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Characterizing DNA methylation patterns in pancreatic cancer genome

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ABSTRACT

We performed a global methylation profiling assay on 1505 CpG sites across 807 genes to characterize DNA methylation patterns in pancreatic cancer genome. We found 289 CpG sites that were differentially methylated in normal pancreas, pancreatic tumors and cancer cell lines. We identified 23 and 35 candidate genes that are regulated by hypermethylation and hypomethylation in pancreatic cancer, respectively. We also identified candidate methylation markers that alter the expression of genes critical to gemcitabine susceptibility in pancreatic cancer. These results indicate that aberrant DNA methylation is a frequent epigenetic event in pancreatic cancer; and by using global methylation profiling assay, it is possible to identify these markers for diagnostic and therapeutic purposes in this disease.

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1. Introduction

Cancer is a complex disease characterized by multiple genetic and epigenetic genomic alterations (Esteller, 2007; Feinberg et al., 2006; Jones and Baylin, 2002). DNA methylation is one of the most important epigenetic alterations and plays a critical functional role in development, differentiation and diseases (Jones and Baylin, 2002). Through the activity of DNA methyltransferases (DNMTs), DNA methylation occurs at the cytosine residue in the context of 5'-CG-3' (CpG dinucleotide) across human genome (Laird, 2003). During the developmental process, DNA methylation plays an essential role in X chromosome inactivation in female somatic cells and in the mono-allelic silencing of parentally imprinted genes (Herman and Baylin, 2003). Once these DNA

methylation patterns are acquired in the early embryo stage, these patterns are inherited and maintained in successive cell generations. Promoter regions are usually enriched with CpG dinucleotides, known as CpG islands; and hypermethylation of these islands correlates with transcriptional silencing of tumor suppressor genes (Herman and Baylin, 2003). Conversely, increased expressions of oncogenes were associated with hypomethylation (Cui et al., 2002). This hypomethylation is known to contribute to cancer cell phenotypes through loss of imprinting (LOI) and genomic instability that characterizes tumors (Cui et al., 2002). Furthermore, tumorigenesis of several cancers was also marked by specific methylation changes in their genomes (Yegnasubramanian et al., 2004). Therefore, it is useful to construct a global methylation profile to discover candidate genes and to predict

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therapeutic outcomes (Shen et al., 2007) and patient survival in cancer (Rosenbaum et al., 2005).

Pancreatic cancer is the fourth most common cause of cancer-related death in the United States, and the death rates for this disease closely mirror incidence rates (Jemal et al., 2007). Lack of early diagnostic biomarkers and ineffectiveness of current therapies for this cancer are among the major factors that contribute to its low survival rate (Jimeno and Hidalgo, 2006). In an effort to identify such biomarkers, many groups have used high-throughput molecular profiling technologies, including oligonucleotide and cDNA arrays and Serial Analysis of Gene Expression (SAGE), to analyze gene expression data (Han et al., 2002; Iacobuzio-Donahue et al., 2002, 2003; Logsdon et al., 2003; Lowe et al., 2007; Ryu et al., 2002). Many candidate genes have been identified through these studies; however, the mechanism for the regulation of these genes is not fully understood. Using the candidate gene approach, Sato et al. (2003) identified seven overexpression genes (CLDN4, LCN2, MSLN, PSCA, S100A4, SFN and TFF2) in pancreatic cancer when compared to normal pancreatic duct were due to hypomethylation. Although the results are encouraging, the limitations for previous studies are the small number of genes examined (Esteller et al., 2001; Sato et al., 2003), which may fail to uncover other candidate genes regulated by DNA methylation in pancreatic cancer. Throughout this paper, we used the word methylation to refer DNA methylation. To address this issue, we have employed a global methylation profiling platform in this work to comprehensively survey a large panel of CpG sites across 800 genes in pancreatic cancer genome. We compared the DNA methylation profiles of the pancreatic tumors and normal tissues in order to unravel methylation markers for diagnostic purposes. We correlated the methylation markers with global gene expression profiles to identify candidate genes that were transcriptional regulated by methylation. By correlating methylation profiles with drug responses, we have identified candidate methylation markers that alter the expression of genes critical to gemcitabine susceptibility in pancreatic cancer.

2. Results

2.1. Methylation profiling in pancreatic cancer cell lines, tumors and normal tissues

To investigate the aberrant patterns of DNA methylation in pancreatic cancer, we employed global analysis to assess the methylation status of 1505 CpG sites across 807 genes in fourteen pancreatic cell lines, thirty pancreatic tumors and seven normal tissues (Supplementary Table 1). The methylation level of each CpG site is represented by the β -value ($0 \leq \beta \leq 1$), where 1 represents fully methylated and 0 represents unmethylated. The Illumina GoldenGate methylation assay (Bibikova et al., 2006) generates the profiles for these biological phenotypes, enabling us to perform global dissection of the aberrant DNA methylation patterns in pancreatic cancer. To our knowledge, this comprehensive database represents the largest survey of methylation status, many target sites per sample and many samples per study, in pancreatic cancer.

2.2. Methylation assay reproducibility

To demonstrate the reproducibility of the high-throughput DNA methylation profiling technique used in this study, biological replicates were profiled on the xenograft samples. We obtained highly reproducible DNA methylation profiles between these biological replicates with an average R^2 of 0.94 ± 0.16 when the β -values were compared. As illustrated in Supplementary Figure 1, when β -values for matching normal (JH033-Normal) and cancer (JH033-Cancer) samples were compared, differential methylation was readily detected.

2.3. Distribution of methylation status in pancreatic cancer and normal genomes

To characterize the global methylation status of pancreatic cancer genome, we first analyzed the distribution of β -values across 1505 CpG sites in pancreatic cancer cell lines, tumors and normal tissues. Globally, the methylation levels of for these samples were distributed bimodally, with higher percentage of CpG sites populated either at the high (hypermethylation) or low (hypomethylation) end of the β -value scale (Figure 1). On average, hypomethylation ($\beta \leq 0.1$) was observed in 38%, 49% and 44% of the studied CpG sites in pancreatic cancer cell lines, pancreatic cancer and normal tissues, respectively. Pancreatic cancer cell lines showed higher (25%) hypermethylation ($\beta > 0.9$) of the CpG sites in this study when compared to 17% and 15% in pancreatic cancer and normal tissues, respectively. This may indicate that the pancreatic cancer cell lines are less stable in their epigenomes. There is not statistically significance between the distribution of methylation sites in the cancer cell lines, cancerous and normal tissues in this study on the global scale; however, each individual tissue and cell line poses unique methylation patterns. Genes located at the two ends are the most interesting in this study, as they are likely regulated by epigenetic events (hypermethylation or hypomethylation) in pancreatic cancer genome.

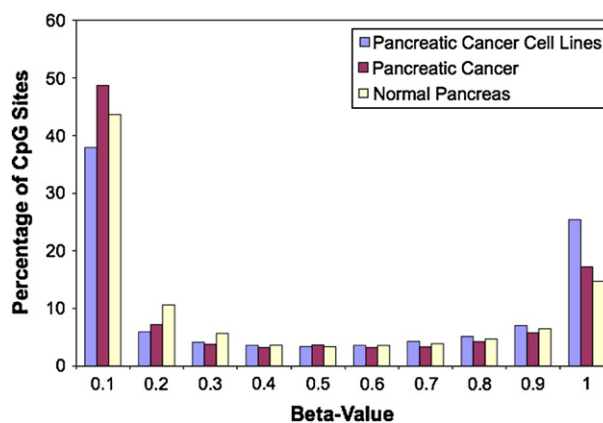


Figure 1 – Distribution of methylation status in pancreatic cancer and normal genomes. Globally, the methylation levels of the pancreatic cancer cell lines, cancerous and normal tissues were distributed bimodally, with higher percentage of CpG sites populated either at the high (hypermethylation) or low (hypomethylation) end of the β -value scale.

