PDLIM4 Repression by Hypermethylation as a Potential Biomarker for Prostate Cancer

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Abstract

Purpose: We analyzed the expression of genes to identify reliable molecular markers in the diagnosis and progression of prostate cancer.

Experimental Design: Gene expression profiling was done using HG-U133 set microarrays in 32 prostate cancer and 8 benign tissues of patients with cancer. Expression levels of 11 genes were selected for quantitative real-time PCR evaluation in 52 prostate cancer and 20 benign tissues. Further, to assess transcriptional inactivation, we analyzed the promoter methylation of genes by quantitative methylation-specific PCR in 62 tumor and 36 benign tissues.

Results: Our results showed a significant down-regulation in the mRNA expression levels of *PRIMA1*, *TU3A, PDLIM4, FLJ14084, SVIL, SORBS1, C21orf63*, and *KIAA1210* and up-regulation of *FABP5, SOX4*, and *MLP* in prostate cancer tissues by TaqMan real-time PCR. Quantitative methylation-specific PCR of *PDLIM4, SVIL, PRIMA1, GSTP1*, and *PTGS2* detected prostate carcinoma with a sensitivity of 94.7%, 75.4%, 47.4%, 89.5%, and 87.7%, and a specificity of 90.5%, 75%, 54.2%, 95.8%, and 90.2%, respectively. Using this panel of methylation markers in combination, we were able to distinguish between prostate cancer and adjacent benign tissues with sensitivities and specificities of about 90% to 100%. Our data provide evidence of transcriptional repression of the putative tumor suppressor gene *PDLIM4* by hypermethylation.

Conclusions: Our analysis revealed differential expression of eight down-regulated and three up-regulated genes, implicating their role in prostate cancer development and progression. We further showed that the hypermethylation of *PDLIM4* gene could be used as a sensitive molecular tool in detection of prostate tumorigenesis.

Gene expression profiling of prostate cancer has identified genes that are differentially expressed in tumor compared with nontumor samples and genes of which expression correlates with tumor grade, metastasis, and disease recurrence (1). However, gene expression analysis alone cannot provide an overall integrative molecular understanding of the genesis and growth of prostate carcinoma. Chromosomal aberrations, translational control of messages, and epigenetic and post-translational modifications all play important roles in the biological behavior of prostate cancer (2–4). Although the list of epigenetic changes in specific genes continues to grow, only a few genes have, thus far, given promising results as potential tumor biomarkers for early diagnosis and risk assessment of prostate cancer (5–12). However, most of the biomarkers reported have shown insufficient sensitivity and specificity for the detection of the entire spectrum of prostate cancer disease that exists in a diagnostic setting. The limited value of established prognostic markers demands identification of new molecular variables of interest in predicting the prognosis of prostate cancer patients. These limitations could potentially be overcome if multiple and specific molecular markers were identified and used simultaneously for diagnosis of prostate cancer (13–17).

Comprehensive gene expression profiling was done in 32 prostate cancer and 8 tumor adjacent benign tissues using HG-U133 oligonucleotide microarrays, and the resulting data were deposited in the Oncomine Cancer Profiling Database (study name, Vanaja_Prostate; http://www.oncomine.org). We analyzed the expression levels of eight down-regulated genes, including supervillin (*SVIL*), proline-rich membrane anchor 1 (*PRIMA1*), *TU3A, FLJ14084, KIAA1210*, sorbin and SH3 domain containing 1 (*SORBS1*), PDZ and LIM domain 4 (*PDLIM4*), and *C21orf63*, and three up-regulated genes, including fatty acid binding protein 5 (*FABP5*), SRY (sex determining region Y)-box 4 (*SOX4*), and myristolated alanine-rich C kinase substrate–like protein (*MLP*), in samples used for microarray analysis and also in an independent set of 32 tissues (20 tumor and 12 benign) to evaluate whether these would

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serve as molecular markers of cancer initiation and progression. Significant deregulation in the expression levels of the genes was found in prostate cancer versus adjacent benign tissues. The promoter methylation status of three of these genes was assessed in 62 prostate cancer tissues and 36 benign prostatic hyperplasia tissues. Our studies show that down-regulation of the expression of *PDLIM4* primarily resulted from methylation in prostate cancer tissues. In addition, partial transcriptional silencing of the expression levels of *SVIL* and *PRIMA1* resulted from methylation. This is the first report depicting epigenetic silencing of *PDLIM4* that could potentially be involved in the pathogenesis of prostate cancer and may have diagnostic value.

