Novel Methylation Biomarker Panel for the Early Detection of Pancreatic Cancer

Joo Mi Yi1,8, Angela A. Guzzetta2, Vasudev J. Bailey3, Stephanie R Downing2, Leander Van Neste7, Katherine B. Chiappinelli1, Brian P. Keely5, Alejandro Stark5, Alexander Herrerra5, Christopher Wolfgang2, Emmanouil P. Pappou2, Christine A. Iacobuzio-Donahue6, Michael G. Goggins6, James G. Herman1, Tza-Huei Wang4,5, Stephen B. Baylin1, and Nita Ahuja1,2,3

Abstract

Purpose: Pancreatic cancer is the fourth leading cause of cancer deaths and there currently is no reliable modality for the early detection of this disease. Here, we identify cancer-specific promoter DNA methylation of BNC1 and ADAMTS1 as a promising biomarker detection strategy meriting investigation in pancreatic cancer.

Experimental Design: We used a genome-wide pharmacologic transcriptome approach to identify novel cancer-specific DNA methylation alterations in pancreatic cancer cell lines. Of eight promising genes, we focused our studies on BNC1 and ADAMTS1 for further downstream analysis, including methylation and expression. We used a nanoparticle-enabled methylation on beads (MOB) technology to detect early-stage pancreatic cancers by analyzing DNA methylation in patient serum.

Results: We identified two novel genes, BNC1 (92%) and ADAMTS1 (68%), that showed a high frequency of methylation in pancreatic cancers (n = 143), up to 100% in PanIN-3 and 97% in stage I invasive cancers. Using the nanoparticle-enabled MOB technology, these alterations could be detected in serum samples (n = 42) from patients with pancreatic cancer, with a sensitivity for BNC1 of 79% [95% confidence interval (CI), 66%–91%] and for ADAMTS1 of 48% (95% CI, 33%–63%), whereas specificity was 89% for BNC1 (95% CI, 76%–100%) and 92% for ADAMTS1 (95% CI, 82%–100%). Overall sensitivity using both markers is 81% (95% CI, 69%–93%) and specificity is 85% (95% CI, 71%–99%).

Conclusions: Promoter DNA methylation of BNC1 and ADAMTS1 is a potential biomarker to detect early-stage pancreatic cancers. Assaying the promoter methylation status of these genes in circulating DNA from serum is a promising strategy for early detection of pancreatic cancer and has the potential to improve mortality from this disease. Clin Cancer Res; 1–12. ©2013 AACR.

Introduction

Pancreatic cancer is a deadly cancer with an overall 5-year survival rate of less than 5% and few improvements in survival over the last 3 decades (1, 2). Pancreatic cancer currently ranks as the fourth leading cause of cancer related death in the United States with an estimated 44,030 new cases and 37,660 deaths in 2011 and its incidence is increasing (3, 4). One of the major factors attributed to the dismal prognosis of pancreatic cancer is delayed diagnosis such that only about 10% of patients are amenable to potential curative surgical resection (4). However, long-term 5-year survival is attainable in selected patients for whom surgical removal of early-stage disease is accomplished (5, 6). Therefore, early detection of pancreatic cancer is thought to be the best modality for improving survival in this lethal disease (7–10). At this time, no reliable screening test currently exists for the early detection of this cancer.

Pancreatic cancer is characterized by multiple genetic and epigenetic changes (11, 12). In recent years, it has become apparent that pancreatic cancer is as much a disease of misregulated epigenetics as it is a disease of DNA mutations (11, 12). In particular, changes in DNA promoter methylation patterns could play a crucial role in tumorigenesis and cancer progression (13). To address the need for both clinical diagnostics and therapeutics, many studies have used DNA methylation analysis of specific genes for

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

Corresponding Authors: Nita Ahuja, Department of Surgery and Oncology, Johns Hopkins University, Baltimore, MD 21287. Phone: 410-502-6136; Fax: 410-502-0987; E-mail: nahuja@jhmi.edu; Stephen B. Baylin, Department of Oncology, Johns Hopkins University, Baltimore, MD 21287, USA. Phone: 410-955-8506; Fax: 410-614-9884; E-mail: sbaylin@jhmi.edu; and Joo Mi Yi, Research Institute, Dongnam Institute of Radiological and Medical Sciences (DIRAMS), Busan, South Korea. Phone: 82-51-720-5139; Fax: 82-51-720-5929; E-mail: jmyi@dirams.re.kr

