Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

Research Article
See perspective p. 1053

Cancer Prevention Research

Global Reactivation of Epigenetically Silenced Genes in Prostate Cancer

Ilsiya Ibragimova[1], Inmaculada Ibáñez de Cáceres[1], Amanda M. Hoffman[1], Anna Potapova[1], Essel Dulaimi[1], Tahseen Al-Saleem[2], Gary R. Hudes[3], Michael F. Ochs[4], and Paul Cairns[1,2]

Abstract

Transcriptional silencing associated with aberrant promoter hypermethylation is a common mechanism of inactivation of tumor suppressor genes in cancer cells. To globally profile the genes silenced by hypermethylation in prostate cancer, we screened a whole genome expression microarray for genes reactivated in the LNCaP, DU-145, PC-3, and MDA2b prostate tumor cell lines after treatment with the demethylating drug 5-aza-2-deoxycytidine and the histone deacetylation–inhibiting drug trichostatin A. A total of 2,997 genes showed at least 2-fold upregulation of expression after drug treatment in at least one prostate tumor cell line. For validation, we examined the first 45 genes, ranked by upregulation of expression, which had a typical CpG island and were known to be expressed in the normal cell counterpart. Two important findings were, first, that several genes known to be frequently hypermethylated in prostate cancer were apparent, and, second, that validation studies revealed eight novel genes hypermethylated in the prostate tumor cell lines, four of which were unmethylated in normal prostate cells and hypermethylated in primary prostate tumors (*SLC15A3*, 66%; *KRT7*, 54%; *TACSTD2*, 17%; *GADD45b*, 3%). Thus, we established the utility of our screen for genes hypermethylated in prostate cancer cells. One of the novel genes was *TACSTD2/TROP2*, a marker of human prostate basal cells with stem cell characteristics. *TACSTD2* was unmethylated in prostatic intraepithelial neoplasia and may have utility in emerging methylation-based prostate cancer tests. Further study of the hypermethylome will provide insight into the biology of the disease and facilitate translational studies in prostate cancer. *Cancer Prev Res; 3(9); 1084–92. ©2010 AACR.*

Introduction

Aberrant DNA hypermethylation of CpG islands in the promoter region of genes is well established as a common mechanism for the transcriptional silencing of tumor suppressor genes in cancer cells and, thus, as an alternative mechanism of functional inactivation (1). The *GSTP1*, *p16^{INK4a}*, *CDH1*, *APC*, and *RASSF1A* tumor suppressor genes as well as a number of other cancer genes have been identified as hypermethylated with associated loss of expression in prostate cancer (2). By definition, a candidate gene approach has resulted in the examination of only a limited number of genes for epigenetic alteration. Many other tumor suppressor and cancer genes important in prostate tumorigenesis likely remain to be identified. A global approach to the identification of epigenetically silenced genes in prostate tumor cells could provide methylation signatures for early detection and for predictive classification studies, identify novel targets for therapy, and lead to further elucidation of the biology of this disease.

One global approach to the identification of epigenetically silenced genes in tumor cells is based on the reversal of epigenetic silencing by drugs such as 5-aza-2-deoxycytidine (5Aza-dC) resulting in reexpression analyzed by well-annotated gene expression arrays. This approach can preferentially identify reexpression of epigenetically silenced genes over methylated CpG islands that do not affect transcription. A proportion of the reexpressed genes will have been silenced by promoter hypermethylation in the untreated tumor cell lines (3–6).

In the present study, we examined the global reactivation of epigenetically silenced genes in prostate cancer by analysis of a gene expression microarray with RNA isolated from four prostate tumor cell lines after treatment with 5Aza-dC and trichostatin A (TSA). Through intuitive selection of upregulated genes followed by validation, we have evidence that at least 20 of 45 genes examined are hypermethylated in prostate cancer, and thus, our screen preferentially selected for epigenetically silenced genes. We report here four genes newly identified

Authors' Affiliations: Departments of [1]Surgical Oncology, [2]Pathology, and [3]Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania and [4]Oncology Biostatistics, Johns Hopkins University, Baltimore, Maryland

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

Corresponding Author: Paul Cairns, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. Phone: 215-7285635; Fax: 215-7282487; E-mail: Paul.Cairns@fccc.edu.

doi: 10.1158/1940-6207.CAPR-10-0039

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

as hypermethylated in primary prostate tumor specimens. Informed analysis of function combined with a pathway and network database analysis supports the relevance of these genes to prostate cancer.

Materials and Methods

Cell lines and drug treatment

Four prostate cancer cell lines, LNCaP, DU-145, PC-3, and MDA2b, were obtained directly from the American Type Culture Collection and were cultured according to the company's recommendations. The four prostate cancer cell lines were treated with 5Aza-dC (Sigma) and TSA (Wako) in a combined treatment. 5Aza-dC was dissolved in PBS as a 5 mmol/L stock solution and stored in aliquots at −80°C. TSA was dissolved in absolute ethanol as a 330 μmol/L stock solution and stored at −20°C. Cells were exposed to 5Aza-dC to a final concentration of 5 μmol/L at 0, 24, and 72 hours over two cell divisions by counting the cells, and then treated with TSA to a final concentration of 500 nmol/L during the 24 hours before RNA extraction. Mock (untreated) cells were cultured with an equivalent volume of PBS alone and, for the 24 hours before RNA extraction, with an equivalent volume of ethanol.

