Frequent Hypomethylation of Multiple Genes Overexpressed in Pancreatic Ductal Adenocarcinoma

Norihiro Sato, Anirban Maitra, Noriyoshi Fukushima, N. Tjarda van Heek, Hiroyuki Matsubayashi, Christine A. Iacobuzio-Donahue, Christophe Rosty, and Michael Goggins

Departments of Pathology [N. S., A. M., N. F., N. T. v. H., M. A. I-D., C. R., M. G.], Oncology [M. G.], and Medicine [M. G.], The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205

ABSTRACT

To investigate the relationship between DNA hypomethylation and gene overexpression in pancreatic cancer, we analyzed the methylation status of a subset of 18 genes previously identified by global gene expression studies as overexpressed in pancreatic cancer tissues compared with normal pancreas. For comparison, we determined the methylation status of 14 genes not known to be overexpressed in pancreatic cancer. Methylation-specific PCR analysis revealed that 19 of these 32 genes were methylated at their 5’ CpGs in normal pancreas. We then analyzed these 19 genes for their methylation pattern in pancreatic cancers and found that all 7 of the genes (claudin4, lipocalin2, 14-3-3z, trefoil factor2, S100A4, mesothelin, and prostate stem cell antigen) that were overexpressed in the neoplastic cells of pancreatic cancers and not expressed in normal pancreatic duct displayed a high prevalence of hypomethylation in pancreatic cancer cell lines and primary pancreatic carcinomas. By contrast, only 1 of 12 genes not overexpressed in pancreatic cancer demonstrated hypomethylation (P = 0.0002). In pancreatic cancer cell lines that retained methylation of 1 or more of the 7 aforementioned overexpressed and hypomethylated genes, treatment with 5-aza-2’-deoxycytidine or with trichostatin A, either alone or in combination, almost invariably reactivated the transcription of each of these 7 genes. These results indicate that gene hypomethylation is a frequent epigenetic event in pancreatic cancer and is commonly associated with the overexpression of affected genes.

INTRODUCTION

Ductal adenocarcinoma of the pancreas is the fifth leading cause of cancer death in the United States (1) and is one of the most aggressive cancers. Despite overall improvements in diagnostic imaging and molecular techniques, most pancreatic cancers are still not diagnosed until after they have metastasized beyond the gland. New sensitive and specific markers for pancreatic cancer are urgently needed (2, 3). In an effort to identify such biomarkers and to better understand the biology of pancreatic cancer, several groups have undertaken large-scale analyses of global gene expression profiles of the disease (4–6). SAGE3 and oligonucleotide and cDNA arrays have been used to identify a large set of genes expressed at higher levels in pancreatic cancer tissues compared with normal pancreas (7–9). However, the mechanism for the overexpression of many of these genes is not known.

The importance of DNA methylation in the transcriptional silencing of cancer-associated genes is increasingly recognized (10). For example, a variety of tumor suppressor, growth regulatory, and mismatch repair genes, including those encoding p16, preproenkephalin, and hMHL14, are inactivated by promoter region hypermethylation in benign and malignant pancreatic neoplasms (11–14). Genome-wide hypomethylation has also been described in various human cancers (15–17) and has been associated with genetic instability characterized by chromosomal aberrations or elevated mutation rates (18, 19). The genome-wide DNA hypomethylation found in cancers is prevalent in normally methylated repeat sequences (reviewed in Ref. 20). DNA hypomethylation at unique sequences could also increase expression of cancer-promoting genes (20). In fact, a correlation between site-specific hypomethylation and transcriptional activation has been observed for several genes such as MAGE (21, 22), S100A4 (23), synuclein γ (24), and other genes (20). In addition, the extent of hypomethylation appears to correlate with tumor grade and with prognosis in certain cancers (25). These findings suggest an important role of DNA hypomethylation in the overexpression of genes in human cancers, but an understanding of the global pattern of hypomethylated genes at multiple loci has been limited by the few genes analyzed to date.

To address this issue, we analyzed the methylation status of a panel of genes that are expressed at higher levels in ductal adenocarcinoma of the pancreas and not known to be expressed in normal pancreatic tissue and compared these methylation patterns to those of a group of genes that are not overexpressed in pancreatic cancer.

