Gene Promoter Methylation in Prostate Tumor–Associated Stromal Cells

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Background: **Gene expression can be silenced through the methylation of specific sites in the promoter region. This mechanism of gene silencing has an important role in the carcinogenesis of prostate and other cancers. Although tumor-associated stromal cells also exhibit changes in gene expression, promoter methylation has not been described in these cells.** ***Methods:*** **Tumor epithelia, tumor-associated stroma and normal epithelia, and stroma adjacent to tumor tissues were isolated from whole-mount prostatectomy specimens (two per patient) of patients (n = 5) with localized prostate cancer and from normal epithelia and stroma from benign prostate hyperplasia specimens (two per patient) from men (n = 5) without prostate cancer by using laser capture microdissection or expression microdissection. The methylation status of three genes important in prostate carcinogenesis, GSTP1, RARβ2, and CD44, were evaluated using quantitative methylation-sensitive polymerase chain reaction.** ***Results:*** **GSTP1 and RARβ2 were methylated in the tumor epithelium of all five prostate cancer patients and in the tumor-associated stroma in four of the five patients. CD44 was methylated in the tumor epithelium from four of the five patients but not in the tumor stroma. GSTP1 and RARβ2 were methylated in normal epithelium of two and four patients, respectively, and in normal stroma of one and two patients, respectively, that were isolated from regions adjacent to the tumors and may have resulted from a tumor-field effect; CD44 methylation was not observed in normal epithelium or stroma. In contrast, normal epithelia and stroma from benign prostate hyperplasia specimens showed no promoter methylation in GSTP1, RARβ2, or CD44.** ***Conclusions:*** **The observation of promoter methylation in the non-neoplastic cells of the prostate tumor microenvironment may advance our understanding of prostate cancer development and progression and lead to new diagnostic and prognostic markers and therapeutic targets.** **[J Natl Cancer Inst 2006;98:255–61]**

Epigenetic mechanisms, such as DNA methylation, are important in normal development and in human carcinogenesis. DNA methylation refers to the covalent bonding of a methyl group to the dinucleotide CpG, catalyzed by DNA methyltransferase (DNMT). The majority of CpG dinucleotides in the genome, which are dispersed across retrotransposons *(1)* or throughout coding regions and introns of genes *(2)*, are methylated in normal cells. However, approximately 15% of CpGs are clustered (CpG islands) in the promoter regions of genes and are normally unmethylated *(3)*. In tumors, promoter CpG islands are often methylated (or hypermethylated), a state that can facilitate tumorigenesis by the transcriptional silencing of tumor suppressors and other regulatory genes.

The methylation of specific genes is associated with the histologic progression of prostate cancer. GSTP1 and RARβ2 are frequently methylated in prostate tumors (>90% of tumors) and in preinvasive lesions, (approximately 50% of high-grade prostate intraepithelial neoplasias) *(4–7)*. Conversely, methylated CD44 and other methylated genes that are associated with aggressive disease phenotypes are not present in high-grade prostate intraepithelial neoplastic lesions but are found with increased frequency in high-grade tumors *(6–8)*.

Stromal components of the tissue microenvironment associated with cancer epithelia are fundamentally different from those of normal tissue. Characteristics of the tumor microenvironment include an activated cellular phenotype (which more readily supports tumor growth), modified extracellular matrix composition, and increased microvessel density *(9–11)*. In many ways, the tumor microenvironment mimics the stroma at the site of wound repair and is characterized by the transition of smooth muscle cells to myofibroblasts *(12)*. Changes in gene methylation patterns may also be involved in this transition. Gene methylation is important in normal organ development and regulates several tissue-, temporal-, and spatial-specific processes *(13–15)*. Under experimental tissue culture conditions, the degree of promoter methylation is dependent on the media and on the conditions under which the cells were grown, showing that the process is closely linked to the cellular environment *(16)*. For example, experimental models using TSUPr1 bladder cancer cells demonstrate that promoter methylation of E-cadherin is substantially modulated by and dependent on whether cells were grown in monolayer or in three-dimensional culture conditions *(16)*.

