MICROARRAYS AND OTHER NEW TECHNOLOGIES

Differential and Epigenetic Gene Expression Profiling Identifies Frequent Disruption of the *RELN* Pathway in Pancreatic Cancers

NORIHIRO SATO,* NORIYOSHI FUKUSHIMA,* RUBENS CHANG,* HIROYUKI MATSUBAYASHI,* and MICHAEL GOGGINS*,†,§

*Departments of Pathology, †Oncology, and §Medicine, The Johns Hopkins Medical Institutions, Baltimore, Maryland

Background & Aims: **Recently described genome-wide approaches robustly detect many candidate genes that are regulated by DNA methylation, but many of these genes do not represent important targets for functional inactivation. Here we used a microarray-based strategy to identify biologically relevant genes associated with epigenetic silencing in pancreatic cancer.** ***Methods:*** **We compared information from differential gene expression analysis with the transcriptional responses to epigenetic modifiers.** ***Results:*** **Using this approach, we identified 7 novel targets for aberrant methylation in pancreatic cancer. One of the genes identified, *RELN* (Reelin), a key regulator of neuronal migration, is frequently silenced in pancreatic cancers, as are several of its downstream mediators. Importantly, small interfering RNA–mediated knockdown of *RELN* in pancreatic cancer cells that retain *RELN* expression resulted in greatly enhanced cell motility, invasiveness, and colony-forming ability. Increased cell motility was also induced by knockdown of downstream components of the *RELN* pathway, including *ApoER2*, *VLDLR*, and *DAB1*. Treatment of pancreatic cancer cells with histone deacetylase inhibitors, valproic acid and suberoylanilide hydroxamic acid, restored the expression of *RELN* and *DAB1* and markedly inhibited their migration.** ***Conclusions:*** **The high prevalence of the silencing of RELN pathway components and its reversal by histone deacetylase inhibitors suggest the importance of this pathway as a diagnostic and therapeutic target for pancreatic cancer.**

Substantial evidence suggests that, in addition to mutational inactivation of tumor suppressor genes, epigenetic gene silencing plays an important role in the development and progression of cancer in humans.[1,2] The discovery of aberrantly methylated genes can form the basis for developing diagnostic markers of cancer or targeted therapeutics.[3–5] This is particularly true for pancreatic cancer because it undergoes frequent alterations in DNA methylation and existing modalities are largely ineffective.[6–10] Microarray-based strategies have been used to screen for genes that are up-regulated by epigenetic modification.[8,11–13] These genome-wide approaches robustly detect many candidate genes that are regulated by DNA methylation[10,12–14]; however, many of these genes do not represent important targets for functional inactivation. In fact, some genes that are identified using this approach in pancreatic cancer cell lines, such as *14-3-3sigma*, are overexpressed and hypomethylated in most primary pancreatic cancers.[15]

In an attempt to identify critical epigenetic targets with biologic significance in pancreatic cancer, we developed a novel microarray-based strategy that combines differential gene expression and epigenetic gene expression profiling. This approach is based on the observation that many genes that are epigenetically silenced and can be reactivated in cancer cell lines by epigenetic-modifying drugs are also underexpressed relative to normal ductal epithelium. This approach was useful for identifying the candidate genes that are most likely targeted for epigenetic silencing in pancreatic cancer. One of the most interesting candidate genes identified by this approach, *RELN* (Reelin), a critical regulator of neuronal migration during brain development, was selected for further investigation for its expression, regulation, and functional relevance in pancreatic cancer.

Abbreviations used in this paper: ApoER2, apolipoprotein E receptor 2; COBRA, combined bisulfite restriction analysis; 5Aza-dC, 5-aza-2′-deoxycytidine; HDAC, histone deacetylase; IPMN, intraductal papillary mucinous neoplasm of the pancreas; MSP, methylation-specific polymerase chain reaction; PKB, protein kinase B; RT-PCR, reverse-transcription polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid; siRNA, small interfering RNA; TSA, trichostatin A; VLDLR, very-low-density lipoprotein receptor; VPA, valproic acid.

0016-5085/06/$32.00
doi:10.1053/j.gastro.2005.11.008

Materials and Methods

Cell Lines and Drug Treatment

Nineteen human pancreatic cancer cell lines were used for the present study. A nonneoplastic immortalized cell line (HPDE) was established from normal human pancreatic ductal epithelium.[16] Primary pancreatic fibroblasts (pancf1, f3, f5) were isolated from chronic pancreatitis or pancreatic cancer tissues as previously described.[9] Cells were treated with 5-aza-2′-deoxycytidine (5Aza-dC; Sigma Chemical Co, St Louis, MO) at 1 μmol/L for 4 days or trichostatin A (TSA; Sigma Chemical Co) at 1 μmol/L for 24 hours. For combined treatment, cells were cultured in the presence of 5Aza-dC (1 μmol/L) for 3 days and were then treated for another 24 hours with TSA (0.5 μmol/L). We also treated Panc1 and AsPC1 with valproic acid (VPA, Sigma Chemical Co) and suberoylanilide hydroxamic acid (SAHA; BioVision, Mountain View, CA) at various concentrations for 24 or 48 hours.