MATERIALS AND METHODS

Cell Lines, Xenografts, and Tissue Samples. Human pancreatic cancer cell lines AsPC1, BxPC3, CAPAN1, CAPAN2, CFPAC1, Hs766T, MiaPaCa2, and Panc1 were obtained from the American Type Culture Collection (Manassas, VA) and Colo357 from ECACC (Salisbury, United Kingdom). Eleven low-passage pancreatic carcinoma cell lines (PL series) were generously provided by Dr. Elizabeth Jaffe. An immortal human pancreatic duct epithelial cell line, HPDE, was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada). Pancreatic cancer xenografts were established from surgically resected primary pancreatic carcinomas (26), and 37 xenografts were randomly selected for this study. A total of 28 frozen pancreatic tissues (8 adenocarcinoma and 20 normal tissues) was obtained from surgical specimens resected at The Johns Hopkins Medical Institutions. In 8 primary pancreatic carcinoma samples and 7 of the 20 normal pancreatic samples, tumor cells and normal duct epithelial cells were selectively microdissected using a LCM system (PixCell II; Arcturus, Mountain View, CA).

cDNA Microarray and Data Analysis. A comprehensive analysis of global gene expression profiles of pancreatic cancer using cDNA microarrays and significance analysis of microarrays was performed as described elsewhere (9). This study involved the analysis of 31 pancreatic cancers, including 14 pancreatic cancer cell lines and 17 primary pancreatic cancers, as well as 5 samples of normal pancreas tissue.

Sequencing Analysis of Bisulfite-treated Genomic DNA. Genomic DNA was isolated from the cell lines and frozen tissues using a DNA isolation kit (Qiagen, Valencia, CA) and subjected to sodium bisulfite treatment. Sequencing of bisulfite-treated DNA samples was performed as described previously (12). Primers were designed to detect the sequence differences between methylated and unmethylated DNA as a result of bisulfite
modification, and each primer pair contained at least three CpG sites to provide optimal specificity. Because knowledge of the regulatory regions of many of the genes overexpressed in pancreatic cancer is not known, we decided to characterize the methylation status of CpGs within a few hundred bp of the transcriptional start site, where CpG methylation has been implicated in transcriptional silencing (27). The primer sequences for bisulfite sequencing and MSP are available upon request.

Oligonucleotide Array Hybridization and Data Analysis. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA). First- and second-stranded cDNA were synthesized from 10 μg of total RNA using T7-(dT)24 primer (Genset Corp., South La Jolla, CA) and SuperScript Choice system (Invitrogen). Labeled cRNA was synthesized from the purified cDNA by *in vitro* transcription reaction using the RNA Transcript Labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY) at 37°C for 6 h and was purified using RNeasy Mini Kit (Qiagen). The cRNA was fragmented at 94°C for 35 min in a fragmentation buffer [40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate]. The fragmented cRNA was then hybridized to the Human Genome U133A chips (Affymetrix, Santa Clara, CA) with 18,462 unique gene/expressed sequence tag transcripts at 45°C for 16 h. The probes were then scanned using a laser scanner, and signal intensity for each transcript (background-subtracted and adjusted for noise) and detection call (present, absent, or marginal) were determined using Microarray Suite Software 5.0 (Affymetrix).

Semiquantitative RT-PCR. RT-PCR was performed with primers for the specific genes (the primer sequences are available upon request) and for GAPDH in duplex reactions. The range of linear amplification for each gene and the GAPDH gene was examined with serial PCR cycles, and the optimal PCR cycles were determined. The relative intensity of mRNA expression for each sample was then corrected for variable RNA recovery using the corresponding GAPDH mRNA measurement as a surrogate for total mRNA.

Inhibition of DNA Methylation and Histone Deacetylase Inhibition. We treated two pancreatic cancer cell lines (MiaPaCa2 and Hs766T) with 5AzadC (Sigma, St. Louis, MO) and TSA (Sigma), either alone or in combination. Cells were exposed continuously to 5AzadC (1 μM) for 4 days or TSA (1 μM) for 24 h. For combined treatment, these cells were cultured in the presence of 5AzadC (1 μM) for 3 days and were then treated for another 24 h with TSA (0.5 μM).

Statistical Analysis. Statistical analysis was performed using Mann-Whitney U nonparametric test, Student’s *t* test, or Fisher’s exact probability test. Differences were considered significant at *P* < 0.05.