2.4. Identifying methylation markers in pancreatic cancer

To investigate whether global DNA methylation in CpG sites can distinguish between normal and pancreatic cancer, we performed unsupervised clustering on the seven matched cancer–tumor pairs on 1505 CpG sites based on their methylation status (β -values). Figure 2a shows the unsupervised clustering dendrogram. It clearly shows that the normal and cancerous tissues can be separated by the global DNA methylation status of these CpG sites. This demonstrates that methylation profiles can be used to distinguish normal samples from cancers.

Next, we used significance analysis of microarrays (SAM) (Tusher et al., 2001) to identify CpG sites (methylation markers) that are differentially methylated in the seven matched normal–tumor pairs. At the false discovery rate equals to 0, a total of 289 CpG sites in 214 genes with an average methylation level at least 2-fold higher or lower in pancreas adenocarcinomas compared to non-adenocarcinoma samples were identified. Of these, 120 and 169 CpG sites were hypermethylated and hypomethylated in the pancreatic cancer samples, respectively (Supplementary Table 2). These CpG sites were deemed as statistical significance methylation markers as their differential methylation status can be used to distinguish pancreatic cancer from normal tissue samples.

2.5. Clustering of independent sample sets based on the identified methylation markers

We next attempted to cluster the pancreatic cancer cell lines and independent pancreatic cancers based on these methylation markers. We employed the hierarchical clustering algorithm to organize these samples based on the CpG methylation status of these markers. Figure 2b shows the dendrogram of these relationships where the length of the branches reflects the relatedness of the samples. Overall, pancreatic tumor samples, cancer cell lines and normal tissues formed three distinct clusters based on the methylation status of these CpG sites. Two tumors (265 and JH034) and one cell line (Panc1) formed a cluster more closely to the normal tissues. One cell line (ASPC1) was clustered together with the tumors. This suggests that the methylation patterns for these markers are different in pancreatic cancer cell lines, tumors and normal samples.

2.6. Hypermethylation sites are enriched in CpG islands

Many studies have showed that methylation patterns are severely disrupted in human tumors. Aberrant hypermethylation of CpG islands in promoter regions represents one of the most frequent epigenetic events associated with gene silencing

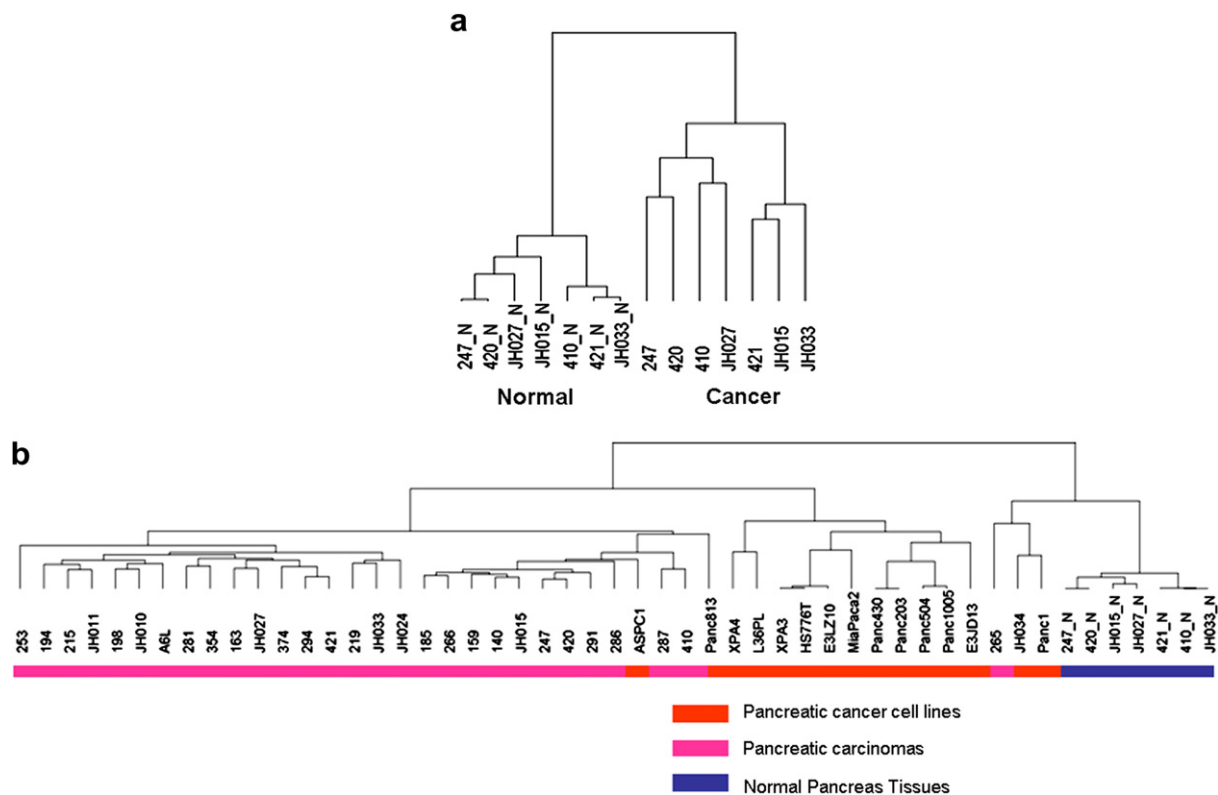


Figure 2 – Unsupervised clustering. (a) Unsupervised clustering of the seven pancreatic cancer–normal pairs based on global methylation profiles. It clearly shows that the normal and cancerous tissues can be separated by the global DNA methylation status of these CpG sites. This demonstrates that methylation profiles can be used to distinguish normal samples from cancers. (b) Unsupervised clustering of the thirty pancreatic cancers, fourteen pancreatic cancer cell lines and seven normal samples based on the methylation profiles of the 289 CpG sites. It shows three distinct clusters where the normal samples are tightly clustered together. The pancreatic cancer cell lines and tumors were clustered together, yet distinct methylation patterns exist between them.

in cancer. For the identified methylation markers, we observed 119/120 (99.2%) hypermethylation sites are located within CpG islands (hypergeometric test, $p < 9 \times 10^{-21}$) (Supplementary Table 2). However, no statistically enrichment of the 57/169 (33.7%) hypomethylation sites are located within CpG islands. We next asked whether there is an enrichment of these methylation markers in the promoter regions. We found 67/120 (55.8%) of the hypermethylation sites are located in the promoter regions compared to the 122/169 (72.2%) of the hypomethylation sites, but neither of them is statistically significance (Supplementary Table 2).

2.7. Methylation markers in distinguishing pancreatic cancers from normal tissues

The 289 methylation markers identified were corresponding to 85 and 129 genes that were hypermethylated and hypomethylated in the pancreatic cancer genome, respectively (Table 1). DNA methylation status for many of these genes was known to be altered in human cancers, including pancreatic cancer (Esteller, 2007). Most notably is the cyclin D2 (CCND2) gene, which is known to be hypermethylated in pancreatic cancer, and is currently being used as a clinical methylation marker to diagnose this disease (Matsubayashi et al., 2003).

