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Advances in Brief

Transcriptional Silencing of Zinc Finger Protein 185 Identified by Expression Profiling Is Associated with Prostate Cancer Progression[1]

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Abstract

We profiled the expression of genes in benign and untreated human prostate cancer tissues using oligonucleotide microarrays. We report here 50 genes with distinct expression patterns in metastatic and confined tumors (Gleason score 6 and 9; lymph node invasive and noninvasive). Validation of expression profiles of 6 genes by quantitative PCR revealed a strong inverse correlation in the expression of zinc finger protein 185 (*ZNF185*), bullous pemphigoid antigen gene (*BPAG1*), and prostate secretory protein (*PSP94*) with progression of prostate cancer. Treatment of prostate cancer cell lines with 5-aza-2′-deoxycytidine (5-Aza-CdR), an inhibitor of DNA methylation, restored *ZNF185* expression levels. Moreover, methylation-specific PCR confirmed methylation of the 5′CpG islands of the *ZNF185* gene in all of the metastatic tissues and 44% of the localized tumor tissues, as well as in the prostate cancer cell lines tested. Thus, transcriptional silencing of *ZNF185* by methylation in prostate tumor tissues implicates the *ZNF185* gene in prostate tumorigenesis.

Introduction

Tumor stage, Gleason score, and preoperative serum prostate specific antigen are currently the only well-recognized predictors of prostate cancer progression. However, these markers cannot reliably identify men that ultimately fail therapy, and they give us no insight into prostate carcinogenesis or potential therapeutic targets for prostate cancer (1). Several groups recently implemented DNA microarrays to analyze differentially expressed genes between corresponding normal and cancer tissues to advance the understanding of the molecular basis of malignancy, and potentially serve as biomarkers or prognostic markers of malignancy (2–6). The altered expression of the genes characteristic of the specific stage of the cancer is now being considered as a supplementary approach to the histopathological work-up of precancerous and cancerous lesions of the prostate (7).

Inactivation of tumor suppressor genes is an important event contributing to the development of neoplastic malignancies. In addition to the classical genetic mechanisms involving deletion or inactivating point mutations, growth regulatory genes can be functionally inactivated by epigenetic alterations, *i.e.,* alterations in the genome other than the DNA sequence itself, which include global genomic hypomethylations (8), promoter hypermethylation of CpG islands (9, 10), histone deacetylations, and chromatin modifications (11, 12). Molecular analysis of tumor-derived genetic and epigenetic alterations may have a profound impact on cancer diagnosis and monitoring for tumor recurrence (10).

The objective of this study was to identify biologically and clinically relevant clusters of genes characteristic of prostate cancer *versus* benign tissues and confined *versus* metastatic prostate cancer using oligonucleotide microarrays. The expression profiles were generated from 5 metastatic prostate tissues, and 23 confined tumors including 12 Gleason score 9 (high grade) and 11 Gleason score 6 (intermediate grade) tumors. In addition, 8 adjacent benign prostatic tissues were also studied. We have shown 50 genes with distinct expression patterns in prostate cancer compared with benign prostatic tissues. Expression levels of prostate secretory protein (*PSP94*), zinc finger protein (*ZNF185*), bullous pemphigoid antigen gene (*BPAG1*), prostate specific transglutaminase gene (*TGM4*), Erg isoform 2 (*Erg-2*), and Rho GDP dissociation inhibitor (*RhoGDI-β*) were validated by Taqman quantitative real-time PCR. Furthermore, analysis of the expression of *ZNF185* in prostate cancer cell lines revealed an increase in the expression by treatment with an inhibitor of DNA methylation, 5-Aza-CdR. MSP indicated *ZNF185* inactivation by CpG dinucleotide methylations in prostate cancer cell lines and cancer tissues. Our studies show that down-regulation of *ZNF185*, *PSP94*, and *BPAG1* with epigenetic alteration of *ZNF185* is highly associated with prostate cancer progression and potentially can be useful as a biomarker for predicting progression of the cancer.

Materials and Methods

Prostate Tissues. Prostate cancer tissue specimens were obtained from patients who had undergone radical prostatectomy for prostate cancer at Mayo Clinic. The Institutional Review Board of Mayo Foundation approved collection of tissues and their use for this study. None of the patients included in this study had received preoperative hormonal therapy, chemotherapy, or radiotherapy. Harvested tissues were embedded in OCT and frozen at −80°C until use. A H&E-stained section was prepared to insure that tumor was present in the tissue used for the analyses. Of 340 tissues available in our tissue bank, we selected tissues that had >80% of the neoplastic cells by histological examination. To examine differential gene expression in intermediate- (Gleason score 6) and high-grade (Gleason score 9) prostatic adenocarcinoma and metastatic tumors, we studied 11 primary stage T2 Gleason score 6 cancers (6 with positive regional lymph nodes and 5 with negative lymph nodes), 12 primary stage T3 Gleason score 9 cancers (6 with positive regional lymph nodes and 6 with negative lymph nodes), and 5 metastatic tumors. Table 1 shows Gleason grade, age, preoperative serum prostate-specific antigen levels, and staging of all of the patients from whom prostate tissues were obtained for this study. Twelve separately collected prostatic tissue samples matched with the cancer tissues (obtained from the same patients) were used as normal controls.

