Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

Human Cancer Biology

Clinical Cancer Research

Genome-Wide Analysis of Promoter Methylation Associated with Gene Expression Profile in Pancreatic Adenocarcinoma

Audrey Vincent[1], Noriyuki Omura[1], Seung-Mo Hong[1], Andrew Jaffe[4], James Eshleman[1,2], and Michael Goggins[1,2,3]

Abstract

Purpose: The goal of this study was to comprehensively identify CpG island methylation alterations between pancreatic cancers and normal pancreata and their associated gene expression alterations.

Experimental Design: We employed methylated CpG island amplification followed by CpG island microarray, a method previously validated for its accuracy and reproducibility, to analyze the methylation profile of 27,800 CpG islands covering 21 MB of the human genome in nine pairs of pancreatic cancer versus normal pancreatic epithelial tissues and in three matched pairs of pancreatic cancer versus lymphoid tissues from the same individual.

Results: This analysis identified 1,658 known loci that were commonly differentially methylated in pancreatic cancer compared with normal pancreas. By integrating the pancreatic DNA methylation status with the gene expression profiles of the same samples before and after treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, and the histone deacetylase inhibitor, trichostatin A, we identified dozens of aberrantly methylated and differentially expressed genes in pancreatic cancers including a more comprehensive list of hypermethylated and silenced genes that have not been previously described as targets for aberrant methylation in cancer.

Conclusion: We expected that the identification of aberrantly hypermethylated and silenced genes will have diagnostic, prognostic, and therapeutic applications. *Clin Cancer Res; 17(13); 4341–54.* ©*2011 AACR.*

Introduction

Pancreatic cancer is the 4th most common cause of cancer death in the United States and has the lowest survival rate for any solid cancer. This particularly poor outcome is due in large part to the late presentation of the disease in most patients. Identifying those at risk of developing pancreatic cancer (1, 2) and developing better diagnostic markers of pancreatic neoplasia (3, 4) could improve the early diagnosis of pancreatic cancer and its precursors (5) and allow more patients to undergo curative surgical resection. Previous studies have shown that aberrant gene hyper- and hypomethylation contributes to pancreatic cancer development and progression (6–11). Furthermore, aberrant methylation increases during neoplastic development among the precursor lesions known as PanINs and intraductal papillary mucinous neoplasms (IPMN; refs. 11, 12). For example, aberrantly hypermethylated genes have been identified in pancreatic cancer by comparing gene expression profiles of pancreatic cancer cells before and after DNA methylation inhibitor treatment (8) and by using promoter (13) and SNP arrays (13, 14). The detection of aberrantly methylated loci relative to normal tissues could improve the diagnosis of pancreatic cancer (3) and may also identify key regulatory genes and pathways that merit therapeutic targeting (15).

We evaluated the accuracy and reproducibility of the methylated CpG island amplification coupled with a genome-wide promoter microarray platform (MCAM) in a pilot study by using the pancreatic cancer cell lines Panc-1 and MiaPaca2 (13, 16). In this study, we used MCAM to more comprehensively define the differential methylated genes between pancreatic cancer cells and normal pancreatic tissues. We then compared the methylation profile of our candidate genes with their global gene expression profile in pancreatic cancer cell lines and normal pancreatic epithelial ductal samples, including global gene expression profiles of cell lines before and after treatment with the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-Aza-dC) and the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA). Our aim

Authors' Affiliations: Departments of [1]Pathology, [2]Oncology, and [3]Medicine, [4]Bloomberg School of Public Health, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins Medical Institutions, Johns Hopkins University, Baltimore, Maryland

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Michael Goggins, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins Medical Institutions, CRBII Room 342, 1550 Orleans St., Baltimore, MD 21231. Phone: 410-955-3511; Fax: 410-614-0671; E-mail: mgoggins@jhmi.edu

doi: 10.1158/1078-0432.CCR-10-3431

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

Translational Relevance

Pancreatic cancer is the 4th leading cause of cancer death in the United States and is characterized by advanced disease at the time of diagnosis and resistance to most therapeutic treatments. Improving our understanding of the molecular mechanisms that contribute to the development of pancreatic cancer at the epigenetic and transcriptional level is crucial for the early diagnosis and therefore the treatment of this deadly disease. In this study, we used cancer cell lines derived from pancreatic ductal adenocarcinoma and compared their DNA methylation profile with pancreatic normal tissue as a reference. We found numerous target genes that are either hypermethylated and silenced or hypomethylated and overexpressed in pancreatic cancer versus normal pancreas and that could be used as building blocks for the development of new diagnosis and prognosis biomarkers, as well as therapeutic tools for pancreatic cancer.

was to identify genes commonly differentially methylated in pancreatic cancer and to identify the subset of aberrantly methylated genes with aberrant gene expression that represent candidate genes undergoing functional disruption in pancreatic cancer.

Materials and Methods

Cell lines and tissue samples

Pancreatic adenocarcinoma cell lines AsPC1, Capan2, MiaPaca2, BxPC3, Capan1, CFPAC1, HS766, Panc1, and Su8686 were cultured under recommended conditions. A32-1, A38-5, Panc215, Panc2.5, Panc2.8, Panc3.014, A2-1, A6L, Panc198, Panc486, and Panc8.13 pancreatic ductal adenocarcinoma cell lines were described previously (17). Immortalized human pancreatic ductal epithelial (HPDE) cells derived from normal human pancreatic ductal epithelium were generously provided by Dr. Ming-Sound Tsao (University of Toronto, Canada). Stored frozen tissues (−80°C) of normal lymphoid tissues (lymphocytes or spleen) were obtained from 3 of the patients from whom we had developed a pancreatic cancer cell line (A32-1, A38-5, and Panc215).

The frozen primary pancreatic cancer tissues, normal pancreatic, and spleen tissues were obtained from patients the time of their pancreatic resection at Johns Hopkins Hospital. Normal pancreatic duct epithelial cells were isolated by using laser capture microdissection from the resected pancreata of 3 patients (mean age, 64 years; range, 59–72 years) who underwent pancreatic resection for benign conditions. Additional normal pancreatic tissues were obtained from patients with pancreatic adenocarcinomas or neuroendocrine neoplasms. DNA was isolated from xenografts of primary pancreatic cancers as previously described (8, 9). Specimens were collected and analyzed with the approval of the Johns Hopkins Committee for Clinical Investigation.

Methylated CpG island amplification procedure and Agilent CpG island microarrays

Methylated CpG island amplification (MCA) was done as described previously (13). The Human CpG Island ChIP-on-Chip Microarray 244K chip interrogates 27,800 CpG islands covering 21 MB with an average of 8 probes per island. Array hybridization was done by the Johns Hopkins SKCCC Microarray Core Facility. Briefly, 2 μg of MCA amplicon were labeled with either Cy3-dUTP or Cy5-dUTP (Perkin Elmer) by using the BioPrime DNA Labeling System (Invitrogen). These dye-labeled amplicons were then mixed and cohybridized to 244K CpG island microarrays, washed, dried, and scanned by using the Agilent G2505B scanner.

Methylation-specific PCR

Bisulfite treatment and DNA amplification were done as described previously (13). Primer sequences are available in Supplementary Table S5.

Affymetrix exon arrays

Cells were treated with 1 μmol/L of 5-Aza-dC (Sigma) for 4 days and 1 μmol/L of the HDAC inhibitor TSA (Sigma) either alone or in combination for 24 hours as previously described (8, 9). Total RNA from cell lines was extracted by using mirVana miRNA Isolation Kit (Ambion) per manufacturer's protocol before DNAase treatment (Ambion).

The Affymetrix Exon Array ST1.0 (Affymetrix) was used to analyze gene expression profiles in untreated and 5-Aza-dC or TSA-treated cell lines as previously described (18, 19).

Data analysis

For MCAM, data were extracted with Agilent Feature Extraction 9.1 software. Methylation-specific sites were called with Agilent Genomic Workbench Standard Edition 5.0.14 software for methylation analysis, which calculates the normalized $\log_2$-signal ratios (NLR) and combined Z scores for each probe. For Affymetrix Exon Array ST1.0, statistical analysis of gene expression array data was completed with Partek Genomic Suite 6.4 software. Raw Affymetrix intensity measurements of all probe sets were background corrected and normalized by the Robust Multichip Average method. Sample relationships were examined by using principal component analysis to reveal any technical effects that would encumber the subsequent analysis. Gene expression intensities were summarized by the 1-step Tukey's biweight method. Two-way ANOVA analysis was done to identify significant expression changes between pancreatic cancer and noncancer samples and between 5-Aza-dC and/or TSA-treated and untreated cells. Gene network and pathway analysis

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

were done by using Ingenuity Pathways Analysis (Ingenuity systems; http://www.ingenuity.com) software. We were in compliance with the Minimum Information about a Microarray Experiment guidelines and have submitted our microarray data set to the Gene Expression Omnibus of National Center for Biotechnology Information. They are accessible through GEO Series accession number GSE21163 and GSE29723.

Immunohistochemistry

The expression of ZBTB16 protein was examined by immunohistochemical labeling of tissue microarrays with anti-ZBTB16 (Invitrogen 34-3,700; 1:100) polyclonal antibody, counterstained with hematoxylin. Immunohistochemical labeling was scored based on intensity; 0 (negative), 1 (weak), or 2 (strong), and on the percentage of positive epithelial cells as 0 (<5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), or 4 (>76%), respectively. A histoscore was generated as the product of intensity multiplied by area and mean histoscores of pancreatic ductal adenocarcinoma and normal ductal epithelial cells were compared by using Paired Student's *t* test.

