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Genome-wide DNA methylation patterns in pancreatic ductal adenocarcinoma reveal epigenetic deregulation of SLIT-ROBO, ITGA2 and MET signaling

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The importance of epigenetic modifications such as DNA methylation in tumorigenesis is increasingly being appreciated. To define the genome-wide pattern of DNA methylation in pancreatic ductal adenocarcinomas (PDAC), we captured the methylation profiles of 167 untreated resected PDACs and compared them to a panel of 29 adjacent nontransformed pancreata using high-density arrays. A total of 11,634 CpG sites associated with 3,522 genes were significantly differentially methylated (DM) in PDAC and were capable of segregating PDAC from non-malignant pancreas, regardless of tumor cellularity. As expected, PDAC hypermethylation was most prevalent in the 5′ region of genes (including the proximal promoter, 5′UTR and CpG islands). Approximately 33% DM genes showed significant inverse correlation with mRNA expression levels. Pathway analysis revealed an enrichment of aberrantly methylated genes involved in key molecular mechanisms important to PDAC: TGF-β, WNT, integrin signaling, cell adhesion, stellate cell activation and axon guidance. Given the recent discovery that SLIT-ROBO mutations play a clinically important role in PDAC, the role of epigenetic perturbation of axon guidance was pursued in more detail. Bisulfite amplicon deep sequencing and qRT-PCR expression analyses confirmed recurrent perturbation of axon guidance pathway genes *SLIT2, SLIT3, ROBO1, ROBO3, ITGA2* and *MET* and suggests epigenetic suppression of SLIT-ROBO signaling and upregulation of *MET* and *ITGA2* expression. Hypomethylation of *MET* and *ITGA2* correlated with high gene expression, which was associated with poor survival. These data suggest that aberrant methylation plays an important role in pancreatic carcinogenesis affecting core signaling pathways with potential implications for the disease pathophysiology and therapy.

What's new?

Based on a large genome-wide scan of DNA methylation, this study reports that global DNA methylation patterns can robustly segregate tumor and non-malignant pancreata. Cancer methylation also affects key pathways in pancreatic carcinogenesis, including TGF-β, WNT, and axon guidance signaling. This study confirms that methylation plays an important role in the development and progression of pancreatic cancer, with implications for both ongoing research and therapeutic development.

Pancreatic ductal adenocarcinoma (PDAC), the predominant form of pancreatic cancer is usually diagnosed at an advanced stage. Optimal treatment involves surgery with adjuvant chemotherapy, however <20% of patients are suitable for operative resection as the majority has either locally advanced or metastatic disease at diagnosis.[1,2] Therapy for advanced disease is largely ineffective and the 5-year survival remains <5%. Consequently, novel therapeutic and early detection strategies are urgently needed.

DNA methylation mostly occurs at cytosine residues in the context of CG dinucleotides (CpG sites) present across the genome, regions rich in these CpG sites are termed CpG islands.[3] Altered DNA methylation is implicated in tumor initiation and progression for several cancer types[4,5] with hypermethylation of CpG islands and promoter regions associated with transcriptional silencing of tumor suppressor genes.[6] Conversely, hypomethylation is associated with overexpression of oncogenes[6] and genomic instability,[7] although the mechanisms are yet to be completely understood.

It is well established that tumors arise through the accumulation of genetic and epigenetic aberrations; the patterns of which differ from cancer to cancer. While recent PDAC exome sequencing studies[8–10] have made significant inroads into identifying the common somatic point mutations and copy number alterations, genome-wide patterns of DNA methylation in PDAC are yet to be fully characterized. To date, DNA methylation has been limited to small number of CpG sites (1,500–244,000) in PDAC xenografts ($n = 30$) or cell lines ($n = 9-14$).[11–13] While these studies suggest that DNA methylation is important in the development and progression of PDAC, further characterization within larger cohorts is needed to determine the scale and the contribution of methylation to molecular mechanisms driving this heterogeneous and complex disease.

This study reports a large scale methylation and expression profiling of a cohort of 167 PDAC and compared them to 29 adjacent nonmalignant pancreas using Infinium 450k methylation arrays (Illumina). We show that aberrant DNA methylation is widespread in pancreatic cancer and is enriched in many of the core cancer-signaling pathways known to be important in pancreatic carcinogenesis. Of particularly, interest was the recurrent hypermethylation/reduced expression of SLIT-ROBO genes, which are key components of axon guidance signaling, a pathway recently implicated in this disease.[10] Furthermore hypomethylation/increased expression of MET and ITGA2 was found to be associated with poor patient survival. Taken together, these data support an orchestrated deregulation of DNA methylation contributing to pancreatic carcinogenesis and an increasing importance of SLIT-ROBO/axon guidance signaling in PDAC.

