The Silencing of MicroRNA 148a Production by DNA Hypermethylation Is an Early Event in Pancreatic Carcinogenesis

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BACKGROUND: The poor prognosis of pancreatic ductal adenocarcinoma (PDAC) is accounted for by the absence of early diagnostic markers and effective treatments. MicroRNAs inhibit the translation of their target mRNAs. The production of microRNAs is strongly altered in cancers, but the causes of these alterations are only partially known. DNA hypermethylation is a major cause of gene inactivation in cancer. Our aims were to identify microRNAs whose gene expression is inactivated by hypermethylation in PDAC and to determine whether this hypermethylation-mediated repression is an early event during pancreatic carcinogenesis. We also sought to investigate whether these differentially methylated regions can serve as a diagnostic marker for PDAC.

METHODS: MicroRNA production was measured by microarray hybridization and reverse-transcription quantitative PCR. The level of DNA methylation was measured by bisulfite mapping and semiquantitative methylation-specific PCR.

RESULTS: We identified 29 microRNAs encoded by genes whose expression is potentially inactivated by DNA hypermethylation. We focused our study on microRNA 148a (miR-148a) and found its production to be repressed, not only in PDAC samples but also in preneoplastic pancreatic intraepithelial neoplasia (PanIN) lesions. More importantly, we found that hypermethylation of the DNA region encoding miR-148a is responsible for its repression, which occurs in PanIN preneoplastic lesions. Finally, we show that the hypermethylated DNA region encoding miR-148a can serve as an ancillary marker for the differential diagnosis of PDAC and chronic pancreatitis (CP).

CONCLUSIONS: We show that the hypermethylation of the DNA region encoding miR-148a is responsible for its repression in PDAC precursor lesions and can be a useful tool for the differential diagnosis of PDAC and CP.

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changes include those in the oncogene \textit{KRAS}^{10} (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), \textit{CDKN2A} (cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)), \textit{TP53} (tumor protein 53), \textit{SMAD4} (SMAD family member 4; alias, \textit{DPC4}, deleted in pancreatic cancer 4), and others \cite{4}. DNA hypermethylation, a major epigenetic modification, has also been reported to be responsible for inactivating the expression of genes [e.g., \textit{CDKN2A}, \textit{PENK} (proenkephalin; alias, preproenkephalin), and others] early in the development of PDAC \cite{6}. More recently, DNA hypermethylation was reported to cause inactivation of microRNA production in several cancers \cite{7}. MicroRNAs, small RNAs that inhibit the translation of their target mRNAs \cite{8}, play important roles in cancer development. The expression profile of microRNA genes is profoundly altered in PDACs \cite{9–11}. We recently demonstrated that the microRNA let7 is frequently downregulated in PDAC samples \cite{12} and that microRNA 21 (miR-21) is overproduced in PDAC precursor lesions \cite{13}.

In this study, we aimed to identify microRNAs encoded by genes whose expression is inactivated by DNA hypermethylation in PDAC, and, more interestingly, to determine whether this alteration occurs early in PDAC development. We also investigated whether these differentially methylated regions (DMRs) of DNA that encode microRNAs can serve as a diagnostic tool for PDAC.

Materials and Methods

TISSUE SAMPLES

The policies of the ethics committee at the University Hospital (CHU) of Toulouse and the Cancéropole Grand Sud-Ouest (France) were rigorously followed throughout the study. After receiving written informed consent, we obtained pancreatic tissue samples from patients undergoing pancreatic surgery (tumor bank collection of the University Hospital of Toulouse). Samples of PDAC tissue and matched adjacent tissue were macrodissected by on-site histopathology faculty. The percentage of tumor cells was >50% in all PDAC samples. Formalin-fixed, paraffin-embedded samples from deidentified human macrodissected samples of nonpathologic pancreatic duct tissues (n = 5) and PanIN-1A (n = 8), PanIN-1B (n = 11), PanIN-2 (n = 10), and PanIN-3 (n = 10) lesions were obtained from the archives of the Department of Pathology at the Dartmouth Hitchcock Medical Center. Leukocytes were obtained from healthy donors.

DNA FROM ENDOSCOPIC ULTRASOUND-GUIDED FINE-NEEDLE ASPIRATION (EUS-FNA) BIOPSES

DNA samples from EUS-FNA biopsies were obtained as previously described \cite{14}. DNA samples from 39 patients (male, 22; female, 16; mean age, 64.4 years) with confirmed PDAC (cytopathologic analysis) and 20 patients (male, 20; mean age, 56 years) with confirmed chronic pancreatitis (CP) (cytopathologic analysis and follow-up) were included in this pilot study.

