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Pancreatic cancer *DNMT1* expression and sensitivity to DNMT1 inhibitors

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Abstract

DNA methyltransferase I (*DNMT1*) is the major methyltransferase responsible for methylating DNA and is overexpressed in many cancers. *DNMT1* is also a therapeutic target for chemotherapy and chemoprevention. We hypothesized that loss of *DNMT1* copy number could result in reduced *DNMT1* levels and greater sensitivity to *DNMT1* inhibitors. We examined *DNMT1* expression in pancreatic cancers by immunohistochemistry and western blotting. We also examined *DNMT1* copy number in 20 pancreatic cancer cell lines using Affymetrix SNP arrays and correlated copy number with *DNMT1* expression. We tested eight pancreatic cancer cell lines with *DNMT1* inhibitors and measured growth inhibition. We identified overexpression of *DNMT1* relative to normal pancreatic duct in 78.7% of pancreatic cancers (37/47) by immunohistochemistry and in 16/20 pancreatic cancer cell lines by western blot. Pancreatic cancer cell lines with loss of *DNMT1* alleles tended to have lower *DNMT1* expression (three of nine cell lines) compared to those without *DNMT1* copy number loss (one of eleven). 5-aza-deoxycytidine (5-Aza-dC) treatment (1–10 uM) depleted *DNMT1* in seven of eight pancreatic cancer cell lines. Three of four pancreatic cancers cell lines with low/normal *DNMT1* expression were sensitive to growth inhibition by low dose 5-Aza-dC (1 uM), whereas only one of four cell lines with high *DNMT1* expression had growth inhibition, and this occurred without evidence of *DNMT1* depletion suggesting a different mechanism for growth inhibition in this cell line. Loss of *DNMT1* alleles may reduce *DNMT1* levels in some pancreatic cancers. Pancreatic cancers with low *DNMT1* expression tend to be more sensitive to low-dose 5-Aza-dc.

Keywords

pancreatic cancer; DNMT1; 5-aza-deoxycytidine; loss of heterozygosity; EGCG

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Introduction

DNA methylation regulates gene expression, genomic imprinting and chromatin structure.¹ During DNA replication, *DNMT1* binds to and methylates the cytosines of palindromic CpG dinucleotides of the daughter strand of newly replicated DNA thereby preserving the parental methylation pattern.² *DNMT1* also interacts with histone proteins to influence chromatin structure.^{3,4} *DNMT1* levels are regulated by transcriptional and posttranscriptional mechanisms^{5,6} and levels vary by the cell cycle.⁷ Aberrant DNA methylation contributes to the development of pancreatic⁸⁻¹⁶ and other cancers,¹⁷⁻²¹ and a likely contributor to the increased DNA methylation in cancer cells is *DNMT1* overexpression.²²⁻²⁵ Both transcriptional activation²⁶ and inhibition of protein degradation are thought to be important in the overexpression of *DNMT1* in cancer cells.^{27,28}

The importance of *DNMT1* in cancer development is further supported by mouse models, as adenomatous polyposis coli (*APC*)-min mice hypomorphic for *DNMT1* are protected from developing intestinal neoplasia.²⁹ However, the resulting DNA hypomethylation increases the risk of liver tumors,²⁹ highlighting the role of both DNA hypermethylation and DNA hypomethylation in cancer development. Global DNA hypomethylation of repetitive DNA is thought to contribute to the development of some cancers by generation of genomic instability and loss of imprinting.^{29,30} Methyl group deficiency such as from low vitamin B₁₂ or folate may increase the risk of developing certain cancers including pancreatic cancer.^{31,32} In addition, pancreatic cancers that acquire defective methylene tetrahydrofolate reductase (*MTHFR*) function by losing *MTHFR* alleles are more hypomethylated and have more chromosomal losses than those without.³⁰ Thus, *DNMT1* alterations have differing consequences for tumor development depending on the tumor type. *DNMT1* expression contributes to cell viability, though other DNMTs compensate when *DNMT1* deficient cells.^{2,33,34} Since DNA hypermethylation often inactivates tumor suppressor genes, *DNMT1* inhibitors have been tested as cancer therapeutics. Cytosine analogues such as 5-Aza-dC are incorporated into DNA during replication and covalently link *DNMT1* to DNA, effectively preventing *DNMT1* from methylating DNA.³⁵ 5-Aza-dC has activity against certain cancers and is approved for the treatment of myelodysplastic syndrome (MDS). Drugs that inhibit DNMT1 without the toxicity of 5-Aza-dC would potentially have more therapeutic applications.³⁶ Tea polyphenols such as epigallocatechin-3-*O*-gallate (EGCG) inhibit DNA methyltransferase-mediated DNA methylation, and act by directly inhibiting DNMTs.^{37,38} EGCG is one of the most abundant components in green tea extract, and has been shown to inhibit cell growth, malignant invasion and metastasis of pancreatic and other cancers and contribute to apoptosis and growth arrest in experimental systems.³⁹