Material and Methods

Patients and sample collection

Samples from 167 tumors and 29 adjacent nonmalignant pancreatic tissues were collected from nontreated PDAC patients who underwent resection surgery. Patients were recruited preoperatively and consented using ICGC ethical

and approved process (Project Number: 2009000745). Frozen sections of the tested samples underwent histological evaluation for tumor and non-neoplastic tissue conformation and tumor content estimation. Samples were collected as part of the Australian Pancreatic Cancer Genome Initiative.

Genomic DNA isolation, bisulfite conversion and 450 K methylation arrays

DNA was extracted from fresh frozen tissues using the All-Prep DNA/RNA Mini Kit (Qiagen). Genomic DNA (500 ng) was bisulfite converted using the EZ DNA methylation Kit (Zimo Research) following the manufacturer's protocol with modification for Illumina Methyaltion arrays. Bisulfite converted DNA was whole genome amplified and hybridized to Infinium Human Methylation 450K BeadChips (Illumina) according to the manufacturer's protocol. Arrays were scanned using an iScan (Illumina). Cellularity of each tumor sample was estimated using SNP array data (Omini1-Quad, V1 and Omini2.5-8,V1.0– Illumina) and qPure tool[14] (Supporting Information Table S1).

Data preprocessing and initial quality assessment

The GenomeStudio v 2011.1 (Illumina) with methylation module (v 1.6.1), was used to process the raw image data. The assay performance was assessed using the "Control Dashboard" in GenomeStudio. Overall sample quality was determined through total number of detected CpGs and the distribution of average beta values for all CpGs. One sample was excluded in this study, as it did not pass these basic quality assessments.

The reproducibility of the data was measured by sample replication across different batches of bisulfite conversion, with a correlation coefficients > 0.99 across replicated samples. Examples of the correlation between replicates are presented in the Supporting Information Figure S1. Supporting Information Figures S2 and S3 show PCA analysis of all 196 samples colored by batch and by cellularity, respectively. No obvious batch effect was observed, main separation is due to the sample type and cellularity (Supporting Information Fig. S3). The methylation array data have been deposited into the Gene Expression Omnibus (Accession number –GSE49149).

Data analysis of 450K methylation arrays

IDAT files were analyzed using the minfi package and subset-quantile within-array normalization (SWAN).[15] Probes on chromosomes X and Y, probes with detection p-value >0.01 in one or more samples and probes with SNP in the 10 last bases or with two or more SNPs in the probe sequence were excluded from analysis. The differential methylation (DM) analysis was performed using 402,496 autosomal CpGs using the "dmpFinder" minfi function. Probes were considered DM if p-value corrected by the Benjamini and Hochberg method was <0.001 and the $\Delta\beta$ (the average beta value in PDAC samples minus the average beta value in nonmalignant samples) was ≥ 0.2. The Wilcoxon rank sum test was used to compare levels of methylation in specific regions of the genome between PDAC and nonmalignant samples.

Clustering analysis

For hierarchical clustering the R function "hclust" with Euclidean distance and complete linkage clustering method was used in combination with "heatmap3" function. The principle component analysis (PCA) and plots were generated using the "pca" function of the R package mixOmics version 4.0-2, (previously named integrOmics,[16] with parameters "center" and "scale" set true).

The recursively partitioned mixture model (RPMM) was used to identify tumor subtypes. The FANNY algorithm was used for initialization and level-weighted of Bayesian information for the split criterion as implemented in the RPMM R package[17] using the 2786 probes most variable (SD ≥ 0.17) across all tumors ($n = 167$).

Pathway analysis

To determine potential biological relevance of CpG sites DM in PDAC pathway analyses were performed using the Ingenuity Pathway Analysis software (Ingenuity Systems; http://www.ingenuity.com) and MetaCore package (Thomson Reuters).

Validation by amplicon deep-sequencing

A subset of 96 PDAC and 15 nontumor samples were used to validate the array data by amplicon deep-sequencing using Ion-Torrent (Life Technologies) or MiSeq (Illumina) sequencers. Primers were design using the MethPrimer[18] program to amplify regions containing CpG sites DM in genes from SLIT-ROBO pathway, ITGA2, MET and genes involved in stellate cell activation TNF, COX2, EGFR and TGFBR1. Primers and amplicon size are presented in the Supporting Information Table S2. Amplicons were pooled and sequenced on Ion Torrent using the 318 chip and 200 bp chemistry or on MiSeq with the Nextera kit. The Ion Torrent data was mapped using TMAP (Life Technologies) and MiSeq data was mapped using BWA backtrack v0.7.5a. For each potential methylated base being interrogated in the amplicon the numbers of reads with methylated cytosines were counted. Percentage of methylation was calculated by (number of reads with methylated C/total reads) $\times$ 100.

Gene expression profiling

RNA from 121 PDAC and 8 nontumor samples was extracted using the AllPrepDNA/RNA Mini Kit (Qiagen). Total RNA (150 ng) was amplified and labeled using the Illumina TotalPrep RNA Amplification Kit (LifeTechnologies). Amplified RNA (750 ng) was hybridized to Illumina human HT12 (V4) arrays following manufacturer's protocol and scanned on Bead Array Reader or iScan (Illumina). The intensities were extracted using the GenomeStudio software (Illumina). Data was background corrected, $\log_2$-transformed

and quantile normalized as implemented in the Lumi package.[19] The gene expression array data have been deposited into the Gene Expression Omnibus (Accession number – GSE36924).

