Epigenetic Down-Regulation of CDKN1C/p57KIP2 in Pancreatic Ductal Neoplasms Identified by Gene Expression Profiling

Norihiro Sato, Hiroyuki Matsubayashi, Tadayoshi Abe, Noriyoshi Fukushima, and Michael Goggins

Abstract

Purpose: Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is an increasingly identified precursor to infiltrating ductal adenocarcinoma. Although our knowledge of the clinical and pathologic features of IPMNs is increasing, the molecular mechanisms underlying these neoplasms remain poorly understood.

Experimental Designs: To provide further insight into the molecular pathobiology of IPMNs, global expression profiling was done to determine genes that are inactivated/down-regulated in IPMNs using oligonucleotide microarrays (Affymetrix).

Results: In total, 300 unique transcripts (217 known genes) were identified as highly underexpressed in 12 IPMNs (<10-fold lower and P < 0.05) compared with five normal pancreatic ductal epithelium samples obtained by laser capture microdissection. The differential expression of a selection of genes was confirmed using reverse-transcription PCR. One of the genes underexpressed at both the transcriptional and protein level in a significant proportion of IPMNs was the cyclin-dependent kinase inhibitor, CDKN1C/p57KIP2. CDKN1C expression was also decreased in many pancreatic cancer cell lines and was restored following treatment with a DNA methylation inhibitor (5-aza-2'-deoxycytidine) or, more potently, with a histone deacetylase inhibitor (trichostatin A). Partial methylation of the CDKN1C promoter CpG island was found in most, but not all, pancreatic cancer cell lines with reduced CDKN1C expression, and was also detectable in IPMNs. Furthermore, a subset of pancreatic cancers showed complete hypomethylation of LIT1, an imprinting control region important for the regulation of CDKN1C expression. Complete hypomethylation in these cancers was the result of deletion of the methylated LIT1 allele at 11p15.5 rather than loss of imprinting.

Conclusions: These findings suggest that CDKN1C is commonly down-regulated in pancreatic ductal neoplasms through a combination of promoter hypermethylation, histone deacetylation, and loss of the maternal allele expressing CDKN1C.

Intraductal papillary mucinous neoplasm (IPMN) is an increasingly recognized noninvasive cystic neoplasm of the pancreas that is characterized by unique clinical, pathologic, and molecular features (1–8). Although most IPMNs are slow-growing and less aggressive compared with conventional ductal adenocarcinoma, early and accurate diagnosis of IPMNs is important because an associated infiltrating adenocarcinoma is frequently identified in the pancreas affected by IPMNs, suggesting that IPMNs evolve into invasive ductal adenocarcinomas (2, 7, 9–11). Recent evidence also suggests that many individuals with an inherited susceptibility to develop pancreatic ductal adenocarcinoma may initially develop IPMNs prior to developing pancreatic adenocarcinoma. In this regard, two recently completed studies have undertaken the screening of asymptomatic individuals at high risk for developing pancreatic neoplasia using endoscopic ultrasound and computed tomography scanning of the pancreas. The first study identified an IPMN and an invasive ductal adenocarcinoma among 37 enrolled individuals (12), and in the second study, five IPMNs were identified including 1 patient who also had a microinvasive adenocarcinoma that arose in a pancreatic intra-epithelial neoplasm (PanIN) among 78 individuals screened. All of these individuals underwent curative resection. These results highlight the importance of IPMNs as a detectable precursor to invasive pancreatic adenocarcinoma. Previously, it had been thought that most pancreatic ductal adenocarcinomas arise from PanINs which are small (<5 mm) lesions because these are the most common lesions found in association with pancreatic ductal adenocarcinomas in pancreatic resections. Although our knowledge of the clinical and pathologic

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4 Canto et al., unpublished observations.
manifestations of IPMNs is increasing (2, 5, 7, 10, 11, 13), the molecular mechanisms underlying these neoplasms remain poorly understood.