MOUSE STRAINS

Conditional LSL K-ras G12D and Pdx1-Cre\textsuperscript{+/−} mouse strains were bred to generate LSL K-ras G12D/Pdx1-Cre\textsuperscript{+/−} (KRAS*) mice, as previously described \cite{5}. The LSL K-ras G12D mouse strain was obtained from the Mouse Models of Human Cancers Consortium Repository (National Cancer Institute). The Pdx1-Cre\textsuperscript{+/−} mouse strain was obtained from the Douglas A. Melton laboratory (Department of Molecular and Cellular Biology, and Howard Hughes Medical Institute, Harvard University) \cite{15}. The IFR150 animal care committee approved all procedures.

REAGENTS AND ANTIBODIES

Acetylated histone H3 and pan-H3 antibodies were purchased from Millipore. 5-Aza-2-deoxycytidine (5-Aza-dC) and trichostatin A (TSA) were purchased from Sigma–Aldrich.

CELL CULTURE

Human PDAC-derived cell lines Capan-1 and Capan-2 (ATCC) were grown in RPMI medium supplemented with 100 mL/L fetal calf serum, 1-glutamine (2 mmol/L), antibiotics (50 mg/L), Fungizone\textsuperscript{®} (0.25 mg/L; Invitrogen) and Plasmocin\textsuperscript{®} (5 mg/L; InvivoGen). Human BxPC-3, Mia PaCa-2, and Panc-1 PDAC-derived cells were grown in DMEM containing 4.5 g/L glucose (Invitrogen), 100 mL/L fetal calf serum, 1-glutamine (2 mmol/L), antibiotics (50 mg/L), and Fungizone (0.25 mg/L). Human pancreatic duct epithelial cells (16) were grown in Defined Keratinocyte-SFM medium (Invitrogen) supplemented with 50 g/L bovine pituitary extract and 5 g/L epidermal growth factor (both from Invitrogen). Cells were treated for 72 h with 2 μmol/L 5-Aza-dC and with either 100 nmol/L (BxPC-3 cells) or 400 nmol/L

\textsuperscript{10} Genes: \textit{KRAS}, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; \textit{CDKN2A}, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); \textit{TP53}, tumor protein 53; \textit{SMAD4}, SMAD family member 4 (alias, \textit{DPC4}, deleted in pancreatic cancer 4); \textit{PENK}, proenkephalin (alias, preproenkephalin); \textit{MUC2}, mucin 2, oligomeric mucin-gel-forming; \textit{MMP9}, matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase); \textit{ACTB}, actin, beta; \textit{GAPDH}, glyceraldehyde-3-phosphate dehydrogenase; \textit{MIR148A}, microRNA 148a; \textit{Pdx1-Cre}, Cre recombinase under the control of the \textit{M. musculus Pdx1} (pancreatic and duodenal homeobox 1) gene promoter; \textit{SPARC}, secreted protein, acidic, cysteine-rich (osteonectin); \textit{TFF2}, tissue factor pathway inhibitor 2; \textit{TGFβ2}, TGFβ-induced factor homeobox 2; \textit{DNMT3B}, DNA (cytosine-5-)methyltransferase 3 beta.
miR-148a Hypermethylation in PDAC Preneoplastic Lesions

(Capan-1, Capan-2, MIA PaCa-2, and PANC-1 cells) TSA.

5-METHYLCYTOSINE QUANTIFICATION BY NEAREST-NEIGHBOR ANALYSIS
The 5-methylcytosine level was quantified by nearest-neighbor analysis as described previously (17). The intensities of 5-methylcytosine and cytosine mononucleotide spots were measured with a PhosphorImager screen and by ImageQuant quantification (GE Healthcare Life Sciences).

HISTONE EXTRACTION AND WESTERN BLOT ANALYSIS
Cells were incubated for 30 min in lysis buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, Protease Inhibitor Cocktail (Roche), and 0.2 mol/L HCl] and centrifuged for 10 min at 11 000 g. The amount of acetylated histone H3 on lysine 9 in response to 5-Aza-dC and TSA treatment was measured by western blot analysis after separation by SDS-PAGE on a 150-g/L acrylamide gel. The amount of total H3 was used as a loading control.