RT-PCR analysis used TaqMan probes for target genes (Life Technologies). cDNA was synthesized using 500 ng of total RNA, random primers and SuperScript III (Life Technologies). Relative gene expression quantification was performed using the ViiA 7 Real Time PCR System V1.2 (Life Technologies) and the ΔΔCT method.

Correlation methylation-gene expression

The relationship between methylation probes and gene expression probes was obtained in the GenomeStudio Methylation module using the vendor lookup table. Spearman correlations were calculated and the *p*-values were corrected for false discovery rate resulting in *q*-values using the R package fdrtool.[20] The correlation was considered significant when *q*-value < 0.05.

Results

Defining a robust methylation signature for PDAC

The clinical features for the cohort ($n = 167$ PDAC) are summarized in Supporting Information Table S3. Tumor content (cellularity) averaged 44%, ranging from 12 to 97% based on high-density SNP arrays and qPURE analysis[14] (Supporting Information Table S1). The distribution of tumor cellularity was characteristic of primary PDACs, which is typified by the presence of an extensive desmoplastic stroma. Sample cellularity is known to affect sensitivity of detecting DM sites, and to assess the influence of cellularity on our ability to detect DM sites, two analyses were carried out: (*i*) tumors with $\geq$ 70% cellularity ($n = 31$) *vs.* adjacent nonmalignant pancreas ($n = 29$) and (*ii*) tumors with $\geq$ 20% cellularity ($n = 140$) *vs.* adjacent nonmalignant pancreas ($n = 29$). A total of 78,162 CpG sites (19.42% of total CpG sites) and 105,516 (26.22% of total CpG sites) showed significant DM in tumors with $\geq$ 70 and $\geq$ 20% cellularity, respectively (adjusted *p*-value < 0.001). Surprisingly, 86% of the DM probes in the high cellularity cohort were also seen in the cohort with cellularity $\geq$ 20% suggesting that PDAC possesses a robust set of methylation changes that were not confounded by cellularity (Supporting Information Fig. S4). To ensure that the most robust set of DM probes in PDAC was used for further analyses, an additional threshold was applied to DM probes ($\Delta\beta \geq 0.2$, where $\Delta\beta$ = the average beta value of PDAC samples minus the average beta value of adjacent nonmalignant pancreas). This approach identified 11,634 DM methylated probes (Supporting Information Table S4), with adjusted *p*-value <0.001 and $\Delta\beta \geq 0.2$ in PDAC samples with 20% or greater tumor cellularity.

This robust set of PDAC DM CpG sites ($n = 11,364$) was not randomly distributed across the genome. The majority of the 5,203 significantly hypomethylated sites were located in gene bodies (58%) and outside CpG islands (88%; Fig. 1*a*). From the 6,431 PDAC hypermethylated sites, 81% were located in CpG islands and 65% located in promoter regions (CpG sites located within 1,500 bp (TSS1500), or 200 bp (TSS200) from the transcription start site, or in the 5′UTR region, or first Exon; Fig. 1*a*). Hypermethylation of probes located in the first exon (Fig. 1*b*) is of particular interest as hyper-methylation of the first exon region silences gene expression in a tighter fashion than TSS regions.[21] Probes in CpG islands were hypermethylated in PDAC compared with nonmalignant pancreas (*p*-value < 0.0001; Fig. 1*c*). Probes in shelf positions ($\geq$2 kb from CpG islands) were hypomethylated in PDAC samples (average beta-value = 0.47 in PDAC and 0.59 in nonmalignant pancreas; Fig. 1*c*); similar to the pattern seen in colon cancer.[6]

Aberrant methylation discriminates PDAC and nonmalignant pancreas independent of tumor cellularity

Hierarchical clustering using the robust set of DM probes ($n = 11,634$) correctly classified 96% (134/140) of PDAC samples (Fig. 2). When 27 samples with <20% cellularity were included in the analysis, PCA analyses and clustering confirmed that the robust DM CpG sites could correctly classify samples 92% (153/167) of the time (Supporting Information Figs. S5 and S6), although the degree of separation was influenced by tumor cellularity (Supporting Information Fig. S6). These results suggest that aberrantly methylated sites have potential applicability as biomarkers for PDAC.

In other cancers (colorectal and gastric), methylation patterns can define clinically significant subtypes.[22,23] To assess if DNA methylation could identify subtypes in PDAC, we performed clustering analysis using RPMM with the top 2,786 most variable probes across all tumor samples. Four clusters were identified (Supporting Information Fig. S7); however, these subgroups did not cosegregate with patient survival (Supporting Information Fig. S8), or other key clinical features (age, gender or tumor grade; data not shown).