Reported genetic alterations in IPMNs include mutations in the Kras2 (14), TP53 (15), and STK11/LKB1 (16) as well as loss of heterozygosity (LOH) of several chromosomal loci (16, 17). Recent evidence suggests that in addition to these genetic alterations, aberrant DNA methylation may contribute to the inactivation of a subset of tumor-suppressor genes in IPMNs, including CDKN2A/p16 (18, 19). Furthermore, two recent studies have evaluated gene expression profiling in IPMNs mainly focusing on genes that are preferentially expressed in IPMNs (20, 21). Using oligonucleotide microarrays, we have previously identified a large number of overexpressed genes in IPMNs as well as a group of genes that are associated with the invasive phenotype of the neoplasms (21). In the present study, we compared gene expression patterns between IPMNs and normal pancreatic ductal epithelium to specifically identify genes down-regulated in these neoplasms. Of the many genes underexpressed in IPMNs, one was the cyclin-dependent kinase inhibitor CDKN1C/p57Kip2. We further investigated potential mechanisms for the down-regulation of CDKN1C in IPMNs and in pancreatic cancer cells.

**Materials and Methods**

**Cell lines, tissue samples, and xenografts.** Human pancreatic cancer cell lines AsPC1, BxPC3, Capan1, Capan2, CPAC1, Hs766T, MiaPaCa2, and Panc1 were obtained from the American Type Culture Collection (Rockville, MD) and Colo357 from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). A nonneoplastic cell line (HPDE) derived from normal human pancreatic ductal epithelium (22) was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Ontario). Fresh frozen tissues of IPMNs and normal pancreata were obtained from surgical specimens resected at The Johns Hopkins Medical Institutions and stored at −70°C with the approval of the Institutional Committee for Clinical Investigation. All frozen sections were carefully evaluated with H&E staining, and IPMNs (intraductal component) and normal pancreatic ducts were selectively microdissected either manually or by laser capture microdissection (Pixcell II LCM system, Arcturus Engineering, Inc., Mountain View, CA), as described previously (21, 23). Pancreatic and biliary cancer xenografts were established in nude mice from surgically resected primary pancreatic carcinomas, and 23 xenografts were used in this study.

**RNA extraction, oligonucleotide array hybridization, and analysis of microarray data.** Total RNA was isolated from IPMNs using Trizol reagent (Invitrogen, Carlsbad, CA), and was purified using RNeasy Mini Kit (Qiagen, Valencia, CA). For microdissected normal ductal epithelial cells, total RNA was extracted using the Picopure RNA isolation kit (Invitrogen, Carlsbad, CA). Total RNA was isolated from IPMNs using Trizol reagent (Invitrogen, Carlsbad, CA) and subjected to two rounds of linear amplification using the RiboAmp RNA amplification kit (Arcturus). Sample preparation and subsequent hybridization to the Human Genome U133A chips (Affymetrix) was carried out according to the manufacturer’s instructions. 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).

Hierarchical cluster analysis was done using dChip (DNA-Chip Analyzer) software (http://www.dchip.org/) after filtering genes with the greatest variation across all samples (SD/mean > 1). The analysis of genes differentially expressed between IPMNs and normal pancreatic duct epithelial cells was done with fold-change analysis and Mann-Whitney U nonparametric test using Data Mining Tool software (Affymetrix).

**Semiquantitative reverse-transcription PCR.** Total RNA was reverse-transcribed using Superscript II (Invitrogen). Reverse-transcription PCR was done using primers specific for six genes (Table 1) in a semiquantitative fashion with primers to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in duplex reactions. The range of amplification for each gene and the GAPDH gene was examined with serial PCR cycles (25-40 cycles), and the optimal PCR cycles were set within the linear amplification range. The relative intensity of CDKN1C mRNA expression was measured by densitometry (Image, NIH, Bethesda, MD) and then corrected for variable RNA recovery using the corresponding GAPDH mRNA measurement as a surrogate for total mRNA.

**Immunohistochemistry.** Five-micrometer sections were cut onto coated slides and deparaffinized by routine techniques. Antigen retrieval was done in 10 mmol/L sodium citrate buffer (pH 6.0) heated at 95°C in a steamer for 20 minutes. After blocking endogenous peroxidase activity with a 3% aqueous H2O2 solution for 5 minutes, the sections were incubated with a monoclonal antibody for p57kip2 (clone 57P06, Lab Vision, Fremont, CA) for 60 minutes. Labeling was detected with the Envision Plus Detection Kit (DAKO, Carpinteria, CA) following the protocol as suggested by the manufacturer, and all sections were counterstained with hematoxylin.