Although *DNMT1* overexpression has been described in several cancers,^{28,40,41} the mechanism for this overexpression is not known. Aberrant signaling induced by mutant *KRAS* has been implicated as a cause of *DNMT1* overexpression, and *KRAS* mutations occur in approximately 90% of pancreatic ductal adenocarcinomas.^{42,43} In addition, both *KRAS* mutations and aberrant promoter methylation are first observed in precursor lesions of the pancreas.^{14,15,44-47} However, *DNMT1* overexpression also occurs in cancers without *KRAS* mutations.²⁸

Another cause of altered protein levels in cancer cells is alterations in chromosome copy number. Pancreatic and other adenocarcinomas undergo widespread chromosomal losses during tumor development.^{48–52} Alterations in gene copy number can influence responses to therapeutic agents. For example, loss of thymidylate synthetase alleles may influence responses to 5-fluorouracil⁵³ and loss of *MTHFR* copies could have adverse effects on folate metabolism.³⁰ Pancreatic and other cancers frequently lose copies of chromosome 19p, the locus of *DNMT1*.^{54,55} We hypothesized that pancreatic cancers with loss of a *DNMT1* allele may have lower *DNMT1* levels than pancreatic cancers without *DNMT1* allelic loss.

In this study we examined the expression of DNMT1 protein in a panel of pancreatic cancers and correlated levels with chromosome 19p copy number and *KRAS* mutation status. We also examined the response of pancreatic cancer lines with low and high DNMT1 expression to the DNMT1 inhibitors 5-Aza-dC and EGCG.

Results

Expression of DNMT1 in pancreatic cancer cell lines

We examined the expression of DNMT1 protein in 20 pancreatic cancer cell lines and two non-neoplastic pancreatic lines (HPDE and HPNE) by western blot (Fig. 1). Sixteen of 20 (80%) of the pancreatic cancer lines had higher DNMT1 levels than HPDE and HPNE after normalizing protein abundance by *GAPDH*. By western blot most of the pancreatic cancers had approximately 2–5 fold higher levels than the reference non-neoplastic cell lines. Four of the pancreatic cancer lines A32-1, Capan1, Su8686 and Panc3.014 had DNMT1 levels approximately the same as that of the non-neoplastic lines.

DNMT1 immunohistochemistry in pancreatic cancers

Next we analyzed DNMT1 expression in pancreatic cancer tissues by immunohistochemistry. DNMT1 labeling was detected in only a minority of normal pancreatic duct and acinar cells. Lymphocytes express sufficient DNMT1 to be observed by immunohistochemistry providing a useful positive control. DNMT1 labeling indicative of overexpression compared to normal pancreas was observed in 37 of 47 pancreatic cancers, a similar proportion to that observed in the pancreatic cancer cell lines. Although cancer samples showed significant higher DNMT1 score than normal control ($p < 0.001$) (Fig. 2), there were no differences in the clinicopathological parameters between DNMT1 expressing and non-expressing cases (data not shown).

Chromosome 19p copy number, *KRAS* mutation and *DNMT1* expression

We hypothesized that one mechanism for differences in the expression of *DNMT1* in different pancreatic cancers would be loss of a *DNMT1* allele through loss of the portion of chromosome 19p containing *DNMT1*. Loss of chromosome 19p is common in pancreatic cancers.^{51,52} We examined the copy number profiles of pancreatic cancer cell lines using Affymetrix SNP arrays looking in particular for evidence copy number loss and allelic loss (loss of heterozygosity or LOH) at the *DNMT1* locus on chromosome 19p13.2. Copy number profiles of eight of these lines was determined using the Affymetrix 50K SNP arrays,⁵³ with Affymetrix 250K SNP arrays used for the remaining lines (Fig. 3). LOH at

