## The High Frequency Aberrantly methylated Targets in Pancreatic Adenocarcinoma Revealed by A Global DNA Methylation Analysis Using MethylCap-seq

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### Abbreviation:

PC: Pancreatic cancer

MethylCap-seq

MSP: Methylation-Specific PCR

BSP: Bisulfite Sequencing PCR

MSRE-qPCR

DMRs: Differentially methylated regions

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### Abstract:

**Purpose:** Extensive reprogramming and dysregulation of DNA methylation is an important feature of pancreatic cancer (PC). Our present study aimed to characterize the genome methylation patterns in various genomic contexts in PC. **Methods:** MethylCap-seq was used to map the differentially methylated regions (DMRs) in pooled samples from 10 PC tissues and from 10 adjacent non-tumor tissues (PN). A selection of DMRs were validated in an independent set of PC and PN samples using MSP, BSP, and MSRE-qPCR. The mRNA and EST expression of the related genes was investigated by RT-QPCR. **Results:** A total of 1,132 hypermethylated DMRs and 627 hypomethylated DMRs were identified in association with CpG islands (CGIs), including regular CGIs and orphan CGIs; 2,955 hypermethylated DMRs and 2,386 hypomethylated DMRs were associated with gene promoters, including promoters with or without CGIs. Moreover, 1,852 hypermethylated DMRs and 1,545 hypomethylated DMRs were found to be associated with CGIs as well as CGI shores. These results suggested that aberrant hypermethylation in PC typically occurs in regions around the transcription start site (TSS). The BSP, MSP, MSRE-qPCR, and RT-qPCR data indicated that the aberrant DNA methylation in PC tissue and in PC cell lines was associated with gene (or related EST) expression. **Conclusions:** Our study characterized the genome-wide DNA methylation patterns in PC and identified DMRs that were distributed among various genomic contexts that might influence the expression of related genes or transcripts to promote PC. These DMRs might be potential diagnostic biomarkers and/or therapeutic targets in PC.

**Keywords:** Pancreatic adenocarcinoma; DNA methylation; MethylCap-seq; Methylome; Orphan CpG Island; CpG island shore;

### Introduction

Pancreatic cancer (PC), a highly malignant tumor of the digestive system, is currently one of the solid tumors with the worst prognosis, with a postoperative 5-year survival rate of less than 25%. Nearly 100,000 people die from PC every year in American and Europe, where PC ranks fourth and fifth in cancer incidence and mortality, respectively [1]. The incidence of PC in China is also demonstrating an increasing trend [2].

From the biological point of view, genetics is predominantly responsible for the stable transfer of hereditary information between generations, whereas the different somatic phenotypes in different tissues and cells are regulated by epigenetics. Because most tumors develop with particular acquired biological phenotypes, epigenetic changes surely play important roles [3]. DNA methylation, a well-studied epigenetic phenomenon, has already been extensively studied in PC. At the gene level, genes such as p14ARF, and p16INK4a [4, 5] were found to have aberrant promoter methylation, leading to abnormalities in gene transcription. At the genomic level, by combining the techniques of methylated CpG island amplification with Agilent 244K Human Promoter ChIP-on-chip microarrays, the genome-wide methylation abnormalities in PC have been dissected [6-7].

The microarray technology platform based DNA methylation studies are usually focused on CpG islands (CGIs), presumably with lower coverage of the whole genome than next-generation sequencing (NGS) technology [8]. Therefore, genome wide methylation discovery of PC by the NGS platform could provide comprehensive description of the DNA methylation profile in other regions, such as CGI shores (2 kb regions flanking a CGI), non-CGI promoter regions and orphan CGIs. Methylation changes in these particular regions have already been suggested to be associated with certain tumor phenotypes [9, 10].