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Translational Relevance

Pancreatic cancer is a deadly cancer with an overall 5-year survival rate of less than 5%. No significant improvements in survival have been observed over the past three decades. Early detection of pancreatic cancer is thought to be the best hope for improving survival in this lethal disease. We have identified a high frequency of methylation of BNC1 and ADAMTS1 in invasive pancreatic cancers at 97%. In addition, using methylation on beads (MOB) technology, BNC1 and ADAMTS1 promoter methylation in pancreatic cancer patient sera can be detected with high sensitivity and specificity. Detection of methylated BNC1 may serve as a new early detection biomarker for the screening of patients with pancreatic cancer and has the potential to improve mortality from this lethal cancer.

application in diagnostics of multiple cancers (13, 14). Such diagnostic tests can, in principle, be used for early detection, for assessing prognosis, as predictors of response to therapy, and as therapeutic targets. Early detection of disease results in an improved clinical outcome for most types of cancer (15, 16). Therefore, much effort has been invested in developing efficient screening technologies for this purpose. Recently, there have been multiple successful reports of DNA methylation screening using various body fluids such as stool for detection of colorectal cancer (17, 18), sputum for lung cancer (19), and urine for prostate cancer (20). However, no screening test is currently available for early detection in pancreatic cancer. This tumor type is often metastatic or locally advanced at initial diagnosis as patients often present with nonspecific gastrointestinal symptoms (21). The development of an early-stage screening modality for pancreatic cancer, for the use in particularly in high-risk groups, would then have a potential impact in reducing mortality from this currently lethal disease.

Any optimal screening technology needs to be simple, easy to conduct, cost-effective, noninvasive, and yield more benefit than harm. Such screening efforts have been in practice for more than a decade for a multitude of various cancers (2). Currently, endoscopic ultrasound has shown promise as a screening tool for pancreatic cancer in high-risk patients, but it is available only in highly specialized centers and is an expensive and invasive modality that needs to be repeated at frequent intervals (22, 23). In the current report, we now define the use of a genome-wide expression array technology to identify novel hypermethylated genes in pancreatic cancer for which assays of methylation status in serum DNA holds robust potential for early detection of this disease.

Materials and Methods

Cell culture, treatment, and transfection

Pancreatic cancer cell lines (Panc1, MIA-PaCa2, PL45, and Capan1) were obtained from American Type Culture Collection and maintained under the recommended conditions. All cell lines were treated with the demethylating agent 5-aza-2’-deoxycytidine (DAC), trichostatin-A (TSA), or saline (control), and cells were collected for DNA and RNA. DKO cells (HCT116 colon cancer cells with genetic disruption of DNMT1 and DNMT3b) were cultured as described previously (24). Full-length BNC1 cDNA was subcloned into the pIREs-neo3 expression vector. Panc-1 and MIA-PaCa2 cells were transfected with the Lipofectamine 2000 Reagent (INVITROGEN) according to the manufacturer’s protocol. Panc-1 and MIA-PaCa2 cells were transfected with a control construct (empty vector) or BNC1-pIRESneo3, selected for 10 days with G418 (500 µg/mL).

Gene expression microarray analysis

Total RNA was harvested from log phase cells using TRIzol (Invitrogen) and the RNeasy kit (QIAGEN) according to the manufacturer’s instructions, including a DNase digestion step. RNA was then used for the Agilent 4 × 44 genome-wide expression array. Data analysis was conducted using previously reported techniques (25).

In vitro cell proliferation, migration, and invasion assays

Panc-1 and MIA-PaCa2 cells were seeded onto 96-well plates (5,000 cells per well), and after 96 hours, the cultures were pulsed for 6 hours with 0.3 µCi [methyl-3H] thymidine (Amersham Life Science) per well. Three independent experiments were carried out. Proliferation was measured using liquid scintillation. Cell migration and invasion assays were conducted using 24-well Transwells (8-µm pore size) coated with (invasion) or without (migration) Matrigel (BD Biosciences). About 20 × 104 Panc-1 and MIA-PaCa2 cells in 1% FBS-DMEM were seeded into the upper chamber, and Dulbecco’s Modified Eagle’s Medium (DMEM) containing 20% FBS was placed in the lower chamber. After 48 hours, cells on the lower surface of the membrane were fixed with methanol and stained with 1% Toluen Blue in 1% borax, and the cells on the lower surface of the membrane were counted with the use of a light microscope. Transwell experiments were assessed in 3 replicate experiments.