RESULTS

The Identification of Genes Overexpressed in Pancreatic Cancer and Validation of Their Expression Patterns. A summary of the overall strategy we used for selecting and analyzing genes and a summary of our results is provided in Fig. 1. We selected a previously published strategy we used for selecting and analyzing genes and a summary of our results in a previous study (27). Differences were considered significant at *P* < 0.05.

Oligonucleotide Array Hybridization and Data Analysis. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA). First- and second-stranded cDNA were synthesized from 10 μg of total RNA using T7-(dT)24 primer (Genset Corp., South La Jolla, CA) and SuperScript Choice system (Invitrogen). Labeled cRNA was synthesized from the purified cDNA by *in vitro* transcription reaction using the RNA Transcript Labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY) at 37°C for 6 h and was purified using RNeasy Mini Kit (Qiagen). The cRNA was fragmented at 94°C for 35 min in a fragmentation buffer [40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate]. The fragmented cRNA was then hybridized to the Human Genome U133A chips (Affymetrix, Santa Clara, CA) with 18,462 unique gene/expressed sequence tag transcripts at 45°C for 16 h. The probes were then scanned using a laser scanner, and signal intensity for each transcript (background-subtracted and adjusted for noise) and detection call (present, absent, or marginal) were determined using Microarray Suite Software 5.0 (Affymetrix).

Semiquantitative RT-PCR. RT-PCR was performed with primers for the specific genes (the primer sequences are available upon request) and for GAPDH in duplex reactions. The range of linear amplification for each gene and the GAPDH gene was examined with serial PCR cycles, and the optimal PCR cycles were determined. The relative intensity of mRNA expression for each sample was then corrected for variable RNA recovery using the corresponding GAPDH mRNA measurement as a surrogate for total mRNA.

Inhibition of DNA Methylation and Histone Deacetylase Inhibition. We treated two pancreatic cancer cell lines (MiaPaCa2 and Hs766T) with 5AzadC (Sigma, St. Louis, MO) and TSA (Sigma), either alone or in combination. Cells were exposed continuously to 5AzadC (1 μM) for 4 days or TSA (1 μM) for 24 h. For combined treatment, these cells were cultured in the presence of 5AzadC (1 μM) for 3 days and were then treated for another 24 h with TSA (0.5 μM).

Statistical Analysis. Statistical analysis was performed using Mann-Whitney U nonparametric test, Student’s *t* test, or Fisher’s exact probability test. Differences were considered significant at *P* < 0.05.

RESULTS

The Identification of Genes Overexpressed in Pancreatic Cancer and Validation of Their Expression Patterns. A summary of the overall strategy we used for selecting and analyzing genes and a summary of our results is provided in Fig. 1. We selected a previously published strategy we used for selecting and analyzing genes and a summary of our results in a previous study (27). Differences were considered significant at *P* < 0.05.
Genomic sequencing was performed on 4 selected genes (pattern of genes overexpressed in pancreatic cancer, bisulfite expressed in Pancreatic Cancer.

To determine the methylation of these 14 genes in pancreatic cancer (data not shown).

Previously described as harboring methylation in normal tissues (36–48). Gene expression data from oligonucleotide or cDNA microarray and SAGE databases showed no evidence for overexpression of any of these 14 genes in pancreatic cancer (data not shown).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
<th>SAGE</th>
<th>RT-PCR</th>
<th>IHC and/or ISH</th>
<th>Previous publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin 4</td>
<td>Tight junction barrier function</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>LCN2, NGAL</td>
<td>Cell regulation</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Mesothelin</td>
<td>tAP-1/1B anchor/cell adhesion</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PSCA</td>
<td>p53-anchored/prostate cancer antigen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SHH4A</td>
<td>Ca-binding protein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Somatic/14-3-3r</td>
<td>G2 cell cycle arrest</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TFF2</td>
<td>Secretory polypeptide/exocytic repair</td>
<td></td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>BarH-like homeobox 1</td>
<td>Homeobox gene; central nervous</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CDH11, type 2, OB-cadherin</td>
<td>Cell adhesion molecule</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>fer-1 (Gaurnothobadis elegans)-like 3</td>
<td>Candidate gene for muscular dystrophy</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Amphiregulin</td>
<td>Epithelial growth factor-family; autocrine growth factor</td>
<td></td>
<td></td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Caveolin 2</td>
<td>Caveolar protein/endocytosis</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cornichon-like</td>
<td>T-cell-related protein</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Inhibin, β-B-subunit</td>
<td>β Subunit of inhibin and activin</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mal, T-cell differentiation 2</td>
<td>Vascular transport</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Met proto-oncogene (HGF receptor)</td>
<td>HGF receptor; tyrosine kinase</td>
<td></td>
<td></td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Thyroid hormone receptor interactor 13</td>
<td>Thyrothrin receptor interacting protein</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TGF, β-induced, 68 k</td>
<td>ECM protein; cellular adhesion</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Genes from literature