In present study, Ten PC tissue and corresponding adjacent tissue samples were pooled separately followed by MethylCap-seq based DNA methylation profile identification process. The methylation profiles of various genomic regions and elements were characterized, including gene-associated CGIs, non-gene-associated CGIs (orphan CGIs), CpG shores and gene promoters without CGIs. Differential methylation status between pancreatic cancers and normal tissues were tested in terms of above genomic regions, therefore, large number of pancreatic cancer specific hyper and hypo-methylated regions were identified. Gene Ontology and KEGG pathway analysis to these aberrantly methylated were conducted to discover aberrant functional modules in the development of pancreatic cancer. The methylation profile constructed by MethylCap-seq was validated by comprehensive procedures based on different techniques and in different samples. Additionally, the regulation role of aberrant methylation of orphan CGIs on the RNA expression was validated by MSRE-qPCR and RT-qPCR in pancreatic cancer cell lines before and after the treatment of 5-Aza-2’-deoxycytidine.

### Materials and Methods

#### Clinical samples

PC tissue samples were collected from 18 patients who had undergone surgical treatment without receiving preoperative chemotherapy or radiotherapy from May 2009 to March 2011 in Renji Hospital, School of Medicine, Shanghai Jiao Tong University. The diagnosis of PC was confirmed by histological examination. Resected tumor tissues and matched normal tissues at least 2 cm away from the tumor tissues were collected during the operation, labeled and stored at -80°C for the future usage. The sixth edition of Tumor, node and metastases (TNM) staging system proposed by the International Union Against Cancer (UICC) was utilized to stage the tumor tissue samples. The study was approved by the medical ethics committee at Renji Hospital, School of Medicine, Shanghai Jiao Tong University. All the patients signed the informed consent form. DNA was isolated from frozen tissues or cell lines using the conventional proteinase K/organic extraction method as previously described [11]. Characteristics of samples were listed in the **Table I**.

#### Genome-wide methylation profiling by MethylCap-seq

Genomic DNA was extracted from 10 PC tissues and 10 matched normal tissues. Equal amounts of DNA from each sample were mixed to form the PC and PN groups. Pooled DNA (1.2 µg) from each group was used to generate the library for MethylCap-seq as previously described [11].

#### Mapping the Sequence Reads and DMR Identification and Annotation

We used BWA alignment tools [12] with the default settings to map the 36 bp single end reads to the hg19 human genome reference assembly [13]. After removing PCR duplicates using Picard, the aligned data were converted, sorted, and indexed using Samtools [14] and Picard (http://picard.sourceforge.net).

Methylation peaks (hypermethylated regions) were identified using MACS in the PC and PN samples as previously described [11].The differentially methylated regions (DMRs) between PC and PN were identified using two methods, MACS [15] and BALM [16], to increase the detective power of MethylCap-seq. To decrease the false positive detection of DMR using BALM, the dual-threshold strategy was applied. A high-confidence threshold (0.975) was utilized in the PC hypermethylated region screening, and a low-confidence threshold (0.950) was utilized in the PN hypermethylated region screening. Then, cancer-specific methylation peaks were defined as hypermethylated regions. Similarly, normal tissue-specific methylation peaks were defined as hypomethylated regions with the reverse settings. Whole genome methylation (methylation of each CpG) was inferred using BALM, which was processed to create a Pearson correlation analysis among all the samples in the R environment. The refSeq genes (UCSC genes) and corresponding CpG islands (CGIs) were downloaded from the Table Browser of the UCSC database [11]. The BED file operations were performed using bed tools [17] and other Perl scripts. All the scripts are available upon email request. The generated genomic methylation profile was uploaded to a public database (Gene Expression Omnibus: GSE54854). Gene ontology analysis was performed using DAVID Bioinformatics Resources 6.7.