Patient samples and study population

Pancreatic tissues were collected from 173 patients with formalin-fixed, paraffin-embedded (FFPE) tissues (Table 1). These included 123 tissue samples from patients with stage I through stage IV pancreatic cancer who underwent primary surgical resection at the Johns Hopkins Hospital (JHH) from 1998 to 2009 (median follow-up of 6.4 years). For comparison, additional FFPE pancreatic tissues were obtained from patients who had undergone pancreatectomy for pancreatic cancer but had the surrounding premalignant lesion called pancreatic intraepithelial neoplasia (PanIN; n = 20) or for pancreatitis (n = 30). Pathology was re-reviewed to confirm histology (C.A. Iacobuzio-Donahue; Table 1). Clinicopathologic characteristics and overall survival were checked using patient medical records.
Total RNA and matched gDNA were obtained from 3 pancreatic cancer patient donors and 4 normal pancreatic tissue donors. (Biochain Institute, Hayward, CA)

Preoperative CA 19-9 levels were investigated in our patient population. A total of 45.1% of patients in our cohort had preoperative CA 19-9 levels measured. The range for normal CA 19-9 at our institution is 0 to 36 U/mL, and values greater than 36 were considered elevated and abnormal.

DNA methylation analysis

Primer pairs for methylation analysis were designed using MSP Primer (http://www.mspprimer.org). All primer sequences are listed in Supplementary Table S1. DNA was extracted using the standard phenol/chloroform extraction method. Bisulfite modification of gDNA was carried out using the EZ DNA Methylation Kit (Zymo Research). Conventional methylation-specific PCR (MSP) was then conducted as previously described on all FFPE samples (26).

Quantitative methylation-specific PCR (qMSP) was conducted on all cell lines and FFPE tissues from normal pancreas (n = 14), chronic pancreatitis (n = 30), PanIN (n = 20), and pancreatic tumors (n = 12). The Power SYBR Green PCR Kit (Applied Biosystems) was used and the amplification conditions consisted of an initial 10-minute denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension for 30 and 60 seconds, respectively. An ABI StepOnePlusReal-Time PCR System was used (Applied Biosystems), and for quantification, the comparative cycle threshold (Ct) method was used, normalizing the Ct values for the indicated gene to the Ct values of GAPDH relative to that of the mean of 4 normal pancreas samples (27).

Pancreatic cancer patient serum samples

Patient serum samples were obtained from individuals with pancreatic cancer before undergoing surgical treatment at the JHH after obtaining informed consent from 2007 to 2009. Matching tumor samples were drawn from the pathology archives of the JHH in accordance with all rules and regulations of the Institutional Review Board (IRB) and as per HIPAA compliance. A total of 42 serum samples were tested from patients with invasive pancreatic cancers and 23 of which had matching FFPE tissue available. In addition, 26 serum samples were obtained from normal, healthy volunteers to serve as controls.

Methylation on beads method

Methylation on beads (MOB) is a recently developed nanotechnology that permits capture, retention, and bisulfite treatment of minute amounts of DNA (28). This type of methodology is ideal for examining DNA in body fluids including stool, sputum, and serum. MOB was conducted as previously described (29, 30). DNA methylation was detected as described above.

Statistical analysis

Unless otherwise noted, experiments were carried out in triplicate and data are presented as the mean ± SEM. For quantitative MSP analysis, BNC1 and ADAMST31 promoter methylation was considered positive if the methylation value was greater than twice that of the average of 4 normal pancreas samples. The Student t-test was used for analyses of [3H]thymidine incorporation and anchorage-independent cell growth. The Mann–Whitney rank-sum test was used to analyze data obtained in the colony formation, migration, and invasion assays. All P values are 2-sided, and P ≤ 0.05 was considered statistically significant. Statistical calculations were carried out using GraphPad Prism4 software.

Table 1. Clinical information for primary pancreatic samples

<table>
<thead>
<tr>
<th>Gender</th>
<th>Normal (n = 4)</th>
<th>PanINs (n = 20)</th>
<th>Stage I (n = 38)</th>
<th>Stage II (n = 78)</th>
<th>Stage III (n = 5)</th>
<th>Stage IV (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>3 (75%)</td>
<td>9 (45%)</td>
<td>9 (23.7%)</td>
<td>44 (57%)</td>
<td>1 (20%)</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Female</td>
<td>1 (25%)</td>
<td>11 (55%)</td>
<td>29 (76.3%)</td>
<td>34 (43%)</td>
<td>4 (80%)</td>
<td>0.0%</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>9 (45%)</td>
<td>3 (7.9%)</td>
<td>4 (5.1%)</td>
<td>1 (20%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>8 (40%)</td>
<td>23 (60.5%)</td>
<td>41 (52%)</td>
<td>4 (40%)</td>
<td>1 (50%)</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>3 (15%)</td>
<td>12 (31.6%)</td>
<td>33 (42.9%)</td>
<td>4 (40%)</td>
<td>1 (50%)</td>
<td></td>
</tr>
</tbody>
</table>

expression analysis was conducted by qPCR using 1 μL of cDNA as template and JumpStart Red Taq DNA Polymerase (SIGMA) for amplification. Quantification of the Ct method was used, normalizing the Ct values for the indicated gene to the Ct values of GAPDH relative to that of the mean of 4 normal pancreas samples (27).
The Cancer Genome Atlas analysis