Results and Discussion

Identification of loci differentially methylated between pancreatic cancer and normal pancreatic tissues

We used Agilent 244K CpG island microarrays to conduct 9 MCAM experiments comparing pancreatic cancer cell lines with normal pancreas. The pancreatic cancer cell lines were compared with pooled laser-captured microdissected normal pancreatic ductal tissues (A32-1, A38-5, Panc215, Panc2.5, Panc2.8, and Panc3.014), to normal pancreatic tissue (MiaPaca2 and Capan2), or to the non-neoplastic pancreatic ductal line HPDE (AsPC1).

To identify differentially methylated probes, we used the previously validated cutoff (13) of ~4-fold (NLR $\geq$ 2 or ≤ -2) differential methylation between pancreatic cancers and normal pancreata (Fig. 1). This cutoff was established by using the same MCAM strategy comparing the pancreatic cancer cell line Panc-1 to the epithelial line, HPDE, and validating the methylation status of 31 genes by using bisulfite sequencing and methylation-specific PCR (MSP). By this criterion, there were 41,606 probes, representing

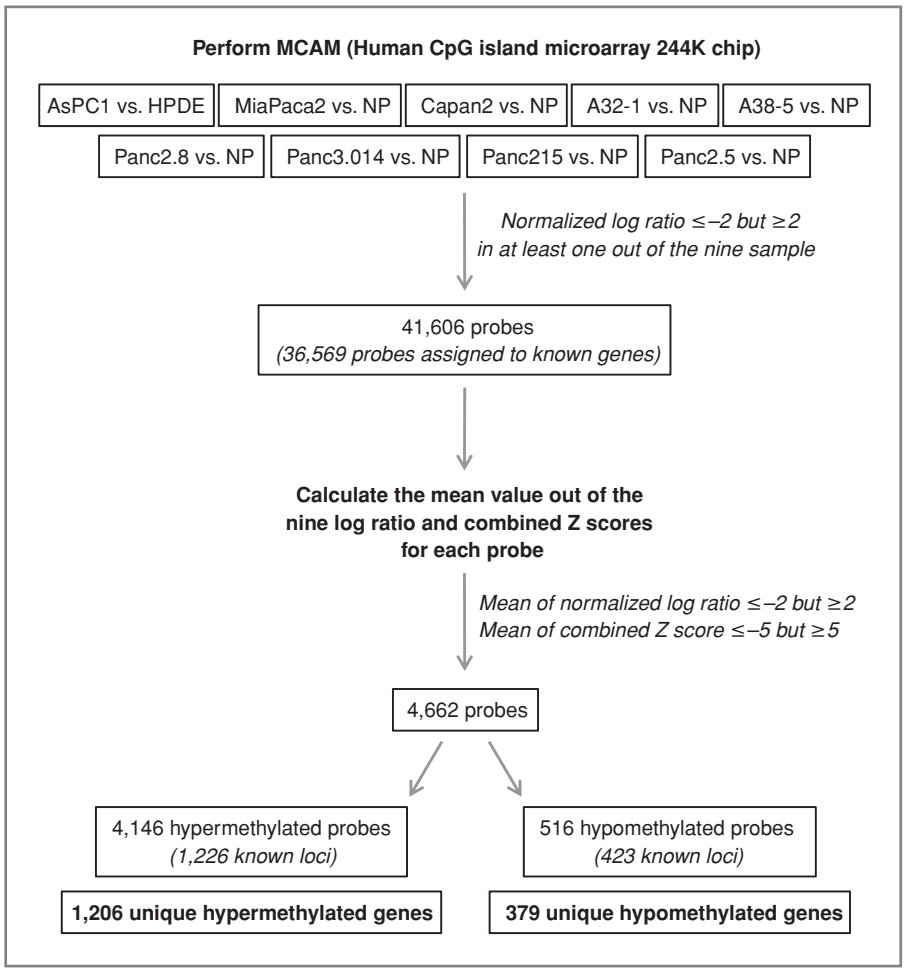

Figure 1. An overview of experimental strategy to evaluate differential DNA methylation in pancreatic cancer cells compared with normal pancreas. NP, normal pancreas.

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

10,012 loci with differential methylation in at least one of the 9 MCAM experiments, a considerably more extensive list than we identified in our pilot MCA experiment by using the 44K array (13). To identify from this list genes commonly methylated in pancreatic cancers, we then calculated the mean of differential methylation ratios of the 9 cancer/normal pairs and the combined Z scores for each of these 41,606 probes and filtered out probes for which the mean of the 9 NLRs was −2 or more but 2 or less (note: Z scores reflects how far a probe log-ratio value is in relation to the Gaussian distributions of probes with similar melting temperatures on the Agilent array). This criterion allowed us to identify 4,672 probes for which the mean combined Z score was either −4.67 or less or 5.1 or more. To refine our gene list, we then filtered out outlier probes for which the mean combined Z scores was −5 or more but 5 or less which left 4,662 probes, corresponding to 1,658 known loci (Supplementary Table S1). Thus, the gene lists identified by using the mean NLR of −2 or more but 2 or less and the mean combined Z scores was −5 or more but 5 or less were almost identical. Among these 1,658 loci, 1,226 were hypermethylated (1,206 genes, Supplementary Table S2) and 423 were hypomethylated (379 genes, Supplementary Table S3) in the pancreatic cancer cell lines compared with normal pancreatic tissue.

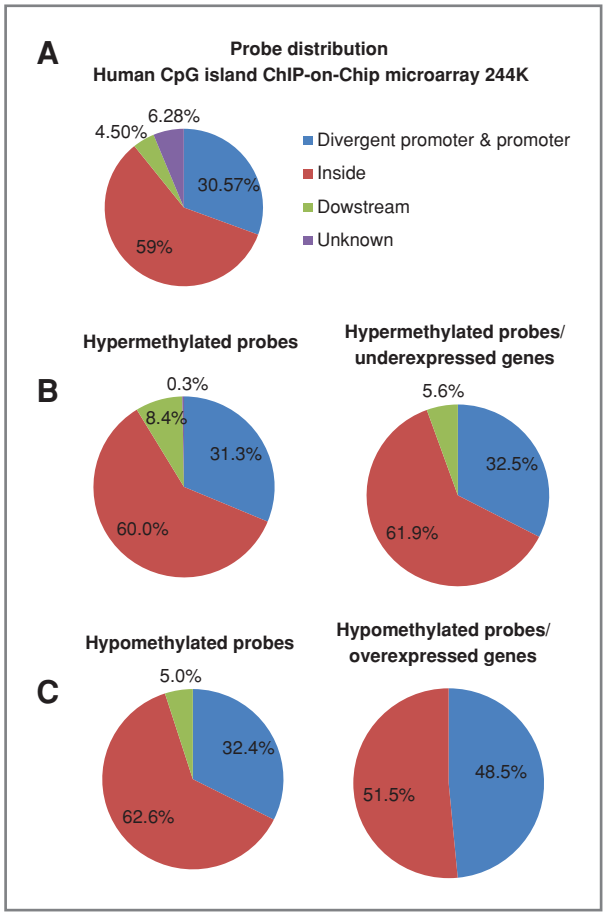

Figure 2. Distribution of Agilent Human CpG island 244K probes covering known genes. A, global distribution. B, distribution of hypermethylated probes. C, distribution of hypomethylated probes.

We found that 60% of hypermethylated probes were located inside known gene bodies, whereas 31.3% were located in their promoters, and 8.42% were located downstream of known genes (Fig. 2B, left). These proportions were very similar among the hypermethylated genes whose expression was downregulated (Fig. 2B, right) and to the overall distribution of probes on the Agilent chip (Fig. 2A). We also found a very similar distribution of hypomethylated probes (Fig. 2C, left). However, when we included only hypomethylated probes that were associated with upregulated genes, we found that 48.5% of probes were located in the promoter region (Fig. 2C, right). This analysis is consistent with the observation that hypermethylation within the gene body and the promoter can result in downregulation, whereas gene upregulation is associated promoter hypomethylation but not gene body hypomethylation.

Because cancer-associated CpG island hypermethylation tends to extensively involve the affected CpG island, we used MSP to confirm differential CpG island methylation identified by MCAM. Specifically, to validate our MCAM results, we used MSP to examine the methylation status of 24 aberrantly hypermethylated genes in 69 pancreatic cancers [43 xenografts of primary pancreatic cancers, 15 primary pancreatic cancer tissues, and 11 cell lines (i.e., BxPC3, Capan1, CFPAC1, HS766, Panc1, Su8686, A2-1, A6L, Panc198, Panc486, and Panc8.13)] and 16 normal pancreatic tissue. We confirmed the differential methylation of 21 of the 24 genes tested (Supplementary Table S4), showing the robustness of our MCAM strategy.

Several genes were methylated in more than 70% of pancreatic cancers and 10% or less of normal pancreata by MSP including *BMI1*, *ID4*, *CACNA1H*, *IRF5*, and *SMOC2*.