Oxytocin receptor gene, proopiomelanocortin, monocarboxylate transporter 3, paired box gene 5, lactate dehydrogenase B, LEP503, cytochrome P450 (CYP19), uncoupling protein homologue, uroplakin 1B, and thyroid transcription factor-1. These 14 genes were chosen for methylation analysis because of their limited tissue distribution and cell lineage-specific expression, and many of them have previously been described as harboring methylation in normal tissues (36–48).

Gene expression data from oligonucleotide or cDNA microarray and SAGE databases showed no evidence for overexpression of any of these 14 genes in pancreatic cancer (data not shown).

Methylation Analysis of Multiple Genes in Normal Pancreas.

We next used MSP to analyze the methylation status of all 32 genes in a panel of normal pancreatic tissues. Overall, 19 (59%) of these 32 genes were methylated in normal pancreatic tissues, and 13 genes were completely unmethylated (Table 1). Of note, normal pancreata that were microdissected manually showed amplification of both methylated and unmethylated alleles for several genes, including mesothelin, TFF2, and claudin4, whereas most of the 7 samples of duct epithelia microdissected by LCM were almost completely methylated (Fig. 2B). Despite the predominant methylation in these LCM-microdissected ductal cells, some of the genes (claudin4, LCN2, 14-3-3r, and TFF2) were unmethylated in an immortal human pancreatic duct epithelial cell line (HPDE). Consistent with this, claudin4, 14-3-3r, and TFF2 mRNA were expressed at variable levels in HPDE by RT-PCR (data not shown). A possible explanation for this discrepancy is that the methylation status of these genes may have been altered during immortalization with the E6 and E7 genes of papillomavirus.

Methylation Analysis of Multiple Genes in Pancreatic Cancer Cell Lines.

The 19 genes that harbored methylation of 5’ CpGs in normal pancreas were then tested for hypomethylation in a panel of 20 pancreatic cancer cell lines. On the basis of the results from normal pancreatic duct epithelium, we defined a gene as hypomethylated if only the unmethylated templates were amplified. MSP analyses revealed that 8 of 19 genes showed hypomethylation in pancreatic cancer cell lines (Fig. 2C). These included 14-3-3r (hypomethylated in 85% of 20 cell lines), claudin4 (85%), LCN2 (85%), TFF2 (65%), S100A4 (50%), mesothelin (40%), PSCA (30%), and CDH11 (20%).
Thus, among the 19 genes normally methylated in the pancreas, all 7 of the genes that we were able to confirm as overexpressed in pancreatic cancer showed hypomethylation in pancreatic cancer cell lines. By contrast, only 1 (CDH11) of 12 genes not overexpressed in pancreatic cancer showed hypomethylation and then only in 4 of 20 pancreatic cancer cell lines (P = 0.0002, Fisher’s exact test).

To determine the pattern of this epigenetic alteration for each cell line, we investigated the hypomethylation profile of these 8 genes in 20 pancreatic cancer cell lines (Fig. 3). Overall, 19 of 20 (95%) pancreatic cancer cell lines showed hypomethylation in at least one gene, and 18 of 20 (90%) had hypomethylation in 2 or more of the target genes. These results suggest that DNA hypomethylation of overexpressed genes is a frequent epigenetic event in pancreatic cancer cell lines. One pancreatic cancer cell line (Hs766T) that lacked hypomethylation in 8 genes in pancreatic cancer cell lines and their level of mRNA expression of the genes that we were able to confirm as overexpressed in pancreatic cancer showed hypomethylation and then only in 4 of 20 pancreatic cancer cell lines (P = 0.0002, Fisher’s exact test).