Materials and Methods

Prostate tissue samples. Surgically resected prostate cancer tissue specimens were obtained from patients who had undergone radical prostatectomy for prostate cancer at Mayo Clinic with Institutional Review Board approval. All the tissues were collected from non-pretreated patients as previously described (18). Prostate cancer specimens were trimmed to obtain tissue sections containing >70% tumor nuclei (by histologic examination) using a cryostat sectioning technique. A H&E-stained section was prepared before and after slides were cut for RNA and DNA isolation for gene expression and methylation analysis. This ensured that tumor was present in the tissues used for analysis with minimal numbers of stromal cells and to eliminate confounding effects of high-grade prostatic intraepithelial neoplasia. Differential expression of genes was analyzed in 12 intermediate-grade (primary stage T2 Gleason score 6 of pattern 3+3) and 15 high-grade (primary stage T3 Gleason score 9 of pattern 4+5) prostatic adenocarcinoma and 5 metastatic tumors along with 8 separately collected nonmalignant adjacent benign prostatic tissue samples as described earlier (18) using Human Genome U133 A&B microarrays representing >39,000 transcripts (Affymetrix, Santa Clara, CA). We validated the expression levels of genes by quantitative real-time PCR in 40 tissues used for microarray analysis and also in an independent set of 10 high-grade (primary stage T3 Gleason score 9 of pattern 4+5) and 10 intermediate-grade (primary stage T2 Gleason score 6 of pattern 3+3) and 12 separately collected nonmalignant benign tissues. To analyze the transcriptional silencing of the genes, we examined promoter methylation in 28 Gleason score 6, 29 Gleason score 9, and 5 metastatic tumors along with 24 tumor adjacent benign tissues obtained by radical retropubic prostectomy and 12 separately collected tissues of benign prostatic hyperplasia without cancer obtained by superpubic prostectomy. Clinical and pathologic information along with GPSM score of patients is shown. GPSM score is a prognostic model using the weighed sum of the pathologic Gleason score, preoperative PSA, seminal vesicle involvement, and marginal status to predict biochemical progression after radical prostatectomy (19). In the GPSM score, points are assigned based on four poor prognostic features consisting of Gleason score + PSA (0-3.9 ng/mL, 0 points; 4-10 ng/mL, 1 point; 10.1-20, 2 points; and ≥20.1, 3 points) + seminal vesicle involvement (2 points) + positive margins (2 points). The 5-year progression-free survival was 94% for scores <5, 60% for 10, and 32% for >12. This simple predictive model allows identification of patients who are at high risk for cancer progression.

Isolation of RNA and quantitative real-time reverse transcription-PCR. RNA was isolated from 72 fresh frozen prostate tissues using 30 sections of 15-μm thicknesses cut with a cryostat as previously described (18). In brief, total RNA was extracted using TRIzol reagent (Life Technologies, Inc., Carlsbad, CA). DNA contamination was removed using DNA-free kit (Ambion, Austin, TX) and RNA cleanup was done using RNeasy Mini kit (Qiagen, Valencia, CA). One microgram of the total RNA was used for first-strand cDNA synthesis.

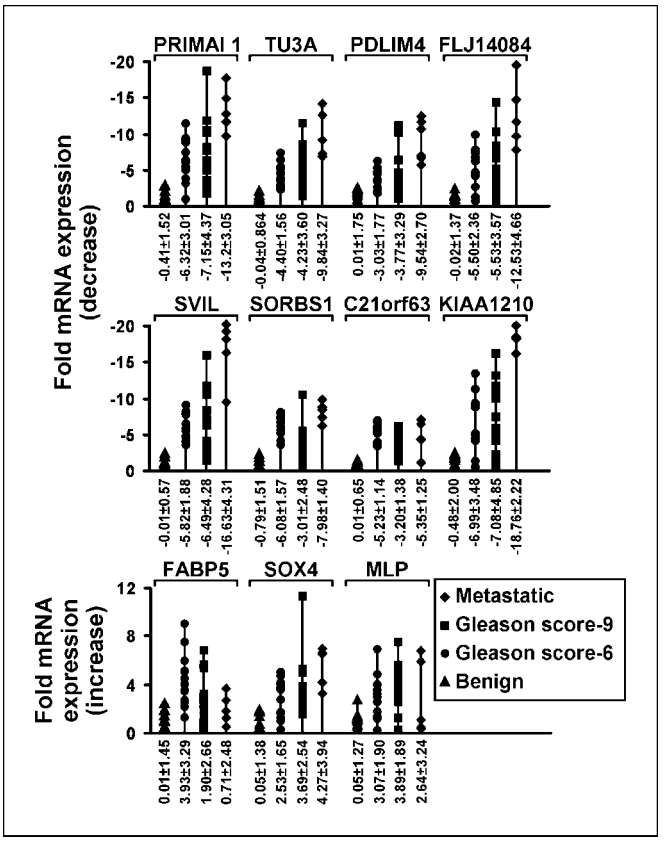

Fig. 1. Expression levels of the genes in prostate tissue samples validated by TaqMan real-time PCR. Values are expressed as the relative fold decrease or fold increase in the mRNA expression with respect to the adjacent benign tissues after normalization to the house keeping gene *GAPDH*. Metastatic tissues, $n = 5$; Gleason score 6 tissues, $n = 12$; Gleason score 9 tissues, $n = 15$; and adjacent benign tissues, $n = 8$. The experiments were repeated twice in triplicate for each sample. Means and SDs are shown on the bottom of X axis.

A high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) was used for conversion of RNA to cDNA. To confirm the differential expression of genes, we selected eight down-regulated and three up-regulated genes for validation by TaqMan real-time reverse transcription-PCR. One microliter of the cDNA and TaqMan real-time primers and probes was used for amplification. A set of primers and a probe for each gene tested was obtained from Applied Biosystems. Assay IDs for the genes are as follows: PDLIM4, Hs00184792_m1; SVIL, Hs00222268_m1; C21orf63, Hs00332708_m1; PRIMA1, Hs00603526_m1; TU3A, Hs00200376_m1; KIAA1210, Hs00393400_m1; FLJ14084, Hs00222179_m1; SORBS1, Hs00248750_m1; MLP, Hs00702769_s1; SOX4, Hs00268388_s1; and FABP5, Hs00870436_s1. All PCR reactions were carried out in TaqMan Universal PCR master mix (Applied Biosytems) with 900 nmol/L of each primer and 250 nmol/L of probe as previously described (18). The relative mRNA expression level of each gene for each patient was normalized for input RNA against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression in the sample. The relative mRNA expression level (CR) of the target gene in each sample was calculated using the comparative cycle time (Ct) method (20) as CR = 2 − (Ct benign target − Ct *GAPDH*) − (Ct tumor target − Ct *GAPDH*).