Aberrantly methylated and differentially expressed genes

We have previously examined gene expression in a set of 6 pancreatic cancer cell lines (A32-1, A38-5, Panc215, Panc2.5, Panc2.8, and Panc3.014) and the pancreatic nonneoplastic line, HPDE, before and after epigenetic treatment (5-Aza-dC, TSA, and the combination) by using Affymetrix ST1.0 Exon Arrays (18). Here, we merged this analysis with the results of our MCAM experiments and compared the baseline and posttreatment gene expression profiles of these 6 pancreatic cancer cell lines focusing on genes shown to be hypermethylated or hypomethylated in our MCAM experiments. We used the 2-way ANOVA analysis to identify expression changes between cancer cells and the nonneoplastic line HPDE. We defined genes as differentially expressed based on a fold-change criteria of ±2-fold. Among the hypermethylated genes, 96 genes were underexpressed in pancreatic cancer cells compared with HPDE cells and the expression of 675 were induced

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

by 5-Aza-dC and/or TSA treatment either alone or in combination (P value <0.05; Supplementary Table S2). 77 genes were both underexpressed and responsive to epigenetic treatment (Table 1). If we employed a more stringent cutoff for underexpressed genes (>3-fold reduction in expression), 37 genes were both underexpressed and responsive to epigenetic treatment (Table 1, bold genes). Among the hypomethylated genes, 24 genes were overexpressed in the pancreatic cancer cell lines compared with nonneoplastic pancreatic duct HPDE cells (fold-change $\geq$2; Table 2) and 243 genes were significantly induced by epigenetic treatment but not necessarily differentially expressed ($P < 0.05$ with a fold-change >2; Supplementary Table S3).

Even by a modest criteria of $\pm$2-fold for differential expression, only a minority of genes displayed methylation-associated alterations in expression compared with those that underwent aberrant methylation (Tables 1 and 2). We previously made this same observation in an MCAM analysis of the Panc-1 versus HPDE cell lines (13). However, when we next examined the relationship between global methylation and gene expression by comparing mean expression levels in the 6 pancreatic cancer cell lines, we found that hypermethylated genes (mean of NLR >1) were significantly less expressed in the pancreatic cancer cells than nonhypermethylated genes (mean of NLR <1, Fig. 3A and B). And conversely, we found that the expression of hypomethylated genes (mean of NLR <$-$1) was significantly higher than nonhypomethylated genes (mean of NLR >$-$1, Fig. 3A and B). These results support the general role of hypermethylation as a mechanism of aberrant silencing in cancer and reinforcing the association between localized promoter hypomethylation and gene overexpression.

In addition, we checked the expression profile of our newly identified hypermethylated and hypomethylated genes previously analyzed by the serial analysis of gene expression (SAGE) method (17). The advantage of this SAGE analysis is that it quantified 1 to 2 million SAGE tags in each of the 24 pancreatic cancer samples 2 laser capture microdissected normal pancreatic duct samples and the nonneoplastic pancreatic duct cell line, HPDE. We defined silenced genes, neoexpressed genes, underexpressed, and overexpressed genes. Silenced genes were defined as genes for which the number of tags was simultaneously less than 1 in more than 50% of the 24 pancreatic cancer samples and more than 10 in both normal samples and were added to Table 1. Underexpressed genes were those with a 2-fold decrease in the number of tags in pancreatic cancer compared with either one of the 2 normal samples (the percentage of pancreatic cancers with underexpression is noted in the SAGE columns of Supplementary Table S2). Neoexpressed genes were defined as genes for which the number of tags was simultaneously more than 10 in more than 50% of the 24 pancreatic cancer samples and less than 1 in both normal samples and were added to Table 2. Overexpressed genes were defined as those with a 2-fold increase in the number of tags in pancreatic cancer compared with either one of the 2 normal samples (the percentage of pancreatic cancer samples with overexpression is noted in Supplementary Table S3).

Genes known to be hypermethylated in pancreatic cancer

Among the list of 1,206 candidate hypermethylated genes, were virtually all of the genes we previously identified by using MCAM on the Agilent 44K microarray and validated by bisulfite sequencing and MSP in Panc-1 cancer cells, that is, *BAI1*, *KCNV1*, *EYA4*, *BNC1*, *HOXA5*, *PAX7*, *SOX14*, *TLX3*, *NRXN1*, *CNTNAP2*, *PKP1*, *ACTA1*, *MDFI*, *EVC2*, *LIN28*, *NRN1*, *PENK*, *FAM84A*, and *ZNF415* (13).

We also confirmed the differential methylation profiles of genes whose epigenetic silencing was revealed by our group and others. These genes include *NPTX2*, *CLDN5*, *LHX1*, *WNT7A*, *FOXE1*, *PAX6*, *BNIP3*, *GADD45B* (8), *HIC1*, *HS3ST2*, *TWIST1* (14), *IRF7*, *CCNA1*, *ALPP*, *CEBPA* (20), *CACNA1G* (21), *CCND2* (22), and *TFPI-2* (23).

Our list included the tumor suppressor genes *STK11* and *WT1*, the transcription factor *RUNX3*, the transcriptional repressor *CBFA2T3*, the secreted glycoprotein and chemorepulsive factor *SLIT2* and the aryl-hydrocarbon receptor repressor, *AHRR*.

Note that our selection criterion was designed to identify genes that are commonly differentially methylated in pancreatic cancer. Thus, several important genes that are infrequently methylated in pancreatic cancer did not meet our commonly methylated filter including *p16/CDKN2A*, *E-cadherin/CDH1*, and *hMLH1* because these genes are uncommon targets in pancreatic cancer (24, 25). Among our 9 pancreatic cancer/normal pancreas methylomes examined, 3 had 1 hypermethylated probe in the *CDKN2A* promoter. Other genes hypermethylated in pancreatic cancer (*RELN*; 7; *MMP1* and *MMP14*; 26) did not meet our filter for commonly methylated genes, although several MCAM probes covering these genes were significant for hypermethylation. Finally, some genes hypermethylated in pancreatic cancer were not identified because their promoters were not interrogated by the MCAM method (e.g., *SPARC*, *MMP3*, *MMP2*, *MMP9*, and *MMP7*).

Genes involved in stem cell pluripotency are hypermethylated in pancreatic cancer

We found DNA hypermethylation of genes involved in stem cell pluripotency, such as the intestinal stem cell marker and chromatin structure regulator *BMI1*, the genes encoding bone morphogenetic proteins BMP3, BMP6, the transcription factors FOXD3, CDX2, UTF1, and T, as well as *NR5A1*, *NR5A2*, *NR2F1*, *NTRK1*, *NTRK2*, *NTRK3*, *NODAL*, *SALL4*, and *SPHK1* genes. In addition, hypermethylation of *FOXA1/A2*, *BMP7*, *IGF2BP1*, *HOXB1*, and *NR2F2* was associated with gene relative underexpression in pancreatic cancer by SAGE analysis (Supplementary Table S2; ref. 17).

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

Table 1. List of hypermethylated and downregulated candidate genes, responsive to epigenetic drug treatment