Aberrant methylation and mRNA expression identifies candidate genes that are epigenetically regulated

The robust set of 11,634 DM CpG sites was mapped onto genomic features associated with 5,668 RefSeq transcripts and 3,522 genes using array vendor's annotations (Supporting Information Table S4), 850 of these genes had been previously reported as DM in previous PDAC methylation studies[11,13,24] (Supporting Information Table S5). In the samples ($n = 129$) with mRNA expression array data available, 33% of aberrantly methylated genes also showed significant methylation-gene expression correlation (*q*-value < 0.05; Supporting Information Tables S6 and S7). Approximately 86% of these genes showed inverse correlations between methylation and gene expression (Supporting Information Table S6) consistent with hypermethylation promoting gene silencing and hypomethylation increasing expression. A set of 178 CpGs had positive correlation between methylation and

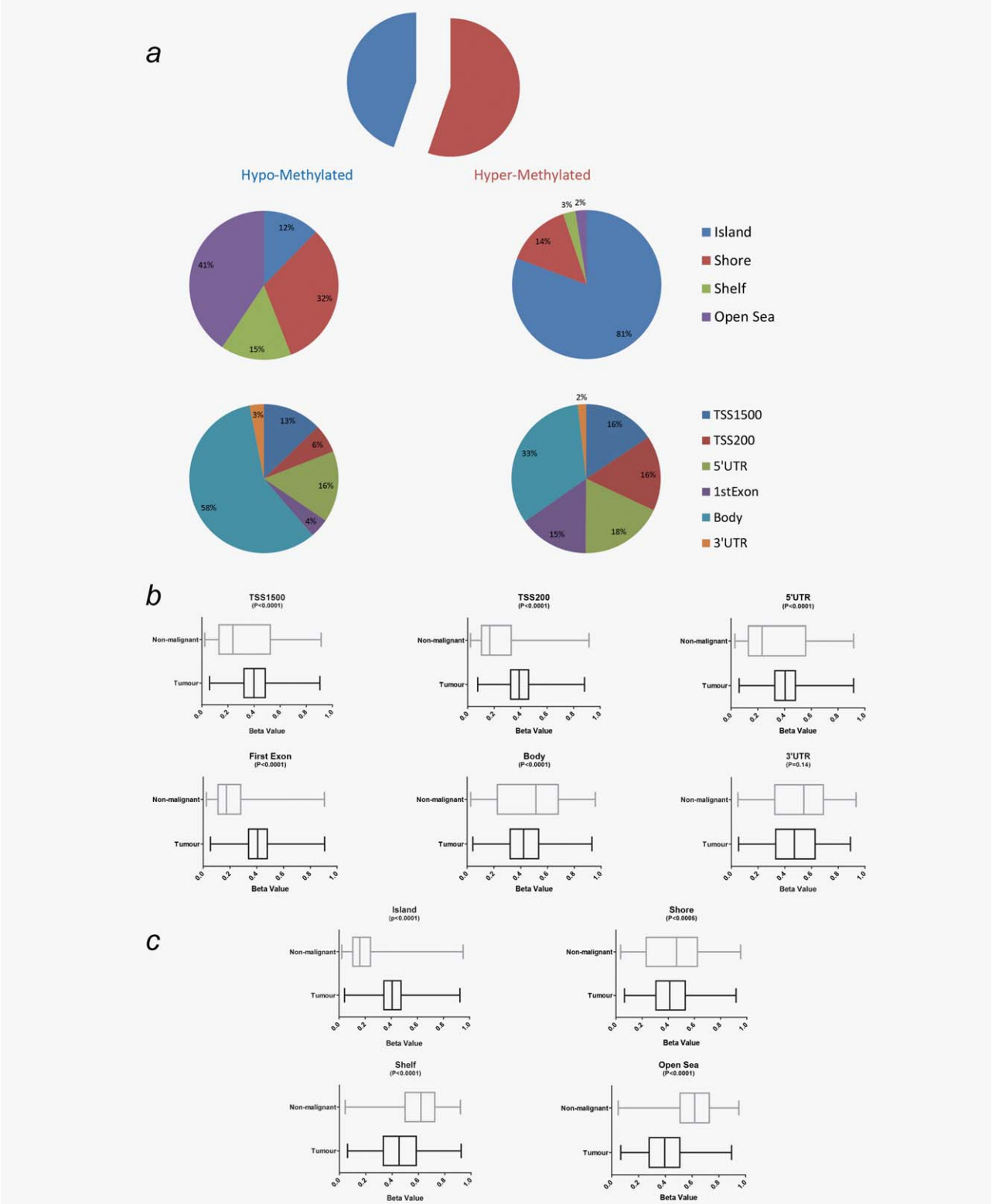

Figure 1. Genomic distribution of DM probes (n = 11,634) in CpG island and other parts of the genome. (*a*) Pie charts show the genomic distribution of hypomethylated and hypermethylated probes across different genomic regions (Island = probes located at CpG islands, Shore probes located less 2kb from CpG island, Shelf = probes located >2k from CpG islands, Open Sea = probes not in island or annotated genes; TSS1500 and