Although previous studies have noted alterations in gene and protein expression in stromal cells associated with tumors *(17,18)*, to our knowledge epigenetic changes in the stromal compartment of the tumor microenvironment of prostate cancer have not been explored. We therefore evaluated the methylation status of three genes involved in prostate carcinogenesis—GSTP1, RARβ2, and CD44—in epithelial and stromal cells from tumor specimens and normal prostate samples. Cells of each type were isolated and extracted from regions of normal and tumor

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tissues within whole mount prostate specimens using laser capture microdissection and expression microdissection.

Materials and Methods

Human Tissues and Cell Lines

Whole-mount prostate specimens were obtained from radical prostatectomy specimens from men ($n = 5$) with clinically localized (organ-confined) prostate cancer who were treated at the National Institutes of Health Clinical Center and the National Naval Medical Center in Bethesda, MD. All patients provided written informed consent, and the research was approved by the National Cancer Institute Institutional Review Board. The prostate glands were placed on ice immediately after removal and transported to the pathology department, where they were inked, sectioned, and placed in 70% ethanol within 1.5 hours after removal. The next day, the tissues were dehydrated with an ethanol and a xylene series. The tissues were then embedded in paraffin as whole-mount sections *(19)*. Benign prostatic hyperplasia specimens were obtained from the Cooperative Human Tissue Network (http://www.chtn.ims.nci.nih.gov). The specimens had been taken from men ($n = 5$) who received a radical prostatectomy and had no histologic evidence of cancer, were flash-frozen, and were mounted in Optimal Cutting Temperature media in a cyromold to generate tissue blocks. LNCaP prostate cancer, PZHPV7 normal prostate epithelial, and PRC30 prostate stromal cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured under the conditions specified by ATCC.

Laser Capture Microdissection of Epithelial and Stromal Cells From Tumor and Normal Prostate Tissue of Patients With Prostate Cancer and Benign Prostate Hyperplasia

Epithelial and stromal tissue samples from areas within and adjacent to tumors and/or histologically normal-appearing tissue were microdissected from the prostate glands of the five men with localized prostate cancer (two samples for each tissue type) using laser capture microdissection with conditions optimized for the selective isolation of specific cell types *(20)*. Normal epithelium and stroma from five patients with benign prostatic hyperplasia were similarly microdissected. An Arcturus Pix Cell II laser system was used with the following parameters: 15 μm shot size, 1 ms duration, power of 30 mW, and approximately 3000 laser shots. All dissections were performed by the same person (J. A. Hanson) to ensure consistency across specimens. Regions of normal, stromal, and tumor tissue for all specimen sections were identified by the study pathologist (J. W. Gillespie). Each tumor epithelia dissection was graded by the study pathologist according to the Gleason scoring system, and the accuracy of the dissections was confirmed by evaluation by a pathologist before and after procurement as well as by examination of the retrieved glands.

Expression Microdissection of Epithelium and Stromal Cells From Tumor and Normal Prostate Tissues of Prostate Cancer Patients

Expression microdissection is a novel tissue microdissection technique that allows for the procurement of specific cells based on molecular targeting *(21)*. Selection of epithelial or stromal compartments was based on immunohistochemical staining with monoclonal mouse anti-cytokeratin AE1/AE3 (DAKO/Cytomation, Carpineria, CA) at 1:50 dilution or with monoclonal mouse anti–smooth muscle actin (Zymed Laboratories, San Francisco, CA) (commercially prediluted), respectively. Five-micron-thick whole-mount specimens from three of the five prostate cancer patients were mounted on charged slides. The tissue was de-waxed twice in xylene and then rehydrated in decreasing concentrations of ethanol. Immunohistochemical staining was performed with the DAKO Envision Plus System with diaminobenzidine (DAB) (DAKO/Cytomation) according to the manufacturer's instructions, except that the DAB stain was 3× concentrated and the incubation time was 20 minutes.