MICRODISSECTION OF PRENEOPLASTIC MURINE TISSUES
Pancreatic tissues were microdissected as previously described (13). Duct lesions were classified according to the WHO classification (2). Five hundred cells were microdissected from cryostat-embedded PanIN lesions generated in a conditional K-ras G12D mouse model (PanIN-1A, n = 6; PanIN-1B, n = 7; PanIN-2, n = 3; and PanIN-3, n = 3). Nonpathologic pancreatic ducts (n = 4) were microdissected from KRAS* and wild-type mice.

EXTRACTION OF GENOMIC DNA AND TOTAL RNA
We extracted genomic DNA from cell lines and ducts with the Wizard Genomic DNA Purification Kit (Promega) and from macro- and microdissected samples with the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems). DNA was extracted from EUS-FNA biopsy samples with the QIAamp DNA Micro Kit (Qiagen), as previously described (14). Extracted DNA was quantified with the ND-1000 NanoDrop spectrophotometer (Thermo Scientific).

RNA was isolated from tumor samples and cell lines with TRIzol® Reagent (Invitrogen). Total RNA was extracted from microdissected samples as previously described (13). RNA quality was assessed with Experion automated electrophoresis technologies (Bio-Rad Laboratories). RNA concentration was measured with the ND-1000 NanoDrop spectrophotometer.

MicroRNA MICROARRAY
PDAC-derived cell lines were treated with 5-Aza-dC and TSA for 72 h. Total RNA was isolated as described above. The Genosensor Corporation used the GenoExplorer™ microRNA labeling kit and GenoExplorer™ Biochips to measure the production of 437 microRNAs. The microRNA data were normalized by dividing the signal intensity of a specific microRNA by the global microRNA signal intensity. This normalization value was then used for calculating the -fold change. The production of each microRNA was measured on 3 different Biochips in triplicate. MicroRNAs that were differentially produced in treated cells vs nontreated cells were identified by statistically significant results in t-tests. As commonly used in similar microRNA microarray studies (18, 19), a -fold change ≥2 was considered biologically relevant.

MEASUREMENT OF MicroRNA AND mRNA PRODUCTION BY QUANTITATIVE REVERSE-TRANSCRIPTION PCR (qRT-PCR)
miRNA and miRNA were reverse-transcribed with the miScript PCR System (Qiagen) as previously described (13). The mRNAs expressed by the MUC2 (mucin 2, oligomeric mucus/gel-forming) and MMP9 [matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)] genes in response to 5-Aza-dC and TSA treatment were measured by quantitative PCR (primers: MMP9 Forward, 5’-CACGCACGCGTGCTCCACCA-3’; MMP9 Reverse, 5’-AACGCTTCACG-3’). Results were normalized to that of ACTB (actin, beta) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by PCR analysis with the primers described previously (20). MicroRNAs were measured with the miScript PCR System and specific primers (Tₘ = 55 °C). Results were normalized to the concentration of U6 RNA, as assessed by PCR analysis with primers U6 Forward (5’-CTCGCTTCCGCA GCACA-3’) and U6 Reverse (5’-AACGCTTCA GGATAGTTTGC-3’) at a melting temperature (Tₘ) of 60 °C and with a StepOne™ Real-Time PCR System (Applied Biosystems). Gene expression was normalized to that of ACTB (actin, beta) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by PCR analysis with the primers described previously (20). MicroRNAs were measured with the miScript PCR System and specific primers (Tₘ = 55 °C). Results were normalized to the concentration of U6 RNA, as assessed by PCR analysis with primers U6 Forward (5’-CTCGCTTCCGCA GCACA-3’) and U6 Reverse (5’-AACGCTTCA GGATAGTTTGC-3’). PCR efficiency was calculated for each set of primers and was >90%.

CpG ISLAND DETERMINATION
A 20-kb genomic DNA fragment encompassing the microRNA coding sequences was analyzed with CpG plot software (http://bioweb.pasteur.fr). We used the following parameter values to report a CpG island: window size, 100 bp; minimum length of an island, 200 bp; window shift increment, 1; minimum observed-to-expected ratio, 0.6; and minimum percentage, 50%.
**BISULFITE MAPPING**