Many hypermethylated genes identified in Table 1 were known to be correlated with various cancers (Esteller, 2007; Esteller et al., 2001; Feinberg et al., 2006), including cadherins (CDH13 and CDH17), transcription factors (HIC1 and HIC2), homeobox proteins (HOXA9 and HOXA11), growth factor binding proteins (IGFBP5 and IGFBP7), SFRP1, DBC1, MOS (Scholz et al., 2005), TMEF2, TIMP3 and WT1 (Satoh et al., 2003a,b). Transcriptional silencing of these genes has been shown to be associated with CpG island promoter hypermethylation in human cancers (Esteller, 2007). This indicates that methylation patterns are unique to specific cancer, and can be used to differentiate between malignancies (Bibikova et al., 2006).

We identified five imprinting genes (CPA4, MEST, MAGEL2, NDN, and SLC22A3) and four X-linked genes (BGN, GPRP, GUCY2F and MAGEA1) that were hypomethylated in the pancreatic cancers as compared to the normal tissues (Table 1). This suggests that LOI and activation of these genes may contribute to pancreas tumorigenesis. Interestingly, hypomethylation of MAGEA1, a male germline specific gene, was also found in many types of tumors (De Smet et al., 2004). Somatic mutation for one of the X-linked genes, GUCY2F, has been observed in breast, lung, colon and pancreatic cancer (Wood et al., 2006). This indicates that, according to the two-hit hypothesis (Herman and Baylin, 2003), some of the tumors may have loss transcriptional repression in GUCY2F and could cause the increased expression of the mutant copy in pancreatic cancer.

We also observed several transcription factors (E2F5, TFF1, TFF2 and ID1) that were hypomethylated in pancreatic cancers as compared to normal samples (Table 1). This suggests that cancer cell cycle may be disrupted through the increased activities of these hypomethylated transcription factors.

2.8. Correlating methylation markers and gene expression profiles

To investigate the relationship between DNA methylation and gene expression in pancreatic cancer genome, we correlated the DNA methylation status of the methylation markers with the global gene expression profiles. We performed linear regression analysis to correlate changes in CpG methylation patterns and basal gene expression levels on the thirty tumor cases. On average, an inverse correlation (-0.45 ± 0.1) was observed between methylation levels and mRNA expression levels (T-test, $p < 0.05$). This demonstrates that increased mRNA expression levels were correlated with hypomethylation or vice versa (decreased mRNA expression levels were correlated with hypermethylation).

From this correlation analysis, aberrant DNA methylation in the 214 genes can be classified into four categories: (a) 63 genes with CpG sites' hypomethylation and increased mRNA expression; (b) 24 genes with CpG sites' hypermethylation and mRNA increased expression; (c) 66 genes with CpG sites' hypomethylation and decreased mRNA expression; and (d) 61 genes with CpG sites' hypermethylation and mRNA decreased expression (Table 1). Genes that showed concordance between methylation patterns and mRNA expression levels (categories (b) and (c)) may suggest that the expressions of these genes were not regulated by DNA methylation. Another possible explanation is that the CpG sites used in this study were limited in covering all the important CpG dinucleotide sites of these genes, therefore, this may missed out some of the inverse correlations observed between methylation patterns and transcript expression levels for these genes. In contrast, genes within the two discordant categories (a) and (d) are the most interesting in this study, as they are likely candidate genes regulated by hypermethylation or hypomethylation. They represent 124 of the 807 (15.4%) genes we investigated in this study.

2.9. Identifying candidate genes regulated by aberrant DNA methylation

To pinpoint the candidate genes regulated by aberrant DNA methylation in pancreatic cancer, we focused on the 124 genes of discordant categories (a) and (d) in Table 1. Using the gene expression profiles of the two normal pancreas cell lines as baseline, we compare these genes in the thirty pancreatic tumors and fourteen cancer cell lines to the normal cell line profiles. We call the expression of a gene is transcriptional silenced by DNA methylation (hypermethylation) if its expression is under-expressed in all the tumors and cancer cell lines when compared to the expression of the normal cell lines. Conversely, if a gene has increased its expression in all the tumors and cancer cell lines when compared to the baseline expressions, then that gene has loss its fidelity in maintaining methylation status (hypomethylation). Using these criteria, we identified 23 and 35 candidate genes that are regulated by hypermethylation and hypomethylation, respectively (Figure 3 and Table 2).

Among the candidate genes listed in Table 2, the overexpression of SFN (also known as 14-3-3- σ) in pancreatic cancer is due to hypomethylation was confirmed by Sato et al. (2003).

Table 1 – Methylation markers in pancreatic cancer genome.

| Chr | Gene | Description | Class ^a |
|-------------------------|---------|---|--------------------|
| Hypermethylation | | | |
| 1 | GSTM2 | glutathione-S-transferase M2 | d |
| | NGFB | nerve growth factor, beta polypeptide precursor | d |
| | PTCH2 | patched 2 | d |
| | TAL1 | T-cell acute lymphocytic leukemia 1 | d |
| 2 | ALK | anaplastic lymphoma kinase Ki-1 | b |
| | DES | desmin | d |
| | FRZB | frizzled-related protein | d |
| | IGFBP5 | insulin-like growth factor binding protein 5 | b |
| | POMC | proopiomelanocortin | b |
| | TMEFF2 | transmembrane protein with EGF-like and two follistatin-like domains 2 | d |
| 4 | CASP3 | caspase 3 preproprotein | b |
| | CASP6 | caspase 6 isoform alpha preproprotein | d |
| | EPHA5 | ephrin receptor EphA5 isoform b | d |
| | FGF2 | fibroblast growth factor 2 | b |
| | FGF5 | fibroblast growth factor 5 isoform 1 precursor | b |
| | HHIP | hedgehog-interacting protein | d |
| | IGFBP7 | insulin-like growth factor binding protein 7 | b |
| | KDR | kinase insert domain receptor (a type III receptor tyrosine kinase) | d |
| | KIT | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog precursor | d |
| | PITX2 | paired-like homeodomain transcription factor 2 isoform c | b |
| 5 | SLIT2 | slit homolog 2 | d |
| | FLT4 | fms-related tyrosine kinase 4 isoform 2 | d |
| 6 | SCGB3A1 | secretoglobin, family 3A, member 1 | d |
| | ESR1 | estrogen receptor 1 | d |
| 7 | EYA4 | eyes absent 4 isoform a | d |
| | COL1A2 | alpha 2 type I collagen | b |
| 8 | FZD9 | frizzled 9 | d |
| | HOXA11 | homeobox protein A11 | d |
| | HOXA9 | homeobox protein A9 isoform b | b |
| | NPY | neuropeptide Y | d |
| | SMO | smoothened | d |
| | TFP12 | tissue factor pathway inhibitor 2 | d |
| | TWIST1 | twist | b |
| | FGFR1 | fibroblast growth factor receptor 1 isoform 1 precursor | d |
| | MOS | v-mos Moloney murine sarcoma viral oncogene homolog | d |
| | NEFL | neurofilament, light polypeptide 68 kDa | d |
| 9 | NRG1 | neuregulin 1 isoform HRG-beta3 | d |
| | PENK | proenkephalin | d |
| | SFRP1 | secreted frizzled-related protein 1 | d |
| | SOX17 | SRY-box 17 | d |
| | TUSC3 | tumor suppressor candidate 3 isoform b | b |
| | APBA1 | amyloid beta A4 precursor protein-binding, family A, member 1 | d |