chromosome 19p was observed in 75.0% (15/20) of these pancreatic cancer lines, but seven of the cancers with 19p LOH did not have 19p copy number loss because of duplication of the retained chromosome. One cell line A32-1 had partial copy number loss with two 19p alleles indicating that the cell line contained a mixed population of cells some with and some without 19p allelic loss. There was no difference in the levels of DNMT1 protein in cell lines with and without 19p LOH. We next compared pancreatic cancers with and without loss of 19p. Nine of 20 (45.0%) of the pancreatic cancer lines had 19p loss and three of them (A32-1, Capan1 and Panc3.014) had low DNMT1 levels. In contrast, only 1 of the eleven lines without copy number loss at 19p had low DNMT1 protein levels (33.3 vs. 9.1%; $p > 0.05$) (Tables 1 and 2). Overall, although the association was not statistically significant, our data is consistent with the hypothesis that 19p copy number loss could cause low DNMT1 levels in some pancreatic cancers, but in other cancers additional mechanisms could still cause *DNMT1* overexpression. Interestingly, we also identified an isolated loss of 19p in the HPDE cell line raising the possibility that copy number loss in this line could contribute to low *DNMT1* expression.

Since mutational activation of the *KRAS* pathway has been implicated as a cause of *DNMT1* overexpression, we also examined the relationship between *KRAS* status and DNMT1 expression level, but found no significant relationship between mutant *KRAS* and DNMT1 expression level. Of the 20 cell lines examined, five were wild-type for *KRAS* and none of these five had low *DNMT1* levels (Tables 1 and 2).

Effects of DNMT1 on global DNA methylation in pancreatic cancer

Since reduced expression of DNMT1 can lead to a loss of DNA methylation, we next examined the relationship between DNMT1 levels and a global measure of DNA methylation using the methylation of the repetitive element LINE1 (a CpG-rich repeat sequence abundant throughout the genome). The percentage methylation of LINE1 elements of each cancer line was determined using COBRA. Interestingly, there was no difference in the percentage of LINE1 methylation among cell lines with elevated DNMT1 (62.0 +/- 12.9%) vs. those with normal or reduced DNMT1 (59.9 +/- 10.8%, Student's t-test, $p > 0.05$) (Tables 1 and 2).

Effects of 5-Aza-dC and EGCG on viability of cells with deficient and proficient DNMT1 in pancreatic cancer

We next determined if the sensitivity of pancreatic cancer cell lines to DNMT1 inhibition correlated with levels of DNMT1. We first tested the DNA methyltransferase inhibitor 5-Aza-dC in eight pancreatic cancer lines, four with low/normal DNMT1 levels and four with high DNMT1 levels. Cells were treated with increasing concentrations of 5-Aza-dC (0, 1, 10 uM) for 48 h. Treatment led to a dose-dependent depletion of DNMT1 in seven of the eight cell lines, with PL3 showing minimal depletion. All seven of these cell lines had partial depletion of DNMT1 at 1 uM concentrations of 5-Aza-dC, and almost complete depletion of DNMT1 was observed in Capan1, A32-1 and CFPAC1 at the highest 5-Aza-dC dose tested (10 uM) (Fig. 4A).

We next examined the effects of 5-Aza-dC on the viability of the same eight cell lines. All the cells were treated with 0.1 to 10 μ M 5-Aza-dC for 48 and 96 h (with fresh drugs added after 48 h) and then the cell proliferation was assessed using the MTS assay. The cell lines with high DNMT1 cells showed little growth inhibition to 48 h of 5-Aza-dC treatment, whereas the four cell lines with low/normal DNMT1 levels had a modest inhibition of growth in response to 5-Aza-dC. One of these one cell lines (Capan1) had ~20% decrease in growth with low-dose 5-Aza-dC (0.5 μ M) (data not shown). After 96 h incubation, Capan1 cell number was reduced by doses as low as of 0.1 μ M ($p < 0.001$) (Fig. 4B). Similarly, the cell lines Su8686 and Panc3.014 which also had low/normal DNMT1 levels were somewhat sensitive to low dose 5-Aza-dC showing an approximately 20% reduction of cell viability at 1.0 μ M ($p < 0.001$, $p < 0.05$, respectively). Thus, at 1.0 μ M 5-Aza-dC concentrations three of the four cell lines with low/normal DNMT1 levels (Capan1, Su8686 and panc3.014) were sensitive to 5-Aza-dC. In contrast, only one of the cell lines with high DNMT1 (PL3) had a similar response to low-dose 5-Aza-dC ($p < 0.05$). Interestingly, PL3 did not show much depletion of DNMT1 with 5-Aza-dC treatment. It is possible that the effective concentration of 5-Aza-dC was lower in this cell line because of drug efflux, or that the cell line retained the ability to produce enough DNMT1 to overcome the effect of the drug. It also suggests that its sensitivity to the drug could be for reasons other than DNMT1 inhibition, although the lack of response of PL3 raises the possibility that 5-Aza-dC sensitivity does not depend on DNMT1 levels. None of the remaining cell lines showed inhibition to 5-Aza-dC even at 96 h. Overall these data support the hypothesis that cancers with low/normal DNMT1 expression are usually more sensitive to 5-Aza-dC than those with high DNMT1 expression.

