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Proteomic Analysis of Chronic Pancreatitis and Pancreatic Adenocarcinoma

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Background & Aims: Markers to differentiate among pancreatic adenocarcinoma, chronic pancreatitis, and normal pancreas would be of significant clinical utility. This study was therefore designed to analyze the proteome of such specimens and identify new candidate proteins for differential diagnosis. **Methods:** A PowerBlot analysis with more than 900 well-characterized antibodies was performed with tissue specimens from patients with chronic pancreatitis, pancreatic adenocarcinoma, and normal pancreas. Differential expression of selected proteins was confirmed on a larger scale by quantitative reverse transcription-polymerase chain reaction and immunohistochemistry using tissue arrays. **Results:** A total of 30 and 102 proteins showed significant deregulation between normal pancreas when compared with chronic pancreatitis and pancreatic adenocarcinoma, respectively, and although a substantial proportion were found similarly dysregulated in both chronic pancreatitis and pancreatic adenocarcinoma, several proteins were identified as potential disease-specific markers. **Conclusions:** A large number of proteins are differentially expressed in chronic pancreatitis and pancreatic adenocarcinoma compared with normal pancreas. Among these, expression analysis of UHRF1, ATP7A, and aldehyde oxidase 1 in combination could potentially provide a useful additional diagnostic tool for fine-needle aspirated or cytological specimens obtained during endoscopic investigations.

With its characteristically severe morbidity and almost unavoidable mortality, pancreatic ductal adenocarcinoma (PDAC) is a serious clinical problem in the Western world.[1] In an effort to identify novel diagnostic and therapeutic targets, many investigators have been exploring the molecular basis of this malignancy, and various approaches for the analysis of the transcriptome have been extensively applied.[2–4] However, very few large-scale investigations into the protein species involved in the pathogenesis of this disease have been performed.[5,6] Because changes at RNA level do not always correlate with protein expression,[7,8] studying proteins directly is required for the reliable identification of novel diagnostic and therapeutic targets.

In a previous study, we analyzed microdissected normal pancreatic ducts and PDAC specimens by 2-dimensional gel analysis and mass spectrometry.[9] In this study, we used the complementary approach of large-scale immunoblotting analysis (BD PowerBlot; Becton Dickinson, Franklin Lakes, NJ) that was successfully exploited in several recent studies.[10–18]

Patients with chronic pancreatitis (CP) have an increased incidence of pancreatic cancer,[19,20] and it can be difficult to distinguish the 2 disease states clinically. We therefore also included CP specimens in our study.

Materials and Methods

Tissue Samples

All pancreatic tissue specimens were obtained from the Human Biomaterials Resource Centre, Hammersmith Hospital Trust, London, and the Cancer Tissue Bank Research Centre in Liverpool. Eight pancreatic intraepithelial neoplasia specimens and 4 pancreatic liver metastases were kindly provided by Drs Teresa A. Brentnall (University of Washington, Seattle, WA) and Makoto Sunamura (Tohoku University, Japan), respectively. All specimens were obtained with full ethical approval from the host institutions. Tissue microarrays were obtained from the Cancer Tissue Bank Research Centre, Liverpool.

BD PowerBlot Array Analysis

BD PowerBlot Western array screening (http://www.bdbiosciences.com/pharmingen/products/product_features.php?key_products=26) was used to analyze 3 groups

Abbreviations used in this paper: AOX1, aldehyde oxidase 1; ATP7A/MNK, ATPase, copper transporting, alpha peptide/Menkes disease protein; CP, chronic pancreatitis; NP, normal pancreas; PDAC, pancreatic ductal adenocarcinoma; UHRF1, ubiquitin-like, containing PHD and RING finger domains 1.

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Table 1. Differentially Expressed Proteins Between Chronic Pancreatitis (CP) and Normal Pancreas (NP)

Protein	HUGO name	Average-fold change[a]	Localization and function	Reported in CP
Up-regulated proteins (n = 15)				
Adaptin d	AP3D1	NC	Cytoplasmic/protein and vesicle transporter	No
A-kinase anchor protein 11	Akap11	NC	Cytoplasmic/PKA regulatory unit binding, cell-cycle control	No
Fatty acid synthase	FASN	NC	Mitochondrial/catalyzes the formation of long-chain fatty acids	No
Hypoxia-inducible factor 1	HIF1A	NC	Nuclear/transcription factor inducing expression of oxygen-regulated genes	No
Insulin-like growth factor 2 receptor	IGF2R	NC	Transmembrane protein/transport of phosphorylated lysosomal enzymes from Golgi complex to lysosomes	No
Plakophilin 3	PKP3	NC	Nuclear/associated with desmosomes; β-catenin family	No
Acetylcholinesterase	ACHE	7.06[b]	Plasma membrane/hydrolyzes acetylcholine	Yes (21)
Bruton agammaglobulinemia tyrosine kinase	BTK	6.28[b]	Cytoplasmic or membrane associated/tyrosine kinase activity, induction of apoptosis by extracellular signals	No
A-Raf	ARAF1	4.4[b]	Cytosolic/proto-oncogene: serine/threonine kinase	No
C-jun/JNK (pT183/pY185) Phosphospecific (54 kilodaltons)	MAPK8	4.19[b]	Cytoplasmic/activated by environmental stress and pro-inflammatory cytokines	Yes (2, 22)
TGF-β–inducible early growth response protein 2	TIEG2	3.13[b]	Nuclear/Kruppel-like transcription factor of Spl family, p18; translation regulatory activity	No
Tubulin, α1	TUBA1	3.1[b]	Cytoplasmic/major constituent of microtubules	Yes (2)
Cofilin	CFL1	2.44[c]	Cytoskeletal and nuclear/actin polymerisation; Rho signaling	No
Ceruloplasmin	CP	2.31[b]	Plasma membrane bound/copper-binding glycoprotein	No
Argininosuccinate synthetase	ASS	2.26[b]	Cytoplasmic/synthetase activity	No
Down-regulated proteins (n = 15)				
Major vault protein (LRP)	MVP	−7.56[b]	Cytoplasmic/nuclear/overexpressed in multidrug-resistant cancer	No
Golgi SNARE (15 kilodalton protein)	BETL1	−7.45[b]	Cytoplasmic/blocked early in transport 1 homolog (*S cerevisiae*)-like	No
Superoxide dismutase 2	SOD2	−7.02[b]	Mitochondrial matrix protein/destroys toxic free radicals	No
Doublecortin	DCX	−6.6[b]	Cytoplasmic/neurogenesis, microtubule binding activity	No
Protein kinase C substrate	PRKCSH	−5.38	Intracellular/binds calcium; involved in protein kinase cascade	No
GRIM-19	GRIM19	−5.2[b]	Mitochondrial/NADH dehydrogenase activity, involved in interferon/retinoic acid induced cell death	No
Mitogen-activated protein kinase 1 (42 kilodaltons)	MAPK1/ERK2	−4.98[b]	Cytosolic/serine-threonine kinase activated by insulin and nerve growth factor	No
Cell division cycle 42	CDC42	−3.25[b]	Plasma membrane/small GTPase; regulation of cell morphology	No
Protein kinase, cAMP dependent, type II, beta	PKARIIb/PRKAR2B	−3.13[b]	Nuclear/cAMP-dependent protein kinase type II β regulatory chain	No
Ndr/serine/threonine kinase 38 (51 kilodaltons)	Ndr/STK38	−2.85[b]	Nuclear/serine-threonine kinase; regulation of cell morphogenesis and proliferation	No
Epithelial protein lost in neoplasm (100 kilodaltons)	EPLIN	−2.7[b]	Cytoplasmic/inhibits actin filament depolymerization and cross-links filaments in bundles	No
BH3-interacting domain death agonist	BID	−2.5[b]	Cytoplasmic/proapoptotic protein	No
Protein-tyrosine phosphatase, nonreceptor type 6 (PTPN6)	PTPIC/SHP1	−2.34[b]	Cytoplasmic/protein tyrosine phosphatase activity	No
Receptor-interacting serine threonine kinase 2	RIPK2/RICK	−2.19[b]	Cytoplasmic/receptor-interacting serine-threonine kinase 2, potentiates caspase 8–mediated apoptosis	No
Peroxisomal dodecenoyl-CoA isomerase	PECI	−2.05[b]	Peroxisomal/binds Acyl-CoA	No

NOTE. Numbers in parentheses are the numbers of the cited references.

NC, not calculable (number/0); PKA, protein kinase A; CAMP, cyclic adenosine monophosphate; NADH, reduced nicotinamide adenine dinucleotide; GTPase, guanosine triphosphatase.

[a]Minus sign indicates down-regulation.

[b]$P < .05$.

[c]$P < .001$.

of lysates, each composed of 5 mg of pooled tissues from 5 normal pancreas (NP), CP, or PDAC samples. Clinical specimens in each disease group were selected to be histologically uniform, with a proportion of neoplastic cellularity in cancer from 50% to 80%. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein transfer, the filters were interrogated with around 900 primary antibodies. Alexa 680–conjugated secondary antibodies (Molecular Probes, Paisley, UK) were then applied. The signal was visualized with an Odyssey Infrared Imaging System and analyzed by densitometry by using PDQuest software (Bio-Rad). After normalization, the data for NP were compared with those for CP and PDAC; in addition, CP was compared with PDAC. The data were organized into levels of confidence with scores of 1–10, depending on the expression level and reproducibility and the intensity and the quality of the signal. All experiments were performed in triplicate. A detailed description of the immunoblotting procedure, a full list of antibodies interrogated, and data analysis are provided as supplementary data (http://sci.cancerresearchuk.org/axp/mphh/gastro05/index.html).

Only the most stringent data with scores of 9 and 10 (1.5 higher overexpression or underexpression in all 9 comparisons from good-quality signals that passed visual inspection) were used in our selection of differentially expressed proteins. In addition, the statistical significance of the selected proteins was assessed by using the paired 2-tailed *t* test with a significance level cutoff set at $P < .05$.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted with the TRIzol protocol (Gibco BRL), and complementary DNA was synthesized from 1

Table 2. Differentially Expressed Proteins Between Pancreatic Cancer (PDAC) and Normal Pancreas (NP) and Chronic Pancreatitis (CP)