Forty pancreatic cancer samples were publically available in The Cancer Genome Atlas (TCGA) with matched expression data via RNASeq version 2 and methylation data via the Illumina Infinium 450 K platform. Level 3 data, which is normalized by TCGA, was used for analysis. Linear regression modeling was used to fit the data and a pairwise, 2-sided Pearson correlation coefficient was calculated and graphed using R: A language and environment for statistical computing (31).

Results

Identification of cancer-specific promoter DNA methylation in pancreatic cancer

To identify novel DNA methylation biomarkers in pancreatic cancer, we used our previously established human transcriptome-wide microarray screen in 4 different human pancreatic cancer cell lines (PL45, Mia-PaCa2, Panc1, and Capan1) to detect genes silenced by promoter hypermethylation (25). This assay uses a genome-wide pharmacologic strategy using DAC and TSA to re-express genes silenced in association with abnormal promoter CpG island DNA methylation (25, 32). We initially identified a total of 1,427 unique genes in all four cell lines that met the criteria based on our prior studies (33): (i) methylation in pancreatic cancer cell lines associated with loss of expression; (ii) presence of gene expression in normal pancreas tissue; (iii) no or low methylation in normal pancreatic tissues (cancer-free tissues); and (iv) methylation present in primary pancreatic cancers. Eight genes (TFPI2, ASCL2, BNC1, TWIST1, BNIP3, ADAMTS1, PNMT, and EVL) fulfilled the above criteria on our initial screen, displaying cancer-specific methylation in primary pancreatic cancers (Fig. 1).

BNC1 and ADAMTS1: Potential DNA methylation biomarker for early detection of pancreatic cancer

We examined the methylation status of all 8 of our candidate genes in a large series of primary pancreatic tumor samples, (n = 123; stages I—IV; Table 1), normal pancreas (n = 4), and premalignant PanINs (n = 20) by MSP. The most frequently methylated gene was BNC1 (91%), followed by ADAMTS1 (67%), TWIST1 (67%), ASCL2 (65%), BNIP3 (49%), TFPI2 (54%), EVL (47%), and PNMT (27%; Fig. 1). Interestingly, 2 of the genes, BNC1 (91%) and ADAMTS1 (67%), that showed frequent methylation in this cohort of primary pancreatic cancer samples also showed frequent methylation in the precursor lesions of pancreatic cancer, PanIN lesion [BNC1: 70% (14 of 20); ADAMTS1: 25% (5 of 20), respectively; Fig. 1].

We verified the abnormal methylation status of BNC1 and ADAMTS1 through quantitative MSP analyses (Fig. 2A) and correlated this with expression patterns for these genes by qPCR (Fig. 2B). Both genes showed lack of endogenous gene expression and significant re-expression after DAC treatment in these cell lines. Treatment with a histone deacetylase inhibitor such as TSA resulted in minimal re-expression except in PL45 for BNC1, which may be regulated both by promoter DNA methylation and by histone modifications (Fig. 2B). We also confirmed promoter-associated CpG island methylation in the BNC1 and ADAMTS1 promoter by bisulfite sequencing analysis in pancreatic cancer cell lines, primary pancreatic cancer samples, normal pancreatic tissue, and in the DNMT1−/− DNMT3B−/− double knockout (DKO), which serves as a negative control. These analyses revealed dense methylation of both BNC1 and ADAMTS1 in the pancreatic cancer cell line and the primary pancreatic cancer and minimal or no methylation in normal pancreas samples and in DKO. These results are all consistent with our conventional and quantitative MSP analyses (Fig. 2C and D).

Little is known about the role of epigenetic alterations in the precursor lesions of pancreatic cancer even though
defining the timing of prevalence of DNA methylation events would be critical information for understanding pancreatic carcinogenesis, for early pancreatic cancer detection as well as for identification of novel targets for chemoprevention (34). Identification of precursor lesions such as PanIN (or pancreatic carcinoma in situ) is thought to represent one of the best targets available for early detection and chemoprevention strategies for pancreatic cancer (35). Specifically, BNC1 was also methylated in the tissues of PanIN-1 (5 of 9, 56%), PanIN-2 (6 of 8, 75%), and PanIN-3 (3 of 3, 100%) during PanIN progression (data not shown).