Isolation of RNA and Gene Expression Profiling. Thirty prostate tissue sections of 15-μm thickness were cut with a cryostat and used for RNA isolation. Total RNA was extracted from frozen tissue sections with TRIzol reagent (Life Technologies, Inc., Carlsbad, CA). DNA was removed by treatment of the samples with DNase I using DNA-free kit (Ambion, Austin, TX), and additional RNA cleanup was performed using RNeasy Mini kit (Qiagen,

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[3] The abbreviations used are: PSP94, prostate secretory protein 94; ZNF185, zinc finger protein 185; BPAG1, bullous pemphigoid antigen gene 1; MSP, methylation-specific PCR; 5-Aza-CdR, 5-aza-2′-deoxycytidine.

Table 1 *Patient data*

Gleason grade/ lymph node	Sample ID	Age	Preop PSA (ng/ml)	Tumor-Node-Metastasis (97)	Metastatic site
6/Negative	6N 1	55	9.4	T2b,N0−	
	6N 2	50	7.5	T2b,N0−	
	6N 3	57	10.3	T2b,N0−	
	6N 4	67	16.7	T2b,N0−	
	6N 5	68	8.1	T2a,N0−	
6/Positive	6P 1	71	17.1	T2b,N1+	
	6P 2	61	5.2	T2b,N0+	
	6P 3	71	41.0	T2b,N0+	
	6P 4	65	7.0	T2a,N0+	
	6P 5	51	14.3	T2b,N0+	
	6P 6	66	23.5	T2b,N0+	
9/Negative	9N 1	67	21.6	T3a,N0−	
	9N 2	65	29.4	T3b,N0−	
	9N 3	65	24.9	T3b,N0−	
	9N 4	54	50.0	T3b,N0−	
	9N 5	59	25.8	T3b,N0−	
	9N 6	71	6.1	T3b,N0−	
9/Positive	9P 1	66	4.5	T3a,N0+	
	9P 2	65	6.69	T3b,N0+	
	9P 3	76	7.6	T3b,N1+	
	9P 4	71	467.0	T3b,N0+	
	9P 5	69	5.6	T3b,N0+	
	9P 6	66	2.9	T3b,N1−	
Metastatic	Met 1	62	0.15		Liver
	Met 2	72	97.3		Peritoneum
	Met 3	49	0.15		Lymph node
	Met 4	60	18.4		Lymph node
	Met 5	68	8.9		Lung

Valencia, CA) according to the manufacturer's protocols. RNA quality was monitored by agarose gel electrophoresis and also on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). High-density oligonucleotide microarrays HG-U95Av2 containing 12,625 sequences of human genes and expressed sequence tags (Affymetrix, Santa Clara, CA) were used in this study. Complementary RNA was prepared, labeled, and hybridized to oligonucleotide arrays as described previously (13). The arrays were scanned with gene array scanner (Agilent Technologies). All of the arrays were scaled to a target intensity of 1500. Raw data were collected and analyzed by using Affymetrix Microarray Suite 5.0 version.

Quantitative Real-Time Reverse Transcription-PCR. To confirm the differential expression of genes from microarray data, we selected 4 down-regulated genes, *ZNF185, PSP94, BPAG1*, and *TGM4,* and 2 up-regulated genes, *Erg-2* and *RhoGDI-β,* for validation by Taqman real-time reverse transcription-PCR in a total of 44 tissues including 36 samples used for microarrays with an additional 4 primary tumors and 4 adjacent benign tissues. One μg of the total RNA was used for first-strand cDNA synthesis. The PCR mix contained 1× reaction buffer [10 mM Tris and 50 mM KCl (pH 8.3)], MgCl2 (5 mM), PCR nucleotide mix (1 mM), random primers (0.08 A260 units), RNase inhibitor (50 units), and avian myeloblastosis virus reverse transcriptase (20 units) in a final volume of 20 μl.

For real-time PCR 1 μl of the cDNA was used in the PCR reactions. Taqman real-time primers and probes were designed using the software Primer Express version 1.5 (PE Applied Biosystems, Foster City, CA) and synthesized at Integrated DNA Technologies (Coralville, IA). The sequences of the primers and probes for each gene are provided in Fig. 2*A*. Probes were labeled at 5′ end with the reporter dye 6-carboxyfluorescein and at 3′ end with a Black Hole Quencher. Probes were purified by reverse-phase high-performance liquid chromatography, and primers were PAGE purified. All of the PCR reactions were carried out in Taqman Universal PCR master mix (PE Applied Biosytems) with 300 nM of each primer and 200 nM of probe in a final volume of 50 μl. Thermal cycling conditions were as follows: 2 min at 50°C, with denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C (melting), and 1 min at 60°C (annealing and elongation). The reactions were performed in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). To evaluate the validity and sensitivity of real-time quantitative PCR, serial dilutions of the oligonucleotide amplicon of the gene in a range of 1 to 1×10^9 copies were used as corresponding standard. Standard curves were generated using the C_t values determined in the real-time PCR to permit gene quantification using the supplied software according to the manufacturer's instructions. In addition, a standard curve was generated for the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (Applied Biosystems; part number 402869) to enable normalization of each gene. Data were expressed as relative copy number of transcripts after normalization.