**Treatment with 5-aza-2′-deoxycytidine and/or trichostatin A.** Two pancreatic cancer cell lines (AsPC1 and BxPC3) were treated with 5-aza-2′-deoxycytidine (Sigma), St. Louis, MO) and trichostatin A (Sigma), either alone or in combination. Cells were exposed continuously to 5-aza-2′-deoxycytidine (1 μmol/L) for 4 days or trichostatin A (1 μmol/L) for 24 hours. For combined treatment, these cells were cultured in the presence of 5-aza-2′-deoxycytidine (1 μmol/L) for 3 days, and were then treated for another 24 hours with trichostatin A (0.5 μmol/L).

**Methylation-specific PCR and bisulfite sequencing analysis.** Genomic DNA was treated with sodium bisulfite (Sigma), purified, and subjected to methylation-specific PCR according to the protocol described previously (24). Briefly, bisulfite-treated DNA was amplified using primers specific for either the methylated or for the unmethylated DNA under the following conditions: 95°C for 5 minutes; 40 cycles of 95°C for 20 seconds, 58°C for 20 seconds, and 72°C for 30 seconds; and a final extension of 4 minutes at 72°C. Primer sequences for CDKN1C promoter were TAG TAC GTC GGT TGT TAA TTG (forward) and CCF ACA CCA ATT TGA AAT AGA (196 bp) and (reverse) CCG AGT GTA GCC AAT GTA GAC (172 bp) and (forward) CTG GTC ATC ACA TGG GAA TG (185 bp) and (reverse) GCC TTT GCA ATC TGG GAA TG (189 bp).

**Table 1. Primer sequences for reverse-transcription PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKNIC</td>
<td>CGG CGA TCA AGA AGC TGT C</td>
<td>GCT TTT GGG GCT CTA AAT TG (189 bp)</td>
</tr>
<tr>
<td>CRISP3</td>
<td>(forward) GTA TAC ACA GGT TGT TTG GTA C</td>
<td>(reverse) AGA AAC TTT GTG TAC TGT AAT G</td>
</tr>
<tr>
<td>SGP28</td>
<td>(forward) ATG GGG AAT TCG TCC AGT TG (185 bp)</td>
<td>(reverse) AGA AAC TTT GTG TAC TGT AAT G</td>
</tr>
<tr>
<td>FLRT2</td>
<td>(forward) GCA TCG CCA GCC TCC TTA C</td>
<td>(reverse) TTT ACA AAG GTG TCC CTT ATG (196 bp)</td>
</tr>
<tr>
<td>SERPING1</td>
<td>(forward) GCC TAC ACA TTG GAATG</td>
<td>(reverse) CTT CTC AAA ATT TTA CAA TGA ATA G (195 bp)</td>
</tr>
<tr>
<td>MDRI</td>
<td>(forward) TCT TCC AAA ATT TTA CAA TGA ATI G</td>
<td>(reverse) AGA CCC GTA ATT TGA TCG ATG</td>
</tr>
<tr>
<td>CFTR</td>
<td>(forward) GCC TTC ATC ACA TTG GAATG</td>
<td>(reverse) AGC AAA TGT GCC AAT GTA TCA AG (172 bp)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(forward) CGG AGT CAA CGG ATT TGA TCG TAT</td>
<td>(reverse) AGC CTT CTC CAT GGT GGT GAA GAC</td>
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TAC G (reverse) for methylated reactions (116 bp). Primer sequences for \textit{LIT1} were GTG TCG TGT TG TTT TTA TGT G (forward) and CAC CAA CCT ACAA TAA CAT C (reverse) for unmethylated reactions (118 bp), and GGT GCC GTC GTT GTT AC (forward) and CAA CCT CAA GTT TAA CAT CG (reverse) for methylated reactions (116 bp).