Oligonucleotide array hybridization

Total RNA used for microarray analysis was isolated from treated and mock-cultured cells using TRIzol reagent (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen), combined with DNase treatment. RNA quality was confirmed by the ratio of 28S and 18S rRNA after agarose gel electrophoresis. Total RNA was reverse transcribed using oligo$(dT)_{24}$ primer and Superscript II reverse transcriptase (Invitrogen) for 1.5 hours at 42°C. cDNA was labeled with Cy3 or Cy5 (Amersham Biosciences) and then hybridized according to the manufacturer's instructions for 18 hours at 42°C on slides with oligonucleotides from 14,802 genes, which were processed and spotted in the DNA Microarray Facility at Fox Chase Cancer Center from 15k human oligonucleotide microarray (MWG-Biotech, Inc.). The Gene list, Gene ID, and Template files for human 15k oligo A can be viewed at http://www.ocimumbio.com/web/arrays/download.asp.

Each cell line was hybridized twice, with a dye-flip replicate using reversed labeling. The hybridized slides were scanned using a GMS 428 scanner (Affymetrix) to generate high-resolution images for both Cy3 and Cy5 channels. Image analysis was done using the ImaGene software (BioDiscovery, Inc.). The spots were identified using an optimized segmentation algorithm. Spots of poor quality, as well as spots with signal levels indistinguishable from the background, were flagged as bad spots. The image data were extracted and analyzed using the Functional Genomics Data Pipeline (7).

Data analysis

Normalization and background correction were done using LOWESS with a width of 0.7 and local background correction. The PC-3 flip array data were removed as quality control measures and its MA plot suggested a poor-quality array. Our goal was to identify genes that changed expression due to methylation in the maximum number of cell lines, as this provided the greatest probability of *in vivo* methylation. We therefore categorized the genes based on their fold change, determining a cutoff of 2-fold (i.e., ratio = 2, log-ratio = 1) between treated and untreated cell lines using the mean value of the dye-flips. We did not correct for disparate values between the dye-flip readings, as we took one large value as indicative of potential methylation in that cell line, and our goal was to count the number of cell lines with such potential methylation. We ranked the genes based on the number of cell lines with potential methylation. We noted several well-known methylation targets, which we took as partial validation of our approach, and pursued the novel genes further.

Specimen collection and DNA extraction

After Institutional Review Board approval and informed consent, primary prostate tumor tissues were obtained from surgical specimens resected at Fox Chase Cancer Center followed by pathologic review and dissection of tumor cell–enriched areas. In this study, we used histologically normal tissue from a prostate with no evidence of cancer obtained from cystoprostatectomy of age-matched bladder cancer patients. DNA was extracted from fresh-frozen tissues or paraffin blocks using a standard technique of digestion with proteinase K followed by phenol-chloroform extraction (8).

Bisulfite modification

Genomic DNA (1 μg) in a volume of 50 μL was denatured by NaOH (0.2 mol/L) for 10 minutes at 37°C and then modified by hydroquinone and sodium bisulfite treatment at 50°C for 17 hours under a mineral oil layer. Modified DNA was purified using Wizard DNA Clean-Up system (Promega). Modification was completed by NaOH (0.3 mol/L) treatment for 5 minutes at room temperature, followed by glycogen, ammonium acetate, and ethanol precipitation (9). Pellets were resuspended in water and stored at −20°C for immediate use or at −80°C for longer-term storage.

Genomic sequencing

A typical 200- to 400-bp fragment containing the promoter CpG island was PCR amplified with bisulfite-modified prostate cancer cell line DNA, normal prostate tissue DNA, and normal lymphocyte DNA for each gene analyzed. The PCR product was loaded into a 1.5% agarose gel, cut out, and cleaned (Qiagen). We used a single PCR amplification for each of the *KRT7*, *TACSTD2*, *SLC15A3*, *GADD45b*, *IFI30*, *ANXA2*, and *AQP3* genes. The primers used for each gene analyzed are given in Supplementary Table S2.

Quantitative real-time methylation-specific PCR

Primer sequences to methylated DNA sequence were designed together with an internal TaqMan probe labeled

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

Table 1. Upregulated genes identified by statistical analysis and database interrogation