TSS200 – probes located within 1500 and 200 bp from transcription start site, respectively; 5′UTR region; first exon; gene body and 3′UTR region). (*b*) Box plot of levels of methylation across gene regions for PDAC samples (n = 140) and adjacent nonmalignant samples (n = 29). Whiskers represent max and min values. (*c*) Methylation levels across CpG island regions for PDAC samples and adjacent nonmalignant pancreatic samples. Whiskers represent max and min values (p-values were computed with Wilcoxon rank sum test). The number of probes in each region are TSS1500 n = 1,596, TSS200 n = 1,443, 5′UTR n = 1,741, first exon n = 1,282, Body n = 3,782, 3′UTR n = 257, Island = 5,329, Shore = 1,888, Shelf = 658 and Open Sea = 1,457.

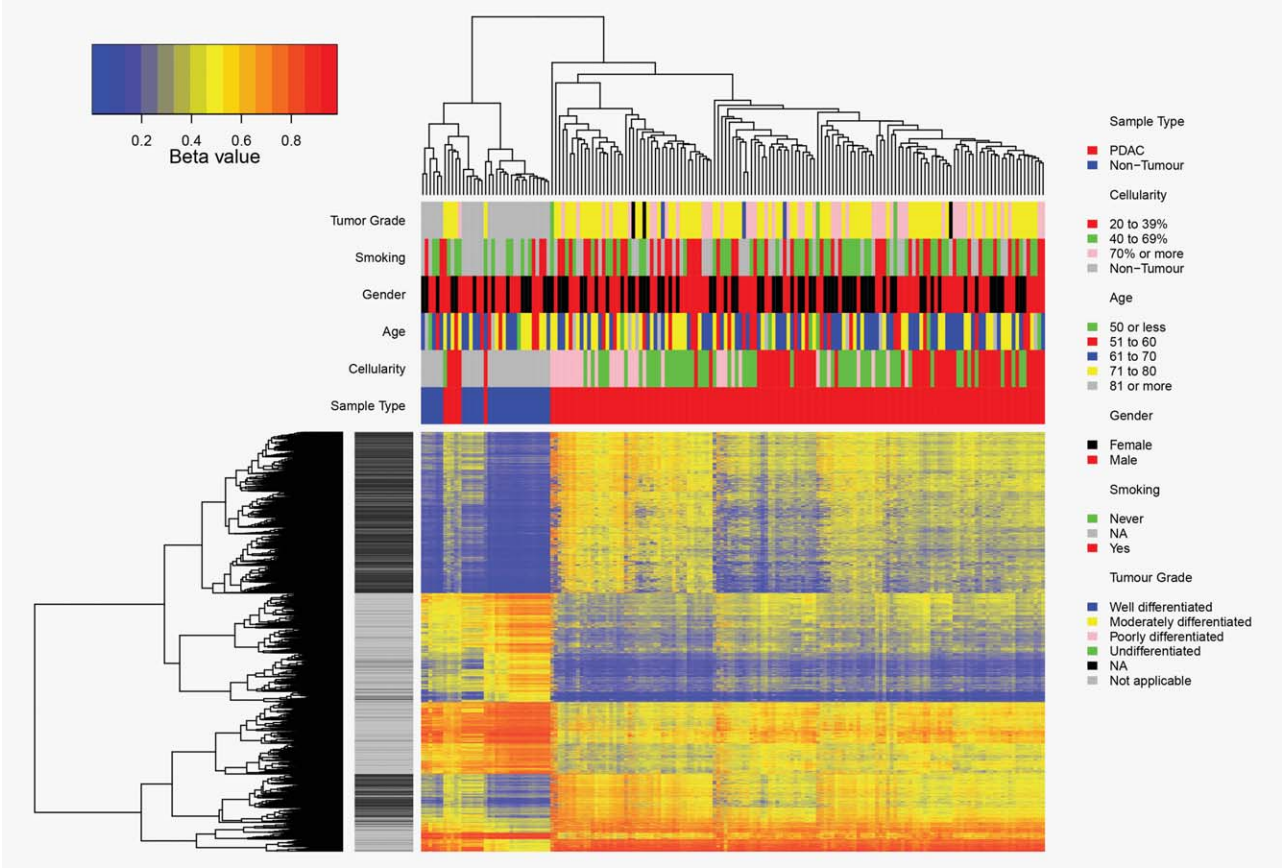

Figure 2. Cluster of PDAC (n = 140) and adjacent nonmalignant samples (n = 29) using 11,634 DM probes (adjusted p-value < 0.001 and $\Delta\beta$ value $\geq$ 0.20). Columns are samples and rows are DM probes. Beta-value equal zero means site is completely unmethylated and one completely methylated. CpG site locations are depicted on the left column of the graph (Black bars = probe located in CpG island, gray bars = probe is in other location of the genome). Clinical information is plotted above the cluster and details presented in the legends.

expression, the majority (n = 128) of which were located in gene bodies (Supporting Information Table S6).