Next, we examined the responses of these same pancreatic cancer lines to another DNMT1 inhibitor, EGCG. DNMT1 protein levels and cell viability were measured in response to a range of concentration of EGCG (0, 5 and 25 μ M for western blot) and (0, 5, 10, 15, 25 and 50 μ M for MTS assay, respectively). In contrast to the response to 5-Aza-dC, there was minimal depletion of DNMT1 protein at the concentrations tested for most of the cell lines (Fig. 4A). The MTS assay indicated a greater sensitivity to EGCG than to 5-Aza-dC (Fig. 4B). There was no clear relationship between the DNMT1 level of cell lines and their EGCG response. This is consistent with the mechanism of inhibition of DNMT1 by direct inhibition rather than by protein depletion. Panc-1 and MiaPaCa2 were the most sensitive to EGCG with MiaPaCa2 showing almost complete inhibition at a concentration of 15 μ M after 96 h in culture.

Discussion

In this study we find that many pancreatic cancers overexpress DNMT1 relative to normal or non-neoplastic pancreas cells. Indeed, a similar fraction of pancreatic cancers were found to overexpress DNMT1 by western blot analysis of pancreatic cancer cell lines (16 of 20, 80.0%) or by immunohistochemistry (37 of 47, 78.7%). We attempted to identify molecular differences between DNMT1 overexpressing and non-overexpressing cell lines. First, we hypothesized that pancreatic cancers with chromosomal loss of a copy of DNMT1 would be more likely to have low DNMT1 levels. We also correlated *DNMT1* expression with *KRAS* mutational status. We found that even *KRAS* wild-type cancers had DNMT1 overexpression and there was no evidence that *KRAS* mutation was associated with DNMT1 levels. For the

chromosome loss analysis, we found that of 20 pancreatic cancers examined for chromosome 19p using Affymetrix SNP arrays, three of nine with 19p copy number loss had low DNMT1 levels, but only one of the eleven cell lines without loss of a DNMT1 locus had low DNMT1 levels. Although this difference did not reach statistical significance, these data are consistent with the hypothesis that copy number loss could result in lower DNMT1 levels if other mechanisms that cause DNMT1 overexpression are not present to compensate for loss of a *DNMT1* allele.

We next determined if low DNMT1 levels influenced pancreatic cancer responses to the *DNMT1* inhibitors, 5-Aza-dC and EGCG. We found that 5-Aza-dC depleted DNMT1 protein in all cell lines except the line PL3 and found that cell lines with high DNMT1 levels showed less growth inhibition than cell lines with low/normal DNMT1. In contrast, although pancreatic cancer growth could be inhibited by EGCG, responses to EGCG did not correlate with DNMT1 levels which is consistent with the mechanism of EGCG inhibition of DNMT1.³⁸ Recent studies have demonstrated that pancreatic cancer cell growth can be inhibited by EGCG.³⁹ It should be noted that both 5-Aza-dC and EGCG are likely to have off target effects besides DNMT1 that could contribute to their responses in vitro. These data are consistent with the hypothesis that there is a dose-dependent depletion of DNMT1 with 5-Aza-dC and pancreatic cancers with low DNMT1 are more likely to deplete their DNMT1 levels at lower doses of 5-Aza-dC than cancers with high DNMT1 levels. Indeed, the level of inhibition of DNMT1 may be critical to the cellular response to 5-Aza-dC, as recent studies suggest that complete depletion of DNMT1 may be required to kill cancer cells.^{34, 60, 61} Our results suggest that by reducing DNMT1 levels chromosome 19p copy number loss could render cancer cells more sensitive to DNMT1 depleting agents such as 5-Aza-dC.

Numerous studies have evaluated the potential therapeutic benefit of using DNMT1 inhibitors to treat cancers. While DNMT1 inhibitors such as 5-Aza-dC have modest activity against myeloid neoplasms, they have not proven to be effective as single agents against solid neoplasms in clinical trials despite the fact that they inhibit cancer cell proliferation and cause cell death in vitro. Further clinical trials are necessary to determine if DNMT1 inhibitors may have more potent effects when used in combination with other chemotherapeutics.⁶² Our results provide preliminary evidence that pancreatic cancer growth inhibition from DNMT1 inhibitors may depend on levels of DNMT1 protein. It would be useful to determine if solid tumor responses to DNMT1 inhibitors were more likely in cancers with low DNMT1 levels.