To elucidate the relationship between the methylation status of hypomethylated genes in pancreatic cancer cell lines and their level of mRNA expression, semiquantitative RT-PCR was performed on the 8 hypomethylated genes. In 7 of 8 genes, the mean expression levels relative to GAPDH were higher in cell lines with hypomethylation than in those without hypomethylation, and the difference was statistically significant for claudin4 (P = 0.01, Mann-Whitney U test), LCN2 (P = 0.008), TFF2 (P = 0.01), S100A4 (P = 0.001), PSCA (P = 0.001), but not for 14-3-3-σ (P = 0.3) or mesothelin (P = 0.1; Fig. 4). For both 14-3-3-σ and mesothelin, we did observe a trend

**Correlation between Methylation Status and mRNA Expression of Genes Hypomethylated in Pancreatic Cancer.** To elucidate the relationship between the methylation status of hypomethylated genes in pancreatic cancer cell lines and their level of mRNA expression, semiquantitative RT-PCR was performed on the 8 hypomethylated genes. In 7 of 8 genes, the mean expression levels relative to GAPDH were higher in cell lines with hypomethylation than in those without hypomethylation, and the difference was statistically significant for claudin4 (P = 0.01, Mann-Whitney U test), LCN2 (P = 0.008), TFF2 (P = 0.01), S100A4 (P = 0.001), PSCA (P = 0.001), but not for 14-3-3-σ (P = 0.3) or mesothelin (P = 0.1; Fig. 4). For both 14-3-3-σ and mesothelin, we did observe a trend
toward an inverse correlation between methylation and expression, but several cell lines that were unmethylated at these genes lacked expression, suggesting that other mechanisms besides hypomethylation were responsible for this lack of expression (49, 50). Because mRNA expression of CDH11 was detected in only 1 of 18 cell lines tested, we were unable to confirm any correlation between hypomethylation of CDH11 and its expression.

To confirm the involvement of methylation in the transcriptional regulation of the 7 genes that we found overexpressed and hypomethylated in pancreatic cancer, we treated two pancreatic cancer cell lines (MiaPaCa2 and Hs766T), which retained methylation of most of these genes (Fig. 3), with the DNA methyltransferase inhibitor (5Aza-dC) and/or the histone deacetylase inhibitor (TSA). Treatment of MiaPaCa2 cells, which harbored methylated 14-3-3-σ, with 5Aza-dC (1 μM for 4 days) led to partial loss of methylation at 5’ CpG sites of this gene and combined treatment with 5Aza-dC and TSA resulted in almost complete loss of methylation, as evidenced by MSP (Fig. 5A). Notably, TSA treatment alone also induced a slight loss of methylation at this gene locus. We also observed that treatment of MiaPaCa2 and Hs766T with 5Aza-dC but not with TSA led to loss of methylation at various degrees at other 6 gene loci (data not shown). We then determined the change in expression of 14-3-3-σ in MiaPaCa2 cells after treatment with 5Aza-dC and/or TSA using quantitative oligonucleotide microarray analysis (Affymetrix U133A chips). The 14-3-3-σ transcript was not detectable (called absent) in mock PBS-treated MiaPaCa2 cells, which is consistent with our RT-PCR result on this cell line (data not shown). Treatment with 5Aza-dC led to a substantial increase (~35-fold) in the 14-3-3-σ expression, whereas TSA treatment slightly increased the expression value (Fig. 5B). Furthermore, combined treatment with 5Aza-dC and TSA resulted in synergistic induction (~80-fold) of 14-3-3-σ expression, suggesting that DNA methylation and histone deacetylation may be cooperatively associated with the transcriptional silencing of this gene.

We also performed semiquantitative RT-PCR to determine the change in expression of all of the 7 hypomethylated genes after 5Aza-dC and/or TSA treatment. Semiquantitative RT-PCR revealed that treatment of MiaPaCa2 and Hs766T with 5Aza-dC or TSA, either alone or in combination, almost invariably reactivated the transcription of each of these 7 genes (Fig. 5C–I). Similar to the finding for 14-3-3-σ, many of the other genes (including claudin4, LCN2, and TFF2) were reactivated synergistically after combined treatment with 5Aza-dC and TSA. These results suggest that the combination of DNA methylation and histone deacetylation may be an important mechanism controlling the transcriptional activity of these hypomethylated genes.