For bisulfite sequencing analysis of \textit{CDKN1C}, a 173-bp fragment in the promoter region was amplified from bisulfite-treated DNA using the primers: GGA ATT GAA ATT ATA TAA TGG (forward) and TCR ATA CCT ACT AAC TAA CTC (reverse). The PCR condition was 95°C for 5 minutes; 40 cycles of 95°C for 20 seconds, 58°C for 20 seconds, and 72°C for 30 seconds; and a final extension of 4 minutes at 72°C. The PCR products were incubated with exonuclease I and shrimp alkaline phosphatase (U.S. Biochemical, Cleveland, OH), and were sequenced using the SequiTherm Excel II cycle sequencing kit (Epicentre Technologies, Madison, WI).

**Results**

**Identification of genes significantly underexpressed in IPMNs.** In the present study, we compared the global gene expression patterns between 12 IPMNs and five normal pancreatic ductal epithelia. For these IPMNs, the intraductal component was selectively microdissected manually for gene expression analysis so that we were able to obtain a high neoplastic cellularity of ~80% to 90% (21). The quality of laser capture microdissected normal ductal epithelial cells was carefully evaluated using the gene expression of a panel of epithelial as well as acinar markers (23). Of 22,283 probe sets featured on the Affymetrix HG-U133A chip, 642 transcripts were found to have greatest variation (SD/mean > 1) across all the 17 samples. Hierarchical cluster analysis with these 642 transcripts identified two major clusters that, as predicted, clearly separated IPMNs from normal pancreatic ductal epithelial samples (Fig. 1). The Affymetrix Data Mining Tool was then used to identify transcripts expressed at significantly lower levels (at levels <10-fold lower and P < 0.05 by Mann-Whitney \textit{U} test) in IPMNs compared with normal ductal epithelial samples. Using this stringent criteria, we identified a total of 300 unique transcripts (217 known genes and 83 uncharacterized sequences, including expressed sequence tags) that are significantly underexpressed in IPMNs relative to normal ducts (a complete list of these 300 transcripts is provided on the web site: http://pathology2.jhu.edu/pancreas/IPMNd300/index.htm).

The large panel of underexpressed genes included those that are functionally involved in a wide spectrum of cellular processes, including cell growth and cell cycle [growth factor receptor-bound protein 14, cyclin-dependent kinase inhibitor 1C (\textit{CDKN1C/p57KIP2})], cell death [clusterin, leucine-rich repeat and death domain–containing protein (\textit{LRDD})], cell adhesion and cell-cell/cell-matrix interactions [cadherin 15, fibronectin leucine-rich transmembrane protein 2 (\textit{FLRT2})], cytoskeletal remodeling (cytokeratin 2, microtubule-associated proteins, myosin 2), proteolytic activity (tissue factor pathway inhibitor-2), calcium homeostasis (calcium binding protein 2, calcium-sensing receptor), and angiogenesis (BAI1-associated protein 2). Of interest, many of the underexpressed genes identified were found to be specifically related to brain/neuronal functions, although the significance of this finding is unclear. These included brain-specific angiogenesis inhibitor 3, brain-specific membrane-anchored protein (\textit{BSMAP}), gamma-aminobutyric acid (\textit{GABA}) A receptor, neuron 1, neurexin 3, neuroglobin, neuroligin, neuronal thread protein, purine-rich element binding protein A (\textit{PURA}), and synaptophysin 1.

We then did semiquantitative reverse-transcription PCR to validate the expression pattern of six selected genes in 12 IPMNs (all of them were analyzed by microarray) and in five normal pancreatic tissues. The genes examined were \textit{CDKN1C}, cysteine-rich secretory protein 3 (\textit{CRISP3/Sgp28}), fibronectin leucine-rich transmembrane protein 2 (\textit{FLRT2}), serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1 (\textit{SERPING1}), ATP-binding cassette, sub-family B (\textit{MDR}1), member 1, P-glycoprotein (\textit{MDR1}), and cystic fibrosis transmembrane conductance regulator, ATP-binding cassette, sub-family C, member 7 (\textit{CFTR}). In all the six genes examined, absent or reduced expression was confirmed in more than half of the 12 IPMNs in contrast to their more abundant expression in normal pancreata (Fig. 2).