#### Methylation analysis

In the present study, BSP was utilized to determine the methylation status at single CpG resolution of DMRs identified by genomic methylation profiling. MSP was performed for qualitative methylation screening in a small set of PC samples. Using MSRE-qPCR, the DNA methylation status in orphan CGIs was quantitatively analyzed in PC cell lines before and after 5-Aza-2’-deoxycytidine treatment and in small samples of clinical PC tissues, as described previously [18]. Approximately 1.0 µg of genomic DNA extracted from PC or PN samples or PC cell lines was bisulfate-treated using EpiTect Kit (Qiagen, Hilden, Germany). Primers for MSP (Methylation-Specific PCR) and BSP(Bisulfite Sequencing PCR) were designed using MethylPrimer, an online primer design tool (http://www.Urogene.org/cgi-bin/methprimer/ methprimer.cg). Methylation-sensitive restriction enzyme-based quantitative PCR (MSRE-qPCR) primers were designed using primer 3 (<http://www.embnet.sk/cgi-bin/primer3_www.cgi>). The sequences of the primers utilized in this study are listed in Supplementary Table S1, S2. Jumpstart Taq (Sigma-Aldrich, St. Louis, MO, USA) was used in BSP and MSP with a 20 µl reaction volume per tube. The BSP and MSP reaction conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 20 s, annealing for 20 s, and 72°C for 20 s; and 72°C for 5 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gels. The PCR products were TA cloned and verified by sequencing. At least 5 clones were sequenced for each BSP reaction.

#### Cell culture and 5-Aza-2-deoxycytidine treatment

Three pancreatic adenocarcinoma cell lines were used: BxPC-3 ATCC, CRL-1687), PANC-1 (ATCC, CRL-1469),and CFPAC-1 (ATCC,CRL-1918). All the cell lines were cultured in RPMI1640supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. All the cell lines were maintained at 37°C in a humidified atmosphere with 5% CO2.

The restoration of gene expression by demethylation was evaluatedin the BxPC-3, CFPAC-1 and CFPAC-1 cell lines. For the CpG demethylation analysis, exponentially growing cells were seeded at a density of 1.5x106 cells/100 mm dish and allowed to attach overnight. The cells were then treated with freshly prepared 5-aza-dC (5.0 µM; Sigma-Aldrich, St. Louis, MO, USA) for 3 days.

#### RNA isolation and Real-time PCR

Total RNA was prepared from cultured cells using Trizol reagent according to the manufacturer’s instructions (Invitrogen,USA) and then reverse transcribed using an oligo(dT) primer and SuperscriptII RNase H-Reverse Transcriptase (Invitrogen, USA). Real-time PCR was performed with primer pairs for the EST expression assay, and GAPDH was used as the internal control.Real-time PCR was performed as follows: 94°C for 3 min followed by 40cycles of 94°C for 10 sec, 62°Cfor 10 sec and 72°C for 15 sec. Real-time qPCR was performed to detect GAPDH expression with a SYBR Green PCR Kit (Applied Biosystems, Foster city, CA, USA) on a ROTOR-GENE 6000 Real-Time PCR System (ROTOR-GENE).

#### Statistical analysis

Statistical calculations were performed using the SPSS statistical software package (Version 13.0; SPSS, Inc. Chicago, IL). The measurement data were analyzed using one-way ANOVA. P<0.05 was considered statistically significant.

## Results

#### Wide-spread aberrant hyper-and hypo-methylation in PC revealed by genomic methylation profiling

The whole genome methylation profiles of the PC and PN samples were successfully established using the MethylCap-seq method. We acquired 33,784,358 raw reads in the PC group and 30,868,151 raw reads in the PN group. By alignment with the human genome (hg19) sequence, 16,267,025 (48.15%) raw reads in the PC group and 15,033,135(48.70%) raw reads in the PN group were uniquely positioned. The reads mapping to 28,691 CGIs defined by UCSC were investigated; 3.57% of the reads in the PC group and 4.25% of the reads in the PN group were positioned at CGIs, with a CGI cover rate of 64.31% in the PC group and 64.36% in the PN group. These data indicated that our experiment provided considerable information regarding genomic CGIs.

In total, 276,442 and 255,743 peaks were found in the PC and PN groups, respectively, presenting an alternate distribution of hypermethylation and hypomethylation of PC-specific features across the chromosomes (**Figure 1A**). An analysis of the hypermethylation peaks approximately 5 kb from the TSS revealed that methylation peaks accumulated near TSS, and more of these peaks were present in the PC group than in the PN group (**Figure 1B**). After deleting the peaks common to both the PC and PN groups (approximately 209,000), 66,807 hypermethylated and 46,815 hypomethylated PC-specific DMRs were identified (**Figure 1C**). Of these PC-specific DMRs, 36,959 hypermethylated DMRs and 25,605 hypomethylated DMRs were located within genes (**Figure 1C**), and 1,131 hypermethylated DMRs and 727 hypomethylated DMRs were associated with CGIs.The hypermethylated and hypomethylated DMRs were divided into 3 categories: TSS, intragenic or intergenic (**Figure 1D**). Subsequently, the location of TSS DMRs and intragenic DMRs were further defined using gene structural annotations of the human genome, such as downstream, enhancer, exon, intron, miRNA, promoter, or 5’ UTR (**Figure 1E**).