Cell lines and 5-aza-deoxycytidine treatment. The human prostate cancer cell lines LNCaP, PC3 (American Type Culture Collection, Rockville, MD), and LAPC4 (a gift from Dr. Charles L. Sawyers, University of California, Los Angeles, CA) were treated with 5% fetal bovine serum in RPMI 1640 medium either with or without 6 μmol/L 5-aza-deoxycytidine (5-Aza-CdR; Sigma Chemicals Co., St. Louis, MO)

Table 1. Significance of differently expressed genes in prostate cancer tissues using oligonucleotide microarrays and validated by quantitative real-time PCR

Gene	Expression array		Quantitative real-time PCR *P* values in different groups							
	t statistics	*P*	ABT-Met-GS6-GS9	Met-GS6-GS9	ABT-Met	ABT-GS6	ABT-GS9	Met-GS6	Met-GS9	GS6-GS9
SORBS1	−8.0964	0.0000	0.0000	0.0002	0.0043	0.0002 (0.0001)	0.0027 (0.0013)	0.0398	0.0052	0.0007 (0.256)
C21orf63	−6.5874	0.0000	0.0000	0.0021	0.0043	0.0002 (0.0001)	0.0001 (0.0001)	0.9580	0.0114	0.0021 (0.496)
SVIL	−8.9672	0.0000	0.0000	0.0044	0.0043	0.0002 (0.0001)	0.0002 (0.0001)	0.0019	0.0040	0.8644 (0.650)
PRIMA1	−4.73	0.0006	0.0000	0.0110	0.0043	0.0004 (0.0001)	0.0003 (0.0001)	0.0027	0.0145	0.8452 (0.226)
PDLIM4	−7.3391	0.0000	0.0000	0.0036	0.0043	0.0002 (0.0001)	0.0001 (0.0001)	0.0019	0.0052	0.3797 (0.496)
FLJ14084	−6.4435	0.0000	0.0000	0.0099	0.0043	0.0006 (0.0002)	0.0004 (0.0003)	0.0052	0.0068	0.8644 (0.405)
TU3A	−4.9284	0.0005	0.0001	0.0098	0.0043	0.0002 (0.0001)	0.0011 (0.0001)	0.0037	0.0088	0.7884 (0.364)
KIAA1210	−4.196	0.0015	0.0001	0.0026	0.0043	0.0010 (0.0001)	0.0022 (0.0001)	0.0019	0.0017	0.9805 (0.364)
SOX4	3.2293	0.0080	0.0012	0.1096	0.0018	0.0061 (0.0002)	0.0002 (0.0001)	0.1021	0.1898	0.1497 (0.049)
MLP	2.8315	0.0163	0.0023	0.4945	0.2723	0.0014 (0.0048)	0.0006 (0.0002)	0.6350	0.5409	0.2614 (0.211)
FABP5	3.4071	0.0059	0.0126	0.0316	0.5101	0.0097 (0.0004)	0.0998 (0.0057)	0.0268	0.0734	0.1243 (0.026)

NOTE: Metastatic tissues (Met; $n = 5$), Gleason score 6 (GS6; $n = 12$), Gleason score 9 (GS9; $n = 15$), and adjacent benign tissues (ABT; $n = 8$) were used. Negative and positive *t* statistic values indicate down-regulation and up-regulation, respectively, of genes in tumor tissues relative to the benign tissues by expression profiling. A Kruskal-Wallis test was done with the TaqMan real-time data to compare the median gene expression levels among different groups. The experiments were repeated twice in triplicate for each sample. Significance of genes in different groups is shown with their *P* values. The values in parentheses indicate the significance of genes validated in an independent set of samples (Gleason score 9, $n = 10$; Gleason score 6, $n = 10$; and benign prostate tissues, $n = 12$).

for 6 days as previously reported (18). For control, normal prostate epithelial cells (PrEC) obtained from Cambrex Bio Science (Walkersville, MD) were cultured with or without 5-Aza-CdR in a prostate epithelial cell medium (PrEGM BulletKit^R, Cambrex Bio Science, Walkersville, MD). Total RNA was isolated from the cell lines and the expression of *PDLIM4*, *SVIL*, and *PRIMA1* was analyzed by TaqMan real-time PCR as described above. For normalization, *GAPDH* was used as an internal control.

DNA isolation and bisulfite conversion. Prostate cancer tissues, including 57 organ confined, 5 metastatic tumors, and 36 benign tissues, were used for genomic DNA isolation. DNA was isolated from 10 tissue sections of 20-μm thickness using the ZR Genomic DNA II kit (Zymo Research Corp., Orange, CA). Five hundred nanograms of the DNA were subjected to sodium bisulfite modification using a Zymo EZ DNA methylation kit (Zymo Research) and the DNA was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Quantitative real-time methylation-specific PCR. Methylation of genomic DNA was measured by fluorescence-based, reverse transcription-PCR (21). In brief, 1 μL of bisulfite-converted genomic DNA was amplified using locus-specific PCR primers flanking an oligonucleotide probe for the genes *PDLIM4*, *SVIL*, and *PRIMA1* (Table 3). PCR amplification was done in a 96-well optical tray with caps. The reaction mixture (25 μL) contained 600 nmol/L of each primer, 200 nmol/L probe, TaqMan Universal PCR master mix (Applied Biosytems), and bisulfite-converted DNA under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Two previously reported genes, *GSTP1* and *PTGS2*, were used as reference. In addition, primers and a probe were used to amplify areas without CpG nucleotides of *ACTB* (β-actin), an internal reference gene (22). Bisulfite-converted universal methylated DNA from Chemicon International (Temecula, CA) served as a positive control and was used to generate a standard curve to quantify the amount of fully methylated regions in each gene. A blank reaction with water substituted for DNA served as negative control. We calculated the ratio of methylation in each sample. The normalized index of methylation (NIM) is defined as the ratio of the normalized amount of converted templates at the promoter of interest to the normalized amount of converted *ACTB* templates in any given sample (23): NIM = [(GENEsample/GENEuniv methyl)/(*ACTB*sample/*ACTB*univ methyl)]; GENEsample is the number of fully methylated copies of the gene of interest in a given sample, GENEuniv methyl is the number of fully methylated copies of the gene of interest in the universally methylated control DNA, *ACTB*sample is the number of