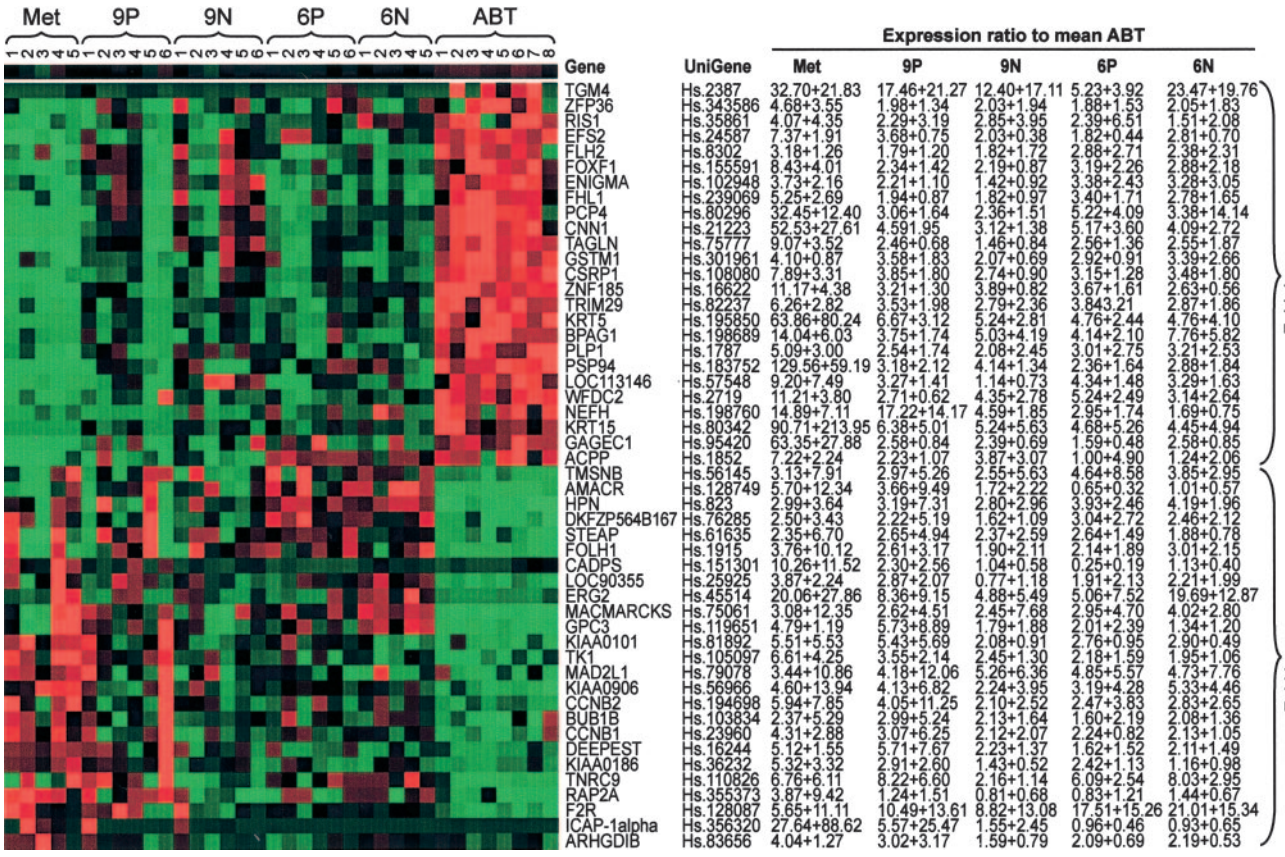
Cell Lines and 5-Aza-CdR Treatment. The human prostate cancer cell lines LNCaP, PC3 (American Type Culture Collection, Rockville, MD), and LAPC4 (a gift from Dr. Charles L. Sawyers, University of California Los Angeles, Los Angeles, CA) were grown in RPMI 1640 supplemented with 5% fetal bovine serum at 37°C and 5% CO_2 until reaching approximately 50–70% confluence. Cells were then treated with 5% fetal bovine serum RPMI 1640 containing 6 μM 5-Aza-CdR (Sigma Chemicals Co., St. Louis, MO) for 6 days, with medium changes on days 1, 3, and 5. Total RNA was isolated from the cell lines, and the expression of the *ZNF185* was analyzed by Taqman real-time PCR as described above. The housekeeping gene *GAPDH* was used as an internal control to enable normalization.