Genomic DNA (100 ng to 1 μg) was treated with sodium bisulfite. In brief, genomic DNA was denatured in 0.3 mol/L NaOH for 15 min at 37 °C. Cytosines were sulfonated in 3.6 mol/L sodium bisulfite (Sigma–Aldrich) and 1 mmol/L hydroquinone (Sigma–Aldrich) overnight at 55 °C. The modified DNA samples were desalted through a column (CpGenome™ Fast DNA Modification Kit; Millipore). The concentrations of modified DNA were measured with a ND-1000 NanoDrop spectrophotometer. We subjected 1–10 ng of the modified DNA to PCR amplification with AmpliTaq Gold® DNA polymerase (Applied Biosystems) and primers miR-148a Forward (5'-TGGGTATTTTGGTTTGGTATT-3') and miR-148a Reverse (5'-ACT ACACCTAAACCCCCCTCTAACC-3') as previously described (21). Thermocycling conditions were as follows: 5 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 15 s at 72 °C; final extension of 10 min at 72 °C. After verification, the PCR products were cloned into pCR.II plasmids (Invitrogen) and sequenced in both orientations (MilleGen). Five to 6 clones were sequenced for each sample. Modified DNA from leukocytes of healthy donors was used as a negative control in the methylation analysis.

**SEMQUANTITATIVE METHYLATION-SPECIFIC PCR (qMS-PCR)**

PCR products obtained for miR-148a bisulfite mapping analysis (see above) were diluted into water (1 volume into 250 volumes). Five microliters was then subjected to qMS-PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) and primers (methylated-specific miR-148a Forward, 5'-TGATGTGTTTTATATCGGTC-3'; unmethylated-specific miR-148a Forward, 5'-TATGATTTGTTTTATTATTGGTT-3'; methylated-specific miR-148a Reverse, 5'-AACACTAAACGA CATTGAGCG-3'; unmethylated-specific miR-148a Reverse, 5'-TATGATTTGTTTTATATTTTCTGTT-3'; unmethylated-specific miR-148a Reverse, 5'-AAC ACTAAACACATCAACAACC-3'). The amplicon size was 235 bp. The PCR reactions were carried out on the StepOne apparatus under the following thermocycling conditions: 40 cycles of 15 s at 95 °C, 30 s at 54 °C (unmethylated) or 57 °C (methylated), and 30 s at 60 °C. Specificity and efficiency were verified for each primer set. The methylation level was calculated with the formula $2^{-\Delta Ct}$, where $\Delta Ct$ is the difference in threshold cycle (Ct) values (i.e., methylated DNA – unmethylated DNA). The methylation level was expressed as a percentage.

**STATISTICAL ANALYSIS**

Results are expressed as the mean (SE). The statistical significance of differences was evaluated with the Student t-test, with a P value <0.05 considered statistically significant. To evaluate diagnostic performance, we used SPSS software (version 15.0) to calculate the area under the ROC curve and the 95% CI. ROC curve analysis was used to determine the cutoff value (an optimal cutoff was determined by setting the threshold for specificity at 90% and then maximizing sensitivity). A specific case was considered positive when the miR-148a hypermethylation level was greater than the cutoff value.

**RESULTS**

**IDENTIFICATION OF MicroRNA INACTIVATED BY DNA HYPERMETHYLATION IN PDAC-DERIVED CELL LINES**

To identify microRNAs whose production was aberrantly inhibited by DNA hypermethylation, we treated 4 PDAC-derived cell lines (BxPC-3, Capan-1, MIA PaCa-2, PANC-1) with a DNA-demethylating agent (5-Aza-dC) and a histone deacetylase inhibitor (TSA). Because each cell line displays a different sensitivity to these drugs, we optimized the 5-Aza-dC and TSA concentrations for each cell line (see Materials and Methods) by measuring the 5-methylcytosine content by nearest-neighbor analysis and the level of acetylated histone on lysine 9 by western blot analysis (see Fig. 1, A and B, in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue7). In parallel, we confirmed the induction of the MUC2 and MMP9 genes, both known to be hypermethylated in the BxPC-3, MIA PaCa-2, and PANC-1 cell lines (22, 23) (see Fig. 1C in the online Data Supplement).

The concentrations of 437 microRNAs in cells treated with 5-Aza-dC and TSA were measured by microRNA microarray hybridization and compared with the corresponding concentrations in untreated cells. We retained only the microRNAs that increased at least 2-fold after 5-Aza-dC and TSA treatment in at least 1 of the PDAC-derived cell lines. Among the 4 cell lines, 55 different microRNAs fulfilled this criterion (Table 1; see Fig. 2A in the online Data Supplement). The 5-Aza-dC/TSA–induced production of a panel of microRNAs that display important functions in cancer progression was further validated by qRT-PCR analysis (see Fig. 2B in the online Data Supplement). Of the 55 microRNA-coding regions, 29 (53%) were located in the vicinity of a CpG island (annotated with an asterisk in Table 1), therefore indicating potentially direct regulation by DNA hypermethylation.