Table 1 (continued)

| Chr | Gene | Description | Class ^a |
|-----|----------|--|--------------------|
| | DBC1 | deleted in bladder cancer 1 | d |
| | FANCG | Fanconi anemia, complementation group G | b |
| | GAS1 | growth arrest-specific 1 | d |
| | TMEFF1 | transmembrane protein with EGF-like and two follistatin-like domains 1 | b |
| 10 | FGF8 | fibroblast growth factor 8 isoform B precursor | d |
| | RET | ret proto-oncogene isoform c | d |
| 11 | ASCL2 | achaete-scute complex homolog-like 2 | d |
| | BDNF | brain-derived neurotrophic factor isoform a preproprotein | b |
| | FGF3 | fibroblast growth factor 3 precursor | d |
| | FLI1 | Friend leukemia virus integration 1 | d |
| | HSD17B12 | steroid dehydrogenase homolog | b |
| | IGF2AS | insulin-like growth factor 2 antisense | d |
| 12 | LMO1 | LIM domain only 1 | d |
| | MYOD1 | myogenic differentiation 1 | d |
| | THY1 | Thy-1 cell surface antigen | d |
| | WT1 | Wilms tumor 1 isoform B | d |
| | CCND2 | cyclin D2 | d |
| | ITPR2 | inositol 1,4,5-triphosphate receptor, type 2 | d |
| 13 | CCNA1 | cyclin A1 | d |
| | FLT1 | fms-related tyrosine kinase 1 | d |
| | FLT3 | fms-related tyrosine kinase 3 | d |
| | SOX1 | SRY (sex determining region Y)-box 1 | d |
| 14 | CHGA | chromogranin A precursor | b |
| | DLK1 | delta-like 1 homolog isoform 1 | b |
| 15 | NTRK3 | neurotrophic tyrosine kinase, receptor, type 3 isoform b precursor | d |
| | RASGRF1 | Ras protein-specific guanine nucleotide-releasing factor 1 isoform 1 | b |
| 16 | CDH13 | cadherin 13 preproprotein | d |
| | HS3ST2 | heparan sulfate D-glucosaminyl 3-O-sulfotransferase 2 | d |
| | MMP2 | matrix metalloproteinase 2 preproprotein | d |
| 17 | MYH11 | smooth muscle myosin heavy chain 11 isoform SM2 | d |
| | TUBB3 | tubulin, beta, 4 | b |
| | ALOX12 | arachidonate 12-lipoxygenase | d |
| 18 | COL1A1 | alpha 1 type I collagen preproprotein | b |
| | GAS7 | growth arrest-specific 7 isoform a | d |
| | HIC1 | hypermethylated in cancer 1 | d |
| 20 | ADCYAP1 | adenylate cyclase activating polypeptide precursor | b |
| | GALR1 | galanin receptor 1 | b |
| 21 | NTSR1 | neurotensin receptor 1 | b |
| 21 | ERG | v-ets erythroblastosis virus E26 oncogene like isoform 2 | d |

(continued on next page)

Table 1 (continued)

| Chr | Gene | Description | Class ^a |
|------------------------|---------|--|--------------------|
| 22 | HIC2 | hypermethylated in cancer 2 | d |
| | SEZ6L | seizure related 6 homolog (mouse)-like precursor | d |
| | TBX1 | T-box 1 isoform A | d |
| | TIMP3 | tissue inhibitor of metalloproteinase 3 precursor | d |
| Hypomethylation | | | |
| 1 | DHCR24 | 24-dehydrocholesterol reductase precursor | a |
| | EPHX1 | epoxide hydrolase 1, microsomal (xenobiotic) | a |
| | HDAC1 | histone deacetylase 1 | a |
| | MUC1 | MUC1 mucin isoform 1 precursor | c |
| | NBL1 | neuroblastoma, suppression of tumorigenicity 1 precursor | a |
| | NES | nestin | c |
| | NID1 | nidogen (enactin) | c |
| | S100A2 | S100 calcium binding protein A2 | c |
| | SFN | stratifin | a |
| | TGFB2 | transforming growth factor, beta 2 | a |
| 2 | CASP8 | caspase 8 isoform A | a |
| | CLK1 | CDC-like kinase 1 isoform 1 | a |
| | GLI2 | GLI-Kruppel family member GLI2 isoform alpha | c |
| | IHH | Indian hedgehog homolog | c |
| | IL1RN | interleukin 1 receptor antagonist isoform 4 | c |
| | UGT1A1 | UDP glycosyltransferase 1 family, polypeptide A1 precursor | c |
| | VAMP8 | vesicle-associated membrane protein 8 | a |
| 3 | ACVR2B | activin A type IIB receptor precursor | a |
| | CCR5 | chemokine (C-C motif) receptor 5 | c |
| | MST1R | macrophage stimulating 1 receptor | a |
| | PPARG | peroxisome proliferative activated receptor gamma isoform 1 | c |
| | TNFSF10 | tumor necrosis factor (ligand) superfamily, member 10 | c |
| 4 | CCKAR | cholecystokinin A receptor | c |
| | CXCL9 | small inducible cytokine B9 precursor | c |
| | IL2 | interleukin 2 precursor | c |
| | IL8 | interleukin 8 precursor | a |
| | SPP1 | secreted phosphoprotein 1 | a |
| 5 | CSF1R | colony stimulating factor 1 receptor precursor | c |
| | CSF2 | colony stimulating factor 2 precursor | a |
| | FGF1 | fibroblast growth factor 1 (acidic) isoform 2 precursor | c |
| | FGFR4 | fibroblast growth factor receptor 4 isoform 1 precursor | c |
| | IL12B | interleukin 12B precursor | c |
| | ITK | IL2-inducible T-cell kinase | a |
| 6 | CCND3 | cyclin D3 | a |
| | FRK | fyn-related kinase | c |
| | NOTCH4 | notch4 preproprotein | c |
| | PPARD | peroxisome proliferative activated receptor, delta isoform 1 | c |
| | SLC22A3 | solute carrier family 22 member 3 | c |
| | SPDEF | SAM pointed domain containing ets transcription factor | a |

Table 1 (continued)

| Chr | Gene | Description | Class ^a |
|-----|-----------|--|--------------------|
| 7 | ASB4 | ankyrin repeat and SOCS box-containing protein 4 isoform a | c |
| | CLDN4 | claudin 4 | a |
| | CPA4 | carboxypeptidase A4 preproprotein | a |
| | EPHB4 | ephrin receptor EphB4 precursor | a |
| | FLJ20712 | hypothetical protein LOC55025 | c |
| | LMTK2 | lemur tyrosine kinase 2 | c |
| | MEST | mesoderm specific transcript isoform a | a |
| | NOS3 | nitric oxide synthase 3 (endothelial cell) | c |
| | PRSS1 | protease, serine, 1 preproprotein | c |
| | SEMA3C | semaphorin 3C | c |
| | TRIP6 | thyroid hormone receptor interactor 6 | a |
| 8 | CDH17 | cadherin 17 precursor | c |
| | DLC1 | deleted in liver cancer 1 isoform 1 | c |
| | E2F5 | E2F transcription factor 5 | a |
| | PLAT | plasminogen activator, tissue type isoform 2 precursor | a |
| | PSCA | prostate stem cell antigen | c |
| | SFTPC | surfactant, pulmonary-associated protein C | c |
| | TNFRSF10A | tumor necrosis factor receptor superfamily, member 10a | a |
| 9 | DAPK1 | death-associated protein kinase 1 | c |
| | LCN2 | lipocalin 2 (oncogene 24p3) | c |
| | SYK | spleen tyrosine kinase | a |
| | VAV2 | vav 2 oncogene | a |
| 10 | ABCC2 | ATP-binding cassette, subfamily C (CFTR/MRP), member 2 | a |
| | CYP2E1 | cytochrome P450, family 2, subfamily E, polypeptide 1 | c |
| | FGFR2 | fibroblast growth factor receptor 2 isoform 12 precursor | c |
| | MAP3K8 | mitogen-activated protein kinase kinase kinase 8 | c |
| | SFTPA1 | surfactant, pulmonary-associated protein A1 | c |
| | SNCG | synuclein, gamma (breast cancer-specific protein 1) | c |
| | TCF7L2 | transcription factor 7-like 2 (T-cell specific, HMG-box) | c |
| 11 | APOA1 | apolipoprotein A-I preproprotein | c |
| | CCND1 | cyclin D1 | a |
| | DDB2 | damage-specific DNA binding protein 2 (48kD) | c |
| | INS | proinsulin precursor | c |
| | MMP1 | matrix metalloproteinase 1 preproprotein | a |
| | MMP7 | matrix metalloproteinase 7 preproprotein | a |
| | PHLDA2 | pleckstrin homology-like domain family A member 2 | a |
| | SPI1 | spleen focus forming virus (SFFV) proviral integration oncogene spi1 | c |
| | TMPPRSS4 | transmembrane protease, serine 4 isoform 1 | a |
| | TRIM29 | tripartite motif protein TRIM29 isoform alpha | c |
| | TSG101 | tumor susceptibility gene 101 | a |