Protein	HUGO name	Average-fold change, PDAC vs NP	Average-fold change, PDAC vs CP	Localization and function	Previously reported in PDAC
Up-regulated proteins (n = 54)					
Adaptin d	AP3D1	NC	1.08	Cytoplasmic/protein and vesicle transporter	No
Fatty acid synthase	FASN	NC	−3.47[a]	Cytoplasmic/catalyzes the formation of long-chain fatty acids	No
HIF-1α	HIF1A	NC	1.29 NS	Nuclear/induction of oxygen-regulated genes	Yes (21)
Ubiquitin-like, containing PHD and RING finger domains	UHRF1/ICBP90	NC	NC	Nuclear/DNA-binding protein; regulates expression of topoisomerase alpha	No
Menkes disease–associated protein	ATP7A/MNK	NC	NC	Membrane ATPase/transmembrane copper transport	No
Plakophilin 3	PKP3	NC	3.67[a]	Nuclear/associated with desmosomes	No
Thrombospondin 2	THBS2	NC	NC	Membrane glycoprotein/mediates cell–cell and cell–matrix interactions	Yes (6, 21, 36)
Kalinin B1 (Laminin β 3)	LAMB3	43.73[b]	33.85[a]	Extracellular/attachment, cell migration	Yes (2, 6, 29)
L-Caldesmon	CALD1	15.07[a]	13.08[a]	Cytoplasmic/actin and myosin binding	Yes (24)
Nucleolysin TIAR	TIAR/TIAL1	14.8[a]	4.96 NS	Cytoplasmic/RNA-binding protein	No
Septin 5 (Peanut-like protein 1)	PNUTL1/CDCREL1	12.66[b]	NC	Nuclear/cell division and cell-cycle related	No
Annexin II	ANXA2	11.59[a]	9.4[a]	Cytoplasmic/calcium-regulated membrane binding	Yes (25)
Actin	ACTA1	11.4[b]	3.11[a]	Cytoplasmic and membrane-associated/cell motility	Yes (26)
Bruton agammaglobulinemia tyrosine kinase	BTK	9.61[a]	1.5 NS	Cytoplasmic and membrane-associated/B-cell ontogeny	No
Annexin I	ANXA1	8.11[b]	14.76[b]	Plasma membrane/calcium and phospholipid-binding protein	Yes (21, 22, 23)
Guanine nucleotide-binding protein	GAT/GNAT2	8.06[b]	4.49[a]	Extracellular/modulator or transducer in transmembrane signaling systems	No
Thrombin	F2	7.32[b]	5.4 NS	Extracellular/coagulation cascade	Yes (27)
DNA excision repair protein	ERCC1	6.9[b]	3.29[a]	Nuclear/DNA repair	No
Retinoblastoma binding protein 4 (46 kilodaltons)	RBBP4	6.59[a]	3.07[a]	Nuclear/mediates chromatin assembly in DNA replication and repair	No
Caveolin 2	CAV2	6.17[b]	1.79 NS	Membranous/scaffolding protein	Yes (21, 23)
Development and differentiation–enhancing factor 1	ASAP1/DDEF1	5.87[a]	1.98 NS	Cytoplasmic and membrane-associated/development and differentiation enhancer	No
Hemopoietic cell kinase	HCK	5.46[a]	8.25[a]	Membranous/coupling the Fc receptor to the activation of the respiratory burst	No
Ras-related protein 2A	RAP2A	5.45[b]	8.52[b]	Plasma/involved in signal transduction	No
Ceruloplasmin	CP	5.08[a]	1.78 NS	Membranous/copper-binding glycoprotein	Yes (28)
IP3 receptor isoform 3	IP3R-3/ITPR3	4.83[a]	3.96[a]	Integral membrane/receptor for inositol 1,4,5-trisphosphate	Yes (2)
Transforming growth factor β1 induced transcript 1	TGFB1I1	4.77[a]	2.53[a]	Membranous/induced by TGFB1; coactivator for the androgen receptor	No
Integrin α2/VLA-2α	ITGA2	4.62[a]	4.29[a]	Type I membrane protein/receptor for laminin, collagen, fibronectin, E-cadherin	Yes (2, 23)
A-Raf proto-oncogene serine/threonine-protein kinase	ARAF1	3.97[b]	−1.1 NS	Membranous/mitogenic signaling	No
p21-Arc	ARPC3	3.90[a]	4.93[a]	Cytoplasmic/actin polymerization	No
Adaptin a	AP2A1	3.88[a]	5.4[a]	Nuclear/protein sorting in Golgi network and/or endosomes	No
Cysteine and glycine-rich protein 1	CRP1/CSRP1	3.77[a]	3.42[a]	Cytoplasmic/neuronal development	Yes (8)
ZIP kinase	DAPK3	3.66[a]	4.52[a]	Membranous/induction of apoptosis	No
Ubiquitin-conjugating enzyme E2 E1	UbcH6/UBE2E1	3.53[a]	2.28[a]	Cytoplasmic/catalyzes the covalent attachment of ubiquitin to other proteins	No
Glutathione S transferase P	GSTP1	3.49[a]	1.74[a]	Nuclear/conjugation of glutathione	No
Signal transducer and activator of transcription 2	STAT2	3.39[a]	2.82[a]	Nuclear/transcription factor, binds to IFN-stimulated response element	No
RAC-α serine/threonine-protein kinase	Pkba/AKT	3.30[a]	ND	Nuclear/protein kinase	Yes (30, 31)
SFRS protein kinase 2 isoform	SRPK2	3.29[a]	1.77 NS	Nuclear/RNA splicing, transferase activity	No
Lamin A/C	LMNA	3.27[a]	3.3[a]	Nuclear/framework for nuclear envelope	No
SHC transforming protein 3	ShcC/SHC3	3.25[a]	3.36 NS	Cytoplasmic/intracellular signalling	No
a-Actinin	ACTN1	3.03[a]	3.9[a]	Cytoplasmic/actin bundling protein	Yes (11)
Tyrosine-protein kinase	CSK	3.02[a]	2.44 NS	Cytoplasmic/phosphorylates src, lyn, and fyn kinases	No
Rho	ARHA	2.97[a]	2.51[a]	Cytoplasmic/signal transduction	Yes (32)
5-Lipoxygenase	ALOX5	2.96[a]	3.39[a]	Cytoplasmic/electron transport, iron-binding activity, oxido reductase activity	Yes (33)
Fascin	FSCN1	2.94[b]	ND	Cytoplasmic/actin bundling activity	Yes (34)
Guanine nucleotide-binding protein	Gb/GNB2	2.83[a]	1.74[a]	Cytoplasmic/transmembrane signaling	No
Retinol binding protein 2	RBP2	2.74[a]	ND	Membranous/transport of retinol	No
Ras-related protein Rab-27A	RAB27A	2.72[a]	2.27[a]	Membranous/GTP binding activity, signal transduction, protein transport	No
Integrin b1	ITGB1	2.70[a]	2.47[a]	Membranous/cell adhesion	Yes (35)
RNA polymerase I–associated factor 53	PAF53	2.55[b]	3.08[a]	Nuclear/catalyzes transcription reaction	No
Beclin	BECN1	2.48[a]	1.87[a]	Membranous/antiviral host defense	No
Centaurin, β2/ACAP2	CENTB2	2.33[a]	2.33 NS	Membranous/GTPase and DNA binding	No
Nudix (36 kilodalton)	NUDT5	2.28[a]	1.7 NS	Membranous/protein binding activity	No
14-3-3e	YWHAE	2.14[a]	2.27[a]	Cytoplasmic/regulator of cell signaling	Yes (37)
Receptor interacting protein 2	RIPK2/RICK	2.07[a]	4.5[a]	Cytoplasmic/activates procaspase 1 and 8 and NFκB	No
Down-regulated proteins (n = 48)					
Aldehyde oxidase 1	AOX1	NC	NC	Cytoplasmic/produces hydrogen peroxide and catalyzes the formation of superoxide	No
Ribosomal protein L22	L22/RPL22	NC	NC	Cytoplasmic/component of 60S subunit	No

(continued on following page)

Table 2. (continued). Differentially Expressed Proteins Between Pancreatic Cancer (PDAC) and Normal Pancreas (NP) and Chronic Pancreatitis (CP)

Protein	HUGO name	Average-fold change, PDAC vs NP	Average-fold change, PDAC vs CP	Localization and function	Previously reported in PDAC
Mitogen-activated protein kinase kinase 3	MAP2K3/MKK3	NC	NC	Cytoplasmic/MAP kinase kinase	No
Serine/threonine kinase 38	STK38/NDR1	NC	NC	Nuclear/regulation of cell morphogenesis and proliferation	No
Occludin	OCLN	NC	NC	Integral membrane protein/formation and regulation of the tight junctions	Yes (37)
Protein disulfide isomerase	PDI/PDIP	NC	NC	Endoplasmic reticulum lumen/catalyzes the rearrangement of disulfide bonds	No
Solute carrier family 1 member 2	SLC1A2/EAAT2	NC	NC	Membranous/transports as a symport with sodium	No
Eukaryotic translation initiation factor 5A	EIF5A	NC	NC	Cytoplasmic/protein biosynthesis	No
Endothelin 1 receptor	EDNRA	NC	NC	Membranous/mediates endothelin 1 action by association with G-proteins	No
Dopamine- and cAMP-regulated neuronal phosphoprotein	PPP1R1B/DARPP-32	−485.35[b]	−383[b]	Cytoplasmic/inhibitor of protein-phosphatase 1	No
Rho GTPase-activating protein 1	ARHGAP1/CDC42GAP	−30[a]	4.53[a]	Cytoplasmic/GTPase-activating protein for rho, rac, and Cdc42	No
Proteintyrosine phosphatase, nonreceptor type 1	PTP1B/PTPN1	−21.41[a]	−44.9[a]	Cytoplasmic/cell growth control and cell response to interferon stimulation	No
Calcineurin	PPP3CA	−17.73[a]	−33.24[a]	Cytoplasmic/calcium-dependent, calmodulin-stimulated protein phosphatase	No
Eukaryotic translation initiation factor 4E	EIF4E	−13.89[a]	−20.45[a]	Cytoplasmic/initiation of protein synthesis	No
Kinesin-like protein 1	Eg5/KIF11	−13.11[a]	−17.61[a]	Nuclear/centrosome separation and establishing of bipolar mitotic spindle	No
Non–POU domain–containing octamer binding	p45nrb/NONO	−10.45[b]	−4.78[a]	Nuclear/RNA- and DNA-binding protein	No
Syncollin	SYCN	−9.5[a]	−13.6[a]	Membranous/calcium-sensitive regulator of exocytosis in exocrine tissues	No
Phosphatidylinositol 3-kinase, regulatory 1	PI3 kinase/PIK3R1	−7.98[a]	−10.31[a]	Cytoplasmic and nuclear/metabolic actions of insulin	Yes (12)
Cell death–regulatory protein	GRIM19	−7.61[a]	−1.46 NS	Mitochondrial inner membrane/proapoptotic	No
Aldehyde dehydrogenase 1A1	ALDH1A1	−7.35[b]	−6.41[b]	Cytoplasmic/oxidative pathway of alcohol metabolism	No
Protein disulfide isomerase related protein	ERP70	−6.66[a]	−4.58[a]	Cytoplasmic/rearrangement of disulfide bonds in proteins	No
Branched-chain aminotransferase 1	BCAT1	−5.99[a]	−4.29[a]	Cytoplasmic/amino acid transaminase	Yes (39)
Synuclein	SNCA	−5.99[a]	−2.07 NS	Cytoplasmic/neuronal plasticity	No
BH3-interacting domain death agonist	BID	−5.85[a]	−2.47 NS	Cytoplasmic/proapoptotic	Yes (38)
Syntaxin	STX6	−5.67[a]	−9.35[a]	Membranous/intracellular trafficking	No
Cyclin-dependent kinase inhibitor 3	KAP/CDKN3	−5.61[b]	−3.71[a]	Cytoplasmic/cell-cycle regulation	No
80K-H	PRKCSH	−5.27[a]	1.009 NS	Cytoplasmic/protein kinase C substrate	No
BCL2-binding protein	BAD	−4.87[a]	−4.61[a]	Cytoplasmic/membranous/proapoptotic	Yes (38)
Orphan nuclear receptor Nurr	NR4A2	−4.65[a]	ND	Nuclear receptor/coactivator of gene transcription	No
B2 bradykinin receptor (41 kilodaltons)	BDKRB2	−4.61[a]	2.01 NS	Membranous/receptor for bradykinin	No
ERK2 (42 kilodaltons)	MAPK1	−4.18[a]	1.19 NS	Cytoplasmic/mitogen-activated protein kinase	Yes (39)
Hsp40	DNAJA1	−4.13[a]	−4.43[a]	Membranous/cochaperone of hsc70; protein import into mitochondria	No
DNA Topo I	TOP1	−3.57[a]	−2.45[a]	Nuclear/controls the DNA topology	No
FIP-2	OPTN	−3.55[a]	ND	Cytoplasmic/inhibits cytolysis induced by TNF-α	No
Adaptin g	AP1G1	−3.49[a]	ND	Cytoplasmic/subunit of clathrin-associated adaptor protein complex 1	No
PKARIIb (53 kilodaltons)	PRKAR2B	−3.45[a]	−2.1[a]	Membrane/cAMP-dependent kinase	No
EMeg32	GNPI	−3.38[a]	−3.04[a]	Membrane/glucosamine-6-phosphate acetyltransferase	No
Lymphocyte-specific protein tyrosine kinase	LCK	−3.3[a]	−3.88[a]	Cytoplasmic/antigen-induced T-cell activation	No
Damage-specific DNA binding protein 1	DDB1	−3.03[a]	−2.09 NS	Nuclear/repair of UV-damaged DNA	No
ERK1 (43 kilodaltons)	MAPK3	−3.02[a]	ND	Membranous/activation by stress and proinflammatory cytokines	Yes (39)
TOK-1	BCCIP	−2.98[a]	−2.26[a]	Nuclear/interacts with BRCA2 and p21	No
Cysteine and glycine-rich protein 2	CSRP2	−2.73[a]	−1.7 NS	Nuclear/cell proliferation, dedifferentiation	No
RONa	MST1R	−2.67[a]	ND	Membranous/receptor for macrophage-stimulating protein	No
DP103/Gemin3	DDX20	−2.51[a]	ND	Cytoplasmic and nuclear/DEAD box protein, putative RNA helicase	No
Ku80	XRCC5	−2.5[a]	−1.99 NS	Nuclear/DNA double-strand break repair	No
SGT1	SUGT1	−2.39[a]	ND	Nuclear/kinetochore function	No
N-Ethylmaleimide–sensitive factor	NSF/SKD2	−2.16[a]	−2.2[b]	Cytoplasmic/vesicle-mediated transport	No
Jun activation domain–binding protein JAB1	CSN5/COPS5	−2.12[a]	ND	Nuclear/subunit of translation initiation factor 3 (eIF3); binds jun-activation domain	No