Function	Gene symbol	Gene name	MCAM: # of hyper-methylated probes	MCAM: # samples with lowest signal ratio >2	MCAM: # samples with mean methylated NLR >2	MCAM: PC vs. lymphs	MCAM: PC vs. spleen and lymphs	GEP: HPDE cells	GEP: Fold change cancer vs. HPDE	SAGE: Silenced (%) in PC	MSP: % methylation NP	MSP: % methylation PC	PcG target	Cancer associated	Bibliography: Reference
G-protein coupled receptors	*FZD2*	frizzled homolog 2 (*Drosophila*)	7	8	7	Yes	Yes	++	−3.40	0	100	94.1			
	NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	11	9	8	Yes	Yes	+	−2.67	0				A	(13)
Enzymes	*CPT1C*	carnitine palmitoyltransferase 1C	4	8	7	Yes		++	−2.24	0				MM	(5)
	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	6	7	6			++	−35.94	0			Yes	CRC	(6)
	HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	8	9	8	Yes		++	−2.00	0				BC*; PC	(7)(8)
	HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	2	8	6			+	1.31	52					
Growth factors	*GAS6*	similar to growth arrest-specific 6; growth arrest-specific 6	1	6	6			++	−3.11	10					
Ion channels	*KCNMA1*	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	2	9	7	Yes	Yes	+	−3.36	0				PrC	(9)
Kinases	*CKB*	creatine kinase, brain	2	5	5	Yes		++	−2.53	0				HM	(10)
	EPHA4	ephrin-A4	3	7	7			++	−2.02	0	31.25	38.25			
Phosphatase	*PPP1R16B*	protein phosphatase 1, regulatory (inhibitor) subunit 16B	2	6	6	Yes	Yes	+	−2.39	0					
Transcription regulators	*BNC1*	basonuclin 1	2	7	6	Yes	Yes	++	−36.07	0			Yes	PC	(11)
	DLX1	distal-less homeobox 1	5	9	5			+	−2.29	0			Yes	LC	(12)
	DLX2	distal-less homeobox 2	4	7	6	Yes	Yes	+	−2.42	0			Yes	A	(13)
	EGR1	early growth response 1	1	5	5			++	−2.03	0				OC	(162)
	FLI1	Friend leukemia virus integration 1	3	6	6	Yes	Yes	+	−2.84	0			Yes	PC	(8)
	FOXF2	forkhead box F2	5	9	8	Yes	Yes	+	1.13	52	25	64.6	Yes	BC	(86)
	FOXG1	forkhead box G1	1	8	8			++	−9.94	0	25	91.1			
	HOXD8	homeobox D8	6	8	6	Yes	Yes	+	−1.42	83			Yes	A	(13)
	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	1	6	6	Yes	Yes	+	−1.39	76	6.25	73.2		CRC	(89)
	INSM2	insulinoma-associated 2	2	7	7	Yes	Yes	+	−2.28	0					
	IRF5	interferon regulatory factor 5	1	5	5			++	−2.16	0	0	77.3	Yes	HC	(161)
	IRF7	interferon regulatory factor 7	1	4	4			++	−2.82	0				PC	(15)
	IRX4	iroquois homeobox 4	24	9	7	Yes	Yes	+	−2.34	0			Yes	CRC	(16)
	SIM1	single-minded homolog 1 (*Drosophila*)	4	7	6	Yes	Yes	+	−3.64	0	15.4	92.8	Yes	BC	(17)
	SOX15	SRY (sex determining region Y)-box 15	2	9	7			+	−7.52	0	31.25	69.4		LC	(160)
	TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	11	9	8	Yes	Yes	++	−2.28	0				LC	(18)
	TWIST1	twist homolog 1 (*Drosophila*)	1	6	6			++	−2.80	0			Yes	PC	(8)
	ZBTB16	zinc finger and BTB domain containing 16	1	5	5			+	−5.97	0	0	35.2	Yes		
	ZNF382	zinc finger protein 382	1	6	6	Yes	Yes	+	−6.43	0				HM	(19)
Transmembrane receptors	*SCARF2*	scavenger receptor class F, member 2	1	6	6	Yes		++	−2.76	0					
	SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	1	6	6			+	1.50	72					
	SFRP1	secreted frizzled-related protein 1	1	6	6			++	−23.78	?	14	86	Yes	RCC	(20)
Transporter	*SLC18A3*	solute carrier family 18 (vesicular acetylcholine), member 3	2	6	6			++	−3.24	0					
	SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	1	4	4			+	−3.82	0				PC	(11)
Protein binding (Cell adhesion and junctions)	*CDH4*	cadherin 4, type 1, R-cadherin (retinal)	2	5	5			+	−4.62	0				CRC, GC	(21)
	CLDN10	claudin 10	1	6	6			+	3.29	52					
	FLRT2	fibronectin leucine rich transmembrane protein 2	5	6	6	Yes		+	−5.23	0				LC	(22)
	ICAM4	intercellular adhesion molecule 4 (Landsteiner-Wiener blood group)	2	8	8			+	−2.09	0			Yes		
	LAMA1	laminin, alpha 1	6	9	6	Yes	Yes	++	−9.13	0				CRC, BC	(163)
	NID1	nidogen 1	2	6	5			++	−3.08	0				CRC, GC	(23)
	NID2	nidogen 2 (osteonidogen)	1	4	4			++	−3.42	0			Yes	CRC, GC	(23)
	PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome); similar to plakophilin 1 isoform 1a	12	9	7	Yes	Yes	++	−8.32	0				PC	(11)
	SPOCK1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	3	7	4	Yes	Yes	++	−6.11	0				LC	(22)
	AMOTL1	angiomotin like 1	1	6	6			++	−2.47	10					

Table 1. List of hypermethylated and downregulated candidate genes, responsive to epigenetic drug treatment (Cont'd)

Gene description			MCAM					GEP		SAGE	MSP		Bibliography		
Function	Gene symbol	Gene name	# of hyper-methylated probes	# samples with lowest signal ratio >2	# samples with mean methylated NLR >2	PC vs. lymphs	PC vs. spleen and lymphs	HPDE cells	Fold change cancer vs. HPDE	Silenced (%) in PC	% methylation NP	% methylation PC	PcG target Cancer associated		Reference
Extracellular matrix	***COL4A1***	collagen, type IV, alpha 1	3	7	7			++	−3.48	0					
	COL4A2	collagen, type IV, alpha 2	1	5	5			++	−5.53	0					
	RECK	reversion-inducing-cysteine-rich protein with kazal motifs	1	4	4			++	−2.56	0				CRC	(25)
	TFPI2	tissue factor pathway inhibitor 2	1	8	8	Yes	Yes	++	−21.06	0			Yes	IPMN	(26)
Other function	***BNIP3***	BCL2/adenovirus E1B 19kDa interacting protein 3	4	6	6			++	−14.62	55				PC	(27)
	BVES	blood vessel epicardial substance	2	7	6	Yes	Yes	++	−9.94	0			Yes	LC	(28)
	CCND2	cyclin D2	1	5	5	Yes		++	−12.69	0			Yes	BC	(29)
	CDT1	chromatin licensing and DNA replication factor 1	2	5	5			++	−2.46	0			Yes		
	DZIP1	DAZ interacting protein 1	1	7	7	Yes	Yes	++	−3.90	0				PC	Omura et al**
	EFHD1	EF-hand domain family, member D1	5	8	8	Yes		++	−7.09	0					
	ELAVL2	ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like 2 (Hu antigen B)	1	6	6			++	−6.90	0			Yes	CRC	(164)
	EVC2	Ellis van Creveld syndrome 2	1	8	8	Yes	Yes	+	−3.57	0				PC	(11)
	GREM1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	3	6	5			++	−2.89	0			Yes	GC, PrC, BC	(30)
	HPCAL4	hippocalcin like 4	1	5	5			++	−2.17	0				CC	(31)
	LTBP2	latent transforming growth factor beta binding protein 2	2	7	7	Yes	Yes	++	−2.36	17				HM	(32)
	PLD5	phospholipase D family, member 5	2	7	6	Yes	Yes	++	−8.08	0					
	RIMS2	regulating synaptic membrane exocytosis 2	5	9	5	Yes	Yes	+	−2.19	0					
	RINT1	RAD50 interactor 1	1	7	7			++	−2.24	0					
	SLIT2	slit homolog 2 (*Drosophila*)	7	9	9	Yes	Yes	++	−2.76	0				PC	(8)
	SMOC2	SPARC related modular calcium binding 2	2	7	7	Yes	Yes	+	−9.32	0	0	81.6			
	SOCS2	suppressor of cytokine signaling 2	1	5	5			+	−2.84	0				BC*	(33)
	WNT5A	wingless-type MMTV integration site family, member 5A	3	6	4	Yes	Yes	+	−2.91	0	0	26.4		CRC	(34)
Unknown function	***BTBD6***	BTB (POZ) domain containing 6	4	6	5	Yes	Yes	++	−4.27	0	81.8	90.6			
	C11orf83	chromosome 11 open reading frame 83	2	6	6			++	−2.35	?					
	C16orf5	chromosome 16 open reading frame 5	1	6	6			++	−2.64	28					
	C1orf61	chromosome 1 open reading frame 61	1	6	6			+	−2.44	0					
	EFS	embryonal Fyn-associated substrate	2	6	6	Yes	Yes	+	−2.38	0				PrC	(9)
	KIAA0495	KIAA0495	4	8	8	Yes	Yes	++	−2.74	0					
	KLHL35	kelch-like 35 (*Drosophila*)	1	6	6			++	−3.90	?				MM	(5)
	TRIM58	tripartite motif-containing 58	3	6	6	Yes	Yes	+	−2.13	0					
	TSPAN4	tetraspanin 4	4	8	8	Yes	Yes	++	−2.30	7					
	ZNF154	zinc finger protein 154	3	9	8	Yes	Yes	+	−2.14	?					
	ZNF415	zinc finger protein 415	1	7	7	Yes		++	−14.14	0				PC	(11)
	ZNF545	zinc finger protein 545	1	6	6			++	−15.77	0					
	ZNF550	zinc finger protein 550	1	6	6			++	−2.57	0				CRC	(164)
	ZNF667	zinc finger protein 667	9	9	8	Yes	Yes	+	−5.48	0					

NOTE: The HPDE column indicates the level of expression of the gene in normal cells: 0, virtually undetectable signal; +, weak expression; ++, strong expression.
All genes from this list were significantly responsive to 5-aza-dC/TSA treatment based on 2-way ANOVA analysis between 5-aza-dC and/or TSA-treated and untreated cancer or noncancer cells and $P < 0.05$.
Bold genes indicate a fold-change lower than 3-fold between cancer cells and HPDE cells. SAGE (serial analysis of gene expression): The numbers indicate the % of pancreatic cancer samples in which the normalized number of tags was less than 1 whereas the number of tags in normal samples was more than 10 (1).
MSP (methylation-specific PCR): % of samples in which methylation was detected by MSP.
Bibliography: Reference numbers refer to the supplementary list of references in the supplementary materials. PcG: Polycomb group protein targets (2–4).
MCAM: Methylated CpG island amplification strategy coupled with microarray platform. PC: Pancreatic cancer; NP: Normal pancreas; Lymphs: Lymphocytes.
GEP: Gene expression profiling with affymetrix exon arrays.
CRC: Colorectal carcinomas; BC: Breast cancer; PrC: Prostate cancer; GC: Gastric cancer; LC: Lung cancer; OC: Ovarian cancer; HC: Hepatocellular carcinoma; RCC: Renal cell carcinoma; CC: Cervical carcinoma; MM: Malignant melanomas; HM: Hematologic malignancies; A: Astrocytomas. *: Biomarkers.** Omura et al, unpublished.
Color coding: 4 — 4 samples with lowest signal ratio more than 2 or with mean normalized log ratio more than 2
5
6
7
8
9 — 9 samples with lowest signal ratio more than 2 or with mean normalized log ratio more than 2