Of these candidate microRNAs, we decided to focus on miR-148a for further study. First, the MIR148A (microRNA 148a) gene is located in close proximity to
2 CpG islands, and its expression was upregulated by 5-Aza-dC and TSA treatment in 3 of 4 PDAC-derived cell lines (see Fig. 2B in the online Data Supplement). Second, miR-148a was shown to be downregulated in pancreatic samples. The decreased expression of this microRNA gene distinguishes PDAC from CP and nonpathologic tissue (10, 11, 24).

**DNA HYPERMETHYLATION CONTROLS MIR148A EXPRESSION IN PDAC CELL LINES**

The DNA region encoding miR-148a is located in close proximity to 2 CpG islands, suggesting a potential for regulation by DNA methylation (Fig. 1A). Sodium bisulfite mapping analysis of the methylation status of the DNA region encoding miR-148a in different PDAC-derived cell lines (Fig. 1B) revealed hypermethylation of this region in 4 of 5 PDAC-derived cell lines, whereas this region was weakly methylated (3.3%) in the Capan-1 cell line. DNA extracted from peripheral leukocytes of healthy donors, in which the miR-148a–coding region is not methylated, was used as a control. In parallel, we measured miR-148a production in these PDAC-derived cell lines (Fig. 1C). We observed that the methylation level of the DNA region of the MIR148A gene was inversely correlated with MIR148A expression, suggesting that DNA hypermethylation is responsible for the repressed production of miR-148a.

We also measured the methylation level in the DNA region encoding miR-148a by qMSP-PCR and showed that the methylation level obtained with this method

| Table 1. List of microRNAs upregulated by at least 2-fold in at least 1 PDAC-derived cell line after 5-Aza-dC and TSA treatment.* |
|---|---|---|---|---|
| BxPC-3 | Capan-1 | MIA PaCa-2 | PANC-1 |
| MicroRNA | -Fold up-regulation | MicroRNA | -Fold up-regulation | MicroRNA | -Fold up-regulation | MicroRNA | -Fold up-regulation |
| miR-494* | 6.2 | miR-16 | 3.8 | miR-203* | 23.8 | miR-563 | 6.7 |
| miR-140 | 5.8 | let-7a1* | 3.7 | miR-29c | 3.8 | miR-382* | 4.4 |
| miR-148a* | 3.6 | miR-99a | 3.6 | miR-30a | 3.7 | miR-181a1 | 3.7 |
| miR-200b* | 2.6 | miR-146b* | 3.4 | miR-148a* | 3.4 | miR-518b | 3.0 |
| miR-564* | 2.2 | miR-106b* | 2.5 | miR-374a | 2.6 | miR-127* | 3.0 |
| miR-513 | 2.0 | miR-30a | 2.4 | miR-195* | 2.5 | miR-143 | 2.7 |
| miR-637* | 2.0 | miR-638* | 2.4 | miR-98 | 2.5 | miR-30b | 2.6 |
| miR-195* | 2.0 | miR-30d | 2.3 | miR-200a* | 2.5 | miR-184 | 2.6 |
| miR-19b1* | 2.3 | miR-17* | 2.5 | miR-148a* | 2.5 |
| miR-29a | 2.2 | let-7i* | 2.4 | miR-146b* | 2.3 |
| miR-135b | 2.2 | miR-30c1 | 2.4 | miR-146a | 2.2 |
| miR-30b | 2.1 | miR-16 | 2.4 | miR-193a* | 2.2 |
| miR-20a* | 2.1 | miR-30b | 2.4 | let-7f1* | 2.1 |
| miR-338* | 2.1 | miR-9-1* | 2.4 | miR-335* | 2.1 |
| miR-595 | 2.1 | miR-30d | 2.3 | miR-133a1 | 2.1 |
| miR-123-1 | 2.0 | miR-93* | 2.2 | miR-9-1* | 2.1 |
| miR-494* | 2.0 | miR-92a1* | 2.1 | miR-518f | 2.0 |
| miR-374a | 2.0 | miR-29b1 | 2.1 | miR-203* | 2.0 |
| miR-29c | 2.0 | let-7c | 2.1 | miR-17* | 2.0 |
| miR-221 | 2.1 | | | |
| let-7d* | 2.1 | | | |
| let-7e* | 2.1 | | | |
| let-7b | 2.0 | | | |
| let-7a1* | 2.0 | | | |
| let-7g* | 2.0 | | | |