(continued on next page)

| Table 1 (continued) | | | |
|---------------------|----------|---|--------------------|
| Chr | Gene | Description | Class ^a |
| 12 | ARHGDIB | Rho GDP dissociation inhibitor (GDI) beta | a |
| | IAPP | islet amyloid polypeptide precursor | a |
| | IFNG | interferon, gamma | a |
| | KRT1 | keratin 1 | a |
| | PTH1LH | parathyroid hormone-like hormone isoform 2 preproprotein | c |
| | PTPN6 | protein tyrosine phosphatase, non-receptor type 6 isoform 2 | a |
| | UNG | uracil-DNA glycosylase isoform UNG1 precursor | a |
| 14 | BMP4 | bone morphogenetic protein 4 preproprotein | c |
| | KIAA0125 | hypothetical protein LOC9834 | a |
| | RIPK3 | receptor-interacting serine-threonine kinase 3 | a |
| 15 | APBA2 | amyloid beta A4 precursor protein-binding, family A, member 2 | c |
| | MAGEL2 | MAGE-like protein 2 | c |
| | NDN | neccin | a |
| | TJP1 | tight junction protein 1 isoform b | a |
| 16 | CARD15 | NOD2 protein | c |
| | CREBBP | CREB binding protein | c |
| | NQO1 | NAD(P)H menadiene oxidoreductase 1, dioxin-inducible isoform c | a |
| | PRSS8 | prolactin preproprotein | a |
| 17 | BRCA1 | breast cancer 1, early onset isoform BRCA1-delta9-11 | c |
| | CRK | v-crk sarcoma virus CT10 oncogene homolog isoform b | a |
| | CSF3 | colony stimulating factor 3 isoform c | c |
| | ITGB4 | integrin beta 4 isoform 1 precursor | a |
| | LIG3 | ligase III, DNA, ATP-dependent isoform alpha precursor | a |
| | NOS2A | nitric oxide synthase 2A isoform 1 | a |
| | SEPT9 | septin 9 | a |
| 18 | SERPINB5 | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5 | a |
| 19 | BAX | BCL2-associated X protein isoform sigma | c |
| | BSG | basigin isoform 1 | a |
| | CEACAM1 | carcinoembryonic antigen-related cell adhesion molecule 1 isoform 1 precursor | c |
| | EMR3 | egf-like module-containing mucin-like receptor 3 isoform b | c |
| | JAK3 | Janus kinase 3 | c |
| | NOTCH3 | Notch homolog 3 | c |
| | PLAUR | plasminogen activator, urokinase receptor isoform 3 precursor | a |
| | PTPRH | protein tyrosine phosphatase, receptor type, H precursor | a |
| 20 | DNMT3B | DNA cytosine-5 methyltransferase 3 beta isoform 2 | a |
| | ID1 | inhibitor of DNA binding 1 isoform a | a |
| | MMP9 | matrix metalloproteinase 9 preproprotein | c |
| | PI3 | elafin preproprotein | c |
| | PTK6 | PTK6 protein tyrosine kinase 6 | c |

| Table 1 (continued) | | | |
|---------------------|---------|---|--------------------|
| Chr | Gene | Description | Class ^a |
| | SRC | proto-oncogene tyrosine-protein kinase SRC | c |
| 21 | B3GALT5 | UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase 5 | c |
| | RIPK4 | ankyrin repeat domain 3 | a |
| | TFF1 | trefoil factor 1 precursor | a |
| | TFF2 | trefoil factor 2 precursor | a |
| 22 | BCR | breakpoint cluster region isoform 2 | a |
| | LIF | leukemia inhibitory factor (cholinergic differentiation factor) | a |
| X | BGN | biglycan preproprotein | c |
| | GRPR | gastrin-releasing peptide receptor | c |
| | GUCY2F | guanylate cyclase 2F | c |
| | MAGEA1 | melanoma antigen family A, 1 | a |

a Classes: (a) genes with CpG sites' hypomethylation and increased mRNA expression; (b) genes with CpG sites' hypermethylation and mRNA increased expression; (c) genes with CpG sites' hypomethylation and decreased mRNA expression; and (d) genes with CpG sites' hypermethylation and mRNA decreased expression.

This supports our approach is valid in identifying candidate genes regulated by aberrant DNA methylation in pancreatic cancer. Cyclin D1 and D3 (*CCND1* and *CCND3*) have been showed to be overexpressed in human pancreatic cancer (Ebert et al., 2001; Gansauge et al., 1997). Here, *CCND1* and *CCND3* were identified as hypomethylated candidate genes, suggesting that overexpression of these cyclins (Ebert et al., 2001; Gansauge et al., 1997) in pancreatic cancer is associated with the loss of methylation. We also identified *MMP7* as a hypomethylated candidate gene, where the overexpression of this gene has been shown to be a useful biomarker in pancreatic cancer (Kuhlmann et al., 2007).

To further characterize the candidate genes regulated by DNA methylation, we used DAVID annotation program to group genes according to their biological functional classes. Eight of the hypomethylated candidate genes (*LIG3*, *CCND3*, *CCND1*, *SFN*, *CLK1*, *NBL1*, *TGFB2*, and *ID1*) were involved in cell cycle and cell proliferation according to Gene Ontology classification. Six of the hypomethylated candidate genes (*CASP8*, *PHLDA2*, *PTPN2*, *TGFB2*, *HDAC1* and *IAPP*) were involved in apoptosis. This suggests that increased expression of these genes due to hypomethylation may contribute to the uncontrollable cell proliferation and genomic instability in pancreatic cancer.

For the hypermethylated candidate genes, five of them (*KIT*, *FLT1*, *FLT3*, *NTRK3* and *EPHA5*) are members of the tyrosine kinase family. This suggests that underexpression of these genes may contribute to the lack of sensitivity in certain tyrosine kinase target drugs, such as imatinib (Chen et al., 2006), in pancreatic cancer.