In conclusion, we find that while many pancreatic cancers overexpress DNMT1, some pancreatic cancers have low/normal DNMT1 levels. Pancreatic cancers with low/normal DNMT1 levels appear to be more sensitive to DNMT1 inhibition by 5-Aza-dC.

Methods

Cell lines and chemicals

Twenty human pancreatic cancer cell lines A32-1, A38-5, AsPC1, BxPC3, Capan1, Capan2, CFPAC1, Hs766T, MiaPaCa2, Panc-1, Panc2.5, Panc2.8, Panc3.014, Panc215, PK8, PK9,

PL3, PL11, Su8686 and TS011 were grown in standard culture conditions. The majority of these cell lines were generated from primary pancreatic cancers at this institution either by Dr. Elizabeth Jaffee (Panc1, Panc2.5, Panc2.8, Panc3.014, PL3, PL11 and TS011) or Dr. James Eshleman (A32-1, A38-5 and Panc215). PK8 and PK9 were obtained from Dr. Akira Hori, University of Sendai Japan, who created these cell lines and the commercial cell lines (AsPC1, BxPC3, Capan1, Capan2, CFPAC1, Hs766T, MiaPaCa2 and Panc1) were obtained from ATCC. The human non-neoplastic pancreatic ductal epithelium (HPDE) cell line and the Nestin-expressing human pancreatic cell line (HPNE) were generously provided by Dr. Ming-Sound Tsao (University of Toronto, Ontario Canada) and Dr. Michel M. Ouellette (University of Nebraska Medical Center, Omaha, NE), respectively. HPNE has a pancreatic fibroblast phenotype. To our knowledge, HPDE is the only non-neoplastic pancreatic duct line available. 5-Aza-dC and EGCG were purchased from Sigma and were dissolved in 1× phosphate-buffered saline (PBS; pH 7.0).

Drug treatment

Eight pancreatic cancer cell lines (A32-1, Capan1, Su8686, Panc3.014, CFPAC1, MiaPaCa2, Panc-1 and PL3) were treated with 5-Aza-dC and EGCG, respectively. Cells in log phase growth were seeded in 10 cm cell culture dishes. After overnight incubation, the cells were cultured with 0.1–10 μM 5-Aza-dC and 5–50 μM EGCG, with drug and media replaced every 48 h.

Western blot

Cells were collected and washed with 1× PBS. Cells were lysed using ultrasound in RIPA buffer (Sigma), and then centrifuged at 13,000 rpm for 30 min. The supernatant was collected, and the protein concentration was determined using the Bio-Rad protein assay reagent. An equal amount of protein (40 μg) from each sample was separated by 4–12% gradient SDS gel electrophoresis (Invitrogen), transferred to nitrocellulose membranes, and incubated in blocking solution (5% milk in TBS with 0.1% Tween 20) for 60 min. Membranes were incubated overnight with a polyclonal antibody against DNMT1 (rabbit-anti DNMT1)^{41,56} (generously provided by Dr. Bill Nelson, Johns Hopkins University) at 1:200 dilution or a monoclonal antibody against *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) at 1:2,000 dilution. Secondary HRP-linked antibodies (Santa Cruz Biotechnology, Amersham Biosciences) were applied at 1:2,000 dilution and proteins were detected using an ECL kit (Amersham Biosciences).

Immunohistochemistry

The expression of DNMT1 protein was examined by immunohistochemical labeling of tissue microarrays (TMAs) (kindly performed by Dr. Angelo DeMarzo and Ms. Jessica L. Hicks, Johns Hopkins University). Antigen retrieval was performed in high temperature target retrieval buffer, heated at 95°C in a steamer for 40 min. After blocking endogenous peroxidase activity with a H₂O₂ solution, the sections were incubated with an anti-DNMT1 polyclonal antibody at 1:1,000 for 45 min. Labelling was detected with the Envision Plus Detection Kit (DAKO, Carpinteria, CA, USA). All sections were counterstained with hematoxylin. The results of immunohistochemical staining were scored based on intensity of

staining as 0 (negative), 1 (weak) or 2 (strong), and based on the percentage of positive epithelial cells as 0 (<5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) or 4 (>76%), respectively. A “histo-score” was generated as the product of intensity multiplied by area.