DNA Isolation and Bisulfite Modification. Genomic DNA was obtained from metastatic, primary, matched benign prostatic tissues and the above mentioned prostate cancer cell lines treated with 5-Aza-CdR, using Wizard genomic DNA purification kit according to the manufacturer's protocol (Promega, Madison, WI). Genomic DNA (100 ng) was modified by sodium bisulfite treatment by converting unmethylated, but not methylated, cytosines to uracil as described previously (14). DNA samples were then purified using the spin columns (Qiagen) and eluted in 50 μl of distilled water. Modification was completed by treatment with NaOH (0.3 M final concentration) for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used for PCR amplification.

MSP. DNA methylation patterns within the gene were determined by chemical modification of unmethylated cytosine to uracil and subsequent PCR as described previously (15) using primers specific for either methylated or the modified unmethylated sequences. The primers used for MSP were shown in Fig. 3*B*. Two sets of primers were designed corresponding to the genomic positions around 210 and 335. Genomic position indicates the location of the 5′ nucleotide of the sense primer in relation to the major transcriptional start site defined in the GenBank accession no. Y09538. The PCR mixture contained 1× PCR buffer [50 mM KCl and 10 mM Tris-HCl (pH 8.3) with 0.01% w/v gelatin], deoxynucleoside triphosphates (0.2 mM each), primers (500 μM), and bisulfite-modified or -unmodified DNA (100 ng) in a final volume of 25 μl. Reactions were hot-started at 95°C for 10 min with the addition of 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). Amplifications were carried out in GeneAmp PCR systems 9700 (Applied Biosystems) for 35 cycles (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C), followed by a final 7-min extension at 72°C. Appropriate negative and positive controls were included in each PCR reaction. One μl of the PCR product was loaded directly onto DNA 500 lab chip and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies).

Results and Discussion

We monitored gene expression profiles of 28 prostate cancer tissues using oligonucleotide microarrays. To identify genes differentially expressed between cancer and benign tissues, we performed a gene-by-gene analysis of the difference in mean log expression between the two groups. Genes were ranked according to intersample variability (SD), and 1850 genes with the most variable expression across all of the samples were median-centered and normalized with respect to other genes in the samples and corresponding genes in the other samples. Genes and samples were subjected to hierarchical clustering essentially as described previously (16). Differential expression of genes in benign and malignant prostate tissues was estimated using an algorithm (13) based on equally weighted contributions from the difference of hybridization intensities (μTumor-μNormal) or (μNormal-μTumor), the quotient of hybridization intensities (μTumor/μNormal) or (μNormal/μTumor), and the result of an unpaired *t* test between expression levels in tumor and normal tissues. We narrowed down the selection criteria to genes that showed a fold change of >2.35 between normal and cancer samples and a $P < 0.001$ by Student's *t* test. We present a cluster of 25 up-regulated and 25