Next, we collected and analyzed a subset of tissue samples from patients diagnosed with noncancerous diseases such as pancreatitis that may have confounding effects for using BNC1 and ADAMTS1 methylation status for early detection of pancreatic cancer. Patients with chronic pancreatitis are at a 2- to 26-fold increased risk for developing pancreatic cancer (4). However, chronic inflammatory conditions may also increase the frequency of methylation as a field defect that may then increase risk of subsequent malignancy (36–38). We compared methylation of BNC1 and ADAMTS1 between different conditions (normal, pancreatitis, PanIN, and invasive cancers) using MSP analysis. As shown in Fig. 3, BNC1 (A) and ADAMTS1 (B) show statistically increased frequency of methylation when comparing normal pancreas tissues and invasive cancers (P < 0.001; both BNC1 and ADAMTS1) as well as between chronic pancreatitis and invasive cancers (P < 0.001; both BNC1 and ADAMTS1). There is a low frequency of BNC1 and ADAMTS1 methylation present in noncancerous disease such as pancreatitis. More interestingly, there is significant quantitative difference between PanINs and invasive cancers on both genes (P < 0.001; both BNC1 and ADAMTS1). However, BNC1 methylation could be detected in the earliest stages of pancreatic carcinogenesis such as PanIN-1, unlike the patterns seen for ADAMTS1 methylation whereby ADAMTS1 methylation is only seen in invasive cancers (data not shown), suggesting that the use of both genes may be optimal to determine the presence of invasive pancreatic cancer.

![Figure 2. A and B, silencing of BNC1 and ADAMTS1 genes in pancreatic cancer cell lines. A, qMSP analysis of BNC1 and ADAMTS1 gene promoter region and correlation with (B) gene expression by qPCR in pancreatic cancer cell lines. IVD, in vitro methylated DNA. DKO HCT116 cells (DNMT1−/− and DNMT3b−/−). Quantitative PCR expression is shown as fold change ± SE relative to mock-treated (M) cells during 5 μmol/L DAC (D) and 300 nmol/L TSA (T) treatments. Normal pancreas and DKO cells were used as controls. (Continued on the following page.)](g)
Correlation of BNC1 and ADAMTS1 methylation and gene expression in pancreatic cancer

We examined whether methylation of promoter-associated CpG-islands of BNC1 and ADAMTS1 is associated with gene silencing by investigating BNC1 and ADAMTS1 mRNA expression in fresh pancreatic cancer tissues (n = 4). Both BNC1 and ADAMTS1 mRNA levels in primary pancreatic cancers were significantly downregulated or silenced compared with normal pancreatic mRNA as well as in DKO cells (P < 0.001; BNC1 and ADAMTS1, t-test; Fig. 4A). We also confirmed, by MSP analysis, that the downregulation of gene expression was associated with DNA methylation of BNC1 and ADAMTS1 in these primary pancreatic cancers.

Normal pancreas had no methylation of either gene but expressed both genes (Fig. 4B). Investigation of methylation and expression in the larger TCGA dataset revealed that ADAMTS1 did not have an adequate number of CpG island probes for evaluation. BNC1 methylation and expression were inversely correlated (correlation coefficient = −0.59, P < 0.001; Fig. 4C).

We also investigated the expression of BNC1 and ADAMTS1 in larger cohorts of primary pancreatic cancers using previously published gene expression microarray data previously in Oncomine (Compendia Bioscience). The Oncomine database is a web-based data mining platform aimed at facilitating gene discovery from genome-wide...
expression analyses in cancer (39). Interestingly, we found that both BNC1 and ADAMTS1 are significantly downregulated in multiple independent primary pancreatic cancers gene expression array datasets (Supplementary Table S2).

**Tumor-suppressive effects of BNC1 gene overexpression in pancreatic cancer cells**

As BNC1 appeared to be the most promising biomarker for early detection of pancreatic cancer, we investigated whether this gene possesses tumor-suppressive effects in pancreatic cancer cells in vitro. We transfected full-length BNC1 into Panc1 and MIA-PaCa2 cells lacking BNC1 expression (Supplementary Fig. S2). We then conducted in vitro colony formation assays and found overexpression with BNC1 full-length gene induced a nearly 2.2-fold (Panc1) and 9-fold (MIA-PaCa2) reduction of G418-resistant colonies (Fig. 5A). In addition, there was a 73% (Panc1) and 82% (MIA-PaCa2) decrease in cell proliferation as measured by [3H] thymidine activity (Fig. 5B and C; left and middle). However, overexpression of BNC1 gene, as compared with control cells transfected with empty vector, had no effect on the migration and invasion capacities of Panc1 and MIA-PaCa2 cells as examined in the Matrigel-coated Transwell membrane assay (Fig. 5B and C; right). Taken together, these data suggest that BNC1 may have tumor-suppressive effects in human pancreatic cancer cells in vitro.