Immunohistochemical staining was performed according to the manufacturer's instructions. The specimens were not counterstained to enhance the contrast between the stained and unstained areas. Expression microdissection was performed with whole-field laser irradiation. The laser system was an Arcturus Pix Cell II with the following parameters: 30 μm shot size, 25–50 ms duration, and power of 50–100 mW.

Quantitative Methylation-Sensitive Polymerase Chain Reaction

The caps (one cap per sample) from both dissection techniques were incubated at 37 °C overnight in 25 μL of lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1% Tween 20, and 1 mg/mL proteinase K) and boiled at 95 °C for 10 minutes to deactivate the proteinase K. The prepared lysate was used directly for subsequent DNA methylation assays.

Gene-specific methylation status was determined using real-time or quantitative methylation polymerase chain reaction (PCR) based on TaqMan chemistry (Applied Biosystems, Foster City, CA) as previously described *(7,22)*. In this technique, methylated sequences are chemically modified and are detected by PCR amplification and hybridization with fluorescently labeled probes. In the modification, bisulfite cleaves the amino group of the cytosine, which converts it to a thymine, whereas the methylated cytosine is protected and remains a cytosine. In the PCR step, the methylated sequences are distinguished with specific PCR primers and/or hybridization probes that anneal to CpG sites in the region of interest.

Bisulfite modification was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Primers and hybridization probes were designed that bind specifically to bisulfite-converted sequences in the CpG islands in the promoters of each of the genes (each assay evaluated four to six individual CpG sites). The primers and probe for each gene assay were described in a previous report (Supplementary Table 1; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue4) *(7)*. Real-time PCR was carried out in a reaction volume of 25 μL using TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). Each PCR mixture contained 300 nM concentrations of each primer, 100 nM probe, and 1× TaqMan buffer. Amplification and detection were carried out using the following profile: one step at 50 °C for 2 minutes, one step at 95 °C for 10 minutes, 50 cycles at 95 °C for 15 seconds, and 60 °C for 1 minutes. The sensitivity and specificity of the

assays were tested by running standards of serial dilutions with known amounts of methylated DNA (25–0.025 ng) from serial dilutions of the methylation-positive prostate cancer cell line (LNCAP) and normal prostate epithelial cell line PZHPV7 (negative for methylation). All samples were within the assay's range of sensitivity and reproducibility based on amplification of an internal reference standard (CT value for ACTB of 38 or less). All samples were run in duplicate with standards and controls on every plate. The quantitative assessment of percent gene methylation was determined by computing the ratio of gene methylation relative to total DNA by extrapolation from a standard curve that was derived by mixing universally methylated DNA (Chemicon International, Temecula, CA) and unmethylated DNA (human placental DNA) (Sigma, St. Louis, MO). The percentages were derived using the following formula: (ng gene/ng ACTB) × 100%; known amounts of percentages of methylated DNA (0%, 10%, 50%, and 100%) were used to evaluate the quantitative precision of the assays. The samples were categorized as unmethylated, low methylation (1%–50%), or high methylation (51%–100%), based on the sensitivity of the assay.

Pyroseqeuncing Assay

To confirm the methylation status of one of the genes (*GSTP1*), we pyrosequenced samples of tumor epithelium and tumor stroma from two patients (98-22 and 98-27) that were acquired by laser capture microdissection. The pyrosequencing assay has been shown to be accurate for quantitative methylation analysis and was conducted as described *(23)*. The PCR product for each pyrosequencing reaction was generated in a 25-μL reaction containing 1× AmpliTaq Gold Master Mix, 2 U of Ampligold Taq (Applied Biosystems), 250 μM concentrations of dNTPs, 0.2 μM forward primer GSTP1-F-5′-GAT TTG GGA AAG AGG GAA AGG T-3′, 0.2 μM reverse primer, GSTP1-R-5′-biotin-GAT GGG GTT TAG AGT TTT TAG TAT GGG-3′, and one-half of a laser capture microdissection sample of ~3000 shots. The amplifications were carried out at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute, and finally by a 10-minute extension at 72 °C. Single-stranded DNA from 8 μL of each PCR sample was generated according to the PSQ 96-sample preparation guide using a vacuum filtration sample device following the manufacturer's instructions (Biotage, Charlottesville, VA). The single-stranded product was annealed to a 0.3 μM concentration of the sequencing primer GSTP1-S-5′-GGA TTT TAG GGA GTT TTT TT-3′, placed at 85 °C for 2 min, and cooled to room temperature. Pyrosequencing was performed on a PSQ96 HS system (Biotage) with the Biotage reagent kit (Biotage) according to the manufacturer's instructions. Raw data were analyzed with the allele quantification algorithm of the provided software.