Table 2. Location of the PCR amplicons used for methylation analysis

Gene name	GenBank accession no.	Amplicon location (GenBank numbering)	Amplicon location (transcription start site)	%GC	ObsCpG/ExpCpG
SVIL	NT_008705	12000242-12000169	−187/−114	76.7	1.012
PRIMA1	NT_026437	75254836-75254751	−318/−233	78.6	0.908
PDLIM4	NT_034772	34008204-34008285	−195/−114	83.1	0.839

NOTE: The GenBank accession number is listed with the corresponding amplicon location within that sequence and the amplicon location relative to the transcription start site. The percentage of guanine-cytosine (%GC) content and CpG observed/expected value of 200 bp encompassing the MethyLight amplicon are indicated for each gene.

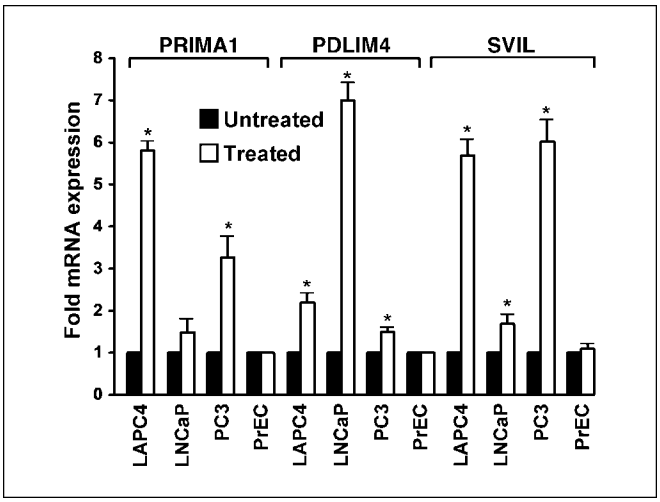

Fig. 2. Expression of mRNA levels in prostate cells lines treated with 6 μmol/L 5-Aza-CdR. Relative fold-decrease in the mRNA expression with respect to the cells without 5-Aza-CdR treatment. Columns, mean of four separate experiments. Bars, SD. *, $P < 0.05$, versus untreated cells.

ACTB copies in a given sample, and *ACTB*univ methyl is the number of *ACTB* copies in the universally methylated DNA. The NIM serves as an index of the percentage of bisulfite-converted input copies of DNA that are fully methylated at the primer and probe hybridization sites.

Statistical analysis of methylation. The CpG island methylation data were obtained from 36 benign and 62 cancerous tissues. Receiver operator characteristic (ROC) curves were generated for each DNA methylation marker; these were summarized by area under the curve (AUC). An AUC value of 100 means the marker is able to perfectly distinguish between benign and cancerous tissues, and a value of 50 indicates the marker is no better at distinguishing between the groups than random chance. Thresholds for each marker were determined that maximized a function of specificity and sensitivity (i.e., made them approximately equal). The values of sensitivity and specificity for the marker at this threshold were determined. Logistic regression was used to determine whether a combination of markers did better than individual markers. The performance of each marker combination was summarized with an ROC curve and AUC using the logistic regression predicted probability of the tissue being cancerous given the value of the markers. Areas under two ROC curves were compared using the method of Delong et al. (24).

Results

Differentially expressed genes in prostate cancer tissues. To identify molecular biomarkers of prostate cancer, we evaluated a panel of deregulated genes (*PRIMA1*, *TU3A*, *PDLIM4*, *FLJ14084*, *SVIL*, *SORBS1*, *C21orf63*, *KIAA1210*, *FABP5*, *SOX4*, and *MLP*) by quantitative real-time reverse transcription-PCR in 40 tissues (Fig. 1) to confirm data from HG-U133 microarrays (data deposited in the Oncomine Cancer Profiling Database under the study Vanaja_Prostate http://www.oncomine.org) and HG-U95Av2 chips (18). These genes were selected because of their moderate to high-level of deregulation in our microarray databases of prostate cancer tissues as well as others (1, 18, 25–32). The expression levels of these genes were consistent with decreased or increased expression in >80% of the tumor tissues; moreover, these genes have been understudied in prostate cancer. An additional independent set of 32 tissues was also used to validate the results. Statistical analysis of expression using U133 chips in prostate cancer over the benign controls and the expression levels in different groups by quantitative PCR is presented in Table 1. A significant decrease in the expression of the down-regulated genes was observed in metastatic and confined tumors with a mean expression of 10- to 20-fold decrease in tumor versus benign tissues. In addition, our data revealed a significant up-regulation of *FABP5*, *SOX4*, and *MLP* in both Gleason score 6 and Gleason score 9 tumors, which is consistent with previous microarray data (29–32). We observed no significant change in the expression levels in Gleason score 6 versus Gleason score 9 tumors. Our analysis confirms the differential expression of these genes and suggests their potential roles in prostate cancer development and progression.