Gene name	Location	Function	Methylation
NM_000228_1—***LAMB3***	1q32.2	Cell communication	(40)
NM_005319_1—H1F2	6p22.2	Nucleosome condensation	Unmethylated
NM_000598_1—***IGFBP3***	7p13	Cell growth and differentiation, apoptosis	(15)
NM_002353_1—**TACSTD2**	1p32.1	Receptor activity	Methylated (LNCaP)
NM_000852_1—***GSTP1***	11q13.2	Transferase activity	(13)
NM_019554_1—***S100A4***	1q21.3	Calcium ion binding	(41)
NM_006332_1—**IFI30**	19p13.11	Immune response	Methylated (LNCaP, PC-3)
NM_004165_1—***RRAD***	16q22.1	Nucleotide binding, GTPase activity	(42)
NM_000574_1—DAF	1q32.2	Complement activation	Unmethylated
XM_027365_1—ARL6IP	16p12.3	Protein transport, cell signaling	Unmethylated
NM_001647_1—***APOD***	3q29	Lipid metabolic process	(31)
NM_014164_1—FXYD5	19q13.12	Cell adhesion	Unmethylated
NM_002306_1—LGALS3	14q22.3	Extracellular matrix organization, biogenesis	(43)
NM_001924_1—*GADD45a*	1p31.2	Apoptosis, DNA repair	(31)
NM_001909_1—CTSD	11p15.5	Proteolysis	Unmethylated
NM_005556_1—**KRT7**	12q13.13	Cytoskeleton organization and biogenesis	Methylated (LNCaP, MDA2b)
NM_003246_1—***THBS1***	15q14	Cell motility and adhesion, inflammation	(44)
AF373867_1—TBX1C	22q11.21	Transcription factor	Unmethylated
NM_004039_1—**ANXA2**	15q22.2	Calcium ion binding	Methylated (LNCaP)
NM_002167_1—ID3	1p36.12	Negative regulation of transcription	(31)
NM_002165_1—ID1	20q11.21	Negative regulation of transcription	Unmethylated
NM_004925_1—**AQP3**	9p13.3	Transport	Methylated (LNCaP)
NM_000610_1—***CD44***	11p13	Cell adhesion	(45)
NM_003687_1—***PDLIM4***	5q23.3	Protein, metal ion binding	(14)
NM_002084—***GPX3***	5q33.1	Glutathione peroxidase activity	(31)
NM_002229_1—JUNB	19p13.13	Transcription factor	(31)
NM_006005_1—WFS1	4p16.1	Putative transmembrane protein	Not done
NM_001953_1—ECGF1	22q13.33	Growth factor	Unmethylated
NM_004417_1—DUSP1	5q35.1	Protein binding	(31, 34)
NM_006111_1—ACAA2	18q21.1	Acetyl-CoA c-acyltransferase activity	Unmethylated
NM_005822_1—DSCR1L1	6p12.3	Central nervous system development	Not done
NM_002204_1—ITGA3	17q21.33	Receptor	Unmethylated
NM_006496_1—GNAI3	17q24.1	Nucleotide binding	Unmethylated
NM_006509_1—RELB	19q13.32	Transcription factor	Unmethylated
NM_005536_1—IMPA1	8q21.13	Phosphatidylinositol biosynthetic process	Not done
NM_006317_1—**BASP1**	5q15.1	Brain acid-soluble protein	Methylated in all four lines

(*Continued on the following page*)

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

Table 1. Upregulated genes identified by statistical analysis and database interrogation (Cont'd)

Gene name	Location	Function	Methylation
AL050044_1—**GADD45b** (hypothetical protein dkfzp566b133)	19p13.3	Apoptosis, regulation of MAPKK activity	Methylated (DU-145, LNCaP)
NM_005737_1—ARL7	2q37.1	Binds and exchanges GTP and GDP	Unmethylated
NM_005919_1—MEF2B	19p13.11	Transcription factor	Not done
NM_001673_1—ASNS	7q21.3	Aspartate and asparagine activity	Unmethylated
NM_005253_1—FOSL2	2p23.2	Transcription factor	Unmethylated
NM_016582_1—**SLC15A3 (PHT2)**	11q12.2	Transporter activity	Methylated in all four lines
NM_021967_1—SERF1A	5q13.2	Nervous system development	Not done
NM_001386_1—DPYSL2	8p21.2	Dihydropyrimidinase activity	Not done
NM_002083_1—GPX2	14q23.3	Response to oxidative stress	Not done

NOTE: The table shows 45 genes that showed at least 2-fold upregulation in at least one of the four cell lines. Gene names in bold indicate genes identified as hypermethylated in prostate cancer cells in this study. Gene names in bold italics indicate genes previously identified as under epigenetic regulation in prostate cancer cells. Location is chromosomal map location. The tumor cell lines identified as hypermethylated by bisulfite sequencing are listed. Numbered references indicate published report of DNA methylation or other epigenetic regulation. Gene function was obtained from Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

Abbreviation: MAPKK, mitogen-activated protein kinase kinase.

with FAM and MGBNFQ for *SLC15A3*, *KRT7*, *GADD45b*, *IFI30*, or *TACSTD2*. *In vitro* methylated normal human genomic DNA, confirmed by bisulfite sequencing to show methylation for the gene to be analyzed, was used as a positive control. The concentration of this DNA was determined and a series of dilutions made for the standard curve. Unmethylated sequence of the *ACTINβ* gene was used as a normalizing control. The percentage of methylated alleles was calculated for a gene based on the standard curve. An Applied Biosystems 7500 Real-Time PCR machine was used for PCR and data were analyzed with SDS 1.3.1 software. *AQP3* and *ANXA2* were assessed by conventional gel-based methylation-specific PCR (MSP). Primer and probe sequences are given in Supplementary Table S2.

Results and Discussion

Selection of reactivated genes for validation

We analyzed differential expression on a 14,802 human gene microarray between RNA from four mock (untreated) prostate cancer cell lines and the same cell lines treated with 5Aza-dC over two cell doubling times and a single dose of TSA 24 hours before harvesting. We combined the 5Aza-dC treatment with TSA because there is a reported synergistic effect on demethylation of DNA (10). A total of 2,997 genes were upregulated at least 2-fold in one or more of the four prostate cancer cell lines analyzed compared with the mock cells. The list of genes was then ranked in descending order from upregulation in all four cell lines to upregulation in one cell line only (Supplementary Table S1). Further data can be found at http://www.ncbi.nlm.nih.gov/geo/ upon publication. The list of ranked upregulated genes was then prioritized for validation by examination of expression status in the normal cell counterpart compared with the tumor cell and the presence and location of a CpG island in the promoter region (11). In addition, known imprinted genes (e.g., IGF2), poorly annotated genes, or genes previously identified by us as upregulated but not to have cancer-specific methylation (e.g., *TGM2* and *GAGE7*; ref. 11) were excluded. Accordingly, we selected the first 45 genes that had higher or equal expression in normal prostate compared with prostate cancer cells according to the Cancer Genome Anatomy Project (CGAP) Serial Analysis Gene Expression (SAGE) database and that contained a CpG island within the promoter by the criteria of Takai and Jones (12) through WebGene analysis of the genomic sequence. Genes that showed no expression in normal cells or did not have a CpG island were excluded from immediate study (Table 1). Importantly, we noted that three of the most frequently methylated genes identified to date in prostate cancer [i.e., *GSTP1* (13), *PDLIM4* (14), and *IGFBP3* (15)] were included in this 45-gene list.