**Identification of CDKN1C/p57KIP2 as a significantly downregulated gene in IPMNs.** Among the genes identified, \textit{CDKN1C} has been considered a putative tumor-suppressor gene because of its involvement in the cancer-predisposing Beckwith-Wiedemann syndrome as well as in various types of sporadic cancers (25–28). Therefore, we further investigated the expression pattern of \textit{CDKN1C} and the potential
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mechanisms for its transcriptional regulation in IPMNs and in pancreatic cancer cell lines.

We first analyzed an independent set of six IPMNs and their matched normal pancreatic tissue samples for CDKN1C expression using semiquantitative reverse-transcription PCR. In four cases (67%), relative CDKN1C expression (normalized by corresponding GAPDH expression) in IPMN was apparently decreased compared with normal pancreas (Fig. 3), confirming frequent down-regulation of CDKN1C mRNA expression in IPMNs.

Next, immunohistochemical analysis of CDKN1C/p57 protein expression was done in a series of 20 IPMNs with different histologic grades. Nuclear staining was considered as indicative of positive expression, as described in previous studies (29, 30). Normal pancreatic ductal epithelial cells were weakly to moderately positive for p57 expression in most cases, although only a small fraction of cells within the ducts or ductules exhibited positive heterogeneous expression (Fig. 4A and B). Islet cells were also strongly positive for p57 immunolabeling and therefore served as an internal control for each PCR reaction.

Fig. 2. RT-PCR analysis of six selected genes in five normal pancreatic tissues and in 12 IPMNs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as an internal control for each PCR reaction.

In all IPMNs, CDKN1C promoter CpG island was unmethylated in the nonneoplastic HPDE that expresses CDKN1C mRNA was strongly expressed in a nonneoplastic pancreatic ductal epithelial cell line (HPDE) but its expression was undetectable or significantly decreased in all pancreatic cancer cell lines tested (Fig. 5A). Whereas previous reports have revealed infrequent mutation of CDKN1C in tumors (31, 32), aberrant hypermethylation of the promoter CpG island has been suggested as a mechanism for inactivation of this gene in certain types of human solid and hematologic cancers (33–36).

In order to determine whether CDKN1C expression in pancreatic neoplasms is regulated by epigenetic mechanisms, we treated two pancreatic cancer cell lines (AsPC1 and BxPC3) where CDKN1C was silenced with a DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine and a histone deacetylase inhibitor trichostatin A, either alone or in combination. In both cell lines, CDKN1C expression was restored after treatment with 5-aza-2′-deoxycytidine, and more potently, with trichostatin A or with the combination of both drugs (Fig. 5B), suggesting the involvement of DNA methylation and histone deacetylation in the CDKN1C silencing. We then determined the methylation status of CDKN1C promoter CpG island in a panel of pancreatic cancer cell lines. Methylation-specific PCR revealed that the CDKN1C promoter was unmethylated in the nonneoplastic HPDE that expresses CDKN1C (Fig. 5C). By contrast, CDKN1C was partially methylated in most, but not all, pancreatic cancer cell lines with absent or reduced CDKN1C expression (Fig. 5C). Using methylation-specific PCR, we also analyzed a panel of 26 IPMNs for their CDKN1C methylation status and detected partial methylation in 10 (38%) cases (Fig. 5D). Furthermore, we did bisulfite sequencing analysis to confirm the methylation status of the CDKN1C promoter in selected samples. Consistent with the results of both the RT-PCR and the methylation analysis, CDKN1C expression was partially silenced in 17 (71%) of 24 evaluable tumors. We also analyzed p57 expression in 18 infiltrating ductal pancreatic adenocarcinomas by immunohistochemistry. Loss of nuclear p57 expression was found in 12 (71%) of 17 evaluable cancers.