Hypomethylation of Multiple Genes in Primary Pancreatic Carcinomas and in Pancreatic Cancer Xenografts. To test whether the aberrant hypomethylation detected in pancreatic cancer cell lines is also present in primary tumors, normal and tumor tissues were separately microdissected from 8 cases with invasive pancreatic adenocarcinoma and analyzed for hypomethylation of 7...
genes (claudin4, LCN2, 14-3-3σ, TFF2, S100A4, mesothelin, and PSCA) by MSP. In all of the 7 genes, hypomethylation was frequently (75–100%) detected in these primary pancreatic cancers compared with their normal counterparts (Fig. 6A). When the relative methylation ratio (the relative proportion of methylated alleles against total intensity of unmethylated and methylated alleles) was measured by densitometry, the mean methylation ratio of tumor DNA was lower than that of the corresponding normal tissue for claudin4 (tumor versus normal, 19 versus 69%, P = 0.002; Student’s t test), LCN2 (15 versus 60%, P = 0.004), 14-3-3σ (19 versus 59%, P < 0.0001), TFF2 (16 versus 61%, P = 0.0002), S100A4 (14 versus 55%, P = 0.008), mesothelin (31 versus 74%, P = 0.007), and PSCA (51 versus 64%, P = 0.2). These findings indicate that hypomethylation of these genes occurs in primary tumors as well as in cancer cell lines.

Finally, we examined the methylation status of the 8 genes hypomethylated in pancreatic cancer cell lines in a series of 37 xenografted primary pancreatic carcinomas. By MSP, hypomethylation (amplification of only unmethylated templates) was detected in 97% of 37 xenografts for claudin4 (tumor versus normal, 19 versus 69%, P = 0.002; Student’s t test), LCN2 (15 versus 60%, P = 0.004), 14-3-3σ (19 versus 59%, P < 0.0001), TFF2 (16 versus 61%, P = 0.0002), S100A4 (14 versus 55%, P = 0.008), mesothelin (31 versus 74%, P = 0.007), and PSCA (51 versus 64%, P = 0.2). These findings indicate that hypomethylation of these genes occurs in primary tumors as well as in cancer cell lines.

Fig. 5. Effect of DNA methylation inhibition and/or histone deacetylase inhibition on mRNA expression of genes frequently hypomethylated in pancreatic cancers. A. MSP analysis of 14-3-3σ in MiaPaCa2 cells treated with 5Aza-dC alone (1 μM for 4 days), TSA alone (1 μM for 24 h), or combination of both (5Aza-dC at 1 μM for 3 days followed by TSA at 0.5 μM for 24 h). B. expression analysis of 14-3-3σ in MiaPaCa2 cells treated with 5Aza-dC and/or TSA determined by oligonucleotide microarrays (Affymetrix U133A chips). Signal intensity, detection call (present, absent, or marginal), and fold-change (relative to mock-treated control) were determined using Microarray Suite 5.0 and Data Mining Tool software (Affymetrix). C–I, semiquantitative RT-PCR analysis of 7 overexpressed and hypomethylated genes in two pancreatic cancer cell lines [MiaPaCa2 (C and E) and Hs766T (D, F, G, H, and I)] after treatment with 5Aza-dC and/or TSA. The band intensities were measured by densitometry and the relative mRNA expression level for each sample (shown in bar graph) was normalized by the corresponding GAPDH expression.

Fig. 6. Methylation analysis of genes hypomethylated in pancreatic cancer cell lines in primary pancreatic carcinomas and in pancreatic cancer xenografts. A, MSP analysis of TFF2, claudin4 (CLDN4), and 14-3-3σ in primary pancreatic carcinomas and the corresponding normal pancreata microdissected from 8 cases with invasive pancreatic adenocarcinoma; N, normal tissue; T, tumor tissue. B, MSP analysis of mesothelin, TFF2, and PSCA in a series of pancreatic cancer xenografts. The PCR products in Lanes U and M indicate the presence of unmethylated and methylated templates, respectively.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2003 American Association for Cancer Research.
hypomethylation of genome-wide hypomethylation might be responsible for the aberrant 7-lycytosine content in a series of tumor cell lines, suggesting that a correlation between the result of genome-wide hypomethylation. Previous investigators found pancreatic cancer, one question we addressed was whether such gene hypomethylation in pancreatic cancer and is associated with the overexpression of af-

gest that DNA hypomethylation is a frequent epigenetic alteration in mRNA expression of these hypomethylated genes. These results sug-
tic pancreas. In addition, among individual pancreatic cancer cell lines, when they are normally methylated and not expressed in nonneoplas-