Reactivation of silenced genes by demethylation treatment. To test whether the underexpressed genes are repressed by methylation and can be reactivated in prostate cancer cell lines, we chose three down-regulated genes, *PDLIM4*, *SVIL*, and *PRIMA1*, for the following studies because of their high frequency of CG content in the predicted promoter regions with CpG islands (Table 2; ref. 33). After treatment of LAPC4, LNCaP, PC3, and PrEC cell lines with an inhibitor of DNA methyltransferase, 5-Aza-CdR, RNA was extracted from cell lines and mRNA levels were quantified using the same primers and probes used in the above TaqMqn real-time PCR

Table 3. Primers and probes used to amplify bisulfite-converted CpG islands of genes by real-time methylation-specific PCR reactions

Gene	5′ to 3′ forward primer	5′ to 3′ TaqMan probe	5′ to 3′ reverse primer
SVIL	CGTTTGGTGGTTTAGTAGAGGGC	FAM-TCGTCGCGCGGGTCGTAAGG-BHQ1	AAACTCGCGCGTCCCC
PDLIM4	CGGGTTGTCGGTAGTCGG	FAM-TTTTTAGAGTTTTTCGAAGTGGGAGGGTCG-BHQ1	AAATTAACCCCGCGACCAA
PRIMA1	CGCGCGGTTAGGCGTA	FAM-TTCGGAGTTATCGCGTTTTGCGTTTC-BHQ1	TCCCGAACCGCTAAACAAAA
GSTP1	AGTTGCGCGGCGATTTC	FAM-CGGTCGACGTTCGGGGTGTAGCG-BHQ1	GCCCCAATACTAAATCACGACG
PTGS2	CGGAAGCGTTCGGGTAAAG	FAM-TTTCCGCCAAATATCTTTTCTTCTTCGCA-BHQ1	AATTCCACCGCCCCAAAC
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	6FAM-ACCACCACCCAACACACAATAACAAACACA-BHQ1	AACCAATAAAACCTACTCCTCCCTTAA