NOTE. Numbers in parentheses are the numbers of the cited references.
NC, not calculable (number/0); ND, no data; NS, not significant; cAMP, cyclic adenosine monophosphate; ATPase, adenosine triphosphatase; IFN, interferon; GTP, guanosine triphosphate; GTPase, guanosine triphosphatase; MAP, mitogen-activated protein; TNF, tumor necrosis factor; TGF, tumor growth factor.
[a]$P < .05$.
[b]$P < .001$.

μg of total RNA by using random primers and the MultiScribe Reverse Transcription kit (Applied Biosystems, Warrington, Cheshire, UK). Quantitative reverse transcription-polymerase chain reaction primers used were as follows: ubiquitin-like, containing PHD and RING finger domains (UHRF1; 145–base pair amplicon) sense, 5′-GCCCGTTCCAGTTGTTCCT-3′; UHRF1 antisense, 5′-AACACCTGTGCCCGAAAGG-3′; ATPase, copper transporting, alpha peptide (ATP7A) (128–base pair amplicon) sense, 5′-GATGATGAGCTGTGTGGCTTGA-3′; ATP7A antisense, 5′-GCTGTTTTACTGTTGTCTCCAGTCA-3′. Reac-

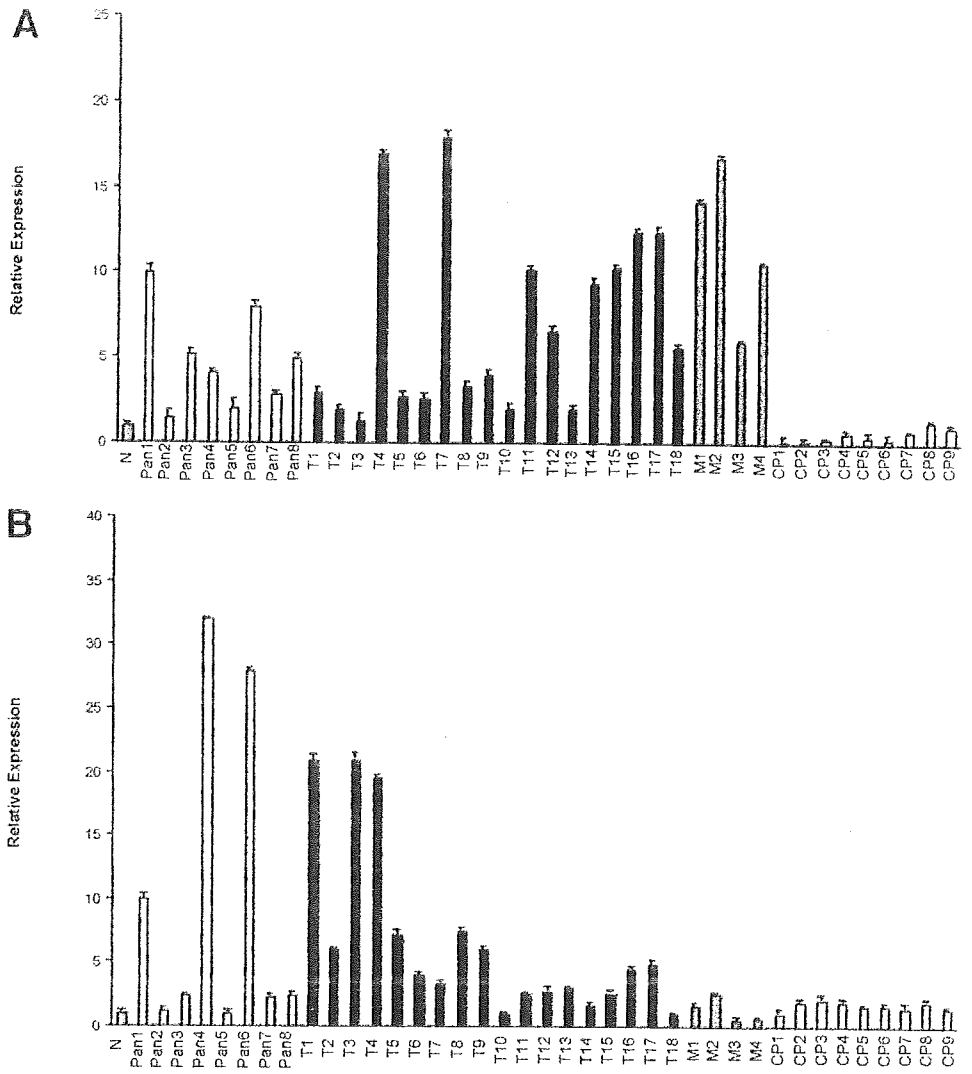

Figure 1. Quantitative reverse transcription-polymerase chain reaction (analysis of UHRF1 (*A*) and ATP7A (*B*). The analyses were performed on 5 pooled normal specimens (N; *gray bar*), 8 pancreatic intraepithelial neoplasia specimens (Pan1–3; *white bars*), 18 primary PDACs (T1–T18; *black bars*), 4 liver metastases (M1–M4; *gray bars*), and 9 cases of CP (CP1–CP9; *white bars*). (*A*) shows that 6 of 8 (75%) pancreatic intraepithelial neoplasia (PanIN) lesions, 14 of 18 (78%) tumor cases, and all 4 metastatic samples showed more than a 2-fold increase of UHRF1 transcript compared with normal pancreas, whereas no up-regulation in CP specimens was noted. For ATP7A, 6 of 8 PanIN (75%) and 11 of 18 (61%) tumor samples showed more than a 2-fold transcript increase, whereas this was not noted in any of the CP and most metastatic specimens (*B*).

tions containing 10 ng of complementary DNA, primers, and SYBR Green sequence-detection reagents (Applied Biosystems) were assayed on an ABI7700 sequence-detection system (Applied Biosystems), and PCR product measured in real time as an increase in SYBR Green fluorescence. Data were analyzed by using the Sequence Detector program v1.9.1 (Applied Biosystems). All the assays were performed in triplicate and normalized with 18S endogenous control. Quantitative gene expression was compared with the average value of all normal samples set arbitrarily at 1.

Immunohistochemical Analysis

The analysis was performed by using a pancreatic cancer–specific tissue microarray with 180 cores, of which 110 were PDAC (55 cases spotted in duplicate). The remaining were normal pancreatic (30 individual cases in duplicate), kidney, and lung cores. Full clinical history (age, sex, tumor size, resection margins, grade, TNM stage, lymph nodes, perineural or vascular invasion, and survival data) was provided. Immunohistochemistry was also performed on CP tissue microarray containing cores representing 24 normal and 24 CP specimens with normal tissue controls as on pancreatic ductal adenocarcinoma tissue microarray.

Immunostaining was performed with anti-UHRF1 and anti–aldehyde oxidase 1 (AOX1) monoclonal antibodies, both diluted 1:200 (all from BD Transduction Laboratory, Cowley, Oxford, UK) by using a Ventana Discovery System (Ventana, Tucson, AZ) according to the protocols provided (http://www.

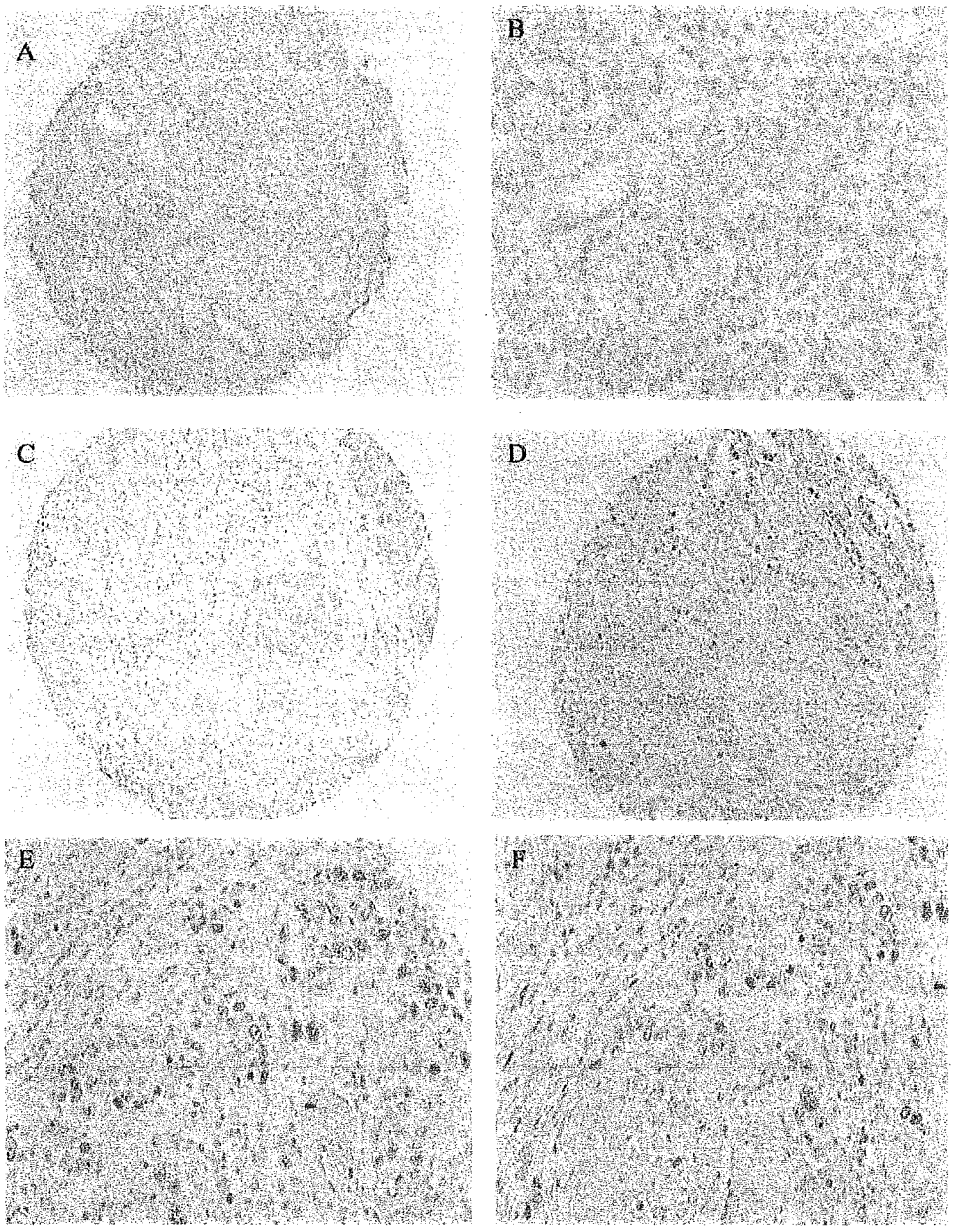

Figure 2. UHRF1 immunohistochemical analysis using pancreatic cancer and chronic pancreatitis tissue microarrays. Out of 34 cores representing individual PDAC cases, 29 (85%) showed nuclear staining in at least 5% of the cells. Among 28 scoreable cores derived from normal pancreatic tissue, only 2 cases (7%) showed positive staining in scattered acinar cells, whereas the remaining 26 (93%) specimens were completely negative. Representative images from normal (*A* and *B*) and chronic pancreatitis (*C*) cases displaying an absence of immunoreactivity from the normal ductal, acinar, and islet cell compartments and 3 pancreatic adenocarcinoma cases (*D–F*) with nuclear UHRF1 expression are shown (original magnification: *A*, *C*, and *D*, 100×; *B*, *E*, and *F*, 200×).

ventanadiscovery.com). UHRF1 was considered positive if immunoreactivity was present in at least 5% of the cell nuclei, and AOX1 was scored as absent, weak, or strong.