It is generally accepted that the methylation of CGIs within a promoter is responsible for gene silencing. However, recent studies have discovered certain types of tumor- and tissue-specific methylation in CGI shores (the 2 kb regions flanking a CGI). In this study, we investigated the methylation status of both CGIs and CGI shores in particular related to gene promoters. This research yielded 1,859 DMRs related to CGIs in the PC group, and these DMRs represented the vast majority of the hypermethylated CGIs in refGene (88%) and of the hypomethylated CGIs in refGene (87%) while orphan CGIs only accounted for 12% (133/1132) and 13% (96/727) of the CGI-related DMRs (**Figure 1F**, Supplementary Table S3, S4). 5,341 DMRs were identified when promoter-related DMRs were considered. We found that the proportion of promoters with CGIs to promoters without CGIs was higher in the hyper-DMR group (21%, 609/2955, Supplementary Table S5) than in the hypo-DMR group (13%, 312/2386, Supplementary Table S6, **Figure 1F**).

Regarding the genes and the manner in which they were disturbed by hyper-DMRs, 527 genes (Supplementary Table S7) were affected by a CGI (483 DMRs) and a CGI shore (502 DMRs), 111 genes (Supplementary Table S8) were affected by a CGI only (108 DMRs), and 1,278(Supplementary Table S9) genes were affected by a CGI shore only (1242 DMRs). In contrast, in analyzing the genes affected by hypo-DMRs, 333 genes (Supplementary Table S10) were affected by both a CGI (305 DMRs) and a CGI shore (315 DMRs), 51 genes (Supplementary Table S11) were affected by a CGI only (47 DMRs), and 1,298 genes (Supplementary Table S12) were affected by CGI shore only (1183 DMRs)( Figure 1G).

#### Gene Ontology and KEGG pathway analysis of the aberrantly methylated genes in PC

It is well accepted that methylation abnormalities within promoters can influence the expression of the corresponding genes. Therefore, we conducted a GO analysis of the genes that showed promoter hyper-or hypo-methylation in the PC group. Statistical significance of p<0.05 indicated gene enrichment in various GO categories (Table 2). We determined that 668 hypermethylated genes (Supplementary Table S13) were enriched in sequence-specific DNA binding (GO:0043565), neuron differentiation (GO:0030182), regulation of transcription and DNA-dependent (GO:0006355), or cell morphogenesis involved in differentiation (GO:0000904); 383 hypomethylated genes (Supplementary Table S14) were enriched in plasma membrane part (GO:0044459), channel regulator activity (GO:0016247), positive regulation of BMP signaling pathway (GO:0030513~), protein homo oligomerization (GO:0051260), or neuron differentiation (GO:0030182). We also identified 111 genes with hypermethylated promoters that were enriched in regulation of transcription term. Among these genes, the methylation status of DLX4, ELAVL2, IRX1, PITX2, SIM2, TBX5, and TFAP2C were subsequently validated in tissue samples.

KEGG pathway analysis of the above mentioned genes revealed that both hyper- and hypo-methylated genes were enriched in neuroactive ligand receptor interaction (hsa04080) as showed in Table 2. Furthermore, several miRNAs were aberrantly methylated in PC. The hypermethylated miRNAs included mir-9-3, mir-9-1, mir-124-3, mir-10b, mir-124-2, mir-718, and mir-203. The hypomethylated miRNAs included mir-210, mir-1469, mir-130b, mir-149, mir-1224, and mir-564 (Table 3).