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

Table 2. List of hypomethylated and upregulated candidate genes

Gene description			MCAM				GEP			SAGE	Bibliography	
Function	Gene symbol	Gene name	# of hypo-methylated probes	# of samples with highest NLR <-2	# of samples with mean methylated NLR <-2	PC vs. lymphs	HPDE Cells	Fold change cancer vs. HPDE	5-aza-dC/TSA response	Neoexpression (%)	Cancer associated	Reference
Enzymes	*DDHD2*	DDHD domain containing 2	1	6	6		++	2.83	S	76		
	HELLS	helicase, lymphoid-specific	1	6	6		++	−1.85	S	90		
	MTRR	5-methyltetrahydrofolate-homocysteine methyltransferase reductase	1	6	6		++	1.11	NS	59		
	PIGF	phosphatidylinositol glycan anchor biosynthesis, class F	1	7	7		++	−1.36	NS	55		
	ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	1	7	7		0	2.26	S	3		
	UBL4A	ubiquitin-like 4A	4	8	8	Yes	++	−1.19	S	72		
Kinases	*DGKH*	diacylglycerol kinase, eta	1	7	7		+	2.68	S	?		
	PIM2	pim-2 oncogene	2	7	7	Yes	++	−1.62	S	59		
Peptidases	*BLMH*	bleomycin hydrolase	1	6	6		++	−1.42	S	62		
	OMA1	OMA1 homolog, zinc metallopeptidase (S. cerevisiae)	1	6	6		?	?	?	55		
	PLAU	plasminogen activator, urokinase	1	6	6		++	4.24	S	0	BC	(35)
Phosphatases	*DUSP4*	dual specificity phosphatase 4	1	6	6		+	2.44	S	0	CRC	(36)
	NT5E	5'-nucleotidase, ecto (CD73)	1	6	6		++	1.13	S	86	GC	(111)
	SYNJ2	synaptojanin 2	2	6	6		++	3.30	S	0		
Transcription regulators	*CREB1*	cAMP responsive element binding protein 1	1	6	6		++	−1.03	S	55		
	HOXB13	homeobox B13	1	6	6		+	4.40	S	7	LC	(12)
	MED22	mediator complex subunit 22	1	6	6		?	?	?	52		
	SIX5	SIX homeobox 5	1	6	6		+	−1.34	S	66		
	SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	1	8	8		++	3.78	S	0	HPDE	(27)
Transmemb. Rec.	*GPC4*	glypican 4	1	6	6	Yes	++	2.13	NS	31		
Transporters	*GJB6*	gap junction protein, beta 6, 30kDa	1	7	7		+	3.89	NS	10		
	SLC16A5	solute carrier family 16, member 5 (monocarboxylic acid transporter 6)	1	8	8		+	4.67	S	0	HC	(37)
	VLDLR	very low density lipoprotein receptor	1	6	6		++	5.75	S	0		
Cell adhesion	*ITGB2*	integrin, beta 2	1	6	6		+	2.50	S	45	GC	(38)
Cytoskeleton	*GRAMD4*	GRAM domain containing 4	1	7	7		++	2.36	NS	?		
Other functions	*APOO*	apolipoprotein O	1	4	4		++	−1.29	S	52		
	ARHGAP21	Rho GTPase activating protein 21	1	6	6		+	2.10	S	0		
	CSTB	cystatin B (stefin B)	1	6	6		++	4.27	S	0	PC	(39)
	MBP	myelin basic protein	1	5	5		+	−1.23	S	86		
	MCF2L	MCF.2 cell line derived transforming sequence-like	5	6	5		+	2.01	S	59		
	MYH14	myosin, heavy chain 14	2	6	6		+	2.54	S	0		
	RASA3	RAS p21 protein activator 3	2	8	6	Yes	++	1.53	S	90		
	RPL10	ribosomal protein L10	3	8	8	Yes	+	2.16	NS	0		
	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	1	7	7		++	−2.07	S	55	MS	(157)
	TRIM37	tripartite motif-containing 37	1	6	6		++	−1.57	NS	62		
Unknown functions	*C21orf56*	chromosome 21 open reading frame 56	1	6	6		+	2.03	S	62		
	CDCP1	CUB domain containing protein 1	1	5	5		++	3.32	S	0	BC	(40)
	FAM8A1	family with sequence similarity 8, member A1	1	7	7		++	−1.48	S	69		
	GLI4	GLI family zinc finger 4	3	6	6		+	1.61	S	86		
	LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	1	6	6		+	3.49	S	76		
	MAPRE2	microtubule-associated protein, RP/EB family, member 2	1	6	6		++	1.91	S	86		
	RNF213	ring finger protein 213	1	6	6		++	2.63	S	0		
	TSSC1	tumor suppressing subtransferable candidate 1	1	6	6		++	1.76	S	66		
	WRB	tryptophan rich basic protein	1	5	5		++	2.26	S	76		

NOTE: Tthe HPDE column indicates the level of expression of the gene in normal cells (0, virtually undetectable signal; +, weak expression; ++, strong expression).
In the 5-aza-dC/TSA column, S indicates a significant response to treatment and NS indicates a not significant response based on a 2-way ANOVA analysis between 5-aza-dC and/or TSA-treated and untreated cancer or noncancer cells and $P < 0.05$.
SAGE (serial analysis of gene expression): The numbers indicate the % of pancreatic cancer samples in which the normalized number of tags was >10 whereas the number of tags in normal samples was <1 (1).
Bibliography: Reference numbers refer to the supplementary list of references in the supplementary materials.
MCAM: Methylated CpG island amplification strategy coupled with microarray platform. GEP: Gene expression profiling with affymetrix exon arrays. PC: Pancreatic cancer; HPDE: human pancreatic ductal epithelial cells.
CRC: Colorectal carcinomas; BC: Breast cancer; GC: Gastric cancer; LC: Lung cancer; HC: Hepatocellular carcinoma; MS: Myelodysplastic syndrome; Lymphs: Lymphocytes.

Color coding:
- 4 — 4 samples with lowest signal ratio more than 2 or with mean normalized log ratio more than 2.
- 5
- 6
- 7
- 8
- 9 — 9 samples with lowest signal ratio more than 2 or with mean Normalized Log Ratio more than 2

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

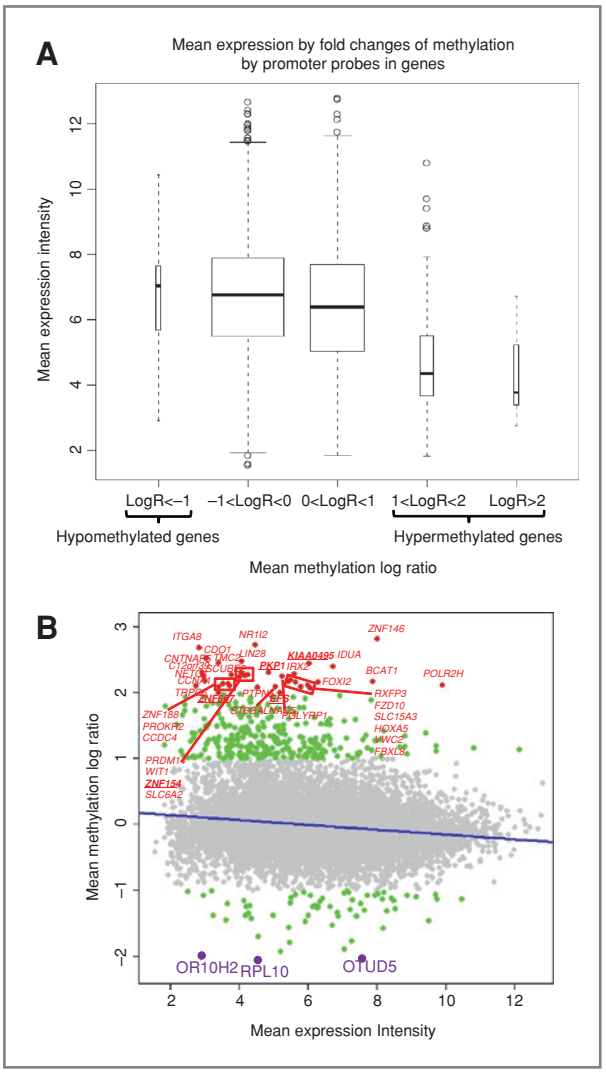

Figure 3. Correlation between gene methylation and gene expression levels in pancreatic cancer samples (A32-1, A38-5, Panc215, Panc2.5, Panc2.8, and Panc3.014). A, boxplots stratifying mean gene expression (intensities values from Affymetrix Exon Array ST1.0) by mean DNA methylation values (NLR from 244K CpG Island Microarrays). For genes with multiple promoter probes the NLRs for each probe were averaged within sample before calculating the promoter region mean at each across the 6 sample pairs. B, scatterplot representing for each gene the mean expression fold-change compared with their methylation profile. The line represents the statistically significant association between mean differential DNA methylation and gene expression.

The WNT signaling pathway is a target of hypermethylation in pancreatic cancer

Interestingly, numerous actors and mediators of the WNT signaling pathway were found to be hypermethylated in the majority of our pancreatic cancer samples and account for an enrichment ratio of 0.167 in our ingenuity pathway analysis. As expected, we found hypermethylation involving genes previously shown to be epigenetically silenced both in pancreatic and other types of cancer (14, 27) and encoding either the WNT ligands *WNT5A*, *WNT7A*, and *WNT9A*, or the cell-surface receptor *FZD9*, as well as the cytoplasmic transducer *APC2*, the nuclear factors *SOX1*, *SOX7*, *SOX14*, and *SOX17*, and the pathway inhibitors *FRZB*, *SFRP1*, *SFRP2*, *KREMEN2*, *NKD2*, and *WIF1*. Finally, we also found hypermethylation of the tumor suppressor candidate *HIC1*, which encodes a transcriptional repressor responsible for abnormal survival circuits through nuclear release of WNT signaling pathway transcription factors (28).