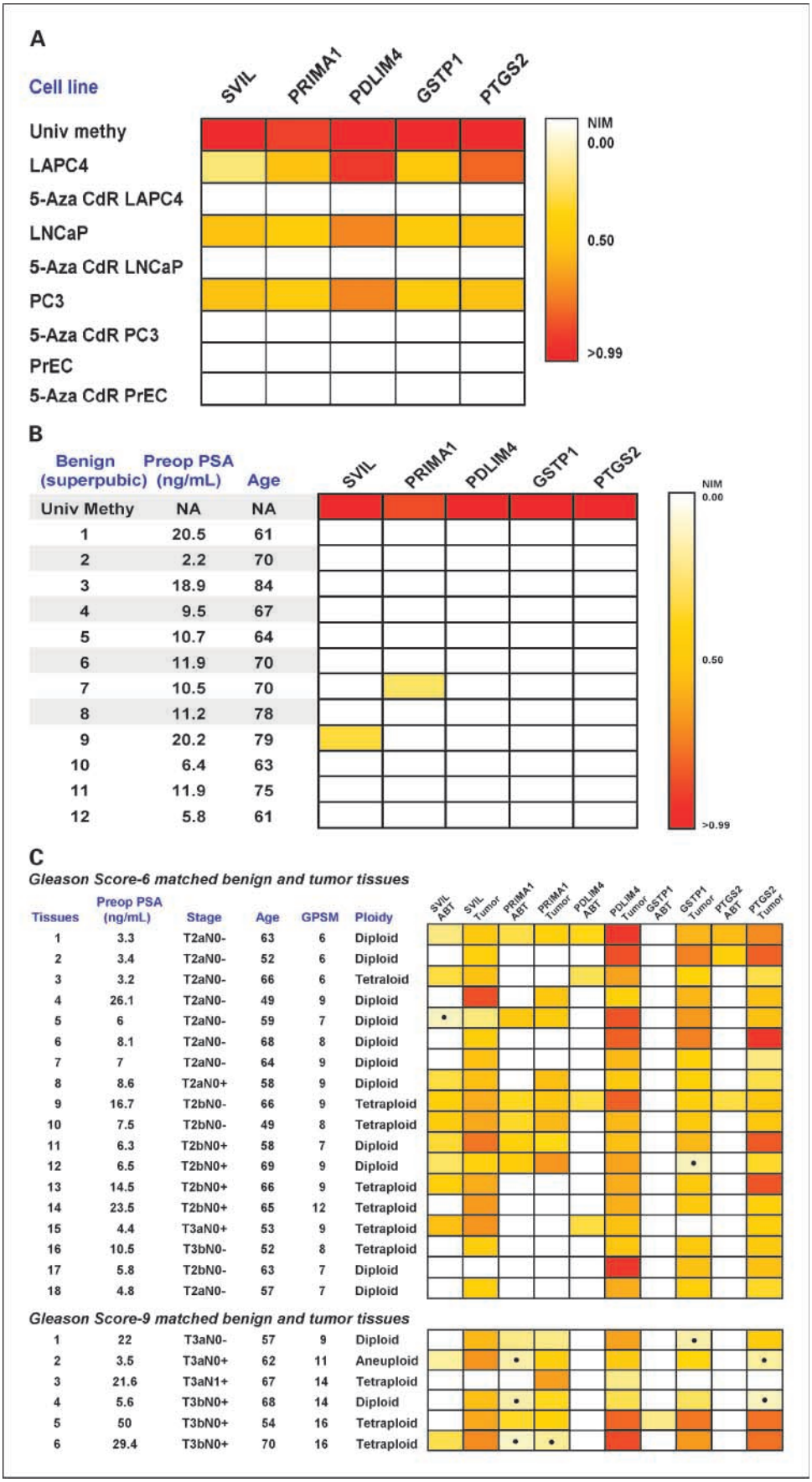

Fig. 3. Normalized methylation index of genes in cell lines treated with and without 5-Aza-CdR (*A*); benign tissues ($n = 12$) obtained from superpubic prostectomy (*B*); and matched tumor and adjacent benign tissues (*ABT*) from patients with Gleason score 6 ($n = 18$) and Gleason score 9 ($n = 6$) tumors (*C*).

quantification. Cell lines with 5-Aza-CdR treatment showed an increase in mRNA levels of *PDLIM4*, *SVIL*, and *PRIMA1* in LAPC4, LNCaP, and PC3 compared with untreated cells (Fig. 2), indicating silencing of gene expression by methylation. In PrEC cells, no significant increase in the gene expression levels was observed with 5-Aza-CdR treatment.

Quantitative assessment of promoter methylation. Quantitative methylation-specific PCR was carried out to assess the degree of methylation of cytosine residues in the 5′ CpG dinucleotides of *PDLIM4*, *SVIL*, and *PRIMA1* in prostate tumors, adjacent benign tissues, and prostate cell lines with and without 5-Aza-CdR treatment using TaqMan primers and probe (Table 3). Two known methylated genes, *PTGS2* and *GSTP1*, were included as references. Treatment of prostate cancer cell lines with 5-Aza-CdR abolished the methylation of the genes in LAPC4, LNCaP, and PC3 cells. A summary of the NIM for the prostate cancer cell lines, benign prostate, and primary and metastatic tissues at each of the five promoter CpG islands is depicted (Fig. 3). The frequencies of methylation of genes in prostate cancer and benign tissues are shown (Table 4). The overall NIM of the genes in tumor tissues was as follows: *PDLIM4* (98%), *PTGS2* (94%), *GSTP1* (90%), *SVIL* (83%), and *PRIMA1* (45%). However, a low prevalence of methylation was detected in benign prostate tissues: *GSTP1* (2.77%), *PTGS2* (8.33%), *PDLIM4* (11.1%), *SVIL* (36.1%), and *PRIMA1* (33.3%).

Sensitivity and specificity of methylation markers for diagnosis of prostate cancer. To assess the potential utility of hypermethylation of genes as molecular markers of prostate cancer, we determined the optimal sensitivity and specificity of methylation by ROC analysis. Optimal thresholds of methylation yielding the maximum sensitivity and specificity for each methylation marker were calculated. This analysis revealed that *PDLIM4*, *GSTP1*, and *PTGS2* had ROC areas under the curve (AUC) of >0.9 with sensitivities and specificities of 85% to 100% (Table 5). To identify combinations of genes that improve the sensitivity and