2.10. Validation by methylation specific PCR (MSP)

We performed methylation specific PCR (MSP) validation on three hypermethylated genes (*DBC1*, *FLT1* and *EYA4*) from Table 1 (candidate genes) in four tumors used in this study (410, 420, JH011 and JH015). Figure 4a shows that the CpG sites

Table 2 – Candidate gene list where their expression is regulated by DNA methylation.

| Gene | Description |
|-------------------------|--|
| Hypermethylation | |
| ALOX12 | arachidonate 12-lipoxygenase |
| DBC1 | deleted in bladder cancer 1 |
| DES | desmin |
| EPHA5 | EPH receptor A5 |
| ERG | v-ets erythroblastosis virus E26 oncogene homolog (avian) |
| ESR1 | estrogen receptor 1 |
| EYA4 | eyes absent homolog 4 (Drosophila) |
| FLT1 | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) |
| FLT3 | fms-related tyrosine kinase 3 |
| GAS7 | growth arrest-specific 7 |
| HS3ST2 | heparan sulfate (glucosamine) 3-O-sulfotransferase 2 |
| KIT | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog |
| MOS | v-mos Moloney murine sarcoma viral oncogene homolog |
| MYH11 | myosin, heavy chain 11, smooth muscle |
| NEFL | neurofilament, light polypeptide 68 kDa |
| NGFB | nerve growth factor, beta polypeptide |
| NTRK3 | neurotrophic tyrosine kinase, receptor, type 3 |
| PENK | proenkephalin |
| PTCH2 | patched homolog 2 (Drosophila) |
| SEZ6L | seizure related 6 homolog (mouse)-like |
| SLIT2 | slit homolog 2 (Drosophila) |
| SMO | smoothened homolog (Drosophila) |
| THY1 | Thy-1 cell surface antigen |
| Hypomethylation | |
| ACVR2B | activin A receptor, type IIB |
| BCR | breakpoint cluster region |
| BSG | basigin (Ok blood group) |
| CASP8 | caspase 8, apoptosis-related cysteine peptidase |
| CCND1 | cyclin D1 |
| CCND3 | cyclin D3 |
| CLK1 | CDC-like kinase 1 |
| CPA4 | carboxypeptidase A4 |
| CRK | v-crk sarcoma virus CT10 oncogene homolog (avian) |
| DNMT3B | DNA (cytosine-5)-methyltransferase 3 beta |
| EPHB4 | EPH receptor B4 |
| EPHX1 | epoxide hydrolase 1, microsomal (xenobiotic) |
| HDAC1 | histone deacetylase 1 |
| IAPP | islet amyloid polypeptide |
| ID1 | inhibitor of DNA binding 1, dominant negative helix-loop-helix protein |
| KIAA0125 | KIAA0125 |
| LIF | leukemia inhibitory factor (cholinergic differentiation factor) |
| LIG3 | ligase III, DNA, ATP-dependent |
| MEST | mesoderm specific transcript homolog (mouse) |
| MMP7 | matrix metalloproteinase 7 (matrilysin, uterine) |
| MST1R | macrophage stimulating 1 receptor (c-met-related tyrosine kinase) |
| NBL1 | neuroblastoma, suppression of tumorigenicity 1 |
| NOS2A | nitric oxide synthase 2A (inducible, hepatocytes) |
| PHLDA2 | pleckstrin homology-like domain, family A, member 2 |
| PLAT | plasminogen activator, tissue |
| PLAUR | plasminogen activator, urokinase receptor |
| PTPN6 | protein tyrosine phosphatase, non-receptor type 6 |
| PTPRH | protein tyrosine phosphatase, receptor type, H |
| SEPT9 | septin 9 |
| SFN | stratifin |

Table 2 (continued)

| Gene | Description |
|--------|--|
| SPDEF | SAM pointed domain containing ets transcription factor |
| TGFB2 | transforming growth factor, beta 2 |
| TJP1 | tight junction protein 1 (zona occludens 1) |
| TSG101 | tumor susceptibility gene 101 |
| VAV2 | vav 2 oncogene |

identified as hypermethylation by the Illumina Bead arrays (high β -values) were indeed methylated, as validated by MSP (Figure 4b). Methylation of *DBC1*, *FLT1* and *EYA4* was observed by MSP in 75% (3/4), 100% (4/4) and 100% (4/4) tumors respectively (Figure 4b). We also performed MSP on two normal pancreatic cell lines (HPDE and HPNE) to validate these methylation changes are specific in tumors. Indeed, we found that the two normal cell lines were not methylated in these genes, except HPNE showed methylation pattern in *DBC1* (Figure 4c). The primers used for the MSP analysis are listed in the Supplementary Table 3.

2.11. Correlating DNA methylation patterns with gemcitabine sensitivity

We next asked whether DNA methylation could be used as molecular markers to predict sensitivity or resistance to chemotherapy. Gemcitabine has been approved as the first-line treatment for pancreatic cancer patients; however, about 75% of patients have minimal benefit from this therapy. Moreover, the genetic factors that predict gemcitabine sensitivity are not fully understood. We hypothesized that aberrant DNA methylation patterns may be used as biomarkers to predict gemcitabine susceptibility in pancreatic cancer. To test this hypothesis, we treated the thirty direct-patient xenograft models with gemcitabine. Relative tumor growth inhibition (TGI) values for these tumors were measured after 28 days of gemcitabine treatment. The thirty xenografts showed varying degrees of gemcitabine sensitivity, ranging from -88% to 59% TGI. For simplicity, in this study, we stratify the tumors into sensitive and resistant groups. We considered tumors with $TGI \leq -20\%$ as sensitive and tumors with $TGI > -20\%$ as resistant to gemcitabine, respectively. Using this cut-off, ten tumors passed the sensitive threshold, and the other twenty tumors were regarded as resistant. Next, we ran SAM on the DNA methylation profiles of the thirty tumors to identify differentially methylated DNA markers in sensitive versus resistant groups. From the SAM analysis, *GSTM1_P266_F* and *ONECU-T2_E96_F*, two DNA markers were identified as statistically significant in distinguishing sensitive from resistant tumors.

We next sought to ask whether these DNA markers can be used as predictor for gemcitabine sensitivity in pancreatic cancer. To test the applicability of these DNA markers, we build a weighted voting predictor from the thirty tumors' methylation profiles (training set) and predict the gemcitabine sensitivity in the twelve pancreatic cancer cell lines (independent test set). The predictor predicts ten and two cancer cell lines as sensitive and resistant to gemcitabine, respectively (Table 3). To validate the prediction of the predictor based on DNA methylation markers, we performed MTT assay on

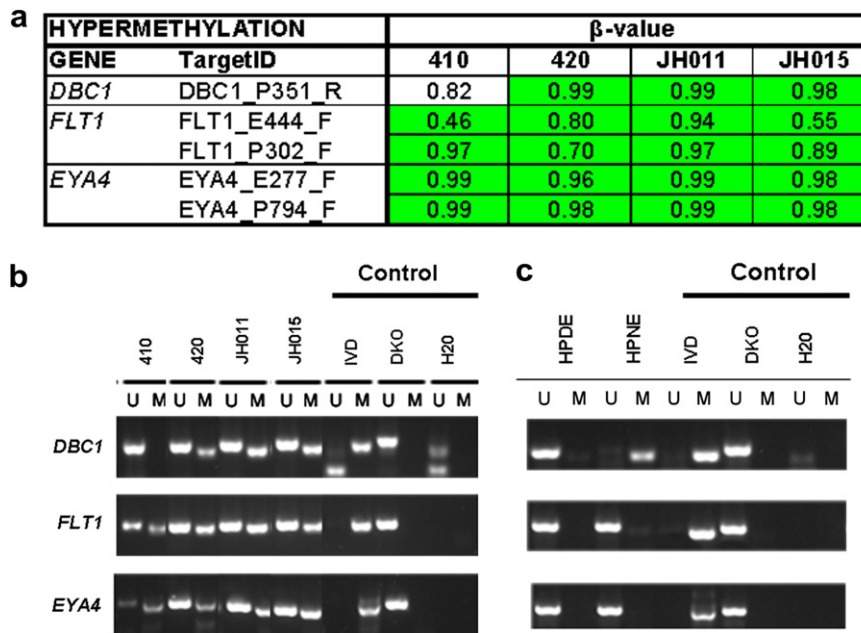


Figure 4 – Validation by the methylation specific PCR. (a) β -values of CpG sites in three candidate genes (*DBC1*, *FLT1* and *EYA4*) as assessed as hypermethylation by the Illumina Bead Array platform. Green color indicates that the methylation calls were validated by MSP method. (b) MSP validation of these genes. U and M columns indicate unmethylated and methylated bands. DKO and IVO were used as the unmethylated (U) and methylated (M) controls. (c) MSP results for the two normal pancreas cell lines on these genes.