We also found DNA hypermethylation surrounding genes of the WNT pathway and overexpressed in different types of cancer, either in the promoter region (*WNT3* and *SOX3*), within the gene body (*FZD1*, *FZD2*, *FZD7*, *SOX15*, and *SOX18*), or both (*FZD10*, *SOX11*, and *MDFI*).

Several hypermethylated WNT pathway genes showed differential gene expression in multiple pancreatic cancer cell lines versus HPDE either by Affymetrix Exon Array (*WNT5A*, *FZD2*, *SFRP1*, and *SOX15*) or by SAGE analysis (*WNT10A*, *SOX17*, *SOX18*, *NKD2*, *WNT18*, *FZD1*, and *FZD7*; Supplementary Table S2; refs. 17, 18).

Eleven other WNT pathway genes showed no differential expression by Affymetrix Microarray or SAGE analysis (Supplementary Table S2; refs. 17, 18), although 5-Aza-dC treatment increased expression of 7 of these 11 genes (*FZD9*, *APC2*, *SOX1*, *SOX3*, *SOX11*, *FRZB*, and *KREMEN2*; Supplementary Table S2), suggesting that DNA hypermethylation influences their expression.

Among the WNT Pathway hypermethylated gene members *WNT5A*, *SFRP1*, and *SOX15* are of particular interest because they had evidence of epigenetic silencing (loss of expression in pancreatic cancers relative to normal pancreas and induced expression with 5-Aza-dC treatment; Supplementary Fig. S1). *SFRP1* is epigenetically inactivated in colorectal and other cancers (29) and suspected to facilitate tumorigenesis by abrogating WNT signaling.

WNT5A is a member of the nontransforming WNT protein family frequently inactivated by tumor-specific methylation in colorectal cancer (30), although opposing roles of WNT5A in cancer have been described (31). For instance, one study found *WNT5A* overexpression in pancreatic cancers (32) whereas our gene expression profiling found underexpression of *WNT5A* compared with HPDE cells (2.9-fold by Affymetrix Exon Array) and compared with normal pancreatic ductal epithelium by SAGE. Another identified hypermethylated and downregulated gene implicated in pancreatic differentiation was *SOX15* (33). To our knowledge, hypermethylation and silencing of *SOX15* have not been previously reported in pancreatic cancer.

The finding that the WNT signaling pathway is a common target of DNA methylation in colon and other cancers led Jones and Baylin (34) to propose that loss of epigenetic gatekeepers such as WNT signaling pathway inhibitors locks cells into stem-cell like states that foster abnormal clonal expansion. The pancreatic cancer

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

genome project provided evidence for aberrant WNT pathway signaling during pancreatic tumorigenesis as somatic mutations in the pathway were occasionally identified in pancreatic cancers (17). In this study, the enrichment of aberrant methylation of the WNT pathway gene members supports a significant role for alterations of this pathway during pancreatic tumorigenesis.

Hypermethylation of cell adhesion molecules during pancreatic carcinogenesis

Numerous genes involved in cell adhesion and junctions were also hypermethylated in pancreatic cancer samples, including members of the cadherin superfamily *CDH2*, *CDH4*, *CDH11*, *PCDH1*, *PCDH10*, *PCDH11Y*, *PCDH17*, *PCDH8*, *PCDHB7*, *PCDHGA6*, *PCDHGB1*, *PCDHGB6*, *PCDHGC4*, and *RET*. Interestingly, the pancreatic cancer genome project identified occasional somatic mutations in cadherins and protocadherins in pancreatic cancers (17). Of these genes, hypermethylation of *CDH4* and *PCDHGB6* was associated with gene underexpression in pancreatic cancers by Affymetrix Array or SAGE data, respectively (Table 1 and Supplementary Table S2).

Hypermethylation of gene clusters and families as a common feature of human cancer

We found that numerous families of genes were simultaneously hypermethylated in our pancreatic cancer samples compared with normal pancreas (Supplementary Table S2, bold genes). These include families of transcription factors and, in particular, homeobox genes clustered on chromosome 7p15.2 (*EVX1*, *HOXA10*, *HOXA11*, *HOXA4*, *HOXA5*, *HOXA7*, and *HOXA9*), on 17q21.32 (*HOXB1*, *HOXB3*, *HOXB5*, *HOXB6*, and *HOXB9*), on 12q13.13 (*HOXC11*, *HOXC12*, *HOXC13*, *HOXC4*, and *HOXC5*), on 2q31.1 (*EVX2*, *HOXD1*, *HOXD10*, *HOXD11*, *HOXD12*, *HOXD4*, *HOXD8*, and *HOXD9*; Supplementary Fig. S2). These also include chromobox genes on 17q25.3 (*CBX2*, *CBX4*, and *CBX8*), Iroquois homeobox genes on 16q12.2 (*IRX3*, *IRX5*, and *IRX6*) and on 5p15.33 (*IRX1*, *IRX2*, and *IRX4*), and SIX homeobox genes on 2p21 (*SIX2* and *SIX3*).

Hypermethylation of clusters of homeobox genes has been reported in other types of cancer, such as lung cancer and astrocytomas (35, 36), but not in pancreatic cancers. Hypermethylation of a gene region such as the homeobox genes is most likely related to deregulation of the well-orchestrated epigenetic machinery involved in the spatiotemporal regulation of these developmental genes. This finding supports other evidence that gene neighborhood is required for an efficient coordination of chromatin modifications during carcinogenesis and developmental processes (37, 38). An important contributor to these epigenetic marks is the Polycomb group of proteins, which has been shown to be responsible for the concerted silencing of homeobox gene clusters. From our hypermethylated candidate gene list, we found 288 known targets of Polycomb proteins (Supplementary Table S2), including families of highly homologous genes that do not belong to clusters, such as LIM homeobox, NK2/3/6 homeobox, paired box, POU class homeobox, sex-determining region Y-box, and T-box transcription factors, which confirms the very tight link between specific hypermethylation and Polycomb protein complexes occupancy in cancer. However, we found aberrant hypermethylation of several other gene family members including members of the G-protein–coupled receptor superfamily, adenylate cyclases, and voltage-dependant calcium and potassium channels. Sequence similarities between these family members are likely responsible for their similar cancer epigenetic patterns. For example, SINE and LINE repetitive elements are enriched at sites of aberrant hypermethylation (38). These sequence-based contributions to aberrant methylation indicate that selection driven by gene silencing need is not responsible for causing methylation marks to arise during tumorigenesis. The fact that only a relatively small proportion of hypermethylated genes showed evidence of downregulation or silencing (*HOXD9*, *HOXD8*, *CBX2*, *IRX2*, *IRX4*, *TBX18*, *SOX15*, *SOX17*, *SOX18*, and *PAX8*; Supplementary Table S2) supports this hypothesis.

Newly identified hypermethylated and underexpressed candidate genes

Of the 81 hypermethylated and underexpressed genes identified by our analysis, 33 have, to our knowledge, not been previously identified as hypermethylated either in pancreatic or other cancers. The differential methylation of several of these genes including *ID4*, *FOXF2*, *FOXG1*, *IRF5*, *SIM1*, *SMOC2*, and *ZBTB16* was confirmed by MSP (Supplementary Table S4). Twelve genes showed gene silencing in pancreatic cancer compared with normal pancreatic duct by SAGE (including *HOXD8*, *BNIP3*, *FOXF2*, and *ID4*), many of which have not been identified as differentially methylated in cancer (Table 1).

Many hypermethylated and underexpressed genes identified have been implicated in tumorigenesis including tumor suppression (*RINT1*; ref. 39), proliferation (*GAS6*, *CDT1*; ref. 40), apoptosis (*IRF5*; ref. 41; *C16ORF5*/*CDIP*; ref. 42), cell adhesion (*CLDN10*, *ICAM4*, *LAMA1*, *AMOTL1*), or transcriptional regulation (*FOXG1*, *INSM2*). Interestingly, although SEMA5A was recently proposed as a marker for aggressive pancreatic cancers, we find most pancreatic cancers underexpressed SEMA5A (43). Other genes of note include the type IV collagen genes *COL4A1* and *COL4A2* which have not been described as aberrantly methylated in cancer.

Finally, to further evaluate genes identified as both hypermethylated and underexpressed by array analysis, we searched for commercially available antibodies to proteins corresponding to genes on our list (Table 1) and selected an antibody to ZBTB16 for further analysis. The transcription factor ZBTB16 (a.k.a PLZF), has been shown to regulate apoptosis and proliferation, as well as

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

the Hox family of genes during development, and has been proposed as a candidate tumor suppressor gene in acute promyelocytic leukemias. By MSP, we confirmed *ZBTB16* promoter methylation in 35.2% of the pancreatic cancer samples tested, whereas none of the DNAs from 16 normal pancreas samples showed methylation. By immunohistochemistry, ZBTB16 protein expression was not detectable in 38 (15.4%) of 247 pancreatic cancers evaluated. Reduced expression relative to normal pancreas was found in the majority of pancreatic cancers (193/211, 94%; mean histoscore 2.7 vs. 6.9, $P < 0.0001$; Supplementary Fig. S3). Interestingly, aberrant methylation and silencing of *ZBTB16* in pancreatic cancers may be functionally significant as it functions to repress miR-221 and miR-222 which is overexpressed in pancreatic cancer cells (44). MiR-221/222 expression is associated with enhanced proliferation and dedifferentiation in melanoma cells (45).