Real-Time Complementary DNA analysis of Epithelial- and Stromal-Specific Markers

RNA was extracted from laser capture–selected tumor epithelium and tumor-associated stromal tissue (~3000 shots each) from two frozen whole-mount specimens and were processed using the Arcturus Micropure RNA kit (Arcturus Bioscience, Mountain View, CA) following the manufacturer's instructions. Complementary DNA (cDNA) was generated using a combination of random primers and poly(dT) and Superscript III (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Epithelial markers (CK19 and CK8), stromal markers (vimentin), and standard reference (ACTB) gene expression assays were run on the Applied Biosystems 7900 Sequence Detector (Applied Biosystems). Assays on Demand (Applied Biosystems) were used for vimentin and ACTB. The primers and probes for CK19 and CK8 are as follows: CK19, sense: 5′-AGG ACC TGC GGG ACA AGA TI-3′, antisense 5′-TGC AGG ACA ATC CTG GAG TTC-3′, 6FAM-TTG GTG CCA CCA TTG-molecular groove binding nonflorescence quencher (MGBNFQ); CK8, sense: 5′-GGG CTG GTG GAG GAC TTC A-3′, anti-sense 5′-AGG ACA AAT TCG TTC TCC ATC TCT-3′; 6FAM-AGG ATG AGA TCA ATA AGC GT-MGBNFQ. A standard curve for the expression of each of the genes was constructed using total RNA from either PZHPV7 normal prostate epithelial cells or PRC30 prostate stromal cells. Serial dilutions (1:1–100 000) of cDNA from each cell line in water were run. Each reaction mixture contained 1× TaqMan buffer, 100 μM primers, and a 300 μM probe in a 10-μL sample volume. The reactions were amplified using the following conditions: 1 minute at 94 °C followed by 40 cycles of 94 °C for 30 seconds and 60 °C for 1 minute. The level of expression of each gene relative to ACTB was calculated using the $\Delta\Delta C_T$ method (http://www.appliedbiosystems.com). All samples were run in duplicate, and mean values were used in the computation.

Results

Laser capture microdissection of epithelial and stromal cells was performed on whole-mount prostate preparations from five prostate cancer patients who were previously characterized as having promoter region hypermethylation *(6)*. Two samples were collected from each prostate cancer patient: tumor epithelium (Fig. 1, B), tumor-associated stroma (stromal regions within tumor area; Fig. 1, C), normal-appearing epithelium, and normal-appearing regions of stromal tissue (located adjacent to the tumor) (Fig. 1, D–F). The microdissections were performed after conditions were optimized (described in "Materials and Methods") to ensure that contamination of stroma with epithelium and vice versa was kept to a minimum. Duplicate epithelium and stroma samples from five patients with benign prostatic hyperplasia (with no evidence of cancer) were similarly dissected and included as controls.