Pathways affected by aberrant DNA methylation in PDAC

First, the entire set of genes (n = 3,522) associated with the robust DM probe set was used for pathway analysis. Ingenuity pathway analysis revealed 25 pathways significantly affected by DNA methylation in PDACs (adjusted p-value < 0.001; Supporting Information Table S8). Axon guidance was one of the most significant (adjusted p-value = 1.91E-05) and was supported by MetaCore pathway analysis (*FDR* = 1.21E-10; Supporting Information Table S9). This pathway was recently implicated in PDAC.[10] Other pathways identified here as enriched for genes aberrantly methylated (Supporting Information Tables S8 and S9) including cell adhesion, hedgehog signaling, TGF-β, integrin signaling and WNT/NOTCH signaling are well known key cancer signaling previously describe to be genetically altered in PDAC.[8] WNT signaling has been reported to be aberrant methylated in PDAC cell lines.[13] Our results confirm that this pathway is aberrantly methylated in this large cohort of PDAC. Stellate cell activation (adjusted p-value = 3.26E-05) another interesting pathway identified here as significantly affected by DNA methylation (Supporting Information Table S8) deserves further investigation due its importance in PDAC. Pancreatic stellate cells are the main fibroblastic cells in PDAC[25] and are known to interact with pancreatic cancer cells creating the fibrotic microenvironment of PDAC.[24] The fibrotic microenvironment of PDAC has been suggested to create a barrier that impairs the delivery of chemotherapeutics and promotes aggressive neoplastic cell behavior.[25] Known genes known to be involved in stellate cell activation (*COX2, TGFBR1*, EGFR, *TNF* and *MET* were hypomethylated in PDAC and confirmed bisulfite amplicon deep sequencing (Supporting Information Figs. S9–S14).

Ingenuity and MetaCore pathway analyses were also performed using 1,158 genes with significant correlation of methylation with mRNA expression levels. A similar enrichment for cell adhesion, apoptosis, invasion and cell proliferation processes was seen in meta-core analysis specifically, cell adhesion: *FDR* = 1.4E-08, WNT signaling: *FDR* = 3.1E-07, axon guidance: *FDR* = 2.0E-05, EMT regulation: *FDR* = 3.3E-05 and integrin mediated cell adhesion: *FDR*=9.3E-03; Supporting Information Table S10). IPA analysis provided

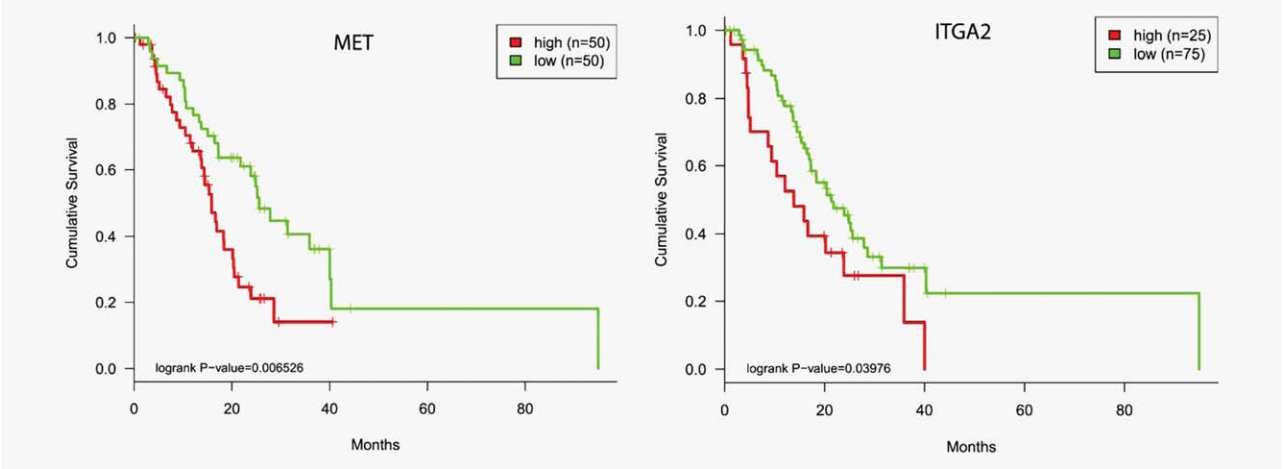

Figure 3. Kaplan-Meier survival curves show cosegregation of gene expression of *ITGA2* and *MET* with clinical outcome. Patients with high mRNA expression of *ITGA2* and *MET* genes showed lower survival. Median survival of patients presenting lower gene expression of *ITGA2* is 21.4 months compared with 13.8 months for high *ITAG2* expressing patients. Patients presenting lower level of *MET* expression have 25.6 months median survival compared with high expressing patients, which median survival is 15.9 months.

further supportive evidence for aberrant methylation of components of axon guidance (adjusted p-value = 9.53E-03) and integrin signaling (adjusted p-value = 4.38E-03; Supporting Information Table S11).