Mechanisms underlying CDKN1C/p57kip2 down-regulation in pancreatic neoplasms. We then explored the potential mechanisms responsible for the down-regulation of CDKN1C in pancreatic neoplasms using a panel of nine pancreatic cancer cell lines. Reverse-transcription PCR revealed that CDKN1C mRNA was strongly expressed in a nonneoplastic pancreatic ductal epithelial cell line (HPDE) but its expression was undetectable or significantly decreased in all pancreatic cancer cell lines tested (Fig. 5A). Whereas previous reports have revealed infrequent mutation of CDKN1C in tumors (31, 32), aberrant hypermethylation of the promoter CpG island has been suggested as a mechanism for inactivation of this gene in certain types of human solid and hematologic cancers (33–36).

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Fig. 3. Semiquantitative RT-PCR analysis of CDKN1C in six IPMNs (Tumor) and their matched normal pancreatic tissues (Normal). The relative intensity of CDKN1C mRNA expression was measured by densitometry and normalized using the corresponding GAPDH mRNA measurement as a surrogate for total mRNA.

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from methylation-specific PCR, the majority of CpG sites analyzed were completely unmethylated in the nonneoplastic HPDE, whereas they were partially methylated in IPMNs (Fig. 5E).

CDKN1C is located within the chromosome 11p15.5 imprinted domain, and CDKN1C expression is thought to be negatively regulated by LIT1, an antisense RNA within the KvLQT1 gene and 220 kb telomeric to CDKN1C (Fig. 6A), although the mechanism by which LIT1 suppresses CDKN1C is unclear (37–39). We therefore determined the possible involvement of loss of imprinting (the absence of maternal methylation) of LIT1 in the CDKN1C down-regulation in pancreatic cancer cells. Using methylation-specific PCR, we found that LIT1 was, as expected, hemimethylated in normal pancreatic tissues.
LOH at the 11p15.5 locus was identified in 9 (39%) of the 23 pancreatic and biliary cancer xenografts with known LOH status at the 11p15.5 locus (40). In total, LOH at this locus (determined by comparing matching xenograft and normal alleles at markers D11S1984 and D11S2362) was identified in 7 (30%) of the 23 xenografts. Of the seven xenografts with LOH, four showed hypomethylation at LIT1, whereas the remaining three contained only methylated LIT1 (Fig. 6C). By contrast, none of the 16 xenografts without LOH had complete hypomethylation or hypermethylation of LIT1 (Fig. 6C). Thus, complete LIT1 hypomethylation was the result of deletion of the LIT1-methylated, maternal CDKN1C-expressing allele and not the result of loss of imprinting and associated hypomethylation of LIT1. Because LOH at 11p15.5 did not seem to target the CDKN1C-expressing allele over the nonexpressing allele, loss of the maternal allele at CDKN1C does not seem to be a selected event. Finally, we investigated the methylation status of LIT1 in peripheral blood lymphocyte DNA from 30 familial pancreatic cancer patients (from kindreds in which three or more family members were affected with pancreatic cancer, at least two of which were first-degree relatives) to test whether LIT1 hypomethylation might occur in such patients as a germ line event. However, we found no evidence for abnormal LIT1 methylation in these familial pancreatic cancer lymphocyte DNAs.

**Discussion**

In an attempt to identify specific genes that are downregulated in IPMNs, we used high-throughput oligonucleotide microarray platform to compare the gene expression patterns between 12 IPMNs and five normal pancreatic ductal epithelial samples purely dissected by laser capture microdissection. This analysis successfully identified a total of 300 transcripts (including 217 known genes) that are markedly (<10-fold lower and P < 0.05) underexpressed in IPMNs relative to normal ductal epithelium. Although a previous study used cDNA microarrays to identify a subset of down-regulated genes in IPMNs compared with only one cell line HPDE (20), our present analysis using a stringent criteria enabled us to identify a larger number of previously unreported genes underexpressed in IPMNs. The identification of genes down-regulated in IPMNs may provide a molecular basis for these neoplasms and, especially, a clue to identify potential tumor-suppressor genes inactivated during pancreatic neoplastic progression. In fact, our approach identified CDKN1C, a previously characterized tumor-suppressor gene, as a target in IPMNs and possibly in pancreatic cancer.