the twelve pancreatic cancer cell lines for gemcitabine exposure at 10 μ M for 72 h. From the MTT analysis, eleven of the twelve cancer cell lines are sensitive to gemcitabine where viable cell mass after gemcitabine treatment is $\leq 50\%$ compared to controls; and one cell line (MiaPaca2) is resistant to gemcitabine with viable cell mass $> 50\%$ compared to controls (Table 3). Hence, the predictor correctly predicts the gemcitabine sensitivity in eleven cell lines, and only missed out one prediction (Panc1) (Table 3).

In the gemcitabine sensitive tumors, *GSTM1_P266_F* has higher methylation level ($\beta_{\text{SEN}} = 0.95 \pm 0.06$) when compared with the resistant tumors ($\beta_{\text{RES}} = 0.72 \pm 0.19$). This higher methylation level may reduce the gene expression of *GSTM1* in the

sensitive cases. Glutathione-S-transferases $\mu 1$ (*GSTM1*) is a key enzyme involved in the glutathione detoxification pathway in mammalian cells. This class of enzyme catalyzed the formation of glutathione conjugates with xenobiotics, toxic superoxides, or antineoplastic agents. Once the conjugates were formed, they can be exported from cells by ATP-dependent glutathione-S-conjugate complex export pump. Some cancer cell line studies have suggested that the activation or elevated of *GST* expression decreases drug effects; however, down-regulation and inhibiting *GST* enhances drug sensitivity (Yang et al., 2006). Indeed, we observed this inversed correlation between *GSTM1* gene expression and methylation levels in these thirty cases (Figure 5), and the effect is more profound in the sensitive cases ($\text{TGI} \leq -20\%$).

The other DNA methylation marker for predicting gemcitabine sensitivity is *ONECUT2_E96_F*, a transcription factor characterized by the presence of a single 'cut' domain and an atypical homeodomain (one cut domain, family member 2). This gene has been suggested to participate in the network of transcription factors required for liver differentiation and metabolism (Jacquemin et al., 1999). However, the role of *ONECUT2* in pancreatic cancer is still unknown. Similar inversed correlation between *ONECUT2* gene expression and methylation patterns was observed in the tumor cases (Figure 5). Our current data suggest that hypermethylation of *GSTM1* and *ONECUT2* may synergized to gemcitabine susceptibility in a subset of pancreatic cancer. (Chen et al., 2006)

Table 3 – Gemcitabine sensitivity predicted by methylation markers and validated by MTT assay.

| Cancer cell lines | Prediction | MTT (% viable cell mass compared to control) | Correct prediction? |
|-------------------|------------|--|---------------------|
| Panc430 | SEN | 10 | Yes |
| XPA3 | SEN | 12 | Yes |
| Panc203 | SEN | 14 | Yes |
| Panc1 | RES | 19 | No |
| Panc1005 | SEN | 21 | Yes |
| E3LZ10 | SEN | 23 | Yes |
| HS776T | SEN | 23 | Yes |
| ASPC1 | SEN | 30 | Yes |
| XPA4 | SEN | 38 | Yes |
| L36PL | SEN | 46 | Yes |
| E3JD13 | SEN | 49 | Yes |
| MiaPaca2 | RES | 79 | Yes |

3. Discussion

In this study, we investigated the methylation status of a relatively large panel of genes in a series of pancreatic cancer cell

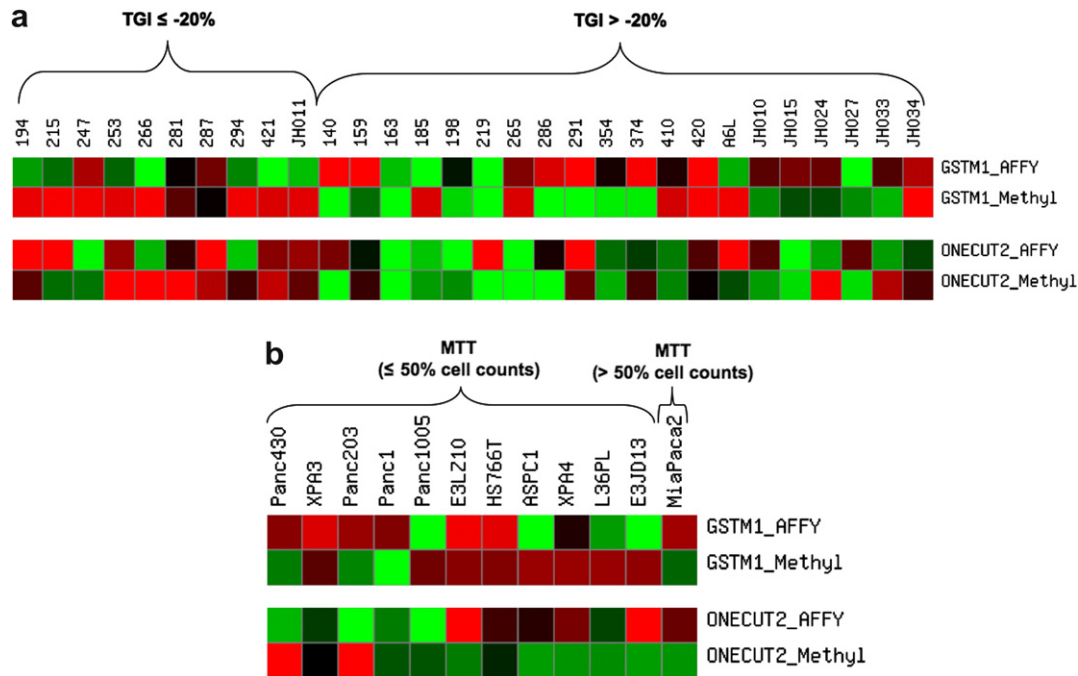


Figure 5 – Correlation of DNA methylation and gene expression levels with gemcitabine sensitivity (a) xenografts models and (b) pancreatic cell lines. *GSTM1_Affy* and *ONECUT2_Affy* represent relative gene expression profiles obtained from Affymetrix GeneChip data. *GSTM1_Methyl* and *ONECUT2_Methyl* represent relative methylation status obtained from the methylation profiling data. Red and green colors indicate high and low expression/methylation, respectively.

lines, primary pancreatic carcinoma and normal pancreas tissues using global methylation profiling assay. By comparing the methylation profiles and gene expression data, we identified a set of candidate genes in pancreatic cancer cell lines and tumors that show an inverse correlation between DNA methylation and mRNA expression. These results suggest that expression changes in these candidate genes may due to abnormal DNA methylation at the promoter regions. Hypermethylation of silencers may also increase the expression of these candidate genes. We found that it is more common to detect hypomethylation of genes that are overexpressed in pancreatic cancer when they are normally methylated and not expressed in non-neoplastic pancreas. This is also supported by the observation of Sato et al. (2003) where DNA hypomethylation is a frequent epigenetic alteration in pancreatic cancer and is associated with the overexpression of affected genes. Several hypermethylated genes identified in this study were also preserved the same methylation pattern in various cancers (Esteller, 2007). Our results strengthen that aberrant DNA methylation is a ubiquitous feature of cancer.