Statistical Analysis

To assess the significance of staining patterns among CP, PDAC, and NP and to explore the associations between the immunoreactivity in tumor samples and clinical data, the Fisher exact test and logistic regression were used, respectively. The analysis was performed within the R statistical environment on a Linux platform.

Results

Table 1 shows that 30 proteins were significantly deregulated in comparison between CP and NP, whereas 102 (54

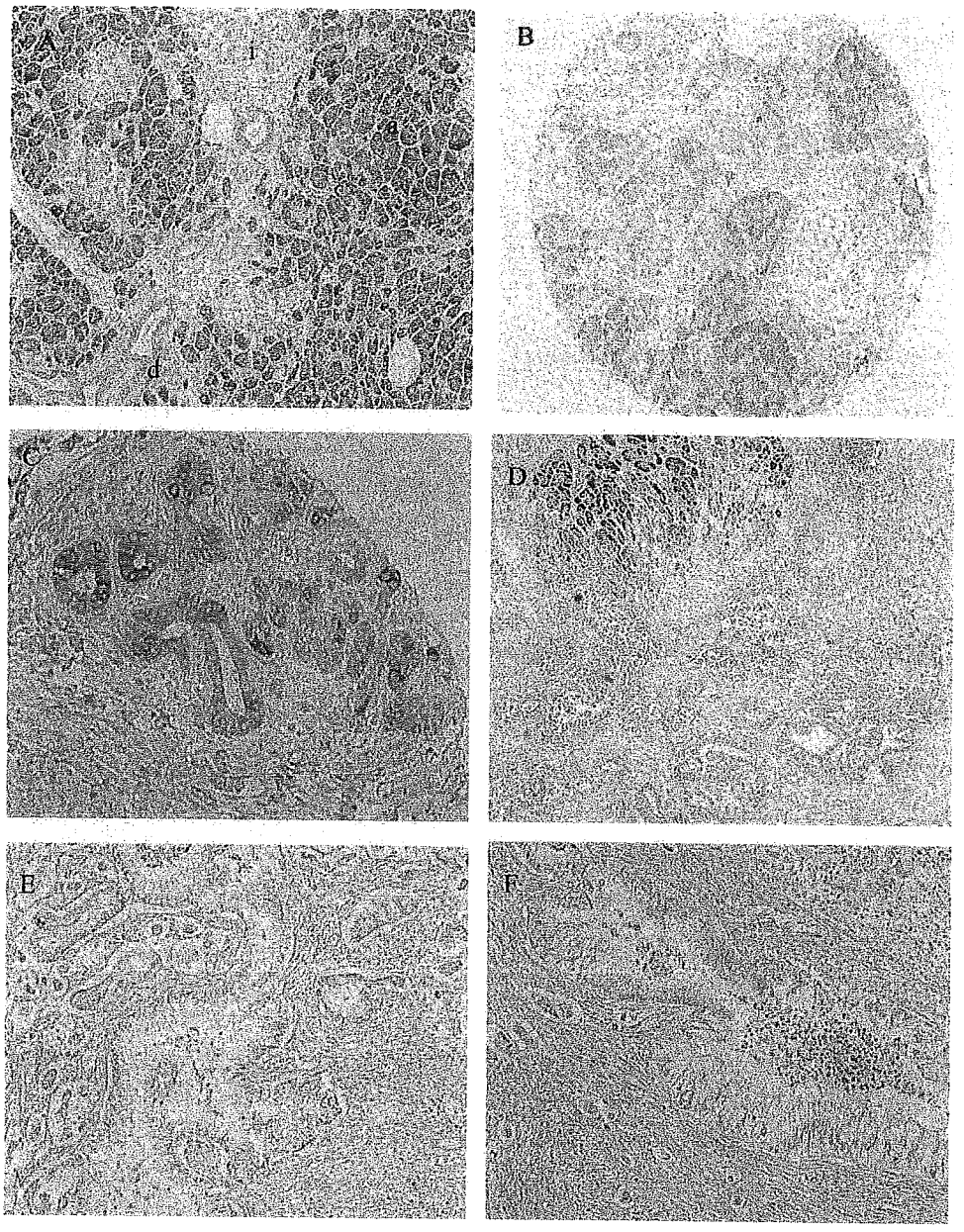

Figure 3. AOX1 immunohistochemical analysis using pancreatic cancer and chronic pancreatitis tissue microarrays. Strong AOX1 expression was seen in approximately 70% of histologically normal pancreatic cores and was present only in the acinar compartment (a) of normal pancreatic tissue and absent in the normal ducts (d) and islets (i) (*A*). Slightly lower intensity of staining was observed in all CP cores (*B*), and it is interesting to note that in all 4 of 24 CP cores that contained acinoductal metaplasia, tubular complexes displayed anti-AOX1 immunoreactivity. This was typically of weak intensity (*C*). Although some immunoreactivity in the remnant acinar elements was noticed in 2 out of 29 scoreable PDAC cores (*D*), the malignant component in all of these cases showed a complete absence of immunoreactivity [representative images are shown in (*E*) and (*F*) (original magnification: *B* and *D*, 100×; *A*, *C*, *E*, and *F*, 200×).

up-regulated and 48 down-regulated) proteins were differentially expressed between PDAC and NP (Table 2). It also shows that only 27 of all deregulated proteins were previously disclosed in earlier studies on PDAC.[2,6,8,12,21–39] Comparison of the tables showed that more than half of the proteins are commonly up-regulated in both CP and PDAC.

Three proteins were selected for more detailed study: UHRF1 and ATP7A, which were overexpressed in PDAC and not present in CP or normal specimens, and AOX1, which was expressed only in NP and CP and not in PDAC. To establish whether UHRF1 and ATP7A protein overexpression was due to transcriptional regulation (AOX1 was

previously shown to be posttranslationally regulated[19], quantitative reverse transcription-polymerase chain reaction was used.

Figure 1 shows increased RNA levels for both UHRF1 (Figure 1*A*) and ATP7A (Figure 1*B*) in a proportion of pancreatic intraepithelial neoplasia samples and in most PDAC specimens, whereas no such up-regulation was seen in CP specimens. The up-regulation of UHRF1 was also maintained in metastatic specimens.

Tissue microarrays were used to confirm the deregulation of UHRF1 and AOX1 proteins. Although we have successfully used anti-ATP7A antibody in both Western blot and immunocytochemical analysis on fresh/frozen tissues (data not shown), we could not obtain reproducible results on formalin-fixed, paraffin-embedded tissues, and these data were therefore excluded.

UHRF1 and AOX1 immunohistochemistry confirmed the immunoblotting data with high statistical significance ($P < .001$). Representative sections for UHRF1 and AOX1 are shown in Figures 2 and 3, respectively. Because of the uniformly high level of immunoreactivity, no significant correlation was found between expression of these proteins and any clinicopathological data.

Discussion

Although multiple gene expression profiling studies of PDAC (several including CP) have been performed previously,[1,2,4–13,40] very few proteomic studies of PDAC tissue specimens have so far been conducted.[13,14] We have now used a large-scale Western blot approach that resulted in identification of several proteins with differential expression among NP, CP, and PDAC, with the additional benefit of immediate availability of antibody reagents.

As shown in Table 2, 76 of 102 (74%) differentially expressed proteins are commonly deregulated between CP and PDAC. These findings are similar to those of Logsdon et al[2] and Binkley et al.[41] Among the commonly overexpressed proteins are actin and associated proteins—namely, α-actinin, L-caldesmon, and p21-Arc—thus suggesting common cytoskeletal modifications that accompany the development of both CP and PDAC. Several common signaling molecules, such as Gat, hemopoetic cell kinase HCK, c-src tyrosine kinase CSK, src homology 2 domain containing transforming protein C3 (SHC3), and pathogen-recognition receptor Gb, as well as several small guanosine triphosphatases (Rho, Rap2A, and Rab27), were also identified. Two proteins involved in apoptosis, Bid and Bad, were found down-regulated in both CP and PDAC specimens, and several oxidative stress–related proteins, including reduced nicotinamide adenine dinucleotide-ubiquinone oxidoreductase B16.6 subunit (GRIM 19) and 5-lipoxygenase (ALOX5), were also commonly deregulated. This significant overlap in protein changes in CP and PDAC is probably due to pathologies seen in both diseases, which include acinar cell degeneration, fibrosis, immune cell infiltration, and ductular hyperplasia. Moreover, both hereditary and sporadic forms of CP show an increased risk of developing PDAC.[20]

Among differentially expressed proteins, 3 were selected for further confirmation by quantitative reverse transcription-polymerase chain reaction and immunohistochemistry on a larger number of clinical specimens: UHRF1 and ATP7A displayed exclusive overexpression in PDAC, whereas AOX1 was present only in NP and CP.

UHRF1 is a transcription factor involved in regulation of topoisomerase IIα during the cell cycle,[42] and higher expression levels of both gene products were recently described in breast cancer.[43] We have found up-regulation of UHRF1 in approximately 80% of PDAC cases at both the RNA and protein levels; moreover, this expression was noted in most pancreatic intraepithelial neoplasia lesions and was retained in all metastatic specimens. Because topoisomerase IIα is known to be up-regulated in PDAC,[44] it is likely that UHRF1 is also involved in topoisomerase IIα regulation in PDACs.

ATP7A is a 160-kilodalton copper-transporting adenosine triphosphatase. Defects in this protein are associated with Menkes disease, an X-linked recessive disorder characterized by growth retardation, neurodegeneration, connective tissue disorders, and death in early childhood.[45] ATP7A is an integral membrane protein that cycles between the trans-Golgi network and relocalizes to the plasma membrane in response to increased copper levels.[46] In plasma, copper is bound to ceruloplasmin.[47] Of note, an increased level of ceruloplasmin was also found in this study (Table 2). Changes in copper and ceruloplasmin levels and tumor growth have already been reported in other tumor types.[48] Because ATP7A overexpression in PDAC seems to be an early event, as suggested by increased transcript levels in most pancreatic intraepithelial neoplasia lesions, the potential role of ATP7A and copper imbalance in PDAC warrants further study.