#### Verification of PC-specific DMRs identified in MethylCap-seq

The accuracy and precision of the profile were validation in two sample sets which included the samples that were pooled in the Methycap-seq library construction and another independent sample set. In one validation vignette, the DNA methylation status of the 10 most significant DMRs which located in the promoter region (P-values<10-15) were evaluated in the same set of PC and PN samples used to create the MethylCap-seq libraries with BSP and MSP techniques, respectively. Four candidate DMR genes which included C5orf38, EMX1, NPR3, VSTM2B, ELAVL2 and TFAP2C were validated to be significant hyper-methylated in PC than that in PN samples with BSP or MSP technique. Another two genes which included, AGAP2 and TRADD were found significantly hypo-methylated in PC groups with MSP technique (representative results are shown in Figure 2A and 2B).

#### Preliminary detection of the DMRs identified by PC genome methylation profiling in limited clinical samples

In another validation vignette, the methylation status of 20 gene-associated DMRs scattered in various genetic elements, such as promoters, miRNAs, introns, exons or CGI shores were analyzed by MSP in the PC and PN testing group. The results revealed that 8 gene-associated DMRs exhibited hypermethylation in at least one of the 8 PC samples but in none of the 5 PN samples. An additional 9 gene-associated DMRs exhibited hypermethylation in at least 2 of the 8 PC samples but in only 1 of the 5 PN samples. DMRs in the promoter regions of TRADD, AGAP2, and FAM115A exhibited a loss of methylation in PC (select results are presented in Figure 2B). The MSP results are presented in Supplementary Table S15.

#### MSRE-qPCR and RT-qPCR validation of the methylation of orphan CGIs and related EST expression in PC cell lines treated with 5-Aza-2’-deoxycytidine

Three PC cell lines were treated with 5-aza-dc. The methylation status of 10 hypermethylated DMRs for partial results, see Figure 3A) in promoter CGIs and orphan CGIs was quantitatively analyzed by MSRE-qPCR (Figure 3B) in the 3 PC cell lines before and after 5-aza-dc treatment. The expression levels of promoter CGI-related genes and orphan CGI-related ESTs were analyzed by RT-qPCR to ascertain the correlation between aberrant DMRs and the corresponding mRNA expression (Figure 3C). The results indicated that the methylation levels of 4 orphan CGIs and 1 promoter CGI were decreased and that the mRNA expression of the related genes or ESTs increased upon 5-Aza-dc treatment, suggesting that the expression of these genes or ESTs might be regulated by DNA methylation. A quantitative analysis of the methylation status of these particular DMRs in an independent set of samples (testing group, 8 PC samples, 5 PN samples and 3 PC cell lines) confirmed the differences in methylation at those 4 DMRs in the clinical samples(Figure 3D).

**Discussion**

Pooling strategies have been utilized innumerous previous genomic studies to investigate the phenotypic similarities in specific models, with the advantage of conserving samples [19, 20]. In the present study, the genome-wide methylation profiles of PC vs.PN tissues were established using two pooled samples.

In the present study, we identified 5,280 hyper-DMRs and 3,488 hypo-DMRs that were closely associated with genes and CGIs in PC compared with PN. The GO analysis of the genes associated with these DMRs revealed that the aberrantly hypermethylated genes primarily belonged to categories related to nucleic acid binding, DNA binding and activation of transcription factors, suggesting that the methylation of the affected genes coupled with the downregulation of RNA expression resulted in the decreased expression of other genes. The research by Helman Eon NSCLC and that by Zhao M demonstrated that methylation-enriched genes exhibited aberrant methylation and RNA expression in multiple tumor types, and these genes were referred to as tumor suppressor genes [21]. The methylation-enriched genes associated with “cell morphogenesis involved in differentiation” (GO: 0000904) may participate in the morphological changes and late-stage differentiation of PC. In contrast, the hypomethylated genes were mostly enriched in “plasma membrane part” (GO:0044459) and “channel regulator activity” (GO:0016247), and these genes have been confirmed to be enriched in other tumor types in genomic methylation studies, suggesting that the abnormal formation of the plasma membrane may be a common feature in tumor differentiation and maintenance[22,23].