The methylation status of GSTP1, RARβ2, and CD44 for each of the laser capture microdissection samples among the five prostate cancer patients was determined (Table 1). GSTP1 and RARβ2 were methylated in the tumor epithelium of all five prostate cancer patients and in the tumor-associated stroma in four of the five patients. CD44 was methylated in the tumor epithelium from four of the five patients but not in the tumor stroma. Many of the samples exhibited robust methylation (>51% of DNA methylated) in both the tumor epithelium and stroma samples taken from the tumor area. To confirm these findings, we conducted GSTP1 quantitative methylation-sensitive PCR and pyrosequencing assays on tumor epithelium and stroma samples from two patients. Both techniques showed 100% methylation in the two tumor epithelium samples, no methylation in one tumor stroma sample, and approximately 30% methylation in the second tumor stroma sample (Supplementary Table 2 and Supplementary

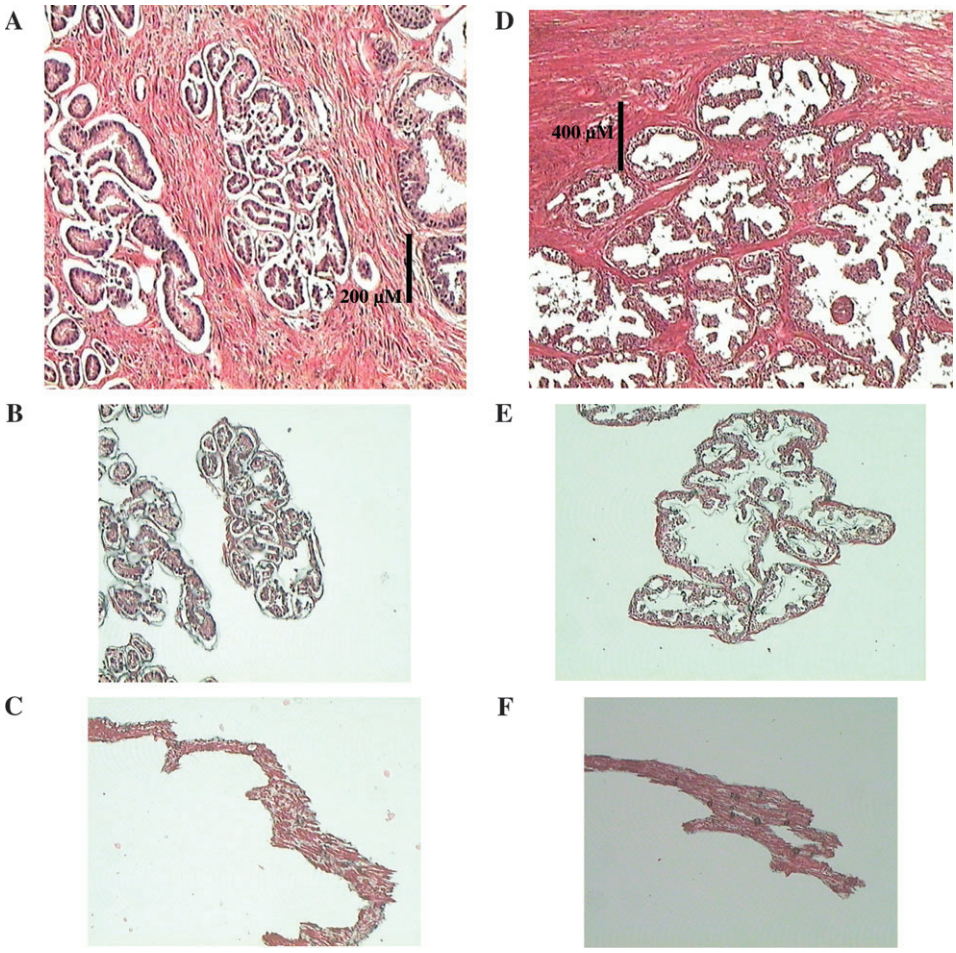

Fig. 1. Representative laser capture microdissected specimens of whole-mount prostatectomy tumor and benign prostate hyperplasia specimens. **A–C**) Prostate cancer tissue before dissection (**A**), dissected tumor epithelium (**B**), and tumor-associated stroma (**C**). **D–F**) Normal tissue before laser capture dissection (**D**), dissected epithelium (**E**), and stroma (**F**). All samples were stained with hematoxylin and eosin.