Gene	UniGene	Expression ratio to mean ABT: Met	9P	9N	6P	6N	
TGM4	Hs.2387	32.70+21.83	17.46+21.27	12.40+17.11	5.23+3.92	23.47+19.76	Fold decrease
ZFP36	Hs.343586	4.68+3.55	1.98+1.34	2.03+1.94	1.88+1.53	2.05+1.83	
RIS1	Hs.35861	4.07+4.35	2.29+3.19	2.85+3.95	2.39+6.51	1.51+2.08	
EFS2	Hs.24587	7.37+1.91	3.68+0.75	2.03+0.38	1.82+0.44	2.81+0.70	
FLH2	Hs.8302	3.18+1.26	1.79+1.20	1.82+1.72	2.88+2.71	2.38+2.31	
FOXF1	Hs.155591	8.43+4.01	2.34+1.42	2.19+0.87	3.19+2.26	2.88+2.18	
ENIGMA	Hs.102948	3.73+2.16	2.21+1.10	1.42+0.92	3.38+2.43	3.28+3.05	
FHL1	Hs.239069	5.25+2.69	1.94+0.87	1.82+0.97	3.40+1.71	2.78+1.65	
PCP4	Hs.80296	32.45+12.40	3.06+1.64	2.36+1.51	5.22+4.09	3.38+14.14	
CNN1	Hs.21223	52.53+27.61	4.591.95	3.12+1.38	5.17+3.60	4.09+2.72	
TAGLN	Hs.75777	9.07+3.52	2.46+0.68	1.46+0.84	2.56+1.36	2.55+1.87	
GSTM1	Hs.301961	4.10+0.87	3.58+1.83	2.07+0.69	2.92+0.91	3.39+2.66	
CSRP1	Hs.108080	7.89+3.31	3.85+1.80	2.74+0.90	3.15+1.28	3.48+1.80	
ZNF185	Hs.16622	11.17+4.38	3.21+1.30	3.89+0.82	3.67+1.61	2.63+0.56	
TRIM29	Hs.82237	6.26+2.82	3.53+1.98	2.79+2.36	3.843.21	2.87+1.86	
KRT5	Hs.195850	63.86+80.24	6.67+3.12	5.24+2.81	4.76+2.44	4.76+4.10	
BPAG1	Hs.198689	14.04+6.03	3.75+1.74	5.03+4.19	4.14+2.10	7.76+5.82	
PLP1	Hs.1787	5.09+3.00	2.54+1.74	2.08+2.45	3.01+2.75	3.21+2.53	
PSP94	Hs.183752	129.56+59.19	3.18+2.12	4.14+1.34	2.36+1.64	2.88+1.84	
LOC113146	Hs.57548	9.20+7.49	3.27+1.41	1.14+0.73	4.34+1.48	3.29+1.63	
WFDC2	Hs.2719	11.21+3.80	2.71+0.62	4.35+2.78	5.24+2.49	3.14+2.64	
NEFH	Hs.198760	14.89+7.11	17.22+14.17	4.59+1.85	2.95+1.74	1.69+0.75	
KRT15	Hs.80342	90.71+213.95	6.38+5.01	5.24+5.63	4.68+5.26	4.45+4.94	
GAGEC1	Hs.95420	63.35+27.88	2.58+0.84	2.39+0.69	1.59+0.48	2.58+0.85	
ACPP	Hs.1852	7.22+2.24	2.23+1.07	3.87+3.07	1.00+4.90	1.24+2.06	
TMSNB	Hs.56145	3.13+7.91	2.97+5.26	2.55+5.63	4.64+8.58	3.85+2.95	Fold increase
AMACR	Hs.128749	5.70+12.34	3.66+9.49	1.72+2.22	0.65+0.32	1.01+0.57	
HPN	Hs.823	2.99+3.64	3.19+7.31	2.80+2.96	3.93+2.46	4.19+1.96	
DKFZP564B167	Hs.76285	2.50+3.43	2.22+5.19	1.62+1.09	3.04+2.72	2.46+2.12	
STEAP	Hs.61635	2.35+6.70	2.65+4.94	2.37+2.59	2.64+1.49	1.88+0.78	
FOLH1	Hs.1915	3.76+10.12	2.61+3.17	1.90+2.11	2.14+1.89	3.01+2.15	
CADPS	Hs.151301	10.26+11.52	2.30+2.56	1.04+0.58	0.25+0.19	1.13+0.40	
LOC90355	Hs.25925	3.87+2.24	2.87+2.07	0.77+1.18	1.91+2.13	2.21+1.99	
ERG2	Hs.45514	20.06+27.86	8.36+9.15	4.88+5.49	5.06+7.52	19.69+12.87	
MACMARCKS	Hs.75061	3.08+12.35	2.62+4.51	2.45+7.68	2.95+4.70	4.02+2.80	
GPC3	Hs.119651	4.79+1.19	5.73+8.89	1.79+1.88	2.01+2.39	1.34+1.20	
KIAA0101	Hs.81892	5.51+5.53	5.43+5.69	2.08+0.91	2.76+0.95	2.90+0.49	
TK1	Hs.105097	6.61+4.25	3.55+2.14	2.45+1.30	2.18+1.59	1.95+1.06	
MAD2L1	Hs.79078	3.44+10.86	4.18+12.06	5.26+6.36	4.85+5.57	4.73+7.76	
KIAA0906	Hs.56966	4.60+13.94	4.13+6.82	2.24+3.95	3.19+4.28	5.33+4.46	
CCNB2	Hs.194698	5.94+7.85	4.05+11.25	2.10+2.52	2.47+3.83	2.83+2.65	
BUB1B	Hs.103834	2.37+5.29	2.99+5.24	2.13+1.64	1.60+2.19	2.08+1.36	
CCNB1	Hs.23960	4.31+2.88	3.07+6.25	2.12+2.07	2.24+0.82	2.13+1.05	
DEEPEST	Hs.16244	5.12+1.55	5.71+7.67	2.23+1.37	1.62+1.52	2.11+1.49	
KIAA0186	Hs.36232	5.32+3.32	2.91+2.60	1.43+0.52	2.42+1.13	1.16+0.98	
TNRC9	Hs.110826	6.76+6.11	8.22+6.60	2.16+1.14	6.09+2.54	8.03+2.95	
RAP2A	Hs.355373	3.87+9.42	1.24+1.51	0.81+0.68	0.83+1.21	1.44+0.67	
F2R	Hs.128087	5.65+11.11	10.49+13.61	8.82+13.08	17.51+15.26	21.01+15.34	
ICAP-1alpha	Hs.356320	27.64+88.62	5.57+25.47	1.55+2.45	0.96+0.46	0.93+0.65	
ARHGDIB	Hs.83656	4.04+1.27	3.02+3.17	1.59+0.79	2.09+0.69	2.19+0.53	

Fig. 1. Expression of 50 significantly regulated genes in 36 prostate tissue samples. Cluster diagram depicting genes that distinguish metastatic (*Met;* $n = 5$) from confined tumors with Gleason score 9 lymph node-positive (*9P;* $n = 6$) or -negative (*9N;* $n = 6$), and Gleason score 6 lymph node-positive (*6P;* $n = 6$) or -negative (*6N;* $n = 5$) prostate cancer and adjacent benign tissues (*ABT;* $n = 8$; *n* represents the number of tissues). Each *row* represents a gene and each *column* a tissue sample. *Red* and *green* represent up-regulation and down-regulation, respectively, relative to the median of the reference pool. *Gray* represents technically inadequate or missing data, and *black* represents equal expression relative to the reference samples. Color saturation is proportional to the magnitude of the difference from the mean. Each gene is labeled by its gene name. Mean and SD of the fold change in the expression levels of genes compared with ABT is shown.

down-regulated genes, which discriminated between normal and cancer tissues (Fig. 1).