AOX1 is a member of a small family of molybdoflavoenzymes that require both the transitional metal molybdenum and the flavin cofactor for their catalytic activity.[49] It oxidizes various substrates, from purines and vitamins to several toxicological and pharmacological substances, including acetaldehyde, a toxic metabolic product of ethanol, and several anticancer drugs.[50] AOX1 also oxidizes retinal into retinoic acid,[51] which is a well-known signaling molecule implicated in the mesenchymal/epithelial interactions in pancreatic development, and is also actively involved in reactive oxygen species metabolism.[52] Our finding of high

AOX1 expression in normal acinar cells, decreased AOX1 expression in CP, retained expression in tubule formations in the course of acinoductal metaplasia, and a complete loss of expression in malignant pancreatic cells is intriguing and requires additional studies.

In summary, we have analyzed protein expression in CP and PDAC and showed involvement of multiple deregulated proteins involved in diverse cellular functions. In addition to proteins showing a common pattern of deregulation, a number of other proteins, such as UHRF1, ATP7A, and AOX1, differ in their expression between CP and PDAC, which points to additional genetic events that lead to the development of cancer.

These and other proteins from our data sets present an invaluable source of potential diagnostic markers that now require a more extensive evaluation in the clinical setting (using needle-aspirated and/or cytological material obtained at endoscopy) to prove their usefulness as differential diagnostic tools.

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Review Article

Molecular mechanisms of pancreatic carcinogenesis

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Pancreatic ductal adenocarcinoma is one of the most fatal malignancies. Intensive investigation of molecular pathogenesis might lead to identifying useful molecules for diagnosis and treatment of the disease. Pancreatic ductal adenocarcinoma harbors complicated aberrations of alleles including losses of 1p, 6q, 9p, 12q, 17p, 18q, and 21q, and gains of 8q and 20q. Pancreatic cancer is usually initiated by mutation of *KRAS* and aberrant expression of SHH. Overexpression of *AURKA* mapping on 20q13.2 may significantly enhance overt tumorigenesity. Aberrations of tumor suppressor genes synergistically accelerate progression of the carcinogenic pathway through pancreatic intraepithelial neoplasia (PanIN) to invasive ductal adenocarcinoma. Abrogation of CDKN2A occurs in low-grade/early PanIN, whereas aberrations of TP53 and SMAD4 occur in high-grade/late PanIN. SMAD4 may play suppressive roles in tumorigenesis by inhibition of angiogenesis. Loss of 18q precedes SMAD4 inactivation, and restoration of chromosome 18 in pancreatic cancer cells results in tumor suppressive phenotypes regardless of SMAD4 status, indicating the possible existence of a tumor suppressor gene(s) other than *SMAD4* on 18q. *DUSP6* at 12q21-q22 is frequently abrogated by loss of expression in invasive ductal adenocarcinomas despite fairly preserved expression in PanIN, which suggests that DUSP6 works as a tumor suppressor in pancreatic carcinogenesis. Restoration of chromosome 12 also suppresses growths of pancreatic cancer cells despite the recovery of expression of DUSP6; the existence of yet another tumor suppressor gene on 12q is strongly suggested. Understanding the molecular mechanisms of pancreatic carcinogenesis will likely provide novel clues for preventing, detecting, and ultimately curing this life-threatening disease. (*Cancer Sci* 2006; 97: 1–7)

Pancreatic cancer is the fifth leading cause of cancer death in men, the sixth in women, in Japan and other developed countries.[1] The five-year survival rate for pancreatic cancer is very low, less than 10%,[1] but both the incidence and mortality of pancreatic cancer are increasing.[2] This information indicates that current interventions to prevent, diagnose, and cure the disease are far from satisfactory. We need to develop novel and efficient procedures to medicate patients with this cancer; this need has driven many researchers to intensive investigations of the molecular mechanisms of the development and progression of pancreatic cancer to detect the molecular clues that are valuable for the invention of novel procedures. This review focuses on the elucidation of current knowledge about the molecular insights of pancreatic carcinogenesis.

Genomic analysis of pancreatic cancer

Pancreatic ductal adenocarcinoma (PDA), the most common type of pancreatic cancer, harbors complicated aberrations of chromosomal alleles, that is, losses in multiple chromosome arms, including 1p, 3p, 4q, 6q, 8p, 9p, 12q, 17p, 18q, and 21q, and gains in 8q and 20q.[3] The aberrations are very characteristic for comparing PDA with other types of cancer, most of which reveal aberrations in fewer numbers of chromosomal regions. The regions where losses occur are suggested to harbor tumor suppressor genes (TSGs); those where gains occur, to harbor oncogenes. Detailed analyses of loss of heterozygosity (LOH) using microsatellite markers indicates several particularly lost regions in 1p, 6q, 9p, 12q, 17p, and 18q; three smallest regions of overlap (SROs) in 6q[4] and two in 12q[5,6] were identified. However, no conclusive candidate TSG has been identified. In 1p, several candidate genes such as *TP73*, *RIZ*, *ICAT*, and *RUNX3* were analyzed,[7–10] but alterations in these genes were rather rare in pancreatic cancer. Future efforts will disclose the conclusion of TSGs in these chromosome arms.

Significant concordance of LOHs were found between 6q and 17p and between 12q and 18q, and LOHs of 12q, 17p and 18q were reported to be associated with poor prognosis of patients with PDA.[11] A study using probes to detect aberrations of specific chromosomal regions including 8q24, 9p21, 17p13, 18q21 and 20q11 by fluorescence *in situ* hybridization in cells in pancreatic juice taken from patients undergoing endoscopic retrograde cholangiopancreatography was performed to test the diagnostic relevance of these allelic aberrations.[8] Aberrations of copy numbers were detected in 70% of patients with pancreatic neoplasms, but no aberrations were detected in any of the patients without them. These results showed that these characteristic allelic aberrations can be used as diagnostic markers for pancreatic cancer.[12]

Promoting molecules

The great majority of PDA cases harbor a gain-of-function mutation of *KRAS*.[13] RAS is a GTP-binding protein involved

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Abbreviations: GTP, guanosine triphosphate; HPDE, human pancreatic duct epithelium; LOH, loss of heterozygosity; MAPK, mitogen-activated protein kinase; MMCT, microcell-mediated chromosome transfer; PanIN, pancreatic intraepithelial neoplasia; PDA, pancreatic ductal adenocarcinoma; SRO, smallest region of overlap; TSG, tumor suppressor gene.

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in growth factor-mediated signal transduction pathways.[14] The mutations of *KRAS* are observed at codons 12, 13 and 61, and the overall frequencies are more than 90% in PDAs. The mutations result in the generation of a constitutively active form of RAS. The constitutively active RAS intrinsically binds to GTP and gives uncontrolled stimulatory signals to downstream cascades involving mitogen-activated protein kinases (MAPKs). Mutations of *KRAS* are frequently observed in pancreatic ductal precursor lesions/pancreatic intraepithelial neoplasia (PanIN). The consistent mutations of *KRAS* in PanIN as well as in PDA indicate that the activation of pathways involving RAS is essential for pancreatic carcinogenesis. However, the mutations of *KRAS* do not appear to be sufficient for the development of PDA. Pancreas-specific endogenous expression of active *Kras*, $Kras^{G12D}$, in genetically engineered mice results in the development of PanIN frequently, but the development of PDA very exceptionally.[15] Transfection of the activated *KRAS* in HPDE cells, the immortalized near-diploid ductal cells derived from normal human pancreas, show partially transformed phenotypes.[16] These results suggest that additional genetic and/or epigenetic events, in addition to the activation of *KRAS*, are necessary for the development of PDA.

SHH is frequently overexpressed in PDAs as well as in PanINs.[17,18] Pancreas-specific overexpression of SHH in genetically engineered mice resulted in the development of PanIN.[17] Gene expression profiling of early PanIN indicated the aberrant expression of foregut markers, which was suggested to be a result of activation of the Hedgehog pathway in the lesion.[19] Suppression of the Hedgehog pathway showed suppressive phenotypes of the cultured pancreatic cancer cells.[17] Hedgehog is a family molecule regulating cell fates in embryogenesis in Drosophila as well as in vertebrates. Activation of the Hedgehog signaling pathway by sporadic mutations or in familial conditions such as Gorlin's syndrome is known to be associated with tumorigenesis in skin, the cerebellum, and skeletal muscle.[18] These pieces of information suggest that the activation of the Hedgehog pathway plays a role at the initial step of the development of PanIN, subsequently progressing to PDA.

Gain of copy number of 20q is frequently observed in PDAs, which indicates a possible existence of oncogene(s) in this chromosome arm.[3] Several candidate oncogenes have been isolated, including *AURKA* locating on 20q13.2.[20] *AURKA* encodes AURKA/STK15/Aurora-A kinase, an essential molecule involved in regulating the functions of centrosomes, spindles, and kinetochores, which are required for proper mitosis of cells.[17] AURKA is overexpressed in various cancer tissues, including PDA, which is associated with a higher grade of tumor and a poorer survival of patients with cancer.[17–23] This overexpression can induce checkpoint disruption by interfering with p53 function and tetraploidization, possibly leading to aneuploidy;[24,25] these may be some of the critical causes for a worsened prognosis. Depletion of AURKA by RNA interference in human pancreatic cancer cells resulted in marked growth suppression *in vitro*, abolishment of tumorigenesity *in vivo*, and synergistic enhancement of cytotoxicity of taxanes, chemotherapeutic agents interfering with the functions of the mitotic spindle.[26] These observations indicate that the overexpression of AURKA plays important roles in the progression of PDA.

Aberrations of suppressive pathways

As discussed in the previous section, PDAs have lost multiple allelic regions hemizygously or homozygously. These regions of loss may harbor tumor suppressor genes. Homozygous deletion of 9p21 is frequently observed in PDA. This region harbors *CDKN2A/INK4A/p16*. This gene is inactivated frequently in PDA by deletion or mutation.[27] Even in PDAs harboring wild-type *CDKN2A*, expression of the gene is transcriptionally silenced by hypermethylation of the promoter, which indicates that CDKN2A is inactivated in virtually all PDAs[28] (Fig. 1). Expression of CDKN2A is lost in moderate/low-grade PanINs.[29,30] Loss of *Cdkn2a/Ink4a* in endogenous $Kras^{G12D}$-expressing mice results in the development of a poorly differentiated sarcomatoid locally invasive carcinoma that is an unusual form in human PDA.[31] The CDKN2A is a cyclin-dependent kinase inhibitor. It binds to CDK4 and prevents interaction between CDK4 and CCND1, which induces cell cycle arrest at G1 phase in cooperation with normal RB function.[32] These pieces of information suggest that the loss of CDKN2A occurs early and enhances the oncogenic potential of activating *KRAS* in pancreatic carcinogenesis.

PDAs have frequently lost 17p13.[5] The region harbors *TP53/p53*, the gene frequently mutated in PDAs.[33] The mutations have been observed as missense or nonsense ones; the former type is more common than the latter in human malignant tumors, including PDA.[34] TP53 is a DNA binding protein functioning as a transcription factor modulating molecules pertaining to variety of functions mainly involved in cell cycle arrest and apoptosis.[35] The missense mutations of TP53 are preferentially observed in its DNA binding domain, which abrogates the binding capacity. The missense-mutated TP53 proteins abnormally accumulated in the nucleus by suppressed turnover, which is observed as if the protein were overexpressed immunohistochemically (Fig. 1). Abnormal accumulation of TP53 is frequently observed in high grade/late PanIN lesions as well.[30] Targeted concomitant endogenous expression of $Trp53^{R172H}$ and $Kras^{G12D}$ to the mouse pancreas revealed the cooperative development of invasive and metastatic ductal carcinoma characterized by loss of wild type allele of *Trp53* and diverse chromosomal instability, which recapitulates human PDA.[36] Missense mutated Trp53 can inhibit Trp63 and Trp73 activity and increase its transformation activity.[37] These observations suggest that the aberration of TP53 function under activated *KRAS* in pancreatic ductal cells induces chromosomal instability and additional genetic aberrations that can advance carcinogenic pathways to invasive ductal carcinoma.