**Detection of DNA methylation in pancreatic cancer patient sera using MOB technology**

As no reliable blood-based screening test exists for patients at high risk of pancreatic cancer, the development of a noninvasive modality for cancer detection may improve mortality in this aggressive cancer (40, 41). Recently, many studies have suggested that cancer-specific DNA methylation patterns can be found in detached tumor cells in bodily fluids and biopsies, or in free-floating DNA from patients with cancer (14, 42). Therefore, we examined, as a pilot study, whether we can detect presence of BNC1 and ADAMTS1 promoter DNA methylation in pancreatic cancer patient sera. We used a highly sensitive nonaoenabled assay, termed MOB, which we have recently developed to improve the sensitivity of methylation detection (28). MOB incorporates DNA isolation and bisulfite conversion in a single-tube process, which minimizes the loss of sample and risk of contamination associated with separate processes.

We tested serum samples from a series of patients with pancreatic cancer, stages I–IV (n = 42) as well as a panel of sera from normal healthy individuals (n = 26). Sensitivity was determined on the basis of the stringent assumption that all patients with pancreatic cancer would have DNA methylation of BNC1 and ADAMTS1, whereas the genes would be unmethylated in all healthy normal volunteers. We found that serum samples from 33 of the 42 patients with cancer had methylation for BNC1, whereas 20 of the 42 showed methylation for ADAMTS1. Overall, for all stages included, the sensitivity was determined to be 79% and 48% for BNC1 and ADAMTS1, respectively. Sensitivity of detection of stage I pancreatic cancers was 90% for both genes. Among the 26 normal serum samples, 3 of the normal samples showed methylation for BNC1, whereas 2 showed methylation for ADAMTS1. Specificity of detection was determined to be 89% for BNC1 and 92% for ADAMTS1 (Table 2). Use of both genes together for early detection improved sensitivity [81%; 95% confidence interval (CI), 69%–93%] but not specificity (85%; 95% CI, 71%–99%) compared with single gene methylation.

**Figure 3.** qMSP analysis of BNC1 and ADAMTS1 using real-time PCR. Normal pancreas (n = 4 normal tissues, n = 10 surrounding normal tissues), chronic pancreatitis samples (n = 30), PanIN 1–3 (n = 20), and stage II tumors (n = 12), respectively. qMSP showed significantly increased frequency of methylation when comparing normal pancreas tissues and invasive cancers (P < 0.001; both BNC1 and ADAMTS1) as well as between chronic pancreatitis and invasive cancers (P < 0.001; both BNC1 and ADAMTS1). qMSP is shown as fold change for methylated signal relative to unmethylated signal. In qMSP analysis, signals for unmethylated (U) and methylated (M) DNA are shown for each sample. Horizontal bar indicates mean methylation level.
detection. Results in the corresponding FFPE tissue samples of pancreatic cancer were higher for both genes, with 100% sensitivity and specificity for \textit{BNC1} and 79.2% sensitivity and 100% specificity for \textit{ADAMTS1}.

### Discussion

In this study, we used a genome-wide pharmacologic approach to identify methylation of cancer-specific genes in pancreatic cell lines. We have used such an approach

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>Estimated value</th>
<th>95% CI</th>
<th>Estimated value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>90% (9/10)</td>
<td></td>
<td>90% (9/10)</td>
<td></td>
</tr>
<tr>
<td>II–IV</td>
<td>32</td>
<td>75% (24/32)</td>
<td></td>
<td>34% (11/32)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>79% (33/42)</td>
<td>66%–91%</td>
<td>48% (20/42)</td>
<td>33%–63%</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Normal</th>
<th>26</th>
<th>Estimated value</th>
<th>95% CI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>89%</td>
<td></td>
<td>92%</td>
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Table 2. Sensitivity and specificity of \textit{BNC1} and \textit{ADAMTS1} in pancreatic cancer patient serum samples.
previously in both colorectal and breast cancers (17, 25, 43). Defining the DNA hypermethylome has been useful to identify not only novel DNA methylation biomarker candidates but also tumor suppressor gene candidates (33). DNA methylation biomarkers have obvious applications in diagnostics (17, 18, 33) but can also contribute indirectly to therapeutics as predictors of response to therapy (28). We initially identified 1,427 genes that are potentially silenced by DNA methylation in pancreatic cancer and then focused on those genes showing cancer-specific methylation in primary tumors (25). In-depth analysis was limited to BNC1 and ADAMTS1, which showed the highest frequency of methylation at 91.8% and 66.7%, respectively, in pancreatic cancer samples. Basonuclin (BNC1) is a zinc finger transcription factor that interacts with the promoters of both RNA polymerases I and II. Bioinformatic analysis suggests that basonuclin target genes may be implicated in chromatin regulation.