We then examined the published literature through the GeneCard (http://www.genecards.org) and PubMed databases, which revealed that 15 genes were previously reported to be hypermethylated in prostate cancer (Table 1). These 15 genes were excluded from immediate analysis. We therefore prioritized the remaining genes for study, and the promoter methylation status of 23 of this set of genes was first validated by direct bisulfite sequencing of the untreated prostate tumor cell line DNA. Eight

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

genes showed extensive methylation (i.e., in a majority of CG dinucleotides; Fig. 1 and Supplementary Fig. S1) in at least one prostate tumor cell line (Table 1). To examine if hypermethylation was specific to neoplastic prostate cells, we performed direct bisulfite sequencing of a normal prostate tissue DNA obtained from the cystoprostatectomy (for bladder cancer) of an age-matched male with no clinical or histopathologic evidence of prostate cancer and also a normal lymphocyte DNA. With the exception of *BASP1*, all genes were unmethylated in the normal prostate tissue DNA (Fig. 1; Supplementary Fig. S1) and unmethylated in the normal lymphocyte DNA.

Frequency of hypermethylation of studied genes

Because there is evidence that transformed tumor cell lines can have more gene methylation than the patient tumor specimen counterparts (16), we next examined the frequency and timing of hypermethylation of the *KRT7*, *TACSTD2*, *SLC15A3*, *GADD45b*, and *IFI30* genes by quantitative real-time MSP of DNA from a set of 19 prostatic intraepithelial neoplasia (PIN) and 35 prostate tumor tissue specimens from patients (Table 2; Fig. 2). The tumors consisted of 18 low and intermediate grade (Gleason ≤7), 16 high grade (Gleason ≥8), and one of unknown grade. Thirteen tumors were stage I or II and 22 tumors were stage III or IV. Hypermethylation of the *ANXA2* and *AQP3* genes was examined by conventional gel-based MSP and verified by bisulfite direct sequencing in a subset of 12 PIN and 20 prostate tumor tissue specimen DNA described above.

The *keratin 7* (*KRT7*) gene was hypermethylated in 10 of 19 (53%) PIN and 19 of 35 (54%) tumors. There was a significant difference ($P = 0.037$) in frequency between 13 of 18 Gleason ≤7 and 5 of 16 Gleason ≥8, but not between stage I-II and stage III-IV prostate tumors (Table 2). Cytokeratins are a subfamily of intermediate filament proteins characterized by biochemical diversity being represented in human epithelial tissues by at least 20 different polypeptides. Expression of particular cytokeratins is often cell type specific. KRT7 is expressed in the ductal epithelium of the genitourinary tract. Expression also varies by course of terminal differentiation (17). Hypermethylation of *KRT7* might indicate lineage differentiation or provide a growth advantage through cell-cell adhesion. A recent study reported a subgroup of clear cell renal cell carcinomas with *KRT7* expression being associated with genetic stability, a distinct global expression signature, and a more indolent clinical course (18). A network of molecular interactions and canonical pathways for *KRT7* formed by Ingenuity Pathways Analysis (IPA) was extensive. Consequently, an example of interaction with one cancer relevant pathway (Wnt signaling) only is shown in Supplementary Fig. S2.

SLC15A3, also known as *PHT2* or *PTR3*, is a member of the SLC15 family of electrogenic transporters that use the proton-motive force for uphill transport of short-chain peptides and peptido-mimetics into a variety of cells (19). Whereas the function of *SLC15A3* in cancer cells is unclear, IPA identified interaction of *SLC15A3* with the NF-κB and the p38 mitogen-activated protein kinase signaling pathways as well as other cancer-related pathways (Supplementary Fig. S2). We found *SLC15A3* to be methylated in 7 of 19 (37%) PIN and 23 of 35 (66%) prostate tumors (Table 2; Fig. 2).

Tumor-associated calcium signal transducer-2 (*TACSTD2*) or *TROP-2* is a cell surface glycoprotein. Its function remains largely unknown; however, it is phosphorylated by protein kinase C, and cross-linking *TACSTD2* with antibodies causes a transient increase in intracellular calcium levels, implying that it has a role in signal transduction (20). A recent report identified two subpopulations of basal cells based on TROP2 expression and reported TROP2 to be a marker of human prostate basal cells with stem cell characteristics (21). Prostate tumors with *TACSTD2/TROP2* hypermethylation and associated loss of expression may identify a distinct subgroup in terms of tumor lineage or tumor behavior. IPA of *TACSTD2* is shown in Supplementary Fig. S2, and interestingly, TACSTD2 interacts with SMARCA4/BRG1, reported to be mutated in tumor cell lines including DU-145 (22) and a component of the SWI-SNF chromatin remodeling complex. In our study, we found *TACSTD2* to be unmethylated in 19 PIN but hypermethylated in 6 of 35 tumors ($P = 0.08$; Table 2; Fig. 2). *TACSTD2* has been reported to be hypermethylated in glioblastomas (23). Current opinion is that patients diagnosed with PIN are not at a higher risk of prostate cancer than are patients diagnosed with benign tumors (24). Thus, a gene methylated in prostate tumors but not in PIN might be a useful marker for the differentiation of PIN from other more aggressive lesions (e.g., intraductal carcinoma). *TACSTD2* may therefore have utility in emerging methylation-based prostate cancer tests (25–28).