Total genomic copy number and LOH analysis

Genomic DNA extracted from pancreatic cancer cell lines, matching normal samples (peripheral blood lymphocytes or immortalized lymphocyte) and non-neoplastic cell lines HPDE and HPNE was digested and hybridized to the Affymetrix GeneChip Human Mapping 250K Sty I array and/or Nsp I array according to the manufacturer’s instruction. Genotype calls were determined by BRLMM algorithm using the Affymetrix Chromosome Copy Number Analysis Tool 4.0 (CNAT 4.0). Genotyping call and probe intensities were imported into Partek® Genomics Suite ver. 6.4 (Partek GS). To create copy number- and LOH-profiles, primary pancreatic cancer line SNP calls were compared to their paired normal sample where available and the AsPC1, BxPC3, Capan2, CFPAC1, Panc-1, Su8686, HPDE and HPNE were compared to the SNP array data of 270 individuals from the HapMap collection. In order to detect regions of copy number gain and loss we applied the Partek Genomic Suite (v6.3) Segmentation tool with segmentation parameters set at: minimum genomic markers: 10, p-value threshold: 0.001, and signal to noise: 0.3. LOH region was detected by Hidden Markov Model with parameters set at maximum probability: 0.99, genomic decay 10, genotyping error rate: 0.1.

Cell viability assay

Cell growth was assessed utilizing a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega), which was done following the manufacturer’s instructions. 3,000 cells in 100 ul medium were plated in each well of 96-well plates and treated as described above. Parallel cell-free reactions were set up and used for background correction. Measurements were performed in six replicates.

DNA isolation and bisulfite treatment

DNA was isolated from cell line samples using the DNeasy Tissue Kit (Qiagen) and bisulfite modification was done as previously described.⁵⁷

KRAS mutation detection

PCR amplification of the *KRAS* gene first exon was performed using an upstream *KRAS* primer (5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′) that encoded a G to C substitution at the first position of codon 11 and a downstream primer 5′-TCA AAG AAT GGT CCT GCA CC-3′.⁵⁸ 15 ul PCR reactions were done with Platinum Taq polymerase. The PCR generated a DNA fragment of 157 nucleotides and the upstream *KRAS* primer generated a BstN1 restriction enzyme site (CCTGG) overlapping the first two nucleotides of codon 12. Upon incubation with BstN1, the fragments containing the wild-type codon 12 sequences were cleaved, resulting in two bands of 128 and 29 nucleotides. Fragments containing mutations at either the first or second positions of codon 12 were not cleaved.

Assay of global DNA methylation

The level of methylation of the long interspersed nucleotide element-1 (LINE1) repetitive elements was measured using combined bisulfite restriction analysis (COBRA) and previously reported primers and PCR conditions. Two separate assays were done and results were averaged.⁵⁹

Statistics

Chi-square tests were used to determine differences in the proportion of copy number loss and *KRAS* mutation between normal and reduced DNMT1 group. The average percentage of LINE1 methylation was compared using Student's t-test. A two-tailed p value of less than 0.05 was used to assess statistical significance. Statistical analysis was performed using the Excel statistics software (Microsoft, Redmond, WA), STATA version 8.2 software.

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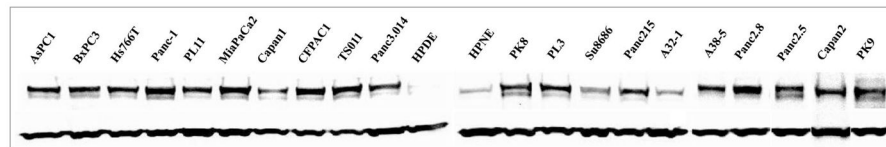


Figure 1.

Expression of DNMT1 in cell lines. Western blot analyses for DNMT1 in pancreatic cancer (20) and non-neoplastic cell lines (2). DNMT1 (~190 kDa) was detected with *GAPDH* (~40 kDa) served as a loading control.