n = 8 n = 19 n = 25 n = 19

* Genomic sequences encoding microRNAs annotated with an asterisk are located in the vicinity (within 20 kb) of a CpG island. miR-148a is highlighted.
correlates with the level obtained by bisulfite mapping (Fig. 2A). Although the miR-148a–coding region was hypermethylated in the BxPC-3, the Capan-2, and, to a lesser extent, the MIA PaCa-2 cell lines, this region is weakly methylated in Capan-1, leukocytes from healthy donors, and nonpathologic human pancreatic ductal epithelial cells (16). Our qMS-PCR analysis showed that 5-Aza-dC and TSA treatment decreased the level of methylation of the miR-148a–coding region, leading to an induction of MIR148A expression (Fig. 2B). Moreover, we observed miR-148a down-regulation in 10 (71.4%) of 14 PDAC samples, compared with nonpathologic adjacent tissues (Fig. 2C), results that are in accord with those of previous studies (10, 11, 24). The methylation level of the miR-148a–coding region was measured by qMS-PCR in the same samples. Spearman correlation analysis revealed an inverse correlation ($r = -0.57; P < 0.05$) between the methylation status of the MIR148A gene and its expression. These results provide evidence that DNA hypermethylation controls miR-148a production and is responsible for its down-regulation in PDAC samples and PDAC-derived cell lines.

**HYPERMETHYLATION OF THE DNA REGION ENCODING miR-148a IS AN EARLY EVENT IN PANCREATIC CARCINOGENESIS**

Although we and others have demonstrated miR-148a down-regulation in PDAC samples [Fig. 2C and (10, 11, 24)], whether this down-regulation occurs as an early event in PDAC carcinogenesis is unknown.

To address this question, we used conditional K-ras G12D mice, a murine model of PDAC precursor PanIN lesions in which the endogenous expression of mutant KRAS in the pancreas is activated by a Pdx1-Cre gene switch.
transgene [Cre recombinase under the control of Mus musculus Pdx1 (pancreatic and duodenal homeobox 1) gene promoter] (5) and that encompasses the entire panel of PanIN lesions (13). We measured the production of miR-148a in microdissected PanIN lesions by qRT-PCR. Nonpathologic ducts surrounding the PanIN lesions from KRAS* mice (KRAS* ducts) were used as a control. We included nonpathologic ducts from LSL K-ras G12D/Pdx1-Cre+/−/− littermates. We observed significant miR-148a down-regulation in the PanIN-1B stage that persisted in PanIN-2 and PanIN-3 lesions, demonstrating that this miR-148a down-regulation occurs at an early stage of PDAC development (Fig. 3A).

PanIN lesions are detected in human pancreas as well and are considered precursors of PDAC. Similarly, we observed miR-148a down-regulation in human PanIN-1B lesions (Fig. 3B). These results confirm the down-regulation of miR-148a in precursor lesions of PDAC.

Our earlier results that DNA hypermethylation is responsible for the repression of miR-148a production in PDAC and PDAC-derived cell lines led us to use qMS-PCR analysis to measure the methylation level of the miR-148a–coding region in our collection of human PanIN lesions. We observed a increased level of methylation in this region that increased significantly with PanIN progression and that was inversely correlated with miR-148a down-regulation (Fig. 3C). This finding strongly suggests that the early repression of miR-148a production in PDAC precursor lesions is provoked by DNA hypermethylation in the region encoding this microRNA.

**The Methylation of the DNA Region Encoding miR-148a Can Be Used as a Marker in the Differential Diagnosis of PDAC and CP**

We tested the hypothesis that the hypermethylated DNA region encoding miR-148a can serve as a diagnostic marker to discriminate PDAC from CP. We successfully determined the methylation level of this DNA region by qMS-PCR in 100% of the patients in the training set (39 patients with proven PDAC and 20 patients with proven CP; Fig. 4A). This analysis revealed that the DNA region encoding miR-148a was significantly more methylated in PDAC samples than in CP samples.
Fig. 2. Methylation level of the DNA region encoding miR-148a in PDAC-derived cell lines, its response to treatment with 5-Aza-dC and TSA, and its relationship to miR-148a production.