Tissue Samples

For microarray analysis, intraductal papillary mucinous neoplasms of the pancreas (IPMNs) and normal pancreatic ducts were selectively microdissected from frozen tissue sections either manually or by laser capture microdissection (Pixcell II LCM system; Arcturus Engineering Inc, Mountain View, CA) as described previously.[17,18] Several cases of infiltrating pancreatic adenocarcinomas and nonneoplastic pancreata were also dissected manually from frozen tissue sections for DNA and RNA extraction. Pancreatic cancer xenografts were established in nude mice from surgically resected primary pancreatic carcinomas, and 19 xenografts were used in this study. A series of 300 consecutive formalin-fixed, paraffin-embedded infiltrating adenocarcinomas of the pancreas from patients who underwent Whipple resection from 1998 to 2003 were arrayed into tissue microarrays. The study was approved by the Johns Hopkins institutional review board.

RNA Extraction, Oligonucleotide Array Hybridization, and Analysis of Microarray Data

We analyzed global gene expression profiling in a total of 34 samples (including cell lines and primary tissues) using oligonucleotide microarrays (Human Genome U133A; Affymetrix, Santa Clara, CA). RNA extraction, sample preparation, and array hybridization were performed as described previously.[8,17,18] Hierarchical cluster analysis was performed using dChip software (http://www.dchip.org) after filtering genes with the greatest variation across all samples (SD/mean >1.5). Differential gene expression between pancreatic neoplasms and normal pancreatic duct epithelial samples was performed with fold-change analysis and *t* test using Data Mining Tool software (Affymetrix). We first compared the gene expression patterns between 17 pancreatic neoplasms and 5 normal pancreatic ductal epithelial samples to identify transcripts that are expressed at significantly lower levels in pancreatic neoplasms. We then combined the expression data from 4 pancreatic cancer cell lines (AsPC1, Hs766T, MiaPaCa2, and Panc1) treated with 5Aza-dC, TSA, or a combination of both drugs. Genes that are expressed at significantly lower levels (less than 5-fold lower and $P < .05$ by *t* test) in pancreatic neoplasms and that are up-regulated (more than 3-fold) in response to these drugs (either alone or in combination) in at least one of these pancreatic cancer cell lines are defined as biologically relevant genes associated with epigenetic silencing in pancreatic cancer. The full data set of Affymetrix gene expression profiles used in this study has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database.

Semiquantitative and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Reverse-transcription polymerase chain reaction (RT-PCR) was performed using primers specific for *RELN*, *CASK*, *ApoER2*, *VLDLR*, and *DAB1* (sequences are available on request). RT-PCR for *RELN* was performed in a semiquantitative fashion with primers to amplify *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) in duplex reactions. The relative intensity of *RELN* messenger RNA (mRNA) expression was measured by densitometry (ImageJ; National Institutes of Health, Bethesda, MD) and then normalized for *GAPDH* expression. The differential expression of *RELN* was confirmed by quantitative real-time RT-PCR using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) on the SmartCycler system (Cepheid, Sunnyvale, CA). Quantitative *RELN* expression was determined by a standard curve from serial dilutions of positive control complementary DNA and then normalized by expression of the housekeeping gene *PGK1*.

Immunoblotting

Whole cell lysates were extracted from cultured cells using a standard protocol. Same amounts of proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with an anti–phospho-Akt1/PKBα (Ser473) (Upstate, Chicago, IL) or with an anti-Akt1/PKBα (Upstate), followed by incubation with appropriate secondary antibodies. Blots were detected using the Amersham (Piscataway, NJ) enhanced chemiluminescence kit.

Immunohistochemistry

Five-micrometer tissue microarray sections were cut onto coated slides, deparaffinized as previously described, and subjected to antigen retrieval in Target Retrieval Solution (DAKO, Carpinteria, CA) heated at 95°C in a steamer for 20 minutes. Immunohistochemical staining was performed in the DAKO Autostainer using a mouse monoclonal antibody for RELN (clone 142, 1:200; Chemicon, Temecula, CA) and Envision Plus Detection Kit (DAKO) according to the protocol suggested by the manufacturer. All sections were counterstained with hematoxylin. Immunohistochemical labeling was