In general we did not observe good correlation in expression of genes between Gleason grades with or without lymph node metastasis, which might be because of the limitation that the expression profiles were not generated from homogeneous cell population. Among the 25 down-regulated genes we identified (Fig. 1), *PSP94, BPAG1, WFDC2, KRT5, KRT15, TAGLN, ZFP 36,* and the genes encoding LIM domain proteins *FLH1*, *FLH2*, and *ENIGMA* are consistent with the expression profiles of the previous studies (2, 7, 17–19). Up-regulation of hepsin, *AMACR*, *STEAP*, *FOLH1*, *RAP2A*, and the unknown gene *DKFZP564B167* are consistent with the data published previously of microarray analysis (2–7, 18, 20, 21). In addition our data also confirm up-regulation of the cell cycle regulated genes *CCNB1, CCNB2, MAD2L1, DEEPEST,* and *BUB1B,* cell adhesion regulator *MACMARCKS,* and unclassified genes *KIAA0186* and *KIAA0906* (5, 7, 17, 21).

We selected *PSP94, ZNF185, BPAG1,* and *TGM4* from the 25 down-regulated genes, and *Erg-2* and *RhoGDI-β* from the 25 up-regulated genes for additional validation by Taqman quantitative PCR. These genes were selected because of their moderate- to high-level expression in prostate cancer. In addition, their potential functions as mentioned below may be highly relevant to prostate cancer biology. Furthermore, except for *PSP94*, their role in prostate cancer biology has not been described. *PSP94* has been shown to be down-regulated in prostate cancer (22) and is the most down-regulated gene in our microarray data.

To validate the expression profiles, Taqman quantitative PCR was performed in duplicate for each sample. The standard curve slope values for all of the genes ranged between −3.58 and −3.20, corresponding to PCR efficiency of >0.9. The Kruskal-Wallis global test was done with the real-time quantitative analysis for all of the genes. A significant decrease in the expression of *ZNF185*, *BPAG1*, and *PSP94* mRNA levels was observed in metastatic *versus* organ confined and localized tumors compared with benign tissues ($P < 0.0001$; Fig. 2*B*). Moreover, the Wilcoxon test was used to compare each tissue type to the adjacent benign tissues. *ZNF185*, *BPAG1*, and *PSP94* showed *P*s < 0.0019 in each group compared with benign tissues.

PSP94 is a highly prostate-specific gene encoding a major prostate secretory protein. Earlier studies reported that both the secretion and synthesis of *PSP94* were reduced in prostate cancer tissues (22). *PSP94* is involved in inhibition of tumor growth by apoptosis (23), and the down-regulation in prostate tumor tissues may be the survival mechanism for cancer cells. Our study suggests that *PSP94* may play a role in prostate cancer progression. *BPAG1* is a 230-kDa hemidesmosomal component involved in adherence of epithelial cells to the basement membrane. Previous studies have shown a loss of *BPAG1* in invasive breast cancer cells (24). The down-regulation of *BPAG1* in our study (>14-fold in metastatic tissues) could be an indicative of an invasive phenotype and might predict the potential of invasive cells to metastasize (25). *Erg-2* is a proto-oncogene known to play an important role in the development of cancer (26). We observed that *Erg-2* expression levels were increased in 16 of 32 (50%) cancer tissues

A

Gene		Primers and Probe	Amplicon bp
ZNF185	FP	TGG ATG AAA GGC AAG GTA AAG AG	84
	RP	TTC TAA AAC TCC CTT AAA GGC AGA CT	
	Probe	CCA AGA TAG GCT GGC TTC CCC CG	
PSP94	FP	AGT GAA TGG ATA ATC TAG TGT GCT TCT AGT	100
	RP	GCA TGG CTA CAC AAT CAT TGA CTA T	
	Probe	CCC AGG CCA GGC CTC ATT CTC CT	
BPAG1	FP	TCG CTG AAA GAG CAC GTC AT	94
	RP	AGC AAT CTA AAA CAC TGC AGC TTG	
	Probe	AAT CAA AGA GAA AGA TAT AAA TTC GTT CCC ACA GCC	
Erg-2	FP	TCC TGT CGG ACA GCT CCA AC	75
	RP	CGG GAT CCG TCA TCT TGA	
	Probe	TGC ATC ACC TGG GAA GGC ACC AAC	