Based on these data, and the recently reported high prevalence of mutations and copy number alterations of axon guidance genes, particular those involved in SLIT-ROBO signaling in PDAC[10] we performed a detailed epigenetic and transcriptomic review to determine whether somatic epigenetic events are enriched in this pathway. Hyper-methylation of *ROBO1, ROBO3, SLIT3, SLIT2* and hypomethylation of *ITAG2* and *MET* (a known interacting partner of *SRGAP1*) were observed in the methylation array data. Deep-amplicon sequencing of bisulfite converted DNA for CpG sites in those genes validated DM (Supporting Information Figs. S9 and S14–S19). Deep-amplicon sequencing also showed that the regions surrounding the CpG sites interrogated in the array have similar levels of hypermethylation or hypomethylation (Supporting Information Figs. S10 and S19) as expected due to the high degree of locally correlated DNA methylation.[26] Arrays and amplicon sequencing results showed high concordance of the methylation levels (average Spearman correlation = 0.88, Supporting Information Fig. S9) validating array results and suggesting epigenetic inactivation of SLIT-ROBO signaling pathway and hypomethylation of *MET* and *ITAG2* in PDAC.

Significant inverse correlations between methylation and array based mRNA expression were observed for *SLIT2, SLIT3, ITGA2* and *MET* genes (Supporting Information Fig. S20) that were validated using qRT-PCR (Supporting Information Fig. S21). Although array mRNA expression levels for *ROBO1 and ROBO3* were at the limit of detection and showed no correlation with arrays (Supporting Information Fig. S22), qRT-PCR analysis demonstrated significant inverse correlation for *ROBO1, ROBO3* genes (p-value $\leq$ 0.05; Supporting Information Fig. S21).

DM and altered mRNA expression of *MET* and *ITGA2* is associated with patient survival

Given that mRNA expression of *ROBO2* and *ROBO3* has recently been associated with poor outcome of PDAC patients[10] and the observed wide spread altered methylation and expression of SLIT-ROBO signaling and their interactive partners, we sought to discern whether these events had any clinical significance. While *SLIT2, SLIT3* and *ITGB4* all showed correlation of methylation and expression levels (Supporting Information Tables S6 and S7), they did not correlate with survival in this cohort (Supporting Information Fig. S23). Interestingly, patients with coordinated hypomethylation and high expression of *MET* and *ITGA2* strongly correlated with poor outcome (p-value = 0.007 and 0.04, respectively; Fig. 3). While these genes are known to be involved in hepatocyte growth factor (HGF)/MET and Integrin signaling pathways, they both signal through *CDC42*, a core signal transduction molecule, which is actively suppressed by normal SLIT-ROBO signaling.

Discussion

This study reports the largest genome-wide scan of DNA methylation in PDAC to date. The large sample size and rigorous statistical threshold led to the identification of DM CpG sites that robustly differentiate PDAC from nonmalignant pancreas, even for samples with low cellularity. These results suggest that DNA methylation may have potential role in the diagnosis of PDAC, and supports further exploration.

The majority of hypermethylated sites are located in CpG islands and promoter regions as previously observed in other cancer types.[6,27] Genes affected by gene-body hypomethylation might be interesting targets for further studies as intragenic methylation is associated with regulation of intragenic promoter activity and alternative transcription in a tissue and

cell specific manner.[28] Of the 3,522 aberrantly methylated genes in PDAC, 850 (Supporting Information Table S5) were previously reported to be aberrantly methylated in pancreatic cancer cell lines.[11–13] This set constitutes almost half of the DM genes identified by Vincent *et al.*[13] where a 244,000 CpG array screen was performed on 9 PDAC cell lines and 3 unmatched laser captured normal pancreatic duct samples. These results suggest that the limitation of cellularity did not seriously confound our study. Furthermore, the strong concordance provides confidence that while the normal tissue control used in this study is not ideal, it is appropriate. Finally is important to note that differences between these two studies warrant further investigation as they may provide insights about the role of DNA methylation in altering other cell types present in the tumor microenvironment and DNA methylation events driven by prolonged cell culture.

Core signaling pathways involved in cell adhesion, apoptosis, invasion and cell proliferation were identified as recurrently aberrantly methylated (Supporting Information Tables S7 and S8). One interesting pathway significantly altered by DNA methylation in PDAC was the stellate cell activation (Supporting Information Table S7). Pancreatic stellate cells are the main fibroblastic cells in PDAC[29] and are known to interact with pancreatic cancer cells creating the fibrotic microenvironment of PDAC. Activated pancreatic stellate cells secrete extracellular matrix proteins that stimulate cancer cell proliferation supporting tumor growth and metastatic spreading, inhibiting apoptosis and enhancing angiogenesis.[24,30] Stromal activation is promoted by multiple cancer cell-derived signaling pathways such as TGF-β, HGF/MET, FGFs, IGF-1 and EGF *via* autocrine and paracrine mechanisms.[25] Members of these pathways were aberrantly methylated in PDAC. *COX2, TGFBR1*, EGFR, *TNF* and *MET* molecules known to be involved in stellate cell activation were hypomethylated in PDAC and confirmed by deep sequencing, suggesting that deregulated DNA methylation may influence stellate activation in PDAC. This cross-talk between stellate and cancer cells could be epigenetically regulated opening new avenues for therapeutic development, as pancreatic stellate cells have been shown to promote epithelial-mesenchymal transition in pancreatic cancer cell lines[31] and fibrotic tissue has been suggested to impact delivery of chemotherapeutics and promote aggressive neoplastic cell behavior in PDAC.[25]