Newly identified hypomethylated and overexpressed candidate genes

Because hypomethylation is associated with gene overexpression in pancreatic and other cancers (9), we also identified candidate hypomethylated genes by using the MCAM assay. Of the 379 unique hypomethylated candidate genes identified, 31 have been described as aberrantly methylated in different cancers (Supplementary Table S3). This list confirmed the aberrant hypomethylation of *NDN* and *SMARCA1* genes, previously described in pancreatic cancer (14). Similarly to hypermethylated genes, some known hypomethylated genes did not meet our criteria for frequent hypomethylation. However, when we examined the NLR for probes covering *MUC4* and *MUC2*, genes previously identified as hypomethylated in pancreatic cancer (46), there was evidence for hypomethylation (NLR ≤ -2) in at least one of the 9 differential methylomes of pancreatic cancer/normal pancreas studied. The promoter regions of other hypomethylated genes in pancreatic cancers (*S100A4*, *LCN2*, *TFF2*, *MSLN CLDN4*, *SFN*, and *PSCA*; 9) were not interrogated by our MCAM method.

Interestingly, while approximately half of the hypermethylated candidate genes had multiple probes meeting criteria for hypermethylation, the vast majority (84.7%) of hypomethylated gene candidates harbored only 1 hypomethylated probe (mean of the 9 NLR ≤ -2 and mean combined Z scores was ≤ -5; Supplementary Table S3).

Our hypomethylated gene list contained chromatin modifiers, including the histone methyltransferases *SETD8* (lysine) and *PRMT1* (arginine), the histone ubiquitin ligase *CTR9*, the histone demethylase *KDM6A*, the chromatin assembly protein HIRIP3, and the histone acetyltransferase EP400. These genes were overexpressed in pancreatic cancer samples compared with HPDE cells and/or normal ductal epithelium by SAGE, and *HIRIP3* was found to be neoexpressed in 41% of pancreatic cancer samples (Supplementary Table S3; ref. 17).

Three oncogenes, *JUNB*, *MYB*, and *FOS* were hypomethylated in our analysis. Although *FOS* undergoes hypomethylation during liver tumorigenesis, *JUNB* and *MYB* have not been previously reported to be hypomethylated. Interestingly, MYB was found to be neoexpressed in 31% of pancreatic cancer compared with normal samples by SAGE (Supplementary Table S3; ref. 17).

Of the 44 hypomethylated genes identified in our analysis and found to be overexpressed by both ExonArray and

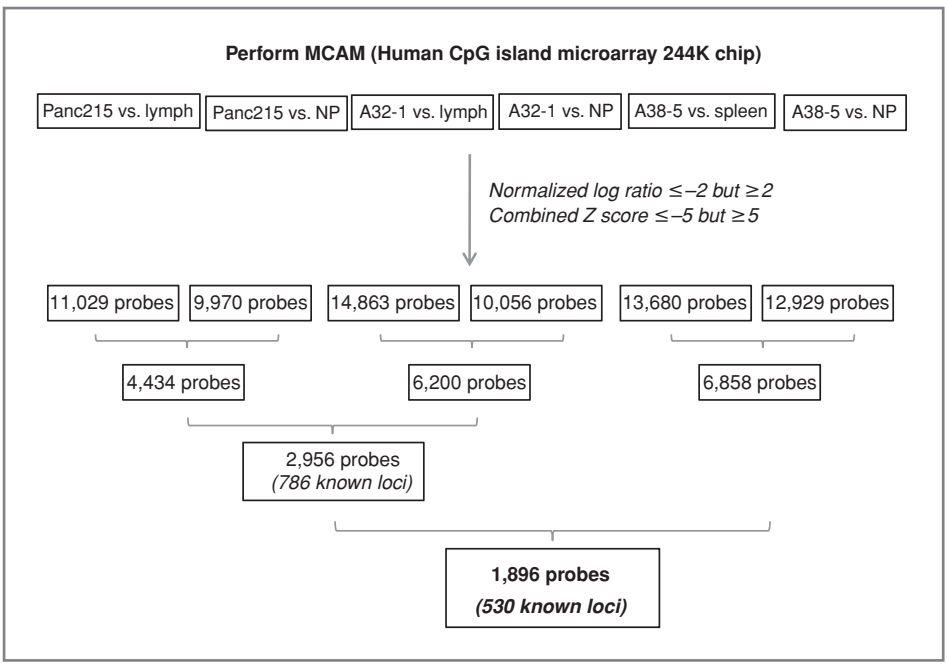

Figure 4. An overview of experimental strategy to evaluate differential DNA methylation in pancreatic cancer cells compared with normal lymphoid tissue. NP, microdissected normal pancreatic ductal epithelium; lymph, peripheral blood lymphocytes.

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

SAGE methods, 34 have, to our knowledge, not been described as aberrantly methylated in cancer. Among them, *DDHD2*, *CREB1*, and *PIM2* have been proposed as oncogenes, whereas *ITGB2* (CD18) and *MAPRE2* are involved in cell invasion and metastasis. *HELLS* is a putative stem cell marker, whereas MTRR restores methionine synthase for folate metabolism and polymorphisms have been associated with pancreatic cancer risk.

Differentially methylated loci of pancreatic cancer samples compared with normal lymphoid tissues

To further validate our list of hyper- and hypomethylated candidate markers, we conducted 3 additional MCAM experiments, in which we compared the methylation profiles of pancreatic cancer cell samples with matched normal DNA either from peripheral blood lymphocytes (A32-1 and Panc215) or from normal spleen (A38-5).

We used the cutoffs described above for the NLR and combined Z score to select probes that were hyper- or hypomethylated in pancreatic cancer cells compared with matched lymphocytes or spleen, respectively (Fig. 4).

We found 11,029 and 14,863 differentially methylated probes in Panc215 and A32-1 samples compared with matched lymphocytes DNA of the patient, respectively. We found 13,680 differentially methylated probes in A38-5 cancer cells compared with their matched normal spleen. We then compared these probes with those differentially methylated in Panc215, A32-1, and A38-5 cancer cells versus the same pooled microdissected normal pancreatic ductal tissue. Because some methylation patterns reflect tissue specific differences, we found more differentially methylated probes when comparing pancreatic cancer DNA with matched lymphoid tissue of a patient than to unmatched normal pancreatic duct. We found 4,434 and 6,200 differentially methylated probes both in cancer compared with lymphocytes and in cancer compared with normal pancreatic tissue, for Panc215 and A32-1 samples, respectively. Moreover, we found differentially methylated 2,956 probes in both Panc215 and A32-1 samples, and both in cancer compared with lymphocytes and cancer compared with normal pancreatic tissue, corresponding to 786 known loci (Fig. 4). Among these loci, 669 unique genes were part of our hypermethylated and hypomethylated candidate lists (Supplementary Tables S2 and S3). These data indicate that while more than half of our differentially methylated candidate genes in pancreatic cancers relative to normal pancreata are also differential methylated relative to peripheral blood lymphocytes, many genes that are methylated in pancreatic cancers are normally methylated in other tissues.

We found differentially methylated 6,858 probes that were both in cancer compared with spleen and cancer compared with normal pancreatic tissue (Fig. 4). Among these probes, 1,896 were also differentially methylated in pancreatic cancer cells compared with normal pancreatic tissue and in pancreatic cancer cells compared with lymphocytes. These corresponded to 530 known loci, and 489 genes present in our hypermethylated and hypomethylated gene lists (Supplementary Tables S2 and S3).

Among these 489 genes, 32 genes show DNA hypermethylation associated with downregulation in cancer cells compared with normal cells (Table 1). These latter genes, with a more specific hypermethylation profiles in pancreatic cancer tissues are likely to be better candidates as pancreatic cancer markers. Indeed, several of these genes have already been proposed as candidate biomarkers of prognosis (*BNC1*; ref. 47; *SLIT2*; ref. 48), or response to treatment (*WNT5A*; 49) in other types of cancer.

Conclusion

In summary, we provide a comprehensive list of candidate aberrantly methylated in pancreatic cancers relative to normal pancreatic duct samples, including novel aberrantly methylated and epigenetically silenced genes validated in pancreatic cancer tissue samples, as well as commonly methylated signaling pathways and gene families. We anticipate that our integrated analysis of pancreatic cancer gene methylation and transcription will facilitate the further investigation of pancreatic cancer biomarkers for diagnostic, prognostic, and therapeutic applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by the National Cancer Institute grants (CA62924, CA120432, and RC2CA148346) and the Michael Rolfe Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 24, 2010; revised April 15, 2011; accepted May 12, 2011; published OnlineFirst May 24, 2011.

References

1. Hruban RH, Canto MI, Goggins M, Schulick R, Klein AP. Update on familial pancreatic cancer. Adv Surg 2010;44:293–311.
2. Jones S, Hruban RH, Kamiyama M, Borges M, Zhang X, Parsons DW, et al. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. Science 2009;324:217.
3. Matsubayashi H, Canto M, Sato N, Klein A, Abe T, Yamashita K, et al. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. Cancer Res 2006;66:1208–17.
4. Parsi MA, Li A, Li CP, Goggins M. DNA methylation alterations in endoscopic retrograde cholangiopancreatography brush samples of patients with suspected pancreaticobiliary disease. Clin Gastroenterol Hepatol 2008;6:1270–8.
5. Canto MI, Goggins M, Hruban RH, Petersen GM, Giardiello FM, Yeo C, et al. Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study. Clin Gastroenterol Hepatol 2006;4:766–81; quiz 665.