B

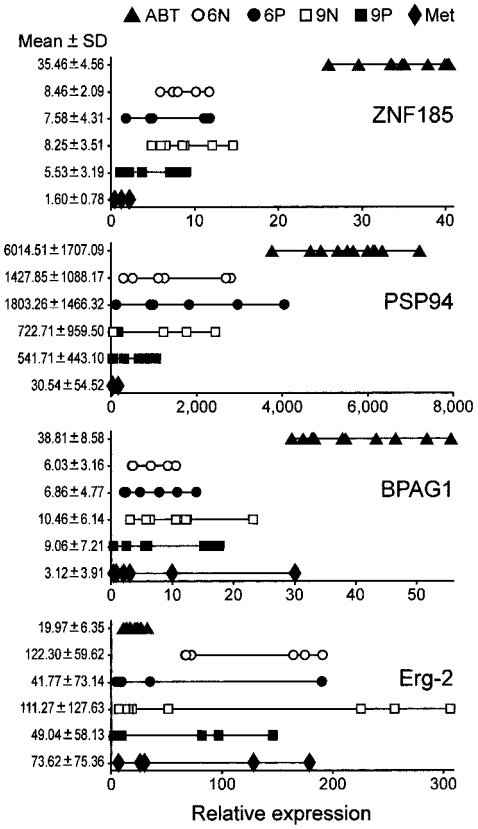
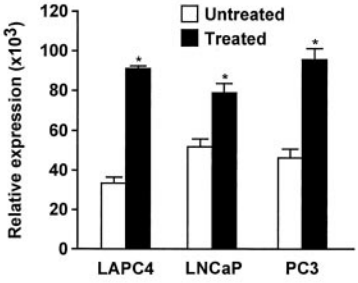


Fig. 2. *A*, forward primer (*FP*), reverse primer (*RP*), and probes used for Taqman real-time PCR. *B*, expression levels of genes *ZNF185, PSP94, BPAG1,* and *Erg-2* as validated by Taqman real-time PCR in 36 samples (28 cancer and 8 benign) used for microarray analysis and an additional 8 samples (4 cancer and 4 benign). Values are expressed as the copy number of the gene relative to glyceraldehyde-3-phosphate dehydrogenase levels. Metastatic tissues (*Met* ◆) $n = 5$, Gleason score 9, lymph node-positive (*9P* ■) $n = 7$ or -negative (*9N* □) $n = 8$ and Gleason score 6, lymph node-positive (*6P* ●) $n = 6$ or -negative tissues (*6N* ○) $n = 6$ and adjacent benign tissues (*ABT* ▲) $n = 12$ were used. (*n* represents the number of tissues). Mean ± SD of relative expression levels of each group is shown on the *left*.

when stringently compared with the highest level of *Erg-2* in 12 adjacent benign tissues. The increase in mRNA levels of *Erg-2* in at least half of the cancer tissues examined indicates a role of *Erg-2* in prostate cancer. These results warrant additional studies of *PSP94*, *BPAG1*, and *Erg-2* in prostate cancer. Furthermore, TGM4 is a prostate tissue specific transglutaminase (type IV) that has been implicated in apoptosis and cell growth (27). *RhoGDI-β* may be involved in cellular transformation (28). Our Taqman PCR study shows that *TGM4* and *RhoGDI-β* levels were not changed significantly in most of the prostate cancer tissues (data not shown).

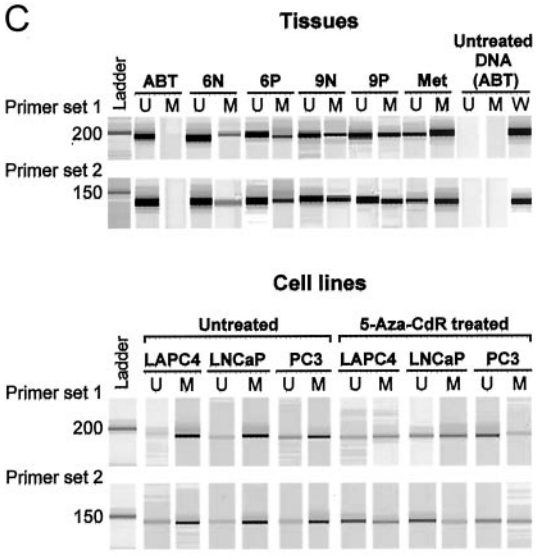
A



B

Primer set			Size (bp)	Genomic position
1 W	FP	GCGCAGTTCCGGGTGTCTGTC	197	210
	RP	GCGGGGAGGACCAGCGTTAG		
1 M	FP	GCGTAGTTTCGGGTGTTTG	197	210
	RP	ACGAAAAAAACCAACGTTAACTA		
1U	FP	GTGTAGTTTTGGGTGTTTGTTAGG	196	210
	RP	CAAAAAAACCAACATTAACTATTCTC		
2 W	FP	CCTGGGACTCCGTCAGACTGG	146	335
	RP	GACAGACACCCGGAACTGCG		
2 M	FP	TTGGGATTTCGTTAGATTGG	145	335
	RP	AACAAACACCCGAAACTACG		
2 U	FP	TGGGATTTTGTTAGATTGGAAAGG	146	333
	RP	CTAACAAACACCCAAAACTACACCA		

C



D

Samples	Total	Methylated (%)
Benign prostatic tissues	12	0 (0)
Gleason Score 6 tumors	11	4 (36.3)
Gleason Score 9 tumors	14	7 (50)
Metastatic tissues	5	5 (100)