Fig. 1; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue4).

Interestingly, GSTP1 and RARβ2 methylation was also observed in several of the histologically normal–appearing epithelia (from two and four patients, respectively) and normal stromal samples (from one and two patients, respectively) located adjacent to the tumor. The majority of these samples showed only partial methylation, but two of them exhibited greater than 51% methylation. These data suggest a possible field effect of gene methylation in the tumor region. CD44 methylation was not observed in normal epithelium or stroma of patients.

To ensure that we were selectively isolating tumor epithelium or stromal cells, gene expression of epithelial-specific markers (CK19 and CK8) was evaluated in tumor epithelial and in tumor stromal samples that were dissected from two prostate cancer patients (Table 2). CK19 and CK8 expression was high in tumor epithelium (1- to 20-fold relative to the ACTB control) and low in tumor stroma (<0.05-fold relative to the ACTB control). In contrast, vimentin expression was much higher in stroma tissue (10- to 50-fold higher in stroma than in epithelium). These data confirm the successful isolation of target cells by laser dissection. We also performed several control experiments to ensure that the laser capture dissection did not recover nontarget cells. These included placing a new cap over the tumor region and firing the laser within acellular regions (i.e., glandular lumens) to mimic dissection. In each control experiment, we did not detect DNA in the methylation assay (results not shown).

To further verify these findings of the presence of methylation in tumor-associated stroma we used expression microdissection to obtain tumor cells and surrounding stroma. Samples of tumor epithelium and associated stroma and normal-appearing epithelium and stroma were obtained from three patients. As was seen with the laser capture–dissected samples, both GSTP1 and RARβ2 were methylated (>51%) in both the tumor stromal and epithelial cells of all three patients (98-22, 98-23, and 98-27; data not shown).

Discussion

We evaluated the methylation of several genes important in prostate carcinogenesis in tumor epithelium and stromal cells in prostate specimens from patients with prostate cancer or benign prostate hyperplasia and found high levels of gene methylation in the tumor epithelium and stromal cells and some methylation in both normal epithelium and stromal cells in normal-appearing tissues located adjacent to tumors. There is evidence in the literature to suggest that changes in the stromal milieu may create a tumor-promoting microenvironment *(24)*. This study, to our knowledge, presents the first evidence that these functional alterations in stroma surrounding tumor may in part be the result of epigenetic gene methylation changes that occur in tumor-associated stromal cells.

GSTP1 and other genes are frequently (>90% of patients) methylated in prostate tumors *(4–7)*, with a differential pattern of gene methylation across the stages of prostate carcinogenesis. For example, GSTP1 and RARβ2 are methylated in preneoplastic precursor lesions (high-grade prostate intraepithelial neoplasms) *(4,6)*,

Table 1. Methylation of GSTP1, RARβ2, and CD44 in epithelial and stromal tissue taken from tumor and histologically normal tissue microdissected from five whole-mount prostate sections*

Patient	Histology	Tissue	Methylation status			Gleason score
			GSTP1	RARβ2	CD44	
98-5	Tumor	Epithelium				7
	Tumor	Epithelium				5
	Tumor	Stroma				–
	Tumor	Stroma				–
	Normal	Epithelium				–
	Normal	Epithelium				–
	Normal	Stroma				–
	Normal	Stroma				–
98-22	Tumor	Epithelium				6
	Tumor	Epithelium				6
	Tumor	Stroma				–
	Tumor	Stroma				–
	Normal	Epithelium				–
	Normal	Epithelium				–
	Normal	Stroma				–
	Normal	Stroma				–
98-23	Tumor	Epithelium				6
	Tumor	Epithelium				6
	Tumor	Stroma				–
	Tumor	Stroma				–
	Normal	Epithelium				–
	Normal	Epithelium				–
	Normal	Stroma				–
	Normal	Stroma				–
98-27	Tumor	Epithelium				9
	Tumor	Epithelium				6
	Tumor	Stroma				–
	Tumor	Stroma				–
	Normal	Epithelium				–
	Normal	Epithelium				–
	Normal	Stroma				–
	Normal	Stroma				–
99-5	Tumor	Epithelium				8
	Tumor	Epithelium				7
	Tumor	Stroma				–
	Tumor	Stroma				–
	Normal	Epithelium				–
	Normal	Epithelium				–
	Normal	Stroma				–
	Normal	Stroma				–