Recent studies have been suggested that aberrant DNA methylation can affect the sensitivity of cancers to chemotherapeutics by altering expression of genes critical to drug response. These studies have showed that methylation of *MGMT* and *CHFR* indicates sensitivity to carmustine and microtubule inhibitors in gliomas and in gastric cancers, respectively (Esteller et al., 2000; Satoh et al., 2003a,b). Shen et al. (2007) found association between *p73* hypermethylation and increased sensitivity to alkylating agents by correlating drug activity with methylation status of 32 CpG island-associated

genes in the NCI-60 cancer cell lines. In this study, we identified DNA methylation of *GSTM1* and *ONECUT2* was associated with gemcitabine sensitivity in pancreatic cancer.

In summary, we demonstrate the use of methylation profiling to identify a list of candidate genes where their expressions are regulated by DNA methylation and demethylation in pancreatic cancer. We also show that integrating high-throughput methylation and gene expression analyses are useful in identifying biomarkers for cancer drug sensitivity.

4. Experimental procedures

4.1. Direct-patient xenografts, normal and pancreatic cancer cell lines

Pancreatic cancer xenografts were established from surgically resected primary pancreatic carcinomas, and thirty xenografts were selected for this study. We also collected seven adjacent normal pancreas tissues from the subset of these xenografts. Fourteen human pancreatic cancer cell lines, Panc1, Panc203, Panc430, Panc504, Panc813, Panc1005, HS766T, MiaPaca2, E3.JD13, E3.LZ10, ASPC1, L3.6PL, XPA3 and XPA4, were used in this study. We used HPNE and HPDE, two non-transformed pancreatic ductal epithelial cell lines, as the normal pancreas cell lines in this study.

4.2. DNA methylation profiling assay

The GoldenGate Methylation Cancer Panel I contains 1505 CpG sites distributed across 807 genes; 1044 CpG sites are located

within CpG islands, and 461 are located outside of CpG islands (Supplementary Table 1). This technology adapts the Illumina high-throughput single nucleotide polymorphism genotyping system to assay the bead-based array platform. Each methylation data site is captured by fluorescent signals from the M (methylated) and U (unmethylated) alleles. To determine the intensity for each CpG site (β), background intensity was subtracted from each data point and the fluorescent signals' ratio from the two alleles is computed as: $\beta = (\max(M,0)) / (|U| + |M| + 100)$. The β -value ($0 \leq \beta \leq 1$) reflects the methylation level of each CpG site where 1 represents fully methylated and 0 represents unmethylated (Bibikova et al., 2006).

4.3. Microarray gene expression profiling data

Gene expressions of the thirty pancreatic xenograft tumors, normal and pancreatic cancer cell lines were profiled using Affymetrix U133 Plus 2.0 gene arrays. Sample preparation and processing procedure were performed as described in the Affymetrix GeneChip® Expression Analysis Manual (Affymetrix Inc., Santa Clara, CA).

4.4. Significance analysis of microarray (SAM)

We used SAM (Tusher et al., 2001) to select genes that show statistically significant changes in their methylation patterns. We obtained the SAM algorithm implemented as a Microsoft Excel add-in from <http://www-stat.stanford.edu/~tibs/SAM/>.

4.5. Hierarchical clustering

We used the Euclidean distance metric on the expression matrix and performed complete linkage hierarchical clustering using Cluster 3.0 (de Hoon et al., 2004) and visualized it in Java TreeView (v1.0.13) (Saldanha, 2004).

4.6. In vivo growth inhibition studies

Six-week-old female athymic nude mice (Harlan, IN, US) were used to evaluate the *in vivo* growth inhibition studies. The xenografts were generated according to previous published methodology (Rubio-Viqueira et al., 2006), and the research protocol was approved by the Johns Hopkins University Animal Care and Use Committee and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. In brief, surgical non-diagnostic specimens of patients operated at the Johns Hopkins Hospital were reimplanted subcutaneously to 1–2 mice for each patient, with 2 small pieces per mouse (F1 generation). Tumors were let to grow to a size of 1.5 cm³ at which point were harvested, divided, and transplanted to another 5 mice (F2 generation). After a second growth passage tumors were excised and propagated to cohorts of 20 mice or more, that constituted the treatment cohort (F3 generation). Tumors in the PancXenoBank are kept as a live bank that is expanded as required for drug testing and biologic studies. Tumors from this treatment cohort were allowed to grow until reaching ~200 mm³, at which time mice were randomized in the following treatment groups: 1) control and 2) gemcitabine 100 mg/kg 2 times a week ip; with five to six mice (at least ten tumors) in each

group. Treatment was given for 28 days. Mice were monitored daily for signs of toxicity and were weighed three times per week. Tumor size was evaluated two times per week by caliper measurements using the following formula: tumor volume = [length × width²]/2. Relative tumor growth inhibition (TGI) was calculated by relative tumor growth of treated mice divided by relative tumor growth of control mice since the initiation of therapy.

4.7. Methylation specific PCR (MSP)

Bisulfite modification of genomic DNA was carried out using the EZ DNA methylation Kit (Zymo Research, Orange, CA). We performed methylation analysis of the promoter regions of various genes using MSP primer pairs covering the putative transcriptional start site in the 5' CpG island with 1 μL of bisulfite-treated DNA as template and JumpStart Red Taq DNA Polymerase (Sigma, St. Louis, MO) for amplification as previously described (1). MSP primers were designed using the online software program MSPPrimer (<http://www.mspprimer.org>). DKO and IVO were used as the unmethylated (U) and methylated (M) controls.

4.8. In vitro drug sensitivity assay

To evaluate the *in vitro* drug sensitivity, pancreatic cancer cell lines treated with gemcitabine were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO). 5000 cells were seeded in 96-well plates, transfected during 48 h, and then exposed to either vehicle or gemcitabine at a concentration of 10 μM for 72 h. Each experiment was performed in sextuplicate independently at least three times.

4.9. Weighted voting predictor

Weighted voting predictor (Golub et al., 1999) was used to distinguish gemcitabine sensitive from resistant cases based on methylation patterns. Let μ_1 (μ_2) and δ_1 (δ_2) represent mean and standard deviation for the sensitive (resistant) cases. Signal-to-noise ratio (S2N) for a marker g is computed as $(\mu_1 - \mu_2) / (\delta_1 + \delta_2)$ and the average mean M_g between two classes is calculated as $(\mu_1 + \mu_2) / 2$. To make the prediction for a new case X , for each methylation marker g , the weight for its vote is computed as $v_g = S2N_g \times (X_g - M_g)$, with a positive value indicating a vote for sensitive and a negative value indicating a vote for resistant. If the total vote for class sensitive (obtained by summing the absolute values of the positive votes over the methylation markers) is greater than the total vote for class resistant (obtained by summing the absolute values of the negative votes), the weighted voting predictor predicts the new case X as sensitive and vice versa.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molonc.2009.03.004.

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