Fig. 3. *A*, expression of *ZNF185* levels in prostate cancer cells treated with 6 μM 5-Aza-CdR for 6 days. Four separate experiments are represented; *bars,* ± SD. *, indicates statistical significance over the untreated cells ($P < 0.05\%$); *B*, the PCR primers [forward primer (*FP*), reverse primer (*RP*)], used for MSP of prostate tissues. *W* represents unmodified or wild type primers. *M,* methylated-specific primers; and *U,* unmethylated-specific primers. Sequence difference between modified primers and unmodified DNA are in *boldface type,* and differences between methylated/modified and unmethylated/modified are *underlined*. *C*, MSP analysis of *ZNF185* DNA in prostate tissue samples and cell lines with and without 5-Aza-CdR treatment. The amplified products were directly loaded onto DNA 500 lab chip and analyzed on Agilent 2100 Bioanalyzer. Molecular size marker is shown at *left*. All DNA samples were bisulfite-treated except those designated untreated. The experiments were repeated twice and the representative band of the PCR product in *Lanes U, M,* and *W* indicates the presence of unmethylated, methylated, and wild-type *ZNF185* DNA, respectively. *D*, summary of the incidence of methylation of *ZNF185* DNA in prostate tissues analyzed by MSP.

We focused on *ZNF185* for additional study because it is a novel LIM domain gene (29), and may play a role in prostate cancer development and progression. LIM domain proteins are known to play an important role in regulation of cellular proliferation and differentiation (30–34). *ZNF185* is located on chromosome Xq28, a chromosomal region of interest as a result of the >20 hereditary diseases mapped to this region. The LIM is a cysteine-rich motif that coordinately binds two zinc atoms and mediates protein-protein interactions. Heiss *et al.* (29) cloned a full-length *ZNF185* cDNA and showed that the transcript is expressed in a very limited number of human tissues with most abundant expression in the prostate. Our observation is the first to identify a relationship of *ZNF185* regulation and cancer. Here we report a significant down-regulation in the expression of ZNF185 gene in all of the prostate cancer tissues compared with benign prostatic tissues (Fig. 1; Fig. 2*B*). The decrease in *ZNF185* expression in prostate tumors indicates that *ZNF185* may play an important role in the development and progression of prostate cancer.

To study the transcriptional silencing of *ZNF185* in prostate cancer, we treated LAPC4, LNCaP, and PC3 prostate cancer cell lines with 5-Aza-CdR, an inhibitor of DNA methyl transferase DNMT1 (34). Treatment with 5-Aza-CdR showed an ~2-fold increase in mRNA levels of *ZNF185* (Fig. 3*A*) indicating that the gene might be partially silenced by methylation. To confirm the transcriptional inactivation, MSP was carried out to assess the methylation status of cytosine residues in the 5′ CpG dinucleotides of genomic DNA in prostate tumors, adjacent benign tissues, and in prostate cell lines with or without treatment with 5-Aza-CdR. Cytosine methylations within CpG dinucleotides were observed in the prostate cancer tissues and cell lines with two sets of primers used for PCR (Fig. 3*C*). A reduction of the methylated band and increase of the unmethylated band in cell lines with 5-Aza-CdR treatment is consistent with the restoration of *ZNF185* mRNA levels after demethylation. (Fig. 3*A*). In most of the tissues samples, DNA not treated with bisulfite (unmodified) failed to amplify with either set of methylated- or unmethylated-specific primers but readily amplified with primers specific for the sequence before modification, suggesting an almost complete bisulfite reaction. Methylation of *ZNF185* was accompanied by amplification of the unmethylated reaction as well. The presence of the unmethylated *ZNF185* DNA could indicate the presence of normal tissues in these nonmicrodissected samples. However, heterogeneity in the patterns of methylation in the tumor itself might also be present. Fisher's unordered test for methylation difference in metastatic, confined tumors and benign tissues was highly significant ($P < 0.0003$). The incidence of methylation in cancer tissues is shown in Fig. 3*D*. Methylation status and down-regulation in the mRNA expression is correlated with higher tumor grade and metastasis. These results suggest that methylation of CpG dinucleotides may be the major factor causing transcriptional inactivation of *ZNF185* and repressing its expression in the prostate cancer tissues.

In summary we show that mRNA expression analysis with oligonucleotide microarrays identified a set of genes that characterize prostate cancer and benign prostatic tissues. We confirmed that a decrease in the expression of genes *PSP94*, *BPAG1*, and *ZNF185* highly correlates with prostate cancer progression. Increase of *Erg-2* levels may suggest its role in the development of prostate cancer. Importantly, this is the first study to identify inactivation of the LIM domain gene *ZNF185* in patients with prostate cancer and in prostate cancer cell lines. This gene may serve as a marker of prostate cancer aggressiveness. In addition, our findings warrant additional investigations of potential transcriptional silencing of *PSP94* and *BPAG1* as prognostic markers for prostate cancer progression, and as potential therapeutic targets for prostate cancer.

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