Figure 5. Functional assays of BNC1 gene in pancreatic cancer cells. A, colony formation in Panc1 and MIA-PaCa2 cells transfected with pcDNA3.1 or BNC1-pcDNA3.1 and grown for 2 weeks in medium containing G418. Results are plotted as the mean colony numbers relative to pcDNA3.1 transfectants in 3 independent experiments (Panc1: *, P = 0.0275; MIA-PaCa2: **, P = 0.0294). B, cell proliferation and invasion assays in Panc1 cells. C, cell proliferation and invasion assays in MIA-PaCa2 cells. Left, BNC1-transfected Panc1 cells (BNC1-pcDNA3.1) were compared with control cells transfected with empty vector (pcDNA3.1). Results are plotted as the mean cells number in three different independent experiments (Panc1: *P = 0.0469, 72 hours; **P = 0.0086, 96 hours; MIA-PaCa2: *, P = 0.0469, 48 hours; **, P = 0.0318, 72 hours; ***, P = 0.0389, 96 hours). Middle, cell proliferation measured by 3H-thymidine incorporation (Panc1: *, P = 0.0286; MIA-PaCa2: **, P = 0.0256). Right, invasion of Panc1 and MIA-PaCa2 cells through Matrigel-coated Transwells relative to control cells transfected with empty vector in 3 independent experiments. NS, not statistically significant.
our studies also found that methylated ADAMTS1 was associated with a worse overall survival as compared with unmethylated ADAMTS1. This was significant by log-rank analysis ($P = 0.03$) as well as by univariate Cox regression analysis (ADAMTS1 unmethylated as reference, ADAMTS1 methylated; OR = 1.6; CI, 1.05–2.52; $P = 0.03$; Supplementary Fig. S3).

Our principal goal in this study was to identify new genes in pancreatic cancer showing tumor-specific methylation, which could serve as early detection biomarkers. The poor accessibility of the pancreas along with the late presentation of symptoms have thwarted attempts at timely detection of this malignancy and contributes to high mortality rates such that pancreatic cancer remains the fourth leading cause of cancer death in both men and women (53). There have been limited improvements in the mortality from this disease over the past 30 years (54). However, cancers such as breast and colorectal cancers, where screening has been in practice for early detection, have seen a decrease in cancer mortality (55). Therefore, development of cancer biomarkers for pancreatic cancer is the best hope for early detection and potentially improving mortality from this lethal cancer (14).

Prior studies have identified possible targets for aberrant DNA methylation in pancreatic cancers. Identification of PanIN using methylation patterns was described by Sato and colleagues with methylation frequency ranging from 13% to 30% for a panel of genes and suggested that their set of genes might also be used for early detection (56). Herein, we suggest that promoter methylation of BNC1 and ADAMTS1 may comprise a more promising method of detection of early-stage pancreatic cancer as they provide a much greater sensitivity. To our knowledge, BNC1 and ADAMTS1 have not been previously described as DNA methylation biomarkers in pancreatic cancers. In addition, we saw that methylation of these genes was able to detect early-stage pancreatic cancers at higher frequencies than the current blood-based test, CA 19-9. In detail, the median CA 19-9 for all patients was 114.5 U/mL. The rate of abnormal and elevated CA 19-9 in all invasive cancers was 70%. This increased with increasing stage, 52% of stage I cancers had an elevated CA 19-9. This rose to 73.7% of stage II cancers and 100% of stage III and IV cancers. In all instances, the combination of methylation of ADAMTS1 and BNC1 showed improved sensitivity compared with CA 19-9 (Table 3). This suggests that methylation of these genes may be useful for screening of high-risk individuals including patients with patients with family history of pancreatic cancer, hereditary pancreatitis, familial syndromes such as BRCA1/2 mutation families, Peutz–Jeghers syndrome or hereditary nonpolyposis colorectal cancer, cystic fibrosis, or familial atypical multiple mole syndrome (57).