IPMNs have recently attracted considerable interest because the disease is being increasingly recognized and because an associated infiltrating adenocarcinoma has often been detected in the pancreas affected by an IPMN (2, 7, 9–11). Because IPMNs exhibit a wide spectrum of malignant potential, accurate preoperative assessment of the grade of malignancy is critical to determine the optimal management for these patients. Currently, however, there are no laboratory markers that can adequately detect IPMNs or differentiate between benign and malignant IPMNs. Conventional cyto logic analysis of pancreatic tissue and serum CA19-9 levels are commonly used in the clinical setting, but they are not able to differentiate between benign and malignant IPMNs (41, 42). The identification and characterization of overexpressed genes as well as underexpressed genes in IPMNs might allow us to identify clinically relevant biomarkers. For example, loss of Dpc4 expression by immunohistochemical labeling has been shown to be a specific marker for adenocarcinoma in biopsy specimens of the pancreas (43). Thus, some of the underexpressed genes identified in IPMNs could represent candidate biomarkers to improve the diagnosis of IPMNs.

CDKN1C is a potent inhibitor of several G1 cyclin complexes, and is a negative regulator of cell proliferation (44, 45). CDKN1C is located on chromosome 11p15.5, a region implicated in sporadic cancers, Wilms tumor, and Beckwith-Wiedemann syndrome, making it a candidate tumor suppressor (44, 46). Indeed, the tumor-suppressive...
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activities of CDKN1C have been established in experimental models (47, 48). In this study, we find that CDKN1C is down-regulated in a significant proportion of IPMNs and pancreatic cancers, supporting its tumor-suppressor role during pancreatic ductal carcinogenesis. Interestingly, a recent study suggested an involvement of CDKN1C up-regulation in transforming growth factor-β-induced cell cycle arrest of human hematopoietic cells (49), raising the possibility that CDKN1C silencing, in addition to frequent genetic inactivation of the transforming growth factor-β signaling pathway (50), might play a role in the loss of responsiveness of pancreatic cancer cells to transforming growth factor-β-induced growth inhibition.

Recent reports have shown that CDKN1C silencing is associated with CpG island hypermethylation of the CDKN1C promoter in gastrointestinal (33, 34) and hematologic cancers (35, 36). However, in a panel of esophageal cancer cell lines, the silencing of CDKN1C was correlated with loss of CpG and histone H3 lysine 9 methylation at LIT1 but not with hypermethylation of its own promoter (51). In the present study, we find partial methylation of the CDKN1C promoter in many of the pancreatic cancer cell lines with reduced CDKN1C expression. Furthermore, CDKN1C expression was robustly re-expressed in two of these cell lines after treatment with epigenetic modifying drugs, suggesting that CDKN1C is transcriptionally regulated, at least in part, through DNA methylation and histone deacetylation. Regarding the imprinting machinery, complete LIT1 hypomethylation was observed in a subset of pancreatic cancer cell lines and xenografts in association with LOH at 11p15.5, but not in xenografts retaining both alleles at this locus, suggesting that loss of imprinting of LIT1 is not a mechanism responsible for CDKN1C down-regulation in pancreatic cancer. Because CDKN1C is expressed predominantly from the maternal allele, selective loss of the maternal allele (which contains the methylated LIT1) is an important mechanism of CDKN1C inactivation (27, 52). We found loss of the maternal CDKN1C-expressing allele in four of the seven pancreatic cancer xenografts with LOH at 11p15.5, but it is not known whether this is a selective event because of the limited number of cases analyzed in this study. Together, our results suggest that CDKN1C down-regulation in pancreatic neoplasms may occur partly through a combination of promoter hypermethylation, histone deacetylation, and loss of the maternal allele expressing CDKN1C.

We identified several other potentially important genes as underexpressed in IPMNs such as MDR1 (53), clusterin (54, 55), CDH19 (56), LRDD/PIDD (57), and tissue factor pathway inhibitor-2 (58). Further investigation is likely to reveal that silencing of many of these genes could promote the neoplastic progression of IPMNs.

In summary, we have identified a large number of genes including CDKN1C, whose expression is down-regulated in IPMNs relative to normal ductal epithelium. Silencing of CDKN1C by epigenetic mechanisms is a common event during IPMN development and may contribute to the development of IPMNs.

References

27. Lai S, Goeplert H, Gillenwater AM, Luma MA.


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