*Gene methylation determined by quantitative methylation-sensitive polymerase chain reaction, as described in "Materials and Methods." Open, gray, and black boxes represent 0, 1%–50%, and 51%–100% methylation, respectively. Duplicate samples of epithelium and stroma were taken from tumor and normal or benign hyperplasia tissue remote from tumor foci. – = not applicable.

whereas CD44 is methylated in association with more advanced disease, i.e., primarily in higher grade tumors *(6–8)*. It is interesting to note that although four of the five prostate cancer patients had CD44 methylation in their tumor epithelium, CD44 methylation was not observed in tumor-associated stromal cells. This difference could be of biologic significance—that is, it may indicate that CD44 methylation in tumor-associated stromal cells is not a normal feature of tumorigenesis. Alternatively, this result could simply reflect the facts that CD44 methylation in tumors is generally less prevalent than GSTP1 and RARβ2 and that we did not evaluate enough samples to observe this phenomenon in the tumor stroma.

Changes in gene promoter methylation in response to the microenvironment have been demonstrated experimentally. Using the in vitro invasion assay, TSUPr1 bladder cancer cells passed through an artificial membrane showed more methylation in the E-cadherin promoter than cells grown in monoculture *(16)*. Replating the cells and allowing them to grow as a monolayer resulted in some loss of methylation. Moreover, cells that were grown as three-dimensional spheroids in Matrigel with impaired cell contact showed E-cadherin demethylation and concomitant up-regulation of transcript. The authors concluded that E-cadherin methylation is dynamic and changes in response to the microenvironment *(16)*. Taken together, these in vitro results suggest that gene promoter methylation in the tumor is not a static condition but changes in concert with the cellular milieu. Our ex vivo data extend this observation to the stromal cells that are associated with human prostate cancer and suggest that the methylation of certain gene promoters in tumor cells and in the adjacent stroma may be important in the pathogenesis of prostate cancer.

The described findings raise several questions regarding the mechanism of aberrant gene promoter hypermethylation in neoplastic and associated stromal cells. At present, it is not known whether tumor cell and stromal cell methylation are inter-dependent or if they are independent responses to the microenvironment.

One plausible mechanism for the aberrant methylation patterns in tumor regions is dysregulation of the machinery that is responsible for this process. Both de novo and maintenance gene methylation occurs predominantly through the action of a

Table 2. Epithelium versus stromal marker specific gene expression in tumor epithelium and stromal samples analyzed by laser capture microdissection

Sample	Gene expression ratio relative to ACTB*		
	CK8	CK19	Vimentin†
Patient 1			
Tumor epithelium	4.0	20.0	0.1
Tumor stroma 1	0.01	0.01	1.0
Tumor stroma 2	0.01	0.05	1.0
Patient 2			
Tumor epithelium	1	1	0.1
Tumor stroma	0.05	0.01	5

*Relative levels computed by the $\Delta\Delta C_T$ method using ACTB as the standard reference gene.

†Vimentin is a stromal marker.

combination of two methyltransferases, DNMT1 and DNMT3b, both of which have altered expression in tumor cells *(25)*. In mouse NIH3T3 fibroblast cells, overexpression of DNMT1 resulted in increased gene methylation that coincided with decreased contact inhibition, increased growth in soft agar, and increased tumorigenicity *(26)*. However, it is not yet known whether these enzymes are similarly dysregulated in tumor-associated stromal cells.