We also conducted a KEGG pathway analysis of the genes with DMRs in their promoter; surprisingly, both hyper-and hypo-methylated genes were enriched in the same pathway, “neuroactive ligand-receptor interaction”, which mainly participates in the endocrine and exocrine functions of cells. Functional abnormalities in these genes have been demonstrated in studies on meningioma and PC [24,25]. Our present study revealedbi-directional methylation changes in this pathway in PC, but the detailed mechanism of action of this pathwayin PC development requires additional research (Table 2).

As an epigenetic factor, miRNAs play an important part in the regulation of cellular biophysical functions, and miRNAs have been shown to be correlated with the apoptosis, invasion, metastasis, recurrence and drug resistance of tumor cells. The regulation of miRNAs by DNA methylation has been extensively studied. In this study, we identified particular miRNAs that were aberrantly methylated in PC. Among these miRNAs, hsa-mir-124-3 has been shown to be hypermethylated in PC and is involved in the progression, metastasis and recurrence of PC [26,27]. mir-130b and mir-210 are two hypomethylated miRNAs that were discovered in this study, and bothof these miRNAs were found to be over expressed in PC and have been correlated with the proliferation and invasion of PC cells[28,29]. Certain miRNAs that we identified as aberrantly methylated in PC have already been studied in other tumor types and are regulated by DNA methylation. For example, hypermethylated miRNAs in PC, such as miR-9-3,mir-9-1,miR-124 and miR-203,are hypermethylated in NSCLC, breast cancer,cervical cancer, and hematological cancer, respectively; hypermethylation decreases miRNA expression, which promotes tumor development and tumor cell proliferation [30,31,32,33].mir-10b, which was found to be hypermethylated in PC in this study, was thought to be an inhibitor of tumor metastasis in animal models

[34] and was found to be over-expressed in PC[35]. Therefore, further studies of the remaining miRNAs that were aberrantly methylated in PC will shed light on the mechanisms of pancreatic carcinogenesis, although the related research is currently very limited.

The top 40 genes,based on p-value, that exhibited methylation changes in the promoter region were selected and examined by MSP in the testing group samples.Of these genes, 18 showed significant differences in methylation between the PC and PN tissues (and/or PC cell lines). Interestingly, among these genes, 7 (DLX4, ELAVL2, IRX1, PITX2, SIM2, TBX5, and TFAP2C) were enriched for the annotation of regulation of transcription(GO:0006355), which corresponds with the results obtained in our previous GO analysis. All of the genes discussed above have been investigated in PC and other tumor types, and their involvement in carcinogenesis has been confirmed.

On both the gene and genome levels, many hypermethylated genes previously reported in PC studies were identified as hyper-DMRs in the present study, including LHX1, FOXE1, PAX6, BNIP3[36], ALPP, CEBPA[37], CACNA1G[38], CCND2[39], BAI1,NRN1, PENK, FAM84A,and ZNF415 [40]. In addition, our study also identified certain other genes that are frequently hypermethylated in different types of cancer, such as RASSF1a, CDKN2A, hHML1, and CDH1[41,42]. Hence, we have established a relatively intact database of abnormal methylation in PC. We also compared our data with those by Goggins[6], who analyzed 9 pairs of PC vs. PN samples using Human CpG Island Microarray 244k chips and obtained (after data filtering with proper thresholds) 1658 differentially methylated known loci. The comparison revealed the following: 1) Regarding the ability to capture aberrantly methylated gene targets, MethylCap-seq identified more targets than the array method in terms of both hypermethylated genes (1983 vs. 1206) and hypomethylated genes (1692 vs. 379) , indicating that “methylated DNA fragment enrichment plus deep sequencing” can uncover more aberrant gene loci, even though it is more labor-intensive and time-consuming. 2) Regarding the genes revealed by both MethylCap-seq and array, there were 737 total genes (Supplementary Table S16) discovered by both methods, which accounts for 46.7% of the total genes recovered. This high recovery rate when comparing the two methods reflects the reliability of the methods for this purpose. However, the unique genes that were identified suggest that these two methods each have their own particular advantages. 3) The high percentage of commonality among the hyper-DMR genes (46.2%) and low percentage of commonality among the hypo-DMR genes (30 genes, 2.9% of all the hypo-DMR genes)(Supplementary Table S17) between the studies by Goggins’ and us might indicate that during PC development, hypermethylation is a more definite and destined process, whereas hypomethylation is somewhat more random or perhaps stochastic. We also compared our current aberrent methylated gene target with that of previous studies. Among all the 3911 DMGs revealed in present study, 728 DMGs were reported by Grimmond（2014）[43]，339 DMGs by Vincent et al(2011)[7] and 55 by Tan et al (2009) [44](Supplementary Table S18). This discrepancy in DMGs number obtained among all the four groups might resulted from different technological platform adapted in each study group，as well as the different ethnic background of patient recruitment. And it also suggests the necessity that the array-based and sequencing-based DNA methylation assay methods should be applied alternatively to complementary each other in genomic level DNA methylation dissection.