Familial pancreatic cancer

Some tumors develop in a hereditary manner; examples include retinoblastoma, familial adenomatous polyposis, breast cancer, neurofibromatosis, and multiple endocrine neoplasia. Such familial syndromes gave valuable clues for the isolation of responsible genes.[38] The isolation of *BRCA2* on chromosome 13 was accelerated by the identification of a homozygous deletion in pancreatic cancer,[39] but the great majority of pancreatic cancers do not harbor mutation in this

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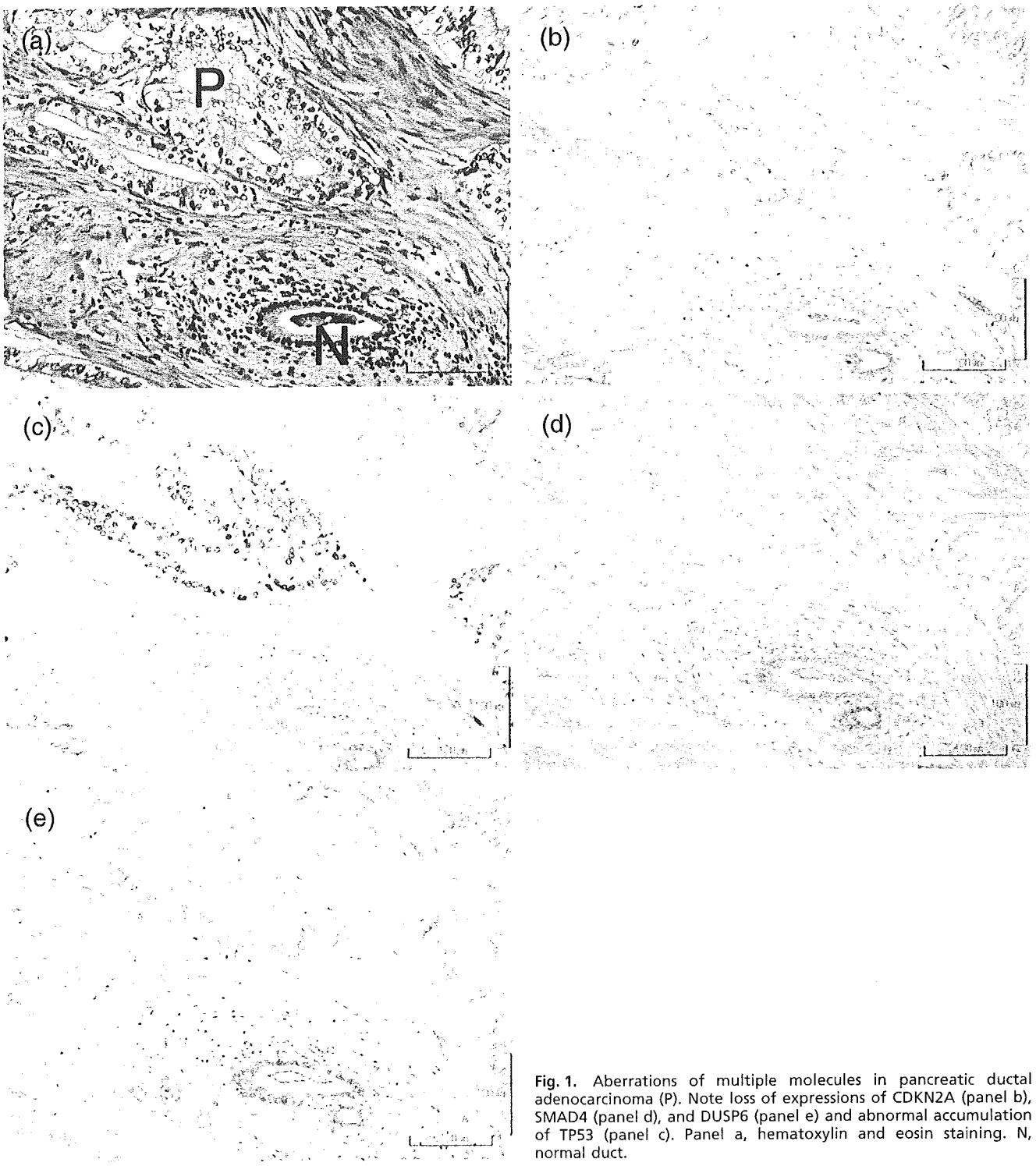

Fig. 1. Aberrations of multiple molecules in pancreatic ductal adenocarcinoma (P). Note loss of expressions of CDKN2A (panel b), SMAD4 (panel d), and DUSP6 (panel e) and abnormal accumulation of TP53 (panel c). Panel a, hematoxylin and eosin staining. N, normal duct.

gene. Several familial pancreatic cancer pedigrees have been reported and positive linkage analysis was detected,[40,41] but isolation of the responsible gene(s) is yet to be accomplished.

Impact of loss of chromosome 18q

Chromosome 18q is frequently deleted hemizygously and/or homozygously in a vast majority of PDAs.[3,5] *SMAD4* was identified in the homozygously deleted region at 18q21.1.[42] SMAD4 is abrogated in approximately 50% of PDAs either by homozygous deletion or mutation.[42,43] Expression of SMAD4 is frequently lost in high-grade/late PanIN lesions as well as in PDA[30,44] (Fig. 1). SMAD4 is a signal mediator involved in the transforming growth factor-β signaling pathway that plays important roles in the negative regulation of cell proliferation, as well as induction of extracellular

matrices, angiogenesis, and immune suppression.[45] SMAD4 comprises a hetero-multimer with SMAD2 and SMAD3, which translocates into the nucleus and functions as a transcription factor cooperating with p300/CBP.[45] Restoration of *SMAD4* in *SMAD4*-deleted pancreatic cancer cells resulted in no alteration of cell growth *in vitro* but the abolition of tumorigenesity in immunodeficient mice due to suppression of angiogenesis, which suggests that SMAD4 functions as a suppressor of tumorigenesis by interfering with interactions between epithelial cells and stromal cells.[46]

Although loss of heterozygosity at chromosome 18q is an overwhelming event in PDAs, occurring in 80–90% of them, the complete SMAD4 inactivation, namely a two-hit mutation, is found in approximately 50%.[42] In intraductal papillary-mucinous neoplasms of the pancreas, one of the precursor types of neoplasms of PDA, loss of 18q is frequently observed despite exclusive preservations of expressions of SMAD4.[12,47] Homozygous deletion telomeric of the *SMAD4* locus is observed in some fractions of PDAs.[48] These observations indicate a possible existence of unknown TSG(s) on 18q. To test this possibility, introduction of an additional copy of chromosome 18 into cultured pancreatic cancer cells with or without SMAD4 inactivation was performed by microcell-mediated chromosome transfer (MMCT).[49] The transferred cells revealed a marked growth retardation, loss of ability for anchorage-independent growth, and modest invasiveness *in vitro*. The *in vivo* tumorigenic ability of the transferred cells was significantly reduced. These results were obtained unanimously throughout the transferred cells despite their different SMAD4 functional status.[49] Moreover, the chromosome 18-transferred cells revealed marked reductions of metastatic ability in experimental *in vivo* models.[50] These observations strongly suggest that a TSG(s), particularly a metastasis-suppressing gene(s), other than *SMAD4*, exists on 18q, and it is involved in pancreatic carcinogenesis.

Tumor suppressor on chromosome 12q

Loss of heterozygosity at chromosome 12q is a frequent aberration in PDAs.[5] Fine mapping of LOH by microsatellite analysis employing markers encompassing the entire long arm of chromosome 12 at every few centi-morgans uncovered two SROs; one at 12q21 and the other at 12q22-q23.1.[6] The mapping of expressed sequence tags in and around these regions to clone candidate tumor suppressor genes resulted in the isolation of *DUSP6/MKP-3* at 12q21-q22.[51] No possible function-affecting mutations were observed, but the *DUSP6* mRNA expressions was strongly suppressed.[51] As shown in Fig. 1, expression of DUSP6 was markedly reduced and/or abolished in PDAs, especially in the poorly differentiated type, despite its fairly good preservation in PanINs.[52] The abrogation of expression of DUSP6 is associated with hypermethylation of a possible control region of the *DUSP6* gene.[53] DUSP6 is a dual specificity phosphatase that specifically binds and dephosphorylates MAPK1, which makes a feedback loop to regulate a physiological activity of MAPK1/ERK2.[54] The cultured pancreatic cancer cells lacking expression of *DUSP6* tend to show constitutively active MAPK1, which suggests

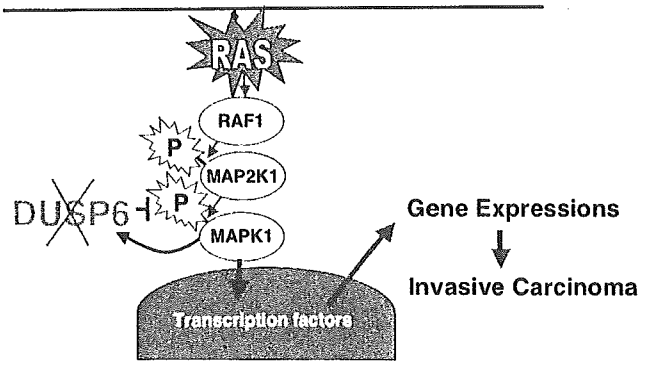

Fig. 2. The RAS-MAPK pathway with abrogation of DUSP6. Active RAS generated by mutated *KRAS* activates downstream cascades including RAF1-MAP2K1-MAPK1. Loss of expression of DUSP6 results in abrogation of the feedback loop between MAPK1 and DUSP6 and leads to constitutive activation of MAPK1, which eventually results in invasive phenotypes.

that loss of function of DUSP6 could induce constitutive activation of MAPK1.[52] Exogenous overexpression of *DUSP6* in *DUSP6*-abrogated pancreatic cancer cells results in growth suppression and the induction of apoptosis.[52] These observations indicate that epigenetic silencing of *DUSP6* is one of the crucial causes of the pathogenesis of PDAs.

How can the abrogation of DUSP6 be interpreted in pancreatic carcinogenesis and progression? As already noted, 80–90% of PDAs harbor the gain-of-function mutation of *KRAS*.[9] *KRAS* encodes RAS, which acts as a molecular switch of downstream signal cascades including RAF1-MAP2K-MAPK1. The mutated *KRAS* generates a constitutively active RAS that hyperstimulates the downstream cascades. In the negative feedback loop manner, the hyperactivated MAPK1 would activate DUSP6, which in turn can suppress the extraordinarily activated MAPK1. However, the abrogation of DUSP6 may result in loss of the feedback loop, which can lead to constitutive activation of MAPK1. The constitutive active MAPK1 may translocate into the nucleus and activate transcription factors that drive numerous effector genes, which could contribute to uncontrolled cell growth and cellular oncogenesis (Fig. 2). From these points of view, DUSP6 functions as a tumor suppressor in the pancreatic carcinogenic pathway that is exclusively surmounted under the activated RAS phenotype.[55] The tumor suppressive activity of DUSP6 is also interpreted by recent reports that include the downregulation of DUSP6 in leukemic cells, involvement in induction of apoptosis by chemotherapy in leukemic cells, suppressive roles in experimental skin carcinogenesis, and involvement in the growth suppression of Jurkat T cells.[56–59] The abrogation of expression of DUSP6 is confined in invasive carcinoma, whereas aberrations of other major suppressive molecules are observed in PanINs.[30] This final finding suggests that DUSP6 functions as a gatekeeper from PanIN to invasive carcinoma, which is independent of other major tumor suppressors (Fig. 3).