A second plausible mechanism for increased gene methylation is that it is secondary to expression alterations in the tumor and associated stromal cells. Changes in gene promoter methylation have been hypothesized to occur through "methylation spreading," i.e., methylation occurring first either on the extreme 5′ or 3′ end of promoter regions and spreading internally over time *(27,28)*. The spreading of methylation across promoter regions is inhibited by specific DNA sequences such as transcription factor binding sites. For example, the introduction of Sp1 sites led to inhibition of de novo DNA methylation of flanking CpG sequences in mouse embryonic carcinoma cells *(29)*. As hypothesized by Turker *(27)*, gene promoter methylation may occur as a process that is initiated as a consequence of reduced gene expression, which leaves unbound DNA vulnerable to chromosome remodeling and induced methylation. In this scenario, the promoter methylation we observed in tumor and stromal cells would have occurred secondary to changes in gene expression that are induced by one, or a combination, of the following: tumor mutations that change gene expression patterns, soluble agents (e.g., growth factors and cytokines), and/or new mechanical stresses of the evolving microenvironment.

Finally, it is possible that methylation-positive stromal cells are derived from the transition of the tumor epithelium itself (containing gene methylation) into cells with a mesenchymal phenotype. It has been postulated that cancer cells can undergo transdifferentiation by epithelial-mesenchymal transition to that of mesenchymal phenotype *(30–32)*. The transdifferentiated cells are associated with increased malignant potential, owing to increased cell motility, migration, and invasion.

The findings of gene methylation in some normal-appearing epithelial and normal stromal cells adjacent to tumor regions suggest a possible field effect. To investigate this possibility in more detail, it will be necessary to perform detailed three-dimensional mapping of gene methylation patterns in prostatectomy specimens. It will be particularly important to carefully define the parameters of such a study because clinical prostate samples exhibit a complex and varied pattern of histopathology. The size, shape, cellular components, and anatomic location of each tumor differ. Moreover, the classification of "normal" epithelial glands within a field of an invasive tumor using light microscopic evaluation is often difficult and must be undertaken cautiously. In the present study, we were able to identify aberrant gene promoter methylation in a subset of the normal epithelial and stromal cell samples immediately adjacent to the tumor region. However, we were not able to further define the characteristics of this potential field effect phenomenon due to the limitations of the specimens available for study. Because we did not document the precise distance of the dissections of histologically normal tissue relative to tumor foci, it is possible that some of the variation in gene methylation observed could be due to normal-appearing samples taken nearer the vicinity of the tumor. If future studies reveal that a methylation field effect does in fact exist, such an effect may have clinical implications. Current core needle biopsy procedures result in a 20% false-negative rate in glands in which cancer exists *(33)*. If tumor regions are surrounded by a zone of aberrant DNA methylation, then assaying for gene promoter methylation may help to identify patients in whom cancer is present but not detected by routine histopathologic analysis.

We carefully evaluated the study design and results to rule out the possibility that the methylation observed in tumor stromal cells was due to experimental artifact. One issue of possible concern was that tumor epithelium could nonspecifically adhere to the laser capture microdissection cap during the dissection of the stromal cells, potentially producing a false positive result. This concern was further heightened by the previous observation that stromal dissections produce substantially less DNA than do epithelial dissections, due to the decreased cellularity of the stroma and the difficulty in recovering DNA from the fibrous collagenous extracellular matrix.

To address this potential limitation, we performed dissections using two separate techniques, evaluated mRNA levels to validate the identity of the recovered cells, and carried out control experiments indicating that nonspecific recovery of cells was not problematic. In addition, the high level of gene methylation of some of the stromal samples suggests that it was not due to contaminating tumor cells. Moreover, CD44 methylation was not observed in any of the stromal samples, irrespective of the strong methylation observed in the tumor cells. For example, patient 98-22 had methylation of all three genes in the tumor epithelium, but only GSTP1 and/or RARβ2 methylation in the stroma.

In summary, our finding of gene methylation in a field of tumor epithelia and tumor-associated stroma has implications for cancer prevention, treatment, and diagnosis. Furthermore, our findings suggest that epigenetic events may play an important role in the development of the activated stroma phenotype.

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NOTES

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