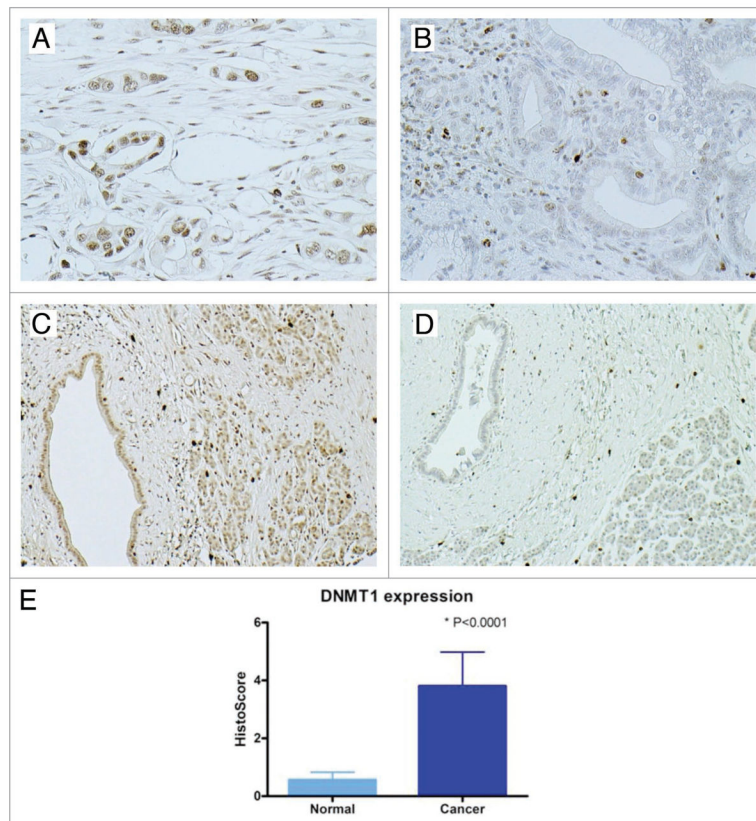


Figure 2.

Immunohistochemical staining of *DNMT1* protein in pancreatic cancer. (A) Nuclear labeling of *DNMT1* and (B) no *DNMT1* labeling in the malignant tumor cells; (C) Expression of *DNMT1* and (D) Lack of *DNMT1* expression in normal ductal epithelial cells; (E) A “Histo-score” was generated as the product of intensity times area.

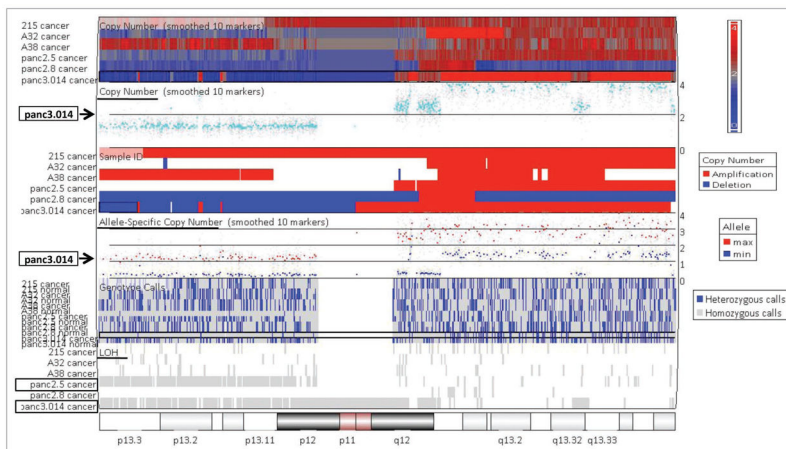
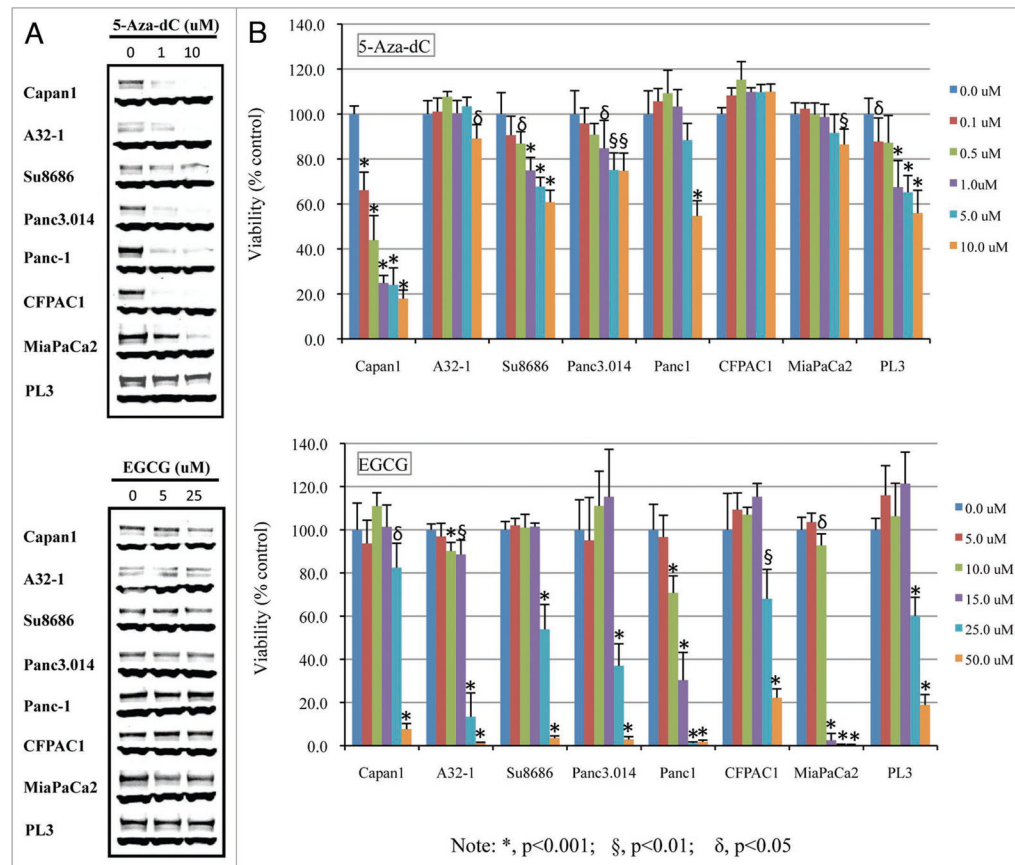