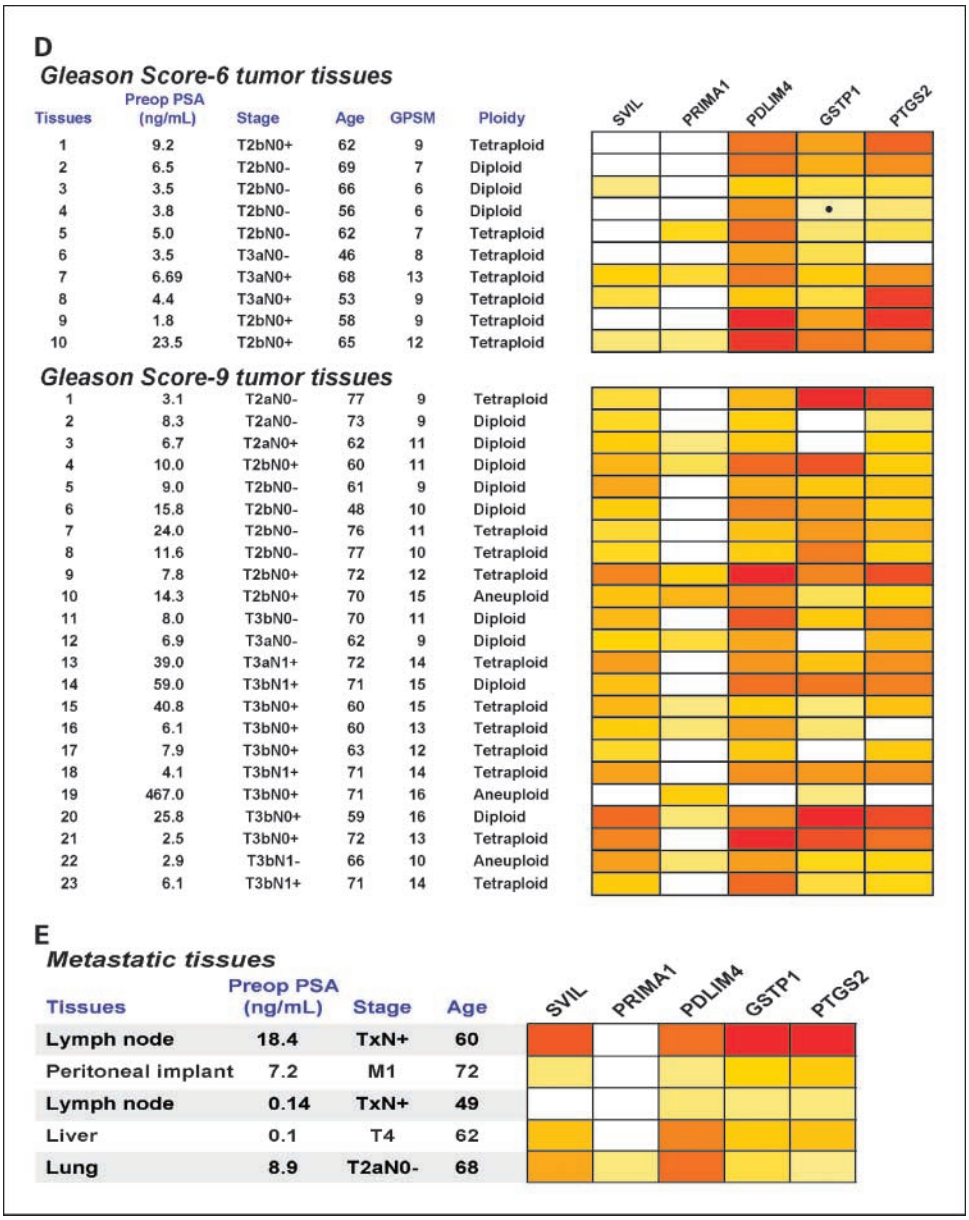

Fig. 3 *Continued.* Normalized methylation index of genes in Gleason score 6 ($n = 10$) and Gleason score 9 ($n = 23$) tumor tissues (*D*) and distant metastatic tissues ($n = 5$; *E*). NIM presented is color scaled from white to red such that white represents an NIM of zero (no methylation detected) and red represents an NIM of >0.99 (>99% of input DNA is methylated). *, NIM values <0.20 but greater than the calculated threshold for each CpG island. Univ. Methy., universally methylated DNA. Clinical and pathologic characteristics of patients are given on the left.

specificity of the individual markers, AUCs of the individual markers were compared with the AUCs of the combination. The statistical significance of the improvement of the combination over each individual marker is indicated by the *P* values. The combination of *GSTP1*/*PDLIM4* showed statistically significant improvements above any individual marker of the pairs. *GSTP1*/*PDLIM4* had a significantly larger AUC value than *GSTP1* alone. Although the combinations of *SVIL*/*PTGS2* and *PDLIM4*/*PTGS2* produced larger AUC values than the individual markers alone, the increase did not achieve statistical significance. When the markers are used in combination, the sensitivities and specificities increased to ≥90% (in most cases). Evaluation of the diagnostic capability of the markers in combination indicates that *PDLIM4* combined with *GSTP1* had the greatest sensitivity and specificity for distinguishing prostate cancer tissue from benign prostate tissue.

Discussion

This study represents an extension of our previous efforts (18) to identify reliable new biomarkers of prostate cancer. In this study, we further analyzed the expression levels of the genes that are deregulated in our microarray analysis using U95Av2 (18) and U133 chips. We validated the mRNA expression levels of eight underexpressed genes (*PRIMA1*, *TU3A*, *PDLIM4*, *FLJ14084*, *SVIL*, *SORBS1*, *C21orf63*, and *KIAA1210*) and three overexpressed genes (*FABP5*, *SOX4*, and *MLP*) in prostate cancer. Our results confirmed that the expression of down-regulated genes was significantly different in patients with intermediate and high-grade prostatic adenocarcinoma versus adjacent noncancerous benign tissues and metastatic versus organ-confined tumors (Table 1). A consistent reduction in the expression of the eight down-regulated genes in prostate cancer suggests their association with cancer development and progression. Deregulation of these genes in prostate cancer is consistent with previous microarray studies (25–32). In contrast to the down-regulated genes, the up-regulated genes had a heterogeneous expression pattern in the tumors tissues. Therefore, in this report we focused on the three most down-regulated genes.

Aberrant DNA methylation of CpG islands in the promoter region of a tumor suppressor gene can repress its transcription (13, 34, 35). Our goal was to determine if the validated genes above are silenced by methylation and can serve as methylation markers to assist in reliable diagnosis of prostate cancer. To explore the mechanism by which these genes might be silenced, we treated prostate cancer cell lines with a demethylating agent 5-Aza-CdR. Aberrant methylation of *PDLIM4*, *SVIL*, and *PRIMA1* was found to correlate with low transcription levels and reactivation was observed after treatment with 5-Aza-CdR. We observed hypermethylation of these genes not only in the prostate cancer cell lines but also in prostate cancer tissues. We therefore conclude that the aberrant methylation of the genes is associated with down-regulation of the gene expression levels in prostate cancer.

SVIL, which is an androgen receptor coregulator and F-actin binding protein, can potentiate androgen receptor activity in nonmuscle cells (36, 37). Down-regulation of *SVIL* expression in prostate cancer was shown in previous reported microarray analyses (26, 30–32). The correlation of *SVIL* down-regulation in prostate cancer with the deregulation of androgen receptor function and tumor progression remains to be determined. *PRIMA1*, a small peptide molecule that binds to p53, was expressed in normal prostate but was down-regulated in primary and metastatic prostate cancer tissues. Detection of *SVIL* and *PRIMA1* methylation in ~50% of the tumor tissues indicates partial silencing of transcriptional levels by promoter methylation. The mechanism contributing to the silencing of these genes needs further evaluation.