Ideally, methods for cancer screening should be easy to conduct, cost-effective, noninvasive, and provide a benefit to patients. Blood-based biomarkers fulfill the first 3 requirements. However, development of such technology requires an ultrasensitive strategy. In a pilot study, we now show that screening with a highly sensitive nano-based technology allows sensitive and specific detection of invasive cancers. Using our 2 biomarkers (BNC1 and ADAMTS1), we are able to detect very early stages of pancreatic cancer, with high sensitivity and specificity. Not only have we achieved, in these first studies, higher sensitivities than previously reported for serum hypermethylation markers, but also our technology has the potential to constitute a cost-effective approach for screening of selected, highest risk individuals in the general population for cancers. In addition, our nano-based MOB detection method

### Table 3. Comparison of BNC1 and ADAMTS1 methylation and CA 19-9

<table>
<thead>
<tr>
<th>Stage</th>
<th>BNC1</th>
<th>ADAMTS1</th>
<th>ADAMTS1 and BNC1 combined</th>
<th>CA 19-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PanIN ($n = 20$)</td>
<td>70%</td>
<td>25%</td>
<td>75%</td>
<td>20%</td>
</tr>
<tr>
<td>Stage I ($n = 38$)</td>
<td>97%</td>
<td>63%</td>
<td>97%</td>
<td>52%</td>
</tr>
<tr>
<td>Stage II ($n = 78$)</td>
<td>96%</td>
<td>82%</td>
<td>96%</td>
<td>73.7%</td>
</tr>
<tr>
<td>Stages III and IV ($n = 7$)</td>
<td>100%</td>
<td>57%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

**NOTE:** The rates of methylation for either BNC1, ADAMTS1, or BNC1 and ADAMTS1 combined were identified for PanIN and invasive pancreatic cancers, stage I, stage II, and stages III and IV. Any methylation was considered to be a positive test. CA 19-9 levels were derived from patients who had evaluation of a CA 19-9 level before surgery. Any value ≥37 U/mL and greater was considered to be positive.
serves to significantly reduce the quantity of serum necessary for analysis (58).

The implementation of BNC1 and ADAMTS1 for screening will require testing in a larger prospective study. We anticipate that the methylation level of these 2 genes (BNC1 and ADAMTS1) may be used alone, or in combination with other standard methods, to characterize the neoplasia. For example, methods for detecting BNC1 or ADAMTS1 promoter methylation could be carried out before or concurrently with testing for mutations common to pancreatic cancer such as KRAS (57), PALB2 (58), or GNAS (59) mutations to improve cancer specificity and sensitivity. Further diagnostic testing could then be initiated to localize the pathology based on their risk profiles including imaging studies such as CT or MRI scans and/or endoscopic studies. As one example, a patient with an extensive family history of pancreatic cancer who tests positive for methylation of BNC1 and ADAMTS1 and also has KRAS mutations could then be tested with CT imaging to evaluate for pancreatic neoplasms and/or endoscopic screening. Moreover, serial screening in serum of these genes methylation status could also provide an estimate of an individual patient cancer risk. However, this would need to be established in future studies.

In conclusion, to our knowledge, this is the first study to describe the use of BNC1 and ADAMTS1 promoter methylation as biomarkers in pancreatic cancer. These genes are methylated at high frequencies in pancreatic cancer and are unmethylated in normal pancreatic tissues. Furthermore, we are able to detect the methylation of these genes in the serum of patients with pancreatic cancer. Our data strongly suggest that BNC1 and ADAMTS1 promoter methylation is potentially useful as a sensitive and specific noninvasive modality for identifying individuals at risk for pancreatic cancer.

Disclosure of Potential Conflicts of Interest
S.B. Baylin has Honoraria from Speakers Bureau of and is also a Consultant/Advisory Board member of Constellation Pharm, MDxHealth, and BioNumerix Pharm. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Study design and drafting of manuscript: J.M. Yi, A.A. Guzzetta, N. Ahuja, S.B. Baylin
Analysis and interpretation of data: J.M. Yi, V.J. Bailey, A.A. Guzzetta, K.B. Chiapponi, N. Ahuja, J.G. Herman, S.B. Baylin
Statistical analysis: A.A. Guzzetta, S.R. Downing
Critical revision of the manuscript for important intellectual content: J.M. Yi, A.A. Guzzetta, V.J. Bailey, K.B. Chiapponi, T.H. Wang, N. Ahuja, J.G. Herman, S.B. Baylin
Technical or material support: S.R. Downing, L. Van Neste, K.B. Chiapponi, N. Ahuja, J.G. Herman, S.B. Baylin
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