methylated at each of the 8 genes found to be hypomethylated a cancer cell. Although normal pancreatic epithelium was predomi-
nantly methylated at each of the 8 genes found to be hypomethylated in pancreatic cancer, we found some variability in the level of meth-

ation profile) by hierarchical cluster analysis (7). The genetic basis for hypomethylation at any of the genes we tested, has been characterized as having an atypical gene expression profile (a normoid gene expression profile) by hierarchical cluster analysis (7). The genetic basis for the normoid gene expression pattern is not known because it has a similar gene mutation pattern to other pancreatic cancers; although Hs766T lacks mutation of p53, it harbors mutated K-ras at codon 61, as well as homzygous deletion of p16 and DPC4 (56).

In considering how DNA hypomethylation may occur during tumor development, two mechanisms warrant consideration. One possibility is that DNA hypomethylation of a gene in a cancer is a remnant of a hypomethylated gene in a pancreatic epithelial cell that evolved into a cancer cell. Although normal pancreatic epithelium was predominantlly methylated at each of the 8 genes found to be hypomethylated in pancreatic cancer, we found some variability in the level of meth-
ylation in the samples of LCM nonneoplastic pancreatic epithelium

Fig. 7. Methylation profiles of 7 overexpressed and hypomethylated genes in a series of 37 pancreatic cancer xenografts determined by MSP.

DISCUSSION

In this study, we investigated the methylation status of a relatively large panel of genes in a series of pancreatic cancer cell lines and primary pancreatic carcinomas. We find that it is common to detect hypomethylation of genes that are overexpressed in pancreatic cancer when they are normally methylated and not expressed in nonneoplas-

cancers. In fact, one pancreatic cancer cell line (Hs766T), which lacked DNA hypomethylation at any of the genes we tested, has been characterized as having an atypical gene expression profile (a normoid gene expression profile) by hierarchical cluster analysis (7). The genetic basis for the normoid gene expression pattern is not known because it has a similar gene mutation pattern to other pancreatic cancers; although Hs766T lacks mutation of p53, it harbors mutated K-ras at codon 61, as well as homzygous deletion of p16 and DPC4 (56).

In considering how DNA hypomethylation may occur during tumor development, two mechanisms warrant consideration. One possibility is that DNA hypomethylation of a gene in a cancer is a remnant of a hypomethylated gene in a pancreatic epithelial cell that evolved into a cancer cell. Although normal pancreatic epithelium was predominantlly methylated at each of the 8 genes found to be hypomethylated in pancreatic cancer, we found some variability in the level of meth-
ylation in the samples of LCM nonneoplastic pancreatic epithelium from seven individuals. In addition, 100% methylation was not always observed in these normal pancreatic epithelia. Therefore, it is possible that some pancreatic epithelial cells harboring hypomethylation at a gene locus before the onset of neoplasia could retain that hypomethyl-

ation throughout neoplastic evolution. However, we do not believe that this is the likely explanation for the gene hypomethylation we observe in pancreatic cancers. First, for several of the loci, the percentage of pancreatic cancers with hypomethylation was ~90%, whereas normal pancreas epithelium was predominantly methylated.

Second, because we found that methylation status is associated with the expression of each of the genes tested, it is likely that if hypo-

HYPOMETHYLATION OF MULTIPLE GENES IN PANCREATIC CANCER

InputElement JPEG: 74x378 to 266x748
methylated originated in a nonneoplastic pancreatic epithelial cell that underwent selection because of other genetic events, one would expect to find that the earliest neoplasms (PanINs) express these genes (57). In contrast, we find that the expression of genes such as S100A4, PSCA, and mesothelin only becomes manifest in late-stage PanINs or in the carcinomas (23, 28, 29). The late expression of genes such as mesothelin and others in PanINs is indirect evidence that hypomethylation is typically not an early feature of pancreatic neoplastic development. Definitive answers to this question will require characterization of hypomethylation patterns of PanINs.