Adrian Bird divided CGIs into three categories, TSS, intragenic and intergenic, and the latter two categories were defined as orphan CGIs [45]. Despite little understanding of the functions of orphan CGIs until recently, studies have shown that orphan CGIs are involved in the regulation of gene transcription, gene printing and non-coding RNA transcription and that they might display tissue-specific methylation profiles [46]. In this study, we investigated the methylation status of particular orphan CGIs in PC. Hypermethylated orphan CGIs have been found in PC. The methylation status of orphan CGIs was closely associated with the transcription levels of nearby un-annotated ESTs. Further studies should be conducted to clarify whether the methylation-regulated ESTs containing orphan CGIs are potential genes or gene elements. It is well known that merely 6.8% of CpGs are located in CGIs. The methylation status and biological functions of the other 93.2% of CpGs have not been adequately studied.The study by Yu indicated that the methylation of CpGs in CGI shores was involved in regulating gene transcription or establishing tissue-specific methylation patterns, and changes in the methylation status of CpGs at these sites might take place at an earlier stage in carcinogenesis than the changesthat occur in promoter CGIs [47]. The methylation changes in all the above regions in PC are indispensable parts of the PC genomic methylation profile and may influence the transcription of related genes and non-coding RNAs and affect tissue-specific cell differentiation, ultimately leading to carcinogenesis.

In summary, the genome-wide methylation profiles of PC vs. PN tissues were established using MethylCap-seq in the present study, revealing globally reprogrammed and deregulated DNA methylation in PC. Compared with PN tissues, there were massive PC-specific changes in terms of aberrant hypermethylation or hypomethylation of CpGs in TSS CGIs, orphan CGIs, CGI shores and promoter regions without CGIs. The above findings will be helpful for elucidating the mechanisms of pancreatic carcinogenesis related tothe DNA methylation-regulated expression of genes and non-coding RNAs. Furthermore, the aberrantly methylated genes in PC identified in this study may be potential biomarker resources for the early diagnosis and treatment of this deadly disease.

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## Figure legends

**Figure 1. Data mining of MethylCap-seq libraries.**A) Chromosomal view of genome-wide hyper-and hypo-methylated DNA in PC compared with PN. Red bar: hypermethylation; Green bar: hypomethylation. B) Hypermethylated peaks around the TSS site in PC compared with PN. Peaks were surveyed in a broad region (from 5 kb downstream to 5 kb upstream of the TSS). C)Differentially methylated regions (DMRs) that were specific for PC. The DMRs are shown according to their inclusion in refGene or CGI definitions. D)The genomic distribution of hyper-DMRs and hypo-DMRs in TSSs, intragenic regions, and intergenic regions. The total number of DMRs is presented at the top of each graph. E) Pie chart of the DMR distribution over the various gene structures based on sole refGene involvement versus both CGI and refGene involvement in PC-specific hyper-and hypo-methylated regions. The genomic context is defined as those found in the UCSC database. F) Pie chart of the PC-selective hyper-and hypo-DMR distribution over orphan CGIs or refGene-related CGIs and over CGI-containing promoters vs. no-CGI promoters. G) Hyper- and hypo-DMRs in PC and their related genes, considering the involvement of various CGI features (CGI, CGI shore, or both CGI and CGI shore).