Figure 3.

Copy number analysis and LOH detection at the *DNMT1* locus (19p13.2) by Affymetrix 250K SNP arrays in pancreatic cancer cell lines (representative shown). Copy number loss was observed in Panc3.014 compared to the matched lymphocytes. LOH appeared on this locus in Panc3.014 and Panc2.5 cell lines.

**Figure 4.**

Response of pancreatic cancer cell lines with deficient and proficient *DNMT1* to the DNA methylation inhibitor 5-Aza-dC and tea polyphenol (EGCG). (A) Effect of increasing concentration of 5-Aza-dC (0, 1, and 10 uM) and EGCG (0, 5, and 25 uM) on expression of DNMT1 protein. *GAPDH* used as a loading control; (B) Effect of DNMT1 on cell viability. Cells were treated with indicated doses of 5-Aza-dC for 96 h (fresh drug added every 48 h) and then cell viability was determined using the MTS assay.

Table 1

Expression of *DNMT1*, loss of copy number and LINE1 methylation in pancreatic cancer cell lines

Cell line	Relative low expression of <i>DNMT1</i>	Allelic loss	Loss of copy number	<i>KRAS</i> status	% LINE1 methylation
Cancer					
A32-1	+	-*	+*	M	71.48
Capan1	+	+	+	M	54.98
Capan2	-	+	+	M	ND
CFPAC1	-	+	+	M	61.28
Panc3.014	+	+	+	M	65.76
Panc-1	-	+	+	M	60.74
PK9	-	+	+	W	77.81
PL3	-	+	+	W	55.00
TS011	-	+	+	M	ND
Panc215	-	-	-	M	57.60
A38-5	-	-	-	M	ND
AsPC1	-	+	-	W	70.72
BxPC3	-	-	-	M	31.94
Hs766	-	+	-	M	53.54
MiaPaCa2	-	+	-	M	71.53
Panc2.5	-	+	-	M	ND
Panc2.8	-	-	-	M	ND
PK8	-	+	-	W	75.29
PL11	-	+	-	W	66.55
Su8686	+	+	-	M	47.34
Non-neoplastic					
HPDE	+	+	+	W	68.69
HPNE	+	-	-	W	64.79

M, mutation type; W, wild-type; ND, not determined;

* , this cell line has partial loss of copy number.

Table 2

Correlation of copy number loss, *KRAS* mutation and LINE1 methylation with low expression of DNMT1 in pancreatic cancer cell lines

	Relative low expression of DNMT1 (+)	Relative low expression of DNMT1 (-)	p value
Loss of copy number (+)	33.3% (3/9)	66.7% (6/9)	
Loss of copy number (-)	9.1% (1/11)	90.9% (10/11)	Chi test, p = 0.178
<i>KRAS</i> mutant type	26.7% (4/15)	73.3% (11/15)	
<i>KRAS</i> wild-type	0.0% (0/5)	100% (5/5)	Chi test, p = 0.197
% LINE1 methylation	59.9 ± 10.8%	62 ± 12.9%	t-test, p = 0.776