Promoter hypermethylation of the growth arrest and DNA damage–inducible, β (*GADD45b*, also referred to as hypothetical protein dkfzp566b133) gene was found by quantitative MSP in one prostate tumor only (Gleason 9, stage IV). Direct bisulfite sequencing of the tumor DNA confirmed this result (data not shown). GADD45 is involved in the regulation of cell cycle arrest and apoptosis and inhibition of cell growth. GADD45 responds to environmental stresses by mediating activation of the p38/c-jun NH_2-terminal kinase pathway (29). A selective IPA of the 10 pathways most strongly associated with *GADD45b* is shown in Supplementary Fig. S2. *GADD45b* has been reported to be hypermethylated in hepatocellular tumors (30). Another GADD45 gene, *GADD45a*, was previously reported as hypermethylated in prostate tumors (31).

The *IFI30*, *ANXA2*, and *AQP3* genes were unmethylated in all PIN and prostate tumor DNA examined. It is possible that analysis of a larger number, or a particular subtype, of primary prostate tumor specimens may yet reveal methylation of these genes. Of the four prostate tumor cell lines, three lines were established from distant metastases and the other, LNCaP, from a lymph node metastasis (32). The source of the cell lines taken together with our observation that these genes were unmethylated in primary

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

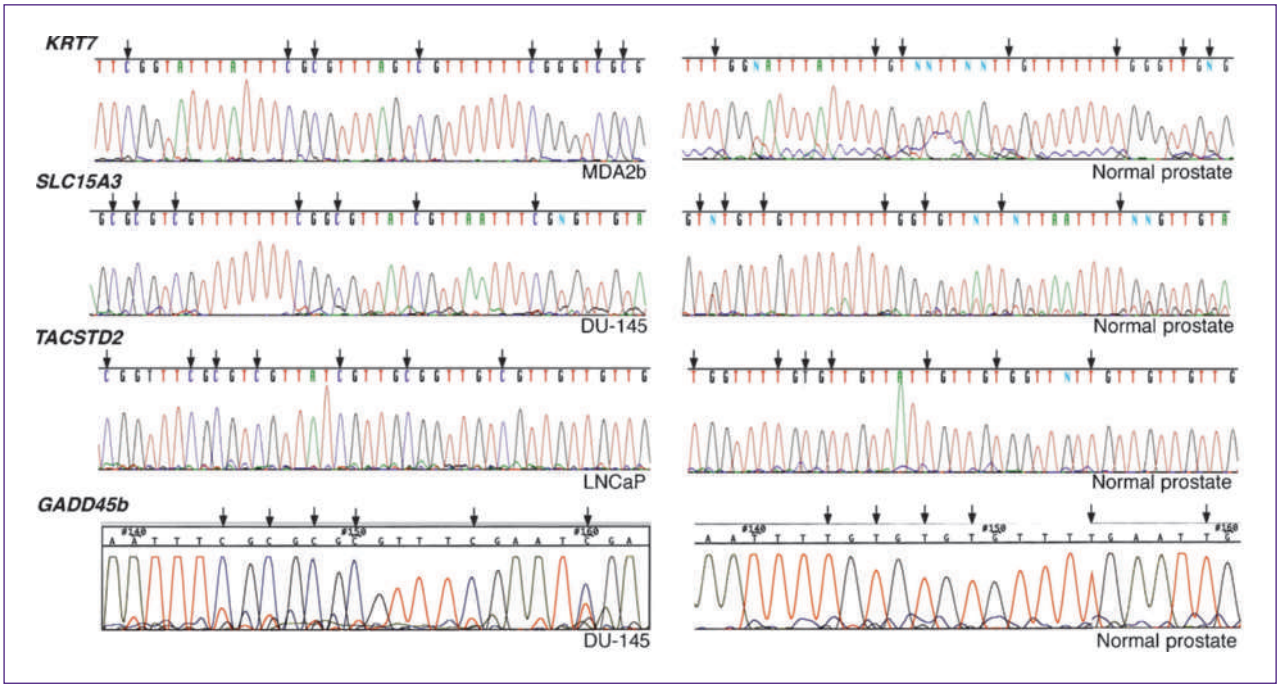

Fig. 1. Bisulfite sequencing of the promoter CpG island of the *KRT7, SLC15A3, TACSTD2*, and *GADD45b* genes in prostate tumor DNA and normal DNA. Representative sequencing of prostate tumor cell line DNA and histologically normal prostate tissue (from an age-matched cystoprostatectomy patient) DNA after bisulfite modification. Unmethylated cytosines (C) are converted to uracil (T). The presence of C preceding a G in the sites indicated by black arrows shows that these cytosines were methylated in the tumor cell line DNA. The presence of T instead of C in the same positions shows that these cytosines were unmethylated in normal prostate tissue DNA.

specimens highlights that the prostate tumor cell lines may be unrepresentative of the disease found in the population. It will be interesting to see if any of these three genes are subsequently identified as aberrantly methylated in other types of cancer.