**Figure 2. Representative results of the BSP and MSP validation of the MethylCap-seq data.** For each gene, the UCSC scheme of the gene locus and the examined promoter regions are shown. A) BSP results. PC, pancreatic cancer; PN, adjacent non-tumor tissue. B) MSP results. 3 PC cell lines (BxPc-3, CFPAC-1, and PANC-1), 8 PC samples (1C, 2C, 5C, 6C, 7C, 8C, 10C, and 311C) and 5 PN samples (1N, 2N, 7N, 10N, and 311N) were evaluated. C: GAPDH-BSP quality and quantity control for the confirmation of bisulfite-converted DNA templates. P: positive control; N:negative control.

**Figure 3.Methylation of CGIs (orphan CGIs or regular CGIs) might influence the expression of putative ESTs or mRNAs.** A) The UCSC scheme of CGIs and the nearby putative ESTs or mRNAs analyzed in this study. B) DNA methylation changes in PC cell lines after treatment with 5-Aza. The GAPDH-BSP product serves as a quality and quantity control for the bisulfite-converted DNA templates.C) EST expression after 5-Aza treatment determined by RT-qPCR. GAPDH mRNA expression was the loading control. D) Box-plot of the quantitative analysis of DNA methylation by MSRE-qPCR in 8 PC and 5 PN samples. P, positive control; N, negative control. The box is defined by 25%/75% quantiles. The methylation levels in the PC and PN samples were compared by one-way ANOVA, and the p-values are indicated.

**Tables: Table 1.Clinical profile of the PC patients recruited in the present study**

PC: pancreatic carcinoma. PN: PC adjacent non-tumor tissue.

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**Table 2. Gene ontology enrichment analysis of aberrant methylation in gene promoters in PC**

****\* logarithmic transformation to the P-value to show the significant level of the differential methylation region (In DMR estimation) or methylation blocks (In MACS). the more of this value, the higher probability was inferred for DMR  or methylation blocks.

**Table 3. Aberrantly methylated miRNAs in PC**

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\* logarithmic transformation to the P-value to show the significant level of the differential methylation region (In DMR estimation) or methylation blocks (In MACS). the more of this value, the higher probability was inferred for DMR  or methylation blocks.

**Supplementary Tables**

**Table title**

**Supplementary Table S1. Analysis of aberrant methylation in pancreatic cancer by BSP and MSRE-qPCR**

**Supplementary Table S2. BSP ,MSRE-qPCR and MSP primers used in this study**

**Supplementary Table S3. PC related hypermethtylated DMR in orphan CGI**

**Supplementary Table S4. PC related hypomethtylated DMR in orphan CGI**

**Supplementary Table S5. PC-related hypermethylated genes that lacks CGI in their promoters**

**Supplementary Table S6. PC-related hypomethylated genes that lacks CGI in their promoters**

**Supplementary Table S7. PC related hyper-DMRs and the involved genes that were affected via both CGIs and CGI shore**

**Supplementary Table S8. PC related hyper-DMRs and the involved genes that were affected via CGIs only**

**Supplementary Table S9. PC related hyper-DMRs and the involved genes that were affected via CGI shores only**

**Supplementary Table S10. PC related hypo-DMRs and the involved genes that were affected via both CGIs and CGI shores**

**SupplementaryTable S11. PC related hypo-DMRs and the involved genes that were affected via CGIs only**

**Supplementary Table S12. PC related hypo-DMRs and the involved genes that were affected via CGI shores only**

**Supplementary Table S13. PC-related hypermethylated CGI in gene promoters**

**Supplementary Table S14. PC-related hypomethylated CGI in gene promoters**

**Supplementary Table S15. MSP validation of 40 targets in clinical samples and cell lines**

**Supplementary Table S16. The hypermethylated genes (loci) recovered by both MethylCap-seq and Microarray 244k chip**

**Supplementary Table S17. The hypomethylated genes (loci) recovered by both MethylCap-seq and Microarray 244k chip**

**Supplementary Table S18. Gene symbol of genes aberrantly methylated in our study and previous studies**