PDLIM4, also called reversion-induced LIM gene (*RIL*), is a PDZ and LIM domain–containing protein (38). It was identified as a potential tumor suppressor gene that is involved in maintenance of normal cell growth. Restoration of the expression of *PDLIM4* was observed in phenotypic revertants of original H-ras transformed cells (39, 40). Furthermore, it has been shown that *PDLIM4* modulates actin stress fiber dynamics through its association with α-actin (38). Our results show that a high rate of promoter methylation of *PDLIM4* could be a primary mechanism to suppress its expression in prostate cancer tissues, which is consistent with several other reports of down-regulation of this gene in prostate cancer (1, 26, 32). Notably, methylation of *PDLIM4* exhibits a high sensitivity for prostate cancer (94.7%) followed by *GSTP1* (89.5%), *PTGS2* (87.7%), and *SVIL* (75.4%). However, *GSTP1* (95.8%) exhibited a better specificity than *PDLIM4* (90.5), *PTGS2* (90.2%), and *SVIL* (75%). Our findings of *GSTP1* methylation in prostate cancer support previous reports (16, 41–43). *PTGS2* was reported to be methylated in prostate cancer (88%) in a relatively large collection of samples (23). Interestingly, the hypermethylation of *PTGS2* was shown to be associated with a higher risk of recurrence of prostate cancer. Our study supports the aberrant methylation of *PTGS2*. However, we were not able to make a conclusion about the association of its methylation with recurrence of the disease because only a small number of patients had recurrence (8 of 62). Moreover, we did not observe a strong correlation between accumulation of methylated target

Table 4. Frequencies of methylation of genes in prostate tissue samples analyzed by quantitative methylation-specific PCR

Samples	Total	Methylated (%)				
		SVIL	*PRIMA1*	*PDLIM4*	*GSTP1*	*PTGS2*
Benign prostatic tissues	36	13 (36.1)	12 (33.3)	4 (11.1)	1 (2.77)	3 (8.33)
Gleason score 6 tumors	28	21 (75)	11 (39.2)	28 (100)	27 (96.4)	27 (96.4)
Gleason score 9 tumors	29	27 (93.1)	16 (55.2)	28 (96.5)	24 (82.7)	26 (89.6)
Metastatic tissues	5	4 (80)	1 (20)	5 (100)	5 (100)	5 (100)

Table 5. Summary of significance of genes as methylation markers individually and in pairwise combinations

Gene	Sensitivity* (95% confidence interval)	Specificity* (95% confidence interval)	AUC (*P*)	Combined AUC compared with individual marker AUC	
				Marker-1, *P*	Marker-2, *P*
GSTP1	89.5 (81.4-94.9)	95.8 (89.5-98.9)	94.3 (<0.001)	—	—
SVIL	75.5 (65.4-83.7)	75.0 (64.9-83.4)	81.8 (<0.001)	—	—
PRIMA1	47.4 (36.9-98.3)	54.2 (43.5-64.6)	54.2 (0.248)	—	—
PDLIM4	94.7 (88-98.3)	90.5 (82.5-97.2)	98.5 (<0.001)	—	—
PTGS2	87.7 (79.3-93.6)	90.2 (75-95.5)	92.9 (<0.001)	—	—
GSTP1 and *SVIL*	91.2 (83.5-96.1)	89.8 (74.8-96.4)	97.0 (<0.001)	0.06	0.01
GSTP1 and *PDLIM4*	98.2 (93-99.8)	98.8 (84.3-100)	98.6 (<0.001)	0.01	0.18
GSTP1 and *PTGS2*	91.2 (83.5-96.1)	94.3 (82.2-98.4)	98.1 (<0.001)	0.03	0.04
SVIL and *PDLIM4*	94.7 (88-98.3)	93.2 (80.5-96.9)	93.7 (<0.001)	0.01	0.51
SVIL and *PTGS2*	87.7 (79.3-93.6)	91.8 (79-96.5)	93.7 (<0.001)	0.04	0.35
PDLIM4 and *PTGS2*	94.5 (88-98.3)	90.5 (84.5-98.9)	98.1 (<0.001)	0.38	0.05

NOTE: Performance of the genes was analyzed by AUC of the individual marker and in combinations. Sensitivity and specificity are reported for the threshold that minimizes sensitivity − specificity. The *P* value indicates the statistical significance of the improvement of the AUC attained by using the combination compared with the individual marker. Marker-1 and marker-2 *P* values compare the AUC of the first/second marker alone to the AUC of the combination.

genes and clinicopathologic factors of prostate cancer. Studies are in progress to elucidate the role of *PDLIM4* and *SVIL* methylation in oncogenic transformation and cancer progression.

Remarkably, the combined use of the methylation status of *PDLIM4*, *GSTP1*, and *PTGS2* improved the theoretical detection rate of prostate adenocarcinoma to 95% to 100% compared with that of each gene tested individually (Table 5). It is noteworthy that this finding was attained without compromising specificity because the definition of the cutoff values took into consideration the highest methylation level detected in benign lesions for each gene. Thus, it may be possible to augment the detection of prostate cancer by analyzing methylation of multiple genes in combination. Moreover, as these genes may be involved in important molecular pathways of carcinogenesis of the prostate (36–38, 44, 45), they could serve as targets for therapies. These findings warrant further validation of these genes in a large series of prospectively collected samples and exploring the implications of these genes in diagnosis and treatment of prostate cancer.

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