An alternative mechanism for the DNA hypomethylation observed in pancreatic cancer is the occurrence of loss of DNA methylation during tumorigenesis. Little is known about the factors that lead to loss of CpG methylation. As is the case for aberrant DNA hypermethylation in cancer, it is not certain at this time if gene-related methylated changes are a cause or consequence of altered transcriptional activity in cancer cells (58). Recently, Di Croce et al. (53) showed that methylation-associated silencing of retinoic acid receptor β2 occurred as a result of binding of oncogenic promyelocytic leukemia-retinoic acid receptor fusion protein to its promoter, whereas the addition of retinoic acid led to partial loss of methylation. Similarly, Bachman et al. (59) demonstrated, in a model system where DNA methyltransferase genes are disrupted in a colorectal cancer cell line, that methylation of histone H3 lysine-9 at the p16 gene locus occurred in conjunction with re-silencing of the gene in the absence of DNA methylation, suggesting that gene silencing because of histone methylation might lead to subsequent DNA methylation. In addition, Hoffman et al. (54) demonstrated that treatment of mouse fibroblasts with a HDAC inhibitor led to partial loss of methylation at the imprinted insulin-like growth factor-2 receptor gene. Cervoni and Szyf (51) have demonstrated that addition of methyl-CpG-binding domain protein-2 and acetylation of histones can induce the demethylation of ectopically methylated DNA. These results and our findings of selective gene hypomethylation raise the possibility that hypomethylation of transcriptionally activated genes in cancer may occur as a consequence of genetic or other events that alter the transcriptional activities of affected promoters.

Aberrant hypomethylation and overexpression of genes such as PSCA and S100A4 may be functionally important in the progression of pancreatic cancer. Overexpression of these genes has been implicated in tumorigenesis and progression of other cancers (60, 61). Importantly, we found that these genes when silenced by methylation became expressed after treatment with 5AzadC or TSA. Therefore, treatment of cancers methylated at these loci with DNA methyltransferase inhibitors or HDAC inhibitors could result in accelerated tumor progression rather than in growth suppression caused by re-expression of silenced tumor suppressor genes (62, 63). Indeed, we have recently found that treatment of pancreatic cancer cell lines with 5AzadC resulted in an increase in their invasive properties in association with an enhanced expression of several matrix metalloproteinases (64). Methylation or gene expression profiling may help to predict the cancers likely to respond to HDAC inhibitors and demethylating agents.

We analyzed hypomethylation patterns in cancer cell lines because it permitted facile comparison of methylation and gene expression patterns in a panel of cancers. In contrast, when primary cancers are studied, it is necessary to completely microdissect the cancer from surrounding stroma, and determining RNA expression on the same primary tissues is difficult. However, aberrant hypermethylation of CpG islands detected in cancer cell lines cannot always be found in primary cancers (65), thus, it is important to confirm such methylation patterns in primary cancers. Failure to find aberrant methylation in primary cancers may arise for several reasons, including failure to use microdissected cancers, tumor heterogeneity, or changes in methylation arising in culture. We have previously reported that the vast majority of aberrantly hypermethylated genes detected in cancer cell lines could also be found in the primary cancers from which they were derived (66). Similarly, Suzuki et al. (67) were able to confirm many of the aberrant hypermethylation patterns first identified in a colon cancer cell line in a panel of primary colorectal cancers. In this study, we were able to confirm that the hypomethylation patterns identified in pancreatic cancer cell lines were also present in microdissected primary pancreatic cancers, as well as in xenografts of primary pancreatic cancers for all 7 genes tested.

In summary, we demonstrate that gene hypomethylation is a common event in pancreatic adenocarcinoma and suggest that hypomethylation associated with overexpression of multiple genes contributes to neoplastic progression.

ACKNOWLEDGMENTS

We thank Dr. Scott E. Kern (The Johns Hopkins Medical Institutions, Baltimore, MD) for providing the DNA samples from pancreatic cancer xenografts.

REFERENCES

HYPOMETHYLATION OF MULTIPLE GENES IN PANCREATIC CANCER


Frequent Hypomethylation of Multiple Genes Overexpressed in Pancreatic Ductal Adenocarcinoma


**Updated version**

Access the most recent version of this article at:

[http://cancerres.aacrjournals.org/content/63/14/4158](http://cancerres.aacrjournals.org/content/63/14/4158)

**Cited articles**

This article cites 65 articles, 30 of which you can access for free at:

[http://cancerres.aacrjournals.org/content/63/14/4158.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/63/14/4158.full.html#ref-list-1)

**Citing articles**

This article has been cited by 40 HighWire-hosted articles. Access the articles at:

[http://cancerres.aacrjournals.org/content/63/14/4158.full.html#related-urls](http://cancerres.aacrjournals.org/content/63/14/4158.full.html#related-urls)

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.