Evidence for epigenetic reactivation of additional genes

Evidence for the specificity and potential of our screen was provided by the inclusion of the *GSTP1* gene known to be hypermethylated in the LNCaP and PC-3 cell lines (13), as well as in the majority of primary prostate tumors, and two other genes, *PDLIM4* (14) and *IGFBP3* (15), reported to be frequently methylated in primary prostate cancer in the 45 selected genes. Because an oligonucleotide probe on an array may not discriminate between alternative splice forms of the same gene, genes that do not display loss of expression of all isoforms with promoter hypermethylation (e.g., *RASSF1A*) will likely not appear upregulated by a global reactivation approach (33). There

Table 2. Frequency of hypermethylation of *KRT7, SLC15A3*, and *TACSTD2* by lesion and histologic grade and stage

	KRT7, n (%)		*P*	*SLC15A3, n* (%)		*P*	*TACSTD2, n* (%)		*P*
	M	U		M	U		M	U	
PIN (19)	10 (53)	9 (47)	1.00	7 (37)	12 (63)	0.051	0 (0)	19 (100)	0.08
Prostate tumors (35)	19 (54)	16 (46)		23 (66)	12 (34)		6 (17)	29 (83)	
Gleason ≤7	13 (72)	5 (28)	0.037	12 (67)	6 (33)	1.00	2 (11)	16 (89)	0.39
Gleason ≥8	5 (31)	11 (69)		11 (69)	5 (31)		4 (25)	12 (75)	
Stage I-II	9 (69)	4 (31)	0.29	9 (69)	4 (31)	1.00	1 (7.7)	12 (92.3)	0.38
Stage III-IV	10 (45)	12 (55)		14 (64)	8 (36)		5 (23)	17 (77)	

NOTE: Fisher's exact test was used to analyze whether the hypermethylation of a given gene was related to tumor grade or stage. Results were declared statistically significant at the 5% significance level. M, number of patients with methylated gene. U, number of patients with unmethylated gene.

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

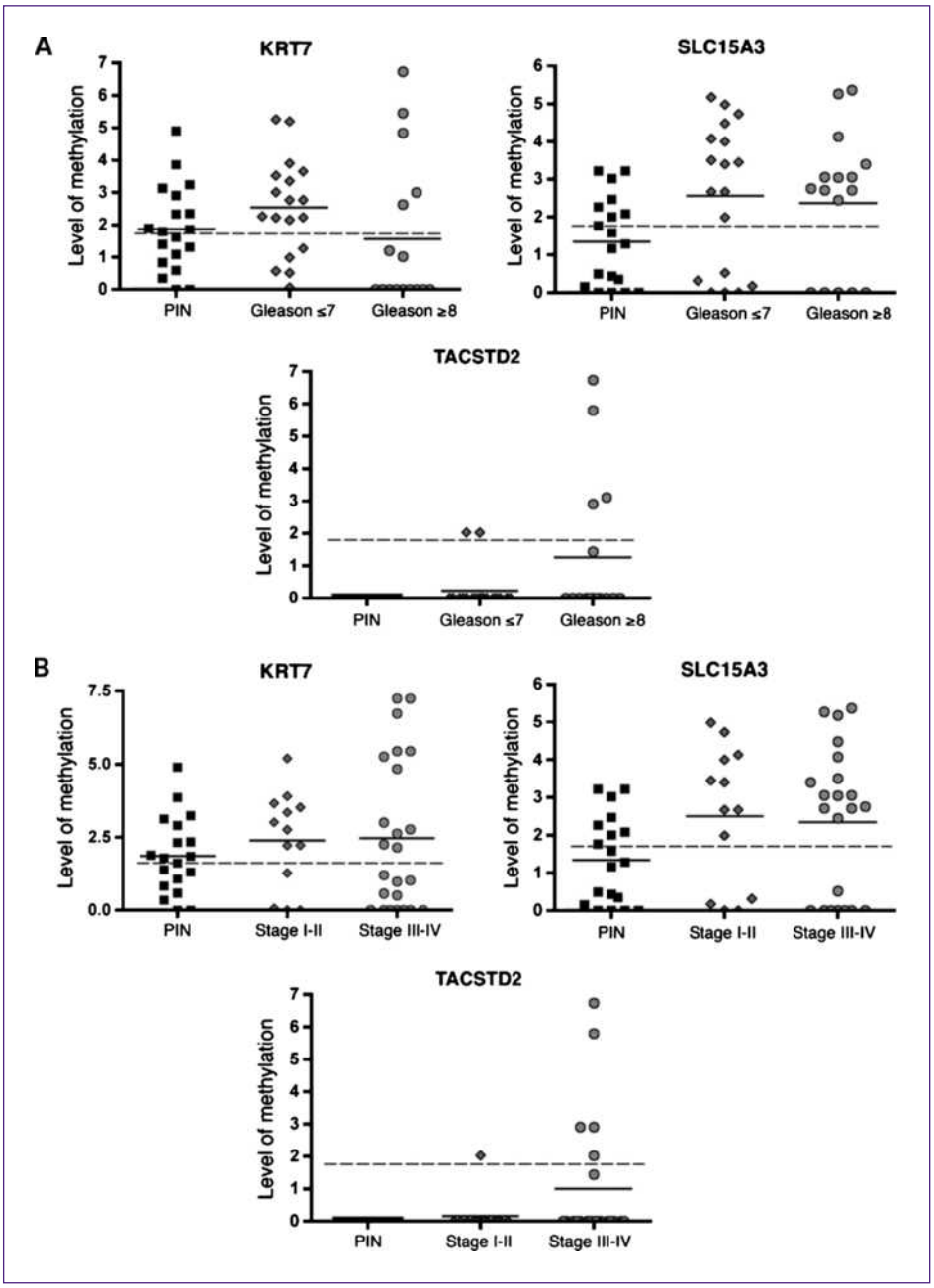

Fig. 2. Distribution plots for the methylation levels of the *KRT7, SLC15A3*, and *TACSTD2* genes by Gleason score (A) and by pathologic stage (B). Quantitative MSP of 19 PIN and 35 primary prostate tumor specimen DNA. The percentage of methylated alleles (PMA) was calculated as the ratio between the PCR amplification product of the gene of interest and the *ACTINβ* reference gene multiplied by 100. The PMA is given as a log value Ln (% + 1). Horizontal solid line, median; dashed line, a PMA of 5%, which we chose as a cutoff point for scoring as methylated.