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

6. Fukushima N, Sato N, Ueki T, Rosty C, Walter KM, Wilentz RE, et al. Aberrant methylation of preproenkephalin and p16 genes in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma. Am J Pathol 2002;160:1573–81.
7. Sato N, Fukushima N, Chang R, Matsubayashi H, Goggins M. Differential and epigenetic gene expression profiling identifies frequent disruption of the RELN pathway in pancreatic cancers. Gastroenterology 2006;130:548–65.
8. Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, et al. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. Cancer Res 2003;63:3735–42.
9. Sato N, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, et al. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res 2003;63:4158–66.
10. Sato N, Matsubayashi H, Abe T, Fukushima N, Goggins M. Epigenetic down-regulation of CDKN1C/p57KIP2 in pancreatic ductal neoplasms identified by gene expression profiling. Clin Cancer Res 2005;11:4681–8.
11. Sato N, Ueki T, Fukushima N, Iacobuzio-Donahue CA, Yeo CJ, Cameron JL, et al. Aberrant methylation of CpG islands in intraductal papillary mucinous neoplasms of the pancreas. Gastroenterology 2002;123:365–72.
12. Hong SM, Kelly D, Griffith M, Omura N, Li A, Li CP, et al. Multiple genes are hypermethylated in intraductal papillary mucinous neoplasms of the pancreas. Mod Pathol 2008;21:1499–507.
13. Omura N, Li CP, Li A, Hong SM, Walter K, Jimeno A, et al. Genome-wide profiling of methylated promoters in pancreatic adenocarcinoma. Cancer Biol Ther 2008;7:1146–56.
14. Tan AC, Jimeno A, Lin SH, Wheelhouse J, Chan F, Solomon A, et al. Characterizing DNA methylation patterns in pancreatic cancer genome. Mol Oncol 2009;3:425–38.
15. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome–-components and functional correlates. Genes Dev 2006;20:3215–31.
16. Estecio MR, Yan PS, Ibrahim AE, Tellez CS, Shen L, Huang TH, et al. High-throughput methylation profiling by MCA coupled to CpG island microarray. Genome Res 2007;17:1529–36.
17. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321:1801–6.
18. Omura N, Griffith M, Vincent A, Li A, Hong SM, Walter K, et al. Cyclooxygenase-deficient pancreatic cancer cells utilize exogenous sources of prostaglandins. Mol Cancer Res 2010;8:821–32.
19. Walter K, Omura N, Hong SM, Griffith M, Vincent A, Borges M, , et al. Overexpression of smoothened activates the sonic hedgehog signaling pathway in pancreatic cancer-associated fibroblasts. Clin Cancer Res 2010;16:1781–9.
20. Kumagai T, Akagi T, Desmond JC, Kawamata N, Gery S, Imai Y, et al. Epigenetic regulation and molecular characterization of C/EBPalpha in pancreatic cancer cells. Int J Cancer 2009;124:827–33.
21. Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 2000;60:1835–9.
22. Matsubayashi H, Sato N, Fukushima N, Yeo CJ, Walter KM, Brune K, et al. Methylation of cyclin D2 is observed frequently in pancreatic cancer but is also an age-related phenomenon in gastrointestinal tissues. Clin Cancer Res 2003;9:1446–52.
23. Sato N, Parker AR, Fukushima N, Miyagi Y, Iacobuzio-Donahue CA, Eshleman JR, et al. Epigenetic inactivation of TFPI-2 as a common mechanism associated with growth and invasion of pancreatic ductal adenocarcinoma. Oncogene 2005;24:850–8.
24. Omura N, Goggins M. Epigenetics and epigenetic alterations in pancreatic cancer. Int J Clin Exp Pathol 2009;2:310–26.
25. Schutte M, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK, et al. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res 1997;57:3126–30.
26. Sato N, Maehara N, Su GH, Goggins M. Effects of 5-aza-2′-deoxycytidine on matrix metalloproteinase expression and pancreatic cancer cell invasiveness. J Natl Cancer Inst 2003;95:327–30.
27. Ying Y, Tao Q. Epigenetic disruption of the WNT/beta-catenin signaling pathway in human cancers. Epigenetics 2009;4:307–12.
28. Valenta T, Lukas J, Doubravska L, Fafilek B, Korinek V. HIC1 attenuates Wnt signaling by recruitment of TCF-4 and beta-catenin to the nuclear bodies. EMBO J 2006;25:2326–37.
29. Caldwell GM, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, et al. The Wnt antagonist sFRP1 in colorectal tumorigenesis. Cancer Res 2004;64:883–8.
30. Ying J, Li H, Yu J, Ng KM, Poon FF, Wong SC, et al. WNT5A exhibits tumor-suppressive activity through antagonizing the Wnt/beta-catenin signaling, and is frequently methylated in colorectal cancer. Clin Cancer Res 2008;14:55–61.
31. McDonald SL, Silver A. The opposing roles of Wnt-5a in cancer. Br J Cancer 2009;101:209–14.
32. Ripka S, Konig A, Buchholz M, Wagner M, Sipos B, Kloppel G, et al. WNT5A–target of CUTL1 and potent modulator of tumor cell migration and invasion in pancreatic cancer. Carcinogenesis 2007;28:1178–87.
33. White P, May CL, Lamounier RN, Brestelli JE, Kaestner KH. Defining pancreatic endocrine precursors and their descendants. Diabetes 2008;57:654–68.
34. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.
35. Rauch T, Li H, Wu X, Pfeifer GP. MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. Cancer Res 2006;66:7939–47.
36. Wu X, Rauch TA, Zhong X, Bennett WP, Latif F, Krex D, et al. CpG island hypermethylation in human astrocytomas. Cancer Res 70:2718–27.
37. Soshnikova N, Duboule D. Epigenetic regulation of vertebrate Hox genes: a dynamic equilibrium. Epigenetics 2009;4:537–40.
38. Estecio MR, Gallegos J, Vallot C, Castoro RJ, Chung W, Maegawa S, et al. Genome architecture marked by retrotransposons modulates predisposition to DNA methylation in cancer. Genome Res
39. Lin X, Liu CC, Gao Q, Zhang X, Wu G, Lee WH. RINT-1 serves as a tumor suppressor and maintains Golgi dynamics and centrosome integrity for cell survival. Mol Cell Biol 2007;27:4905–16.
40. Liontos M, Koutsami M, Sideridou M, Evangelou K, Kletsas D, Levy B, et al. Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. Cancer Res 2007;67:10899–909.
41. Hu G, Barnes BJ. IRF-5 is a mediator of the death receptor-induced apoptotic signaling pathway. J Biol Chem 2009;284:2767–77.
42. Brown L, Ongusaha PP, Kim HG, Nuti S, Mandinova A, Lee JW, et al. CDIP, a novel pro-apoptotic gene, regulates TNFalpha-mediated apoptosis in a p53-dependent manner. EMBO J 2007;26:3410–22.
43. Sadanandam A, Varney ML, Singh S, Ashour AE, Moniaux N, Deb S, et al. High gene expression of semaphorin 5A in pancreatic cancer is associated with tumor growth, invasion and metastasis. Int J Cancer 127;1373–83.
44. Kent OA, Mullendore M, Wentzel EA, Lopez-Romero P, Tan AC, Alvarez H, et al. A resource for analysis of microRNA expression and function in pancreatic ductal adenocarcinoma cells. Cancer Biol Ther 2009;8:2013–24.
45. Felicetti F, Errico MC, Bottero L, Segnalini P, Stoppacciaro A, Biffoni M, et al. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. Cancer Res 2008;68:2745–54.
46. Vincent A, van Seuningen I. Mucins, epigenetics and cancer. In: van Seuningen I, editor. The epitheliul mucins: structure/function roles in cancer and inflammatory diseases. Kerala: Research Signpost; 2008. p. 95–108.

Published OnlineFirst May 24, 2011; DOI: 10.1158/1078-0432.CCR-10-3431

47. Morris MR, Ricketts C, Gentle D, Abdulrahman M, Clarke N, Brown M, et al. Identification of candidate tumour suppressor genes frequently methylated in renal cell carcinoma. Oncogene 29;2104–17.
48. Tseng RC, Lee SH, Hsu HS, Chen BH, Tsai WC, Tzao C, et al. SLIT2 attenuation during lung cancer progression deregulates beta-catenin and E-cadherin and associates with poor prognosis. Cancer Res 2010;70:543–51.
49. Ford CE, Ekstrom EJ, Andersson T. Wnt-5a signaling restores tamoxifen sensitivity in estrogen receptor-negative breast cancer cells. Proc Natl Acad Sci U S A 2009;106:3919–24.

AACR American Association for Cancer Research

Clinical Cancer Research

Genome-Wide Analysis of Promoter Methylation Associated with Gene Expression Profile in Pancreatic Adenocarcinoma

Audrey Vincent, Noriyuki Omura, Seung-Mo Hong, et al.

Clin Cancer Res 2011;17:4341-4354. Published OnlineFirst May 24, 2011.