(A), Methylation level of the miR-148a–coding region in human pancreatic duct epithelial cells (HPDE), PDAC-derived cell lines, and leukocytes, as measured by qMS-PCR. Results represent the mean of 3 quantifications performed in duplicate and are expressed as the percentage of methylation. (B), The methylation level of the miR-148a–coding region and miR-148a production in Capan-2 cells in response to 5-Aza-dC and TSA treatment were measured by qMS-PCR and qRT-PCR, respectively. Data represent the mean of 3 quantifications performed in duplicate, and the methylation level and miR-148a production are expressed as a percentage of the corresponding values for untreated Capan-2 cells (100%, filled bars). *P < 0.05. (C), miR-148a production in 14 PDAC samples was measured by qRT-PCR and compared with the values for matching nonpathologic tissues. The U6 RNA concentration was used for normalization. Data are presented as the mean of 3 different quantifications performed in duplicate, and the production of miR-148a in PDAC samples is expressed as a percentage of that in adjacent tissues. The methylation level of the miR-148a–coding region was measured by qMS-PCR. Results represent the means of 3 quantifications and are expressed as the difference in the percentage of methylation between samples of PDAC and adjacent tissue. *P < 0.05.
miR-148a production and methylation level of the DNA region encoding miR-148a in PDAC precursor PanIN lesions.

(A), miR-148a production in murine nonpathologic ducts and preneoplastic PanIN lesions as measured by qRT-PCR. Nonpathologic ducts (normal duct) and PanIN lesions were obtained from KRAS<sup>*</sup> mice. Nonpathologic duct samples obtained from LSL K-ras G12D/Pdx1-Cre<sup>−/−</sup> littermates were used as a control. The level of U6 RNA was used for normalization. Results represent the mean of 2 different quantifications and are expressed as 2<sup>−ΔCt</sup>. *P < 0.05.

(B), miR-148a production in human nonpathologic ducts (normal duct) and preneoplastic PanIN lesions as measured by qRT-PCR. The level of U6 RNA was used for normalization. Results are the mean of 2 different quantifications and are expressed as 2<sup>−ΔCt</sup>. *P < 0.05.

(C), Methylation levels of the miR-148a–coding region in nonpathologic ducts (normal duct) and preneoplastic PanIN lesions as measured by qMS-PCR. Results are the mean of 3 quantifications and are expressed as the percentage of methylation of the miR-148a–coding region. *P < 0.05.

(A), The DNA methylation level of the miR-148a–coding region was measured by qMS-PCR analysis of DNA extracted from EUS-FNA samples from 20 patients with proven CP and 39 patients with proven PDAC. The results represent the mean of 3 different quantifications and are expressed as the percentage of methylation. (B), ROC curve for the miR-148a methylation level in PDAC and CP patients.

(mean miR-148a methylation, 4.25% and 1.21%, respectively; P < 0.05). We calculated the area under the ROC curve (0.719; 95% CI, 0.587–0.852; P < 0.05) and determined 3% to be the best cutoff value for diagnostically differentiating PDAC from CP at a specificity set at 90%. The corresponding sensitivity, positive predictive value, and negative predictive value of the test were 46.2%, 90.4%, and 47.4%, respectively (Fig. 4B).
Discussion

The expression profiles of microRNA genes are substantially altered in cancers (25). Loss or gain of microRNA gene expression is a crucial event in carcinogenesis. DNA hypermethylation is one cause of inactivation for numerous tumor suppressor genes and has been identified more recently as a cause of inactivation of microRNA gene expression (7, 19, 26). In this study, we identified 55 microRNAs that were upregulated after 5-Aza-dC and TSA treatment in at least 1 of 4 PDAC-derived cell lines (Table 1). A recent study that used a similar approach with Mia PaCa-2 and PANC-1 cells identified a different panel of microRNAs (18). Although certain microRNAs (miR-29a, miR-30b, and so on) are common to the 2 studies, the discrepancy between these 2 studies can be explained either by the different duration of the 5-Aza-dC treatment and its concentration or by the use of different techniques for microRNA microarray hybridization and/or normalization. Of these 55 microRNAs, the coding sequences for 29 (53%) of these microRNAs were located in the vicinity of a CpG island, therefore strongly suggesting direct repression by DNA hypermethylation. Of these 29 candidates, we focused our study on miR-148a for 3 reasons. First, the production of miR-148a was upregulated in response to 5-Aza-dC and TSA treatment in 3 of 4 PDAC-derived cell lines. Second, previous studies demonstrated a miR-148a down-regulation in PDAC that can distinguish PDAC from CP (11, 24). Third, miR-148a down-regulation has also been observed in other types of cancer, suggesting an important role for this microRNA in cancer development (19, 21, 27). We then demonstrated that aberrant DNA hypermethylation is the cause of the repression of miR-148a production in PDAC-derived cell lines and PDAC samples, compared with adjacent samples of nonpathologic tissue (Figs. 1 and 2). These findings are in accordance with previous studies that showed hypermethylation of the region encoding miR-148a in other cancer samples associated with the appearance of lymph node metastasis (19, 21). More interestingly, we further showed that this aberrant hypermethylation of the miR-148a–coding region occurs early in human PDAC precursor PanIN lesions (Fig. 3). Importantly, hypermethylation of the miR-148a–coding region, which correlates with a repression of miR-148a production, appears at the PanIN-1B stage. It is not unexpected that this early epigenetic event in PDAC carcinogenesis is also involved in the metastatic propensity of other cancers, as suggested by Lujambio et al. (19). Indeed, there are several examples of genes implicated in the regulation of invasion [e.g., SPARC, secreted protein, acidic, cysteine-rich (osteonectin); TFPI2, tissue factor pathway inhibitor 2] that are silenced by hypermethylation in pancreatic preneoplastic lesions (28–30). Together, these findings favor an important role for the loss of MIR148A expression in PDAC development.