Because the precise localization of *DUSP6*, the candidate TSG in 12q, is outside of SRO in this region,[51] MMCT-mediated introduction of chromosome 12 was performed, and it was found that the hybrid cells showed growth suppression

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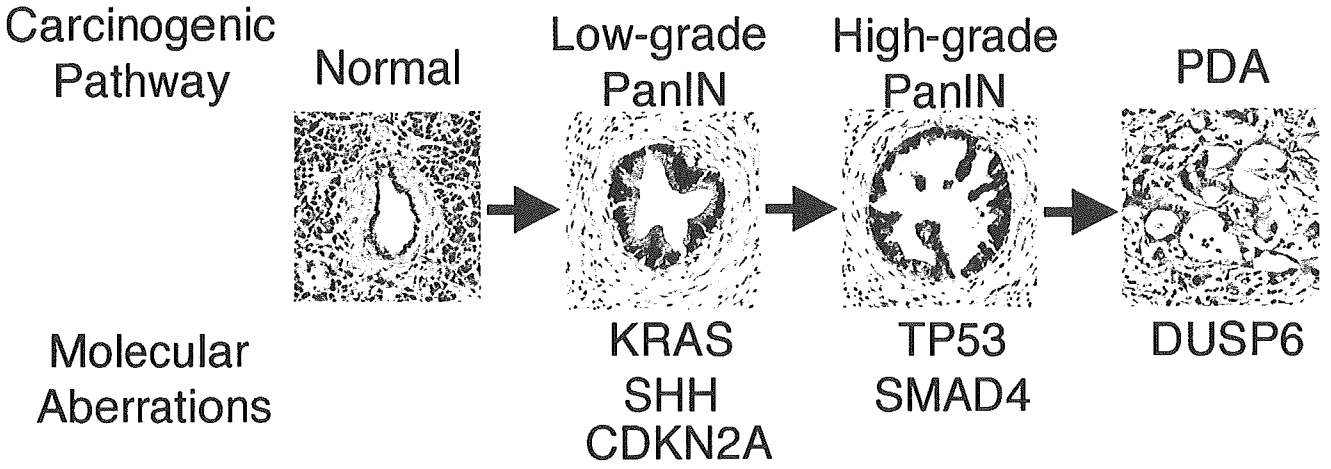

Fig. 3. Molecular pathways of pancreatic carcinogenesis. Activation of KRAS and SHH along with inactivation of CDKN2A contribute to the formation of low-grade pancreatic intraepithelial neoplasia (PanIN). Additional inactivation of TP53 and SMAD4 contributes to one step up the carcinogenesis stairs; the tumors turn into high-grade PanIN. Finally, inactivation of DUSP6 leads to pancreatic ductal adenocarcinoma. PDA, pancreatic ductal adenocarcinoma.

in vivo through angiogenesis inhibition.[60] Microarray analysis revealed that expression of *DUSP6* remained at the suppressed level even in hybrid cells.[60] Therefore, a TSG(s) beside *DUSP6* is hidden in 12q that should be unveiled in future investigations.

Conclusion

PDAs harbor complicated combinations of aberrations of alleles. These aberrations are distinctive in pancreatic ductal adenocarcinoma and are useful as diagnostic markers. The promotion of pancreatic carcinogenesis is obviously initiated by mutation of *KRAS* and aberrant expression of SHH. Overexpression of *AURKA* mapping on 20q13.2 may significantly enhance overt tumorigenesity. Aberrations of tumor suppressor genes synergistically accelerate the progression of carcinogenesis through PanIN to PDA. Abrogation of CDKN2A occurs in low-grade/early PanIN, whereas aberrations of TP53 occur in high-grade/late PanIN; they may play different roles in the progression of carcinogenesis. SMAD4 may play a suppressive role in tumorigenesis by inhibiting angiogenesis. Restoration of chromosome 18 in pancreatic cancer cells results in tumor suppressive phenotypes regardless of SMAD4 status, which suggests the possible existence of a yet-to-be discovered TSG(s) in addition to *SMAD4*. *DUSP6* at 12q21-q22 is frequently abrogated by loss of expression in invasive ductal adenocarcinomas despite fairly preserved expression in PanINs, which suggests that DUSP6 is a tumor suppressor functioning as a gatekeeper of pancreatic carcinogenesis. Restoration of chromosome 12 in pancreatic cancer cells has revealed tumor suppressive phenotypes *in vivo* without recovery of *DUSP6* expression; a buried TSG(s) in 12q is awaiting our discovery. At present, the major pancreatic carcinogenic pathway can be modeled by involving these key molecules (Fig. 3). There is a possibility of innovation for accurate and effective diagnosis using the cells obtained from the pancreatic juice and the molecules in this schema. For this purpose, we must improve this schema by adding more molecules. The understanding of the molecular mechanisms of pancreatic carcinogenesis will likely provide novel clues for preventing, detecting and ultimately curing patients with this life-threatening disease.

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A Novel Cancer Testis Antigen That Is Frequently Expressed in Pancreatic, Lung, and Endometrial Cancers

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Abstract

Purpose: To isolate cancer testis antigens that are expressed in pancreatic cancers and may be useful in clinical applications.

Experimental Design: To efficiently isolate cancer testis antigens, a testis cDNA library was immunoscreened (SEREX) with serum from a patient with pancreatic ductal adenocarcinoma. The expression of isolated antigens in various cancer cell lines and tissues was evaluated by reverse transcription-PCR and Northern blot analyses. The immunogenicity of the antigen in cancer patients was evaluated by detection of the IgG antibody in sera from patients with various cancers.

Results: Of the three clones isolated through screening of a total of 2×10^6 cDNA library clones, one clone (KU-CT-1) was found to be expressed in various cancers but only in testis among normal tissues, indicating that it was a novel cancer testis antigen. The *KU-CT-1* gene is located on chromosome 10p12 and produces two splice variants, which encode proteins of 397 and 872 amino acids, respectively. KU-CT-1 was expressed in pancreatic cancer tissues (3 of 9, 33%), lung cancer tissues (9 of 24, 38%), and endometrial cancer tissues (7 of 11, 64%). Specific serum IgG antibodies were detected in 3 of 20 pancreatic cancer patients, 2 of 12 endometrial cancer patients, 1 of 18 colon cancer patients, and 1 of 10 prostate cancer patients but not detected in 30 healthy individuals.

Conclusions: KU-CT-1 is a new cancer testis antigen that is expressed in pancreatic, lung, and endometrial cancers and may be useful for diagnosis and immunotherapy for patients with various cancers.

Pancreatic cancer is a particularly problematic cancer, because early diagnosis is difficult and the tumor is relatively resistant to surgery, radiotherapy, and chemotherapy. Therefore, new diagnostic and therapeutic methods are needed for this cancer. Immunotherapy may be a potential treatment, because various immunotherapies seem to improve the prognosis of patients with pancreatic cancer (1). For the development and improvement of immunotherapy, identification of human tumor antigens is important for evaluation of antitumor immune responses, mechanisms of tumor escape, and more effective immunization (2). However, only a few pancreatic cancer tumor antigens have been identified, including MUC-1 (3), K-ras (4), HER-2/*neu* (5), and p53 (6).

A variety of human tumor antigens have been identified to date, particularly in melanoma. These include tumor-specific antigens derived from genetic alterations in tumor cells, such as β-catenin and CDK4; tissue-specific proteins, such as PSA, gp100, and proteinase 3; cancer testis antigens, such as MAGE and NY-ESO-1; and antigens that are overexpressed in tumor cells, such as WT1, HER-2/*neu*, survivin, and hTERT. In pancreatic cancers, recognition of mutated K-ras, HER-2/*neu*, p53, and MUC-1 by either $CD8^+$ or $CD4^+$ CTLs has been reported (1, 5, 7).

Cancer testis antigens that are expressed in various cancers and normal germ line tissues, such as testis, placenta, and ovary, are a promising class of tumor antigens due to their limited expression in germ line tissues. They are often expressed in MHC class I–negative cells, such as spermatogonia and spermatocytes (8). However, most cancer testis antigens identified to date are expressed relatively rarely in pancreatic cancer (9–14), although SSX-4, SCP-1, and GAGE are reported

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to be expressed relatively frequently in pancreatic cancer tissues. Cancer testis antigens have previously been isolated by various methods, including cDNA expression cloning with tumor-reactive CTLs, patients' sera (SEREX), or cDNA subtractions (15). Among these methods, SEREX seems to be effective for the isolation of cancer testis antigens, and various cancer testis antigens, including MAGEs, SSX2, SCP-1, and CAGE, have been isolated either using cDNA libraries made from tumor cells or testis tissues.

In this study, we attempted to isolate additional cancer testis antigens, which are expressed in pancreatic cancers and are immunogenic in patients with pancreatic cancer, using the SEREX method with a testis cDNA library and serum from a patient with pancreatic ductal adenocarcinoma. One of the isolated novel cancer testis antigens (KU-CT-1) was found to express only in the testis among normal tissues but was expressed frequently in various cancer tissues and, particularly, in pancreatic, lung and endometrial cancers. Furthermore, the IgG antibody specific for this antigen was detected in sera from patients with various cancers, including pancreatic cancer, but not in sera from healthy individuals. Thus, KU-CT-1 may be useful diagnostically and in immunotherapy for patients with various cancers.

Materials and Methods

Cell lines and tissues. The pancreatic ductal adenocancer cell lines MIAPaCa2, ASPC1 (from the American Type Culture Collection, Manassas, VA), Panc-1, PK1, PK8, PK9, PK45p, and PK59 (Tohoku University, Sendai, Japan); the colon cancer cell lines Colo201, Colo320, SW837, RCM-1, WiDr, DLD-1, and LOVO (JCRB, Osaka, Japan); the stomach cancer cell lines MKN1, MKN7, MKN28, MKN46, and MKN74 (JCRB); the esophageal cancer cell lines TE1, TE2, TE3, TE4, TE5, TE6, TE7, TE8, TE9, TE10, TE11, TE12, TE13, TE14, and TE15 (Tohoku University); the endometrial cancer cell lines Ishikawa, SNGH, EM-tn, EM-kg, SKG3b SNGW (Keio University, Tokyo, Japan), and Hec1b (Kitazato University, Tokyo, Japan); the bladder cancer cell lines BC47, KU1, KU7, T24, and Vmcub1 (Keio University); the renal cell cancer cell lines Saito, RCC6, RCC7, RCC8, RCC9 (Surgery Branch, National Cancer Institute, Bethesda, MD), Caki, ACHN, 769P, 786O, and A498 (American Type Culture Collection); the prostate cancer cell lines JAC1, PC3, DU145, and LNCap; the breast cancer cell lines HS578 and MDA231 (Keio University); the lung cancer cell lines: LU99 (large cell cancer), LK2, EBC1, and LC-1sq (squamous cell cancer), and SBC2, RERF-LC-MA, RERF-LC-OK, RERF-LC-MS, VMRC-LCD, VMRC-LCP, ABC-1, LK-2, and A549/JCRB (adenocancer); the melanoma cell lines SKmel23, 888mel, A375mel, 1363mel, 928mel, 624mel, 586mel, 526mel, 624Amel, 397mel, and 1362mel (Surgery Branch, National Cancer Institute); the chronic myeloid leukemia cell line K562; the acute myeloid leukemia cell lines Kasumi-1 and HL60; the Burkitt's lymphoma cell line Daudi; and the T-cell lymphoma cell line, Molt4(JCRB) were maintained in our laboratory. Cultured fibroblasts and activated T cells were generated from patients with informed consent at Keio University and cultured in RPMI with 10% fetal bovine serum. Melanocytes were kindly provided by Dr. Honjou (Morinaga Institute of Biological Science, Yokohama, Japan). Total RNA from normal tissues, including brain, heart, lung, stomach, small intestine, colon, pancreas, liver, kidney, bladder, prostate, testis, ovary, uterus, placenta, spleen, thymus, bone marrow, skeletal muscle, adipose tissue, retina, mammary gland, salivary gland, fetal brain, fetal liver, and fetal thymus, were purchased from Clontech (Palo Alto, CA). Total RNA from esophagus was purchased from Biochain Institute, Inc. (Hayward, CA). The pancreatic ductal adenocancer, malignant melanoma, endometrial cancer, stomach cancer, colon cancer, renal cell cancer, and lung cancer samples used in this study were surgically resected at Tokoku University hospital, Keio University Hospital, or the National Cancer Center, after informed consent was obtained according to the institution guidelines. These specimens were stored at −80°C until use.

cDNA expression cloning with patient serum antibody (SEREX). Normal testis tissue was obtained from a 76-year-old man who was clinically diagnosed with advanced prostate carcinoma (clinical stage D2) and had undergone bilateral castration at Keio University Hospital in 1998. Total RNA was extracted from human normal testis tissue using a guanidine isothiocyanate/CsCl gradient. Polyadenylate + RNA was then purified twice with latex beads (Oligotex-dT 30 super mRNA Purification kit; Takara Shuzo, Kyoto, Japan), and 5 μg of polyadenylate + RNA was used for construction of a cDNA library. First-strand synthesis was done using an oligo-dT primer with an internal *Xho*I cleavage site and 5-methyl-CTP. The cDNA was ligated to *Eco*RI adapters and was digested with *Xho*I. These cDNA fragments were directly inserted into a *λZAP II* (Stratagene, La Jolla, CA), bacteriophage expression vector, and packaged into phage particles, which were transformed in *Escherichia coli*. The primary size of the library was 1.2×10^7 plaque-forming units.