was evidence from the literature for epigenetic regulation of several other genes in the 45 studied. The references for such genes are given in Table 1. In our list (Table 1), there were also additional genes reported by several groups to be upregulated after epigenetic reactivation but with no evidence of methylation in prostate tumor cells (e.g., *DUSP1*; refs. 31, 34). Such genes may be upregulated by demethylation and reactivation of another gene or may result from a stress response to the drug treatment. In general, our study compares well to the "hit rate," that is, the ratio of genes hypermethylated among genes upregulated after demethylation reported in other global reactivation studies (5, 6, 31). We did not place emphasis on the higher fold of reactivation because we considered this to be arbitrary given the current limited understanding of the degree of reexpression necessary to restore the normal function of a particular gene.

The prostate cancer cell hypermethylome

The average total number of genes methylated with functional significance in the prostate tumor cell is unknown but might be reasonably estimated as several

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

hundreds (35, 36). Several prostate cancer global methylation studies have been reported. Yu et al. compared the results from PC-3, DU-145, and LNCaP lines on an oligonucleotide-based methylation array to an expression array (37) and reported a number of genes methylated by array analysis, some of which were replicated by nonquantitative conventional MSP analysis but not by direct bisulfite sequencing. Lodygin et al. performed a global epigenetic reactivation of the same three prostate tumor cell lines and identified a number of known, as well as novel, genes methylated in prostate cancer from the subset of 50 genes examined (31). Several of the genes in the ranked selection by Lodygin were also in our selected list. Their study did not perform quantitative MSP or examine normal cells for imprinted or tissue-specific methylated genes. Hoque et al. also studied epigenetic reactivation in the same three lines as well as the 22Rv1 line. They selected 45 reactivated genes, of which 9 were previously reported to be hypermethylated in cancer cells; 16 of the remaining 36 were methylated in the cell lines and 8 in primary prostate tumors of which 3 did not have methylation in normal prostate cells (38). Chung et al. used methylated CpG island amplification coupled with representational difference analysis of PC-3, DU-145, and LNCaP, and validation by combined bisulfite restriction analysis and pyrosequencing identified six novel methylated genes in primary prostate tumors that had significantly higher methylation than in adjacent normal prostate tissue (39).

Summary

In summary, we report for the first time four novel genes with promoter hypermethylation in primary prostate tumor specimens but that are unmethylated in normal cells. One of these genes, *TACSTD2/TROP2*, is a potential marker for detection or diagnosis of prostate cancer rather than for PIN only, as well as a prostate stem cell marker (21). The specificity of our screen for hypermethylated genes is further supported by the reactivation of *GSTP1* and other genes known to be frequently methylated in prostate cancer in our selection. Further mining of the data provided here (Supplementary Table S1) as well as emerging technologies will increase our knowledge of the prostate hypermethylome. Such studies should lead to further understanding of the biology of prostate tumorigenesis and the identification of further hypermethylated genes as candidate markers for the diagnosis and prognosis of prostate cancer.

Disclosure of Potential Conflicts of Interest

P. Cairns is a paid consultant to Oncomethylome Sciences. The terms of this arrangement are being managed by Fox Chase Cancer Center in accordance with its conflict of interest policies. The other authors disclosed no potential conflicts of interest.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 02/22/2010; revised 03/30/2010; accepted 04/14/2010; published OnlineFirst 08/10/2010.

References

1. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.
2. Maruyama R, Toyooka S, Toyooka KO, et al. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 2002;8:514–9.
3. Cairns P. 5′-Azacytidine expression arrays. Methods Mol Biol (Clifton, NJ) 2009;507:165–74.
4. Liang G, Gonzales FA, Jones PA, Orntoft TF, Thykjaer T. Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2′-deoxycytidine. Cancer Res 2002;62:961–6.
5. Suzuki H, Gabrielson E, Chen W, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 2002;31:141–9.
6. Yamashita K, Upadhyay S, Osada M, et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell 2002;2:485–95.
7. Grant JD, Somers LA, Zhang Y, Manion FJ, Bidaut G, Ochs MF. FGDP: functional genomics data pipeline for automated, multiple microarray data analyses. Bioinformatics 2004;20:282–3.
8. Sambrook J, Russell DW. Molecular cloning. A laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2001.
9. Herman JG, Graff JR, Myöhänen S, BD N, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.
10. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999;21:103–7.
11. Ibanez de Caceres I, Dulaimi E, Hoffman AM, Al-Saleem T, Uzzo RG, Cairns P. Identification of novel target genes by an epigenetic reactivation screen of renal cancer. Cancer Res 2006;66:5021–8.
12. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002;99:3740–5.
13. Lee W-H, Morton RA, Epstein JI, et al. Cytidine methylation of regulatory sequences near the π-class glutathione *S*-transferase gene accompanies human prostatic carcinogenesis. Proc Natl Acad Sci U S A 1994;91:11733–7.
14. Vanaja DK, Ballman KV, Morlan BW, et al. PDLIM4 repression by hypermethylation as a potential biomarker for prostate cancer. Clin Cancer Res 2006;12:1128–36.
15. Perry AS, Loftus B, Moroose R, et al. *In silico* mining identifies IGFBP3 as a novel target of methylation in prostate cancer. Br J Cancer 2007;96:1587–94.
16. Smiraglia DJ, Rush LJ, Fruhwald MC, et al. Excessive CpG island hypermethylation in cancer cell lines versus primary human malignancies. Hum Mol Genet 2001;10:1413–9.
17. Omary MB, Ku NO, Strnad P, Hanada S. Toward unraveling the complexity of simple epithelial keratins in human disease. J Clin Invest 2009;119:1794–805.
18. Mertz KD, Demichelis F, Sboner A, et al. Association of cytokeratin 7 and 19 expression with genomic stability and favorable prognosis in clear cell renal cell cancer. Int J Cancer 2008;123:569–76.
19. Daniel H, Kottra G. The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Arch 2004;447:610–8.
20. Fornaro M, Dell'Arciprete R, Stella M, et al. Cloning of the gene encoding Trop-2, a cell-surface glycoprotein expressed by human carcinomas. Int J Cancer 1995;62:610–8.
21. Goldstein AS, Lawson DA, Cheng D, Sun W, Garraway IP, Witte ON.