The identification of the mRNA targets of miR-148a will provide valuable information to better understand the consequences of the loss of miR-148a in PDAC development. To date, only a few mRNA targets have been described for miR-148a in other cancers. The mRNA of TGIF2 (TGFβ-induced factor homeobox 2) gene, which is overexpressed in ovarian cancer, was demonstrated to be targeted by miR-148a (19). The mRNA of pregnant X receptor, a major transcription factor regulating the inducible expression of a variety of transporters and drug-metabolizing enzymes, has also been demonstrated to be a miR-148a target (31). Interestingly, the mRNA of the DNMT3B [DNA (cytosine-5-)-methyltransferase 3 beta] gene, which encodes a DNA methyltransferase enzyme, is another known target of miR-148a (32). It is therefore tempting to speculate that the down-regulation of miR-148a observed early in preneoplastic lesions leads to an up-regulation of DNMT3B and consequently to the inactivation of tumor suppressor genes.

DMR detection is commonly used in clinics for cancer diagnosis (33), and the detection of DMRs in resected tumor samples has previously been described to diagnose PDAC (34). Unfortunately, only 15% of PDAC patients are eligible for surgical resection. As previously mentioned, miR-148a down-regulation distinguishes PDAC samples from CP samples (11, 24). Despite the improvements in imaging techniques, the differential diagnosis of PDAC and CP remains arduous in practice, especially in cases of the pseudotumoral form of CP. EUS-FNA biopsy is a safe and effective technique for diagnosing and staging PDAC; however, measurement of RNA production can be problematic because of the poor quality of the RNA extracted from these biopsies [(24) and our own experience]. EUS-FNA biopsy allows cytoligic diagnosis and the detection of genetic alterations, such as mutation of the KRAS gene (14). Although a similar approach has been used to discriminate breast cancer from benign lesions (35), our study is the first to demonstrate the feasibility and potential value of qMS-PCR quantification of a specific DMR region in DNA extracted from EUS-FNA samples of patients with pancreatic ailments. Indeed, we have shown that the methylation level of the miR-148a–coding region is significantly higher in PDAC samples than in CP samples (4.25% vs 1.22%, P < 0.05; Fig. 4). The weak level of methylation compared with that in PDAC-derived cell lines (Figs. 1 and 2) can be easily explained by the lower content of cancerous cells in EUS-FNA biopsy samples. Our goal is to use this marker as a diagnostic tool.
for PDAC rather than as a screening tool. For a specificity goal of ≥90%, we determined a cutoff value for miR-148a hypermethylation of 3% with a sensitivity at 46.2% (Fig. 4). We are aware that the accuracy of our test remains below that routinely achieved by cytopathology analysis. It is important to specify, however, that the differential diagnosis of PDAC and CP in the patients included in this study was established by EUS-FNA cytopathologic analysis, and despite the high specificity (100%), cytotologic analysis with a sensitivity of only 83% is not a gold standard for the differential diagnosis of PDAC and CP (14). To measure the accuracy of our 2 tests (i.e., cytopathology and analysis of methylation levels in the miR-148a–coding region) or the performance of a combination of both methods will require study with resected tumors and pathology analysis. In this retrospective study, 2 of 20 patients with a CP diagnosis presented with a MIR148A methylation level greater than the cutoff threshold of 3%. We cannot exclude the possibility that these 2 patients with diagnosed CP actually have high-grade PanIN lesions or will develop PDAC in the near future.

In conclusion, we point out the potential value of this marker in the differential diagnosis of PDAC and CP. When cytology results are inconclusive, the use of this marker may avoid the need for additional invasive biopsies. Moreover, we envision combining study of the methylation level of the miR-148a–coding region with cytopathology analysis to improve the diagnosis of PDAC.

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