruban RH, Iacobuzio-Donahue C, Wilentz RE, Goggins M and Kern SE. (2001). *Cancer J.*, **7,** 251–258.
Iacobuzio-Donahue CA, Ashfaq R, Maitra A, Adsay NV, Shen-Ong GL, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M and Hruban RH. (2003a). *Cancer Res.*, **63,** 8614–8622.
Iacobuzio-Donahue CA, Maitra A, Olsen M, Lowe AW, Van Heek NT, Rosty C, Walter K, Sato N, Parker A, Ashfaq R, Jaffee E, Ryu B, Jones J, Eshleman JR, Yeo CJ, Cameron JL, Kern SE, Hruban RH, Brown PO and Goggins M. (2003b). *Am. J. Pathol.*, **162,** 1151–1162.
Iacobuzio-Donahue CA, Maitra A, Shen-Ong GL, van Heek T, Ashfaq R, Meyer R, Walter K, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M and Hruban RH. (2002). *Am. J. Pathol.*, **160,** 1239–1249.
Izumo A, Yamaguchi K, Eguchi T, Nishiyama K, Yamamoto H, Yonemasu H, Yao T, Tanaka M and Tsuneyoshi M. (2003). *Oncol. Rep.*, **10,** 515–525.
Jabara S, Christenson LK, Wang CY, McAllister JM, Javitt NB, Dunaif A and Strauss III JF. (2003). *J. Clin. Endocrinol. Metab.*, **88,** 484–492.
Jhappan C, Gallahan D, Stahle C, Chu E, Smith GH, Merlino G and Callahan R. (1992). *Genes Dev.*, **6,** 345–355.
Jones SE and Jomary C. (2002). *BioEssays*, **24,** 811–820.
Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H and Dorken B. (2002). *Blood*, **99,** 3398–3403.
Kalus I, Schnegelsberg B, Seidah NG, Kleene R and Schachner M. (2003). *J. Biol. Chem.*, **278,** 10381–10388.
Kamiguchi H, Hlavin ML and Lemmon V. (1998). *Mol. Cell Neurosci.*, **12,** 48–55.
Kern SE, Hruban RH, Hidalgo M and Yeo CJ. (2002). *Cancer Biol. Ther.*, **1,** 607–613.
Klein AP, Hruban RH, Brune KA, Petersen GM and Goggins M. (2001). *Cancer J.*, **7,** 266–273.
Kowitz A, Kadmon G, Eckert M, Schirrmacher V, Schachner M and Altevogt P. (1992). *Eur. J. Immunol.*, **22,** 1199–1205.
Li D, Zhu J, Firozi PF, Abbruzzese JL, Evans DB, Cleary K, Friess H and Sen S. (2003). *Clin. Cancer Res.*, **9,** 991–997.
Livak KJ and Schmittgen TD. (2001). *Methods*, **25,** 402–408.
Logsdon CD, Simeone DM, Binkley C, Arumugam T, Greenson JK, Giordano TJ, Misek DE, Kuick R and Hanash S. (2003). *Cancer Res.*, **63,** 2649–2657.
Luttges J, Feyerabend B, Buchelt T, Pacena M and Kloppel G. (2002). *Am. J. Surg. Pathol.*, **26,** 466–471.

Mahlamaki EH, Barlund M, Tanner M, Gorunova L, Hoglund M, Karhu R and Kallioniemi A. (2002). *Genes Chromosomes Cancer*, **35,** 353–358.
Maillard I and Pear WS. (2003). *Cancer Cell*, **3,** 203–205.
Miao X, Sun T, Wang Y, Zhang X, Tan W and Lin D. (2004). *Cancer Res.*, **64,** 2680–2683.
Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM and Leach SD. (2003). *Cancer Cell*, **3,** 565–576.
Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP and Radtke F. (2003). *Nat. Genet.*, **33,** 416–421.
Ohhashi K, Murakami F and Maruyama M. (1982). *Prog. Dig. Endosc.*, **203,** 348–351.
Rubin D, Warshaw AL, Southern JF, Pins M, Compton CC and Lewandrowski KB. (1994). *Surgery*, **115,** 52–55.
Ryu B, Jones J, Blades NJ, Parmigiani G, Hollingsworth MA, Hruban RH and Kern SE. (2002). *Cancer Res.*, **62,** 819–826.
Ryu B, Jones J, Hollingsworth MA, Hruban RH and Kern SE. (2001). *Cancer Res.*, **61,** 1833–1838.