Recent data suggest a role for genes involved in axon guidance pathway in tumorigenesis and tumor progression for a variety of cancer types including PDAC[10] and this pathway is being explored as a potential biomarker and therapeutic target in breast cancer.[32] Our results suggest additional perturbation of axon guidance through epigenetic inactivation of the SLIT-ROBO signaling, a major component of axon guidance. SLIT-ROBO signaling modulates HGF/MET and WNT signaling activity, both known to play important roles in carcinogenesis.[33] Dallol *et al.*[34] identified hyper-methylation of the *ROBO1* gene in 19% of breast carcinomas and 18% of clear cell renal cell carcinomas. Narayan *et al.*[35] showed hypermethylation of *SLIT1, SLIT2, SLIT3, ROBO1* and *ROBO3* genes in cervical cancer, suggesting that the hypermethylation of SLIT-ROBO signaling genes may be associated with cervical cancer progression. Interestingly, Vincent *et al.*[13] identified aberrant methylation of *ROBO1, ROBO3, SLIT2* and *SLIT3* in pancreatic cell lines. In addition, inactivation of the SLIT-ROBO pathway is associated with increased metastasis in melanoma cells.[36] Over-expression of *SLIT2* significantly decreased invasion and migration in breast cancer cell lines and mice injected with *SLIT2* expressing cells have a reduction of breast carcinoma incidence of 60%.[37]

Biankin *et al.*[10] identified genomic aberrations of *ROBO*1 in 11% (10% loss and 1% mutation) and *SLIT2* in 10% (7% loss and 3% mutation) of PDAC samples. Here for a subset of 58 tumors that exome or whole genome sequencing data was available (ICGC portal: http://dcc.icgc.org/) we identified tumors with loss of one allele, non-silent mutation or hypermethylation in *ROBO1, ROBO3, SLIT2* and *SLIT3* genes. Similar levels of loss and mutation rate previously reported[10] were observed in this small cohort (Supporting Information Fig. S24). A small proportion of the tumors presented mutation or loss of one allele and hypermethylation; however, the data presented here suggest that hypermethylation of SLIT-ROBO is a more wide spread mechanism of inactivation of this pathway. From the 58 tumors 48% showed hypermethylation of all four genes *(ROBO1, ROBO3, SLIT2* and *SLIT3;* Supporting Information Fig. S25). The hypermethylation of these genes and significant negative correlation with gene expression suggests epigenetic inactivation of SLIT-ROBO signaling in PDAC and constitutes an alternative mechanism to disrupt this pathway. The importance of DNA methylation cooperating with other genetic mechanisms to alter key signaling pathways critical to cancer development is well established.[22,38] Hypermethylation has been shown to be a preferred mechanism for inactivation of tumor suppressor in other cancers,[39] suggesting that tumor suppressor genes with a low incidence of mutations can use epigenetic inactivation more frequently.

Both *MET* and *IGTA2* genes were hypomethylated in PDAC and this correlated inversely with gene expression using arrays (q-value = 1.35E-08 and 4.10E-10, respectively) and RT-PCR (Supporting Information Fig. S21). In addition, high gene expression correlated with poor patient survival (Fig. 3). *MET* encodes a tyrosine kinase receptor for the HGF and is a poor prognostic indicator in other cancer types.[40] *MET* promotes a broad range of promalignant processes including cell motility, cell proliferation, invasion and metastasis.[40] The potential role of *ITGA2* in tumorigenesis is less well understood. *ITGA2* gene encodes the alpha subunit of a transmembrane receptor for collagen (*ITGA2/ITGB1*), which is implicated in cell adhesion and migration.[41] ITGA2 was found previously to be upregulated in PDAC in a meta-analysis of four independent studies.[42] Finally, both *MET* and *ITGA2* signal *via CDC42*,[43–45] the key effector molecule that is normally suppressed by active SLIT-ROBO signaling. Epigenetic or other mechanisms of suppression of SLIT-ROBO

signaling would amplify the effect of elevated expression of *MET* and *ITGA2*.

In summary, this large cohort screen provides evidence that aberrant DNA methylation and altered gene expression targets key pathways important to PDAC tumorigenesis and provides several candidate genes for future investigations. Taken together these data suggest an orchestrated epigenetic deregulation that plays a role in promoting pro-malignant cues that contribute to the aggressive nature of pancreatic cancer.

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