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics. Proc Natl Acad Sci U S A 2008; 105:20882–7.

22. Wong AK, Shanahan F, Chen Y, et al. BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Res 2000;60:6171–7.
23. Kim TY, Zhong S, Fields CR, Kim JH, Robertson KD. Epigenomic profiling reveals novel and frequent targets of aberrant DNA methylation-mediated silencing in malignant glioma. Cancer Res 2006;66:7490–501.
24. Epstein JI, Herawi M. Prostate needle biopsies containing prostatic intraepithelial neoplasia or atypical foci suspicious for carcinoma: implications for patient care. J Urol 2006;175:820–34.
25. Cairns P, Esteller M, Herman JG, et al. Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. Clin Cancer Res 2001;7:2727–30.
26. Harden SV, Sanderson H, Goodman SN, et al. Quantitative GSTP1 methylation and the detection of prostate adenocarcinoma in sextant biopsies. J Natl Cancer Inst 2003;95:1634–7.
27. Hoque MO, Topaloglu O, Begum S, et al. Quantitative methylation-specific polymerase chain reaction gene patterns in urine sediment distinguish prostate cancer patients from control subjects. J Clin Oncol 2005;23:6569–75.
28. Tokumaru Y, Harden SV, Sun DI, Yamashita K, Epstein JI, Sidransky D. Optimal use of a panel of methylation markers with GSTP1 hypermethylation in the diagnosis of prostate adenocarcinoma. Clin Cancer Res 2004;10:5518–22.
29. Takekawa M, Saito H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 1998;95:521–30.
30. Qiu W, Zhou B, Zou H, et al. Hypermethylation of growth arrest DNA damage-inducible gene 45 β promoter in human hepatocellular carcinoma. Am J Pathol 2004;165:1689–99.
31. Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H. Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res 2005;65:4218–27.
32. van Bokhoven A, Varella-Garcia M, Korch C, et al. Molecular characterization of human prostate carcinoma cell lines. Prostate 2003;57:205–25.
33. Shames DS, Girard L, Gao B, et al. A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies. PLoS Med 2006;3:e486.
34. Rauhala HE, Porkka KP, Tolonen TT, Martikainen PM, Tammela TL, Visakorpi T. Dual-specificity phosphatase 1 and serum/glucocorticoid-regulated kinase are downregulated in prostate cancer. Int J Cancer 2005;117:738–45.
35. Costello JF, Frühwald MC, Smiraglia DJ, et al. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000;25:132–8.
36. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006; 314:268–74.
37. Yu YP, Paranjpe S, Nelson J, et al. High throughput screening of methylation status of genes in prostate cancer using an oligonucleotide methylation array. Carcinogenesis 2005;26:471–9.
38. Hoque MO, Kim MS, Ostrow KL, et al. Genome-wide promoter analysis uncovers portions of the cancer methylome. Cancer Res 2008; 68:2661–70.
39. Chung W, Kwabi-Addo B, Ittmann M, et al. Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. PLoS ONE 2008;3:e2079.
40. Sathyanarayana UG, Padar A, Suzuki M, et al. Aberrant promoter methylation of laminin-5-encoding genes in prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 2003;9:6395–400.
41. Rehman I, Goodarzi A, Cross SS, et al. DNA methylation and immunohistochemical analysis of the S100A4 calcium binding protein in human prostate cancer. Prostate 2007;67:341–7.
42. Suzuki M, Shigematsu H, Shivapurkar N, et al. Methylation of apoptosis related genes in the pathogenesis and prognosis of prostate cancer. Cancer Lett 2006;242:222–30.
43. Ahmed H, Banerjee PP, Vasta GR. Differential expression of galectins in normal, benign and malignant prostate epithelial cells: silencing of galectin-3 expression in prostate cancer by its promoter methylation. Biochem Biophys Res Commun 2007;358: 241–6.
44. Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. J Pathol 2004;202:233–40.
45. Verkaik NS, van Steenbrugge GJ, van Weerden WM, Bussemakers MJ, van der Kwast TH. Silencing of CD44 expression in prostate cancer by hypermethylation of the CD44 promoter region. Lab Invest 2000;80:1291–8.

Published OnlineFirst August 10, 2010; DOI: 10.1158/1940-6207.CAPR-10-0039

Cancer Prevention Research

Global Reactivation of Epigenetically Silenced Genes in Prostate Cancer

Ilsiya Ibragimova, Inmaculada Ibáñez de Cáceres, Amanda M. Hoffman, et al.

Cancer Prev Res 2010;3:1084-1092. Published OnlineFirst August 10, 2010.