Sand JA, Hyoty MK, Mattila J, Dagorn JC and Nordback IH. (1996). *Surgery*, **119,** 275–280.
Sato N, Fukushima N, Maitra A, Iacobuzio-Donahue CA, Van Heek NT, Cameron JL, Yeo CJ, Hruban RH and Goggins M. (2004). *Am. J. Pathol.*, **164,** 903–914.
Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, Hruban RH and Goggins M. (2003a). *Cancer Res.*, **63,** 3735–3742.
Sato N, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, Rosty C and Goggins M. (2003b). *Cancer Res.*, **63,** 4158–4166.
Sato N, Rosty C, Jansen M, Fukushima N, Ueki T, Yeo CJ, Cameron JL, Iacobuzio-Donahue CA, Hruban RH and Goggins M. (2001). *Am. J. Pathol.*, **159,** 2017–2022.
Sato N, Ueki T, Fukushima N, Iacobuzio-Donahue CA, Yeo CJ, Cameron JL, Hruban RH and Goggins M. (2002). *Gastroenterology*, **123,** 365–372.
Sausville EA. (2004). *Nat. Med.*, **10,** 234–235.
Schachner M. (1997). *Curr. Opin. Cell Biol.*, **9,** 627–634.
Sessa F, Solcia E, Capella C, Bonato M, Scarpa A, Zamboni G, Pellegata NS, Ranzani GN, Rickaert F and Kloppel G. (1994). *Virchows Arch.*, **425,** 357–367.
Seth P, Porter D, Lahti-Domenici J, Geng Y, Richardson A and Polyak K. (2002). *Cancer Res.*, **62,** 4540–4544.
Solcia E, Capella C and Kloppel G. (1997). *Tumors of the Pancreas*. Armed Forces Institute of Pathology: Washington, DC, pp. 220–223.
Sorianello E, Fritz S, Beyer C, Hales DB, Mayerhofer A, Libertun C and Lux-Lantos V. (2002). *Eur. J. Endocrinol.*, **147,** 387–395.
Sperti C, Pasquali C, Guolo P, Polverosi R, Liessi G and Pedrazzoli S. (1996). *Cancer*, **78,** 237–243.
Sperti C, Pasquali C, Pedrazzoli S, Guolo P and Liessi G. (1997). *Am. J. Gastroenterol.*, **92,** 672–675.
Su GH, Bansal R, Murphy KM, Montgomery E, Yeo CJ, Hruban RH and Kern SE. (2001). *Proc. Natl. Acad. Sci. USA*, **98,** 3254–3257.
Terris B, Blaveri E, Crnogorac-Jurcevic T, Jones M, Missiaglia E, Ruszniewski P, Sauvanet A and Lemoine NR. (2002). *Am. J. Pathol.*, **160,** 1745–1754.
Thompson LD, Becker RC, Przygodzki RM, Adair CF and Heffess CS. (1999). *Am. J. Surg. Pathol.*, **23,** 1–16.
Thor G, Probstmeier R and Schachner M. (1987). *EMBO J.*, **6,** 2581–2586.
Ueki T, Toyota M, Skinner H, Walter KM, Yeo CJ, Issa JP, Hruban RH and Goggins M. (2001). *Cancer Res.*, **61,** 8540–8546.
Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH and Goggins M. (2000). *Cancer Res.*, **60,** 1835–1839.
van der Heijden MS, Yeo CJ, Hruban RH and Kern SE. (2003). *Cancer Res.*, **63,** 2585–2588.
Warshaw AL, Compton CC, Lewandrowski K, Cardenosa G and Mueller PR. (1990). *Ann. Surg.*, **212,** 432–443 discussion 444–445.
Yoshizawa K, Nagai H, Sakurai S, Hironaka M, Morinaga S, Saitoh K and Fukayama M. (2002). *Virchows Arch.*, **441,** 437–443.
Zagouras P, Stifani S, Blaumueller CM, Carcangiu ML and Artavanis-Tsakonas S. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 6414–6418.
Zamboni G, Kloppel G, Hruban RH, Longnecker DS and Adler G. (2000). *World Health Organization Classification of Tumours, Pathology & Genetics, Tumor of the Digestive System* Aaltonen LA (ed). Oxford University Press: UK, pp. 234–236.
Zamboni G, Scarpa A, Bogina G, Iacono C, Bassi C, Talamini G, Sessa F, Capella C, Solcia E, Rickaert F, Mariuzzi GM and Kloppel G. (1999). *Am. J. Surg. Pathol.*, **23,** 410–422.