Immunoscreening with serum IgG antibody was done as previously described (16), using serum from a patient with pancreatic ductal adenocarcinoma (stage IVb) who was treated at Tokoku University Hospital in 1998. Briefly, 1 mL of serum from the patient was diluted 1:15 with 5% skimmed milk and 0.1% sodium azide. This mixture was reacted with lysates of *E. coli* overnight and then diluted 1:200 in 5% skimmed milk in TBS. The testis cDNA library was expressed in XL-1 blue MRF' strain with a titer of 1.5×10^4 per dish, and a total of 2.0×10^6 clones were screened. After DNA sequencing of the isolated clones, the clones were analyzed by comparison with genetic databases at the National Center for Biotechnology Information.

Reverse transcription-PCR and Northern blot analysis. Total RNAs were extracted from various cell lines and from tumor specimens by the guanidine isothiocyanate gradient method. Total RNAs derived from normal tissues were purchased from CLONTEC Lab, Inc. (Palo Alto, CA). cDNAs were synthesized from 5 μg of total RNA by reverse transcriptase (Moloney murine leukemia virus Reverse Transcriptase *RNase H*(−), Promega Corp., Madison, WI), and 30-cycle PCR was done at an appropriate annealing temperature for each primer set, with cDNA, an Ex Taq kit (Takara Shuzo), and the following primers: TW1, sense 5'-GTAAGGGAGAACCAAGTAGGGA-3', antisense 5'-CCAGCTCTT-CACATTCATCGAC-3'; TW2, sense 5'-ACTGATGATTCTCCCAAGCCC-3', antisense 5'-GGAAACACGGTAAGGCAGAAG-3'; TW3, sense 5'-GATT-CAGCAGACGGGGGG-3', antisense 5'-CTGCTGCTTCCCGTACCT-3'.

Northern blot hybridization was done using total RNAs from cancer cell lines, normal tissues, and cultured normal cells: 10 μg per lane of total RNA was fractionated by electrophoresis in a 1.0% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-XL, Amersham Biosciences Corp., Piscataway, NJ). ^{32}P-labeled gene-specific cDNA probes were prepared using a High Prime DNA Labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). Prehybridization was done at 68°C for 1 hour, and hybridization with the radioisotope-labeled cDNA probes was done at 68°C for 2 hours. QuickHyb Hybridization Solution (Stratagene) was used for this process. The nylon membranes were washed twice for 5 minutes at room temperature with 2 × SSC in 0.1% SDS and then washed twice for 30 minutes at 60°C with 0.1 × SSC in 0.1% SDS. Detection of the radioisotope was done using a BAS-2500 or BAS-5000 analyzer (Fujifilm, Tokyo, Japan).

Detection of antigen-specific IgG antibodies in sera from patients with various cancers and from healthy individuals. Screening of IgG antibodies specific for each antigen in sera from patients with various cancers and pancreatitis and from healthy individuals was done by an immunoscreening method similar to that used for the isolation of antigens. Informed consent was obtained from all the subjects. All sera were diluted 1:100, and each phage containing an antigen clone was mixed 2:1 with negative control phages that did not contain a cDNA insert, to clarify the positive signals.

Immunoprecipitation with a protein produced by **in vitro** *transcription/translation.* *In vitro* transcription/translation was done using a Single Tube Protein System 3, T7 (Takara Shuzo). Briefly, isolated KU-CT-1L cDNA clone was amplified by 30 cycles of PCR using a Pyrobest kit (Takara Shuzo) with sense primer 5′-GATGGGTAAAAAGATAAAGAA-GG-3′ and antisense primer 5′-TCACTGCATGGCCATGTTTGTTAA-3′. Incubation with End Conversion mix was done at 22°C for 5 minutes. Four units of T4 DNA ligase, pT7 Blue-2 Blunt Vector, and the reaction products were then mixed and incubated at 22°C for 1 hour. The cDNA template was amplified by 30 cycles of PCR using an Ex Taq kit (Takara Shuzo) with the 20-mer (5′-CAGCTATGACCATGATTACG-3′) and the KU-CT-1L-specific antisense primer. Amplified cDNA template was used for *in vitro* transcription and translation reactions with ^{35}S-labeled methionine. Translated KU-CT-1L proteins were separated using 10% SDS-PAGE, and the radioactivity was detected using a BAS-2500 or BAS-5000 analyzer (Fujifilm).

Immunoprecipitation was done with *in vitro* translated ^{35}S-labeled KU-CT-1L protein: 10 μL of sera were mixed with 2 mg of protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ) in TBS buffer [10 mmol/L Tris-HCl (pH 7.4), 140 mmol/L NaCl], incubated overnight at 4°C, and then washed in immunoprecipitation buffer [10 mmol/L Tris-HCl (pH 8), 500 mmol/L NaCl, 0.1% NP40] thrice. The pellet was resuspended in immunoprecipitation buffer and incubated with 5 μL of *in vitro* translated protein at 4°C for 2 hours. The resin was washed with immunoprecipitation buffer five times. The protein absorbed by the resin was dissolved in SDS gel-loading buffer and then incubated at 100°C for 5 minutes. The labeled KU-CT-1L protein was detected using a BAS-2500 or BAS-5000 analyzer (Fujifilm) after separation using 10% SDS-PAGE.

Results

Isolation of a novel cancer testis antigen by SEREX using serum from a patient with pancreatic ductal adenocarcinoma. To isolate cancer testis antigens, which are immunogenic in patients with pancreatic cancer, a total of 2.0×10^6 clones from a normal testis cDNA library were immunoscreened by the SEREX method, using serum from a patient with pancreatic ductal adenocarcinoma. Three cDNA clones were isolated (Table 1). TW1 (KIAA1107) and TW2 [a zinc finger DAZ-interacting protein 1 (DZIP1), KIAA0996] were found to be expressed in various normal tissues, based on serial analysis of gene expression databases and reverse transcription-PCR analysis. However, reverse transcription-PCR analysis indicated that TW3 (located on chromosome 10p12 and giving hypothetical protein FLJ32827; see below) was expressed only in testis tissue among 27 normal tissues and cultured noncancer cells, including fibroblasts, melanocytes, and activated T cells (a representative result is shown in Fig. 1A). However, TW3 was expressed in PK1 pancreatic cancer, MKN1 stomach cancer, TE4 esophageal cancer, Ishikawa endometrial cancer, RCC6 renal cell cancer, K1S lung adenocancer, and 526mel melanoma among the cancer cell lines tested and was expressed in pancreatic, endometrial, and lung cancers among the cancer tissues. Among the cell lines, TW3 was expressed in 2 of 8 pancreatic ductal adenocancer cell lines (Panc-1 and PK1); 2 of 5 stomach cancer cell lines (MKN1 and MKN46); 4 of 15 esophagus cancer cell lines (TE2, TE4, TE11, and TE12); 4 of 7 endometrial cancer cell lines (Ishikawa, EM-tn, Hec1b, and SNGW); 6 of 10 renal cancer cell lines (RCC6, RCC7, RCC8, Saito, ACHN, and A498); 1 of 4 prostate cancer cell lines (DU145); 7 of 12 lung cancer cell lines (adenocarcinoma: RERF-LC-MS, VMRC-LCD, VMRC-LCP, and A549; squamous cell carcinoma: EBC1, LK-2, and LC-1sq); 1 of 5 leukemia and lymphoma cell lines (K562); and 1 of 11 melanoma cell lines (526mel) but was not expressed in colon, bladder, and breast cancer cell lines. Among the cancer tissues tested, TW3 was expressed in 3 of 9 pancreatic ductal adenocarcinoma tissues (33%), 7 of 11 endometrial cancer tissues (64%), 1 of 8 renal cell cancer tissues (13%), and 9 of 24 lung cancer tissues (38%; Table 2). Northern blot analysis showed a main band of ~3.0 kb and a faint band of 1.5 kb in testis and cancer tissues (PA1 pancreatic cancer and EM2 endometrial cancer; Fig. 1B and C). These results indicate that TW3 is a novel cancer testis antigen that is frequently expressed in pancreatic cancers, endometrial cancers, and lung cancers. TW3 was subsequently renamed as KU-CT-1.

Isolation and characterization of full-length KU-CT-1 cDNA. The sequence of the isolated 1,452-base TW3 clone containing a stop codon and an additional polyadenylate signal in the 3′ region completely matched the sequence of the 1,223 bp at the 5′ end of a 2,831-bp hypothetical protein, FLJ32827. Comparison with genome databases showed that TW3 and FLJ32827 are splicing variants from a gene on chromosome 10p12 (Fig. 2). They seem to correspond to the 3.0-kb main band and 1.5-kb minor band detected by the Northern blot analysis, because KU-CT-1 encodes a protein of 397 amino acids and FLJ32827 contains 872 amino acids; these proteins were named KU-CT-1S (TW3) and KU-CT-1L (FLJ32827). The *KU-CT-1* gene contains 20 exons over about 110 kb on chromosome 10p12, whereas KU-CT-1S includes of the first 11 exons of KU-CT-1, and KU-CT-1L has 19 exons (lacking exon 11 of KU-CT-1).

The first exon contained a sequence corresponding to the Kozak rule AGGATGG sequence, and the last exon (exon 11 in KU-CT-1S and exon 20 in KU-CT-1L) contained a stop codon and an additional polyadenylate signal, AATAAA. KU-CT-1 does not have a leader sequence or a putative transmembrane domain, indicating that it is an intracellular protein. PROSITE analysis showed that the common region of KU-CT-1 has four Armadillo/β-catenin-like repeats, five N-glycosylation sites, a cyclic AMP–dependent and cyclic guanosine 3′,5′-monophosphate (GMP)–dependent protein kinase phosphorylation site, two protein kinase C phosphorylation sites, nine casein kinase

Table 1. Antigens isolated by SEREX using a testis cDNA library and serum from a pancreatic cancer patient

Clone ID	Length (kb)	Identity	Accession number	Chromosomal location
TW1	2.2	KIAA1107 protein	XM034086	Chromosome 1
TW2	2.2	Zinc-finger protein DZIP1	XM014934	Chromosome 13
TW3	1.5	Hypothetical protein FLJ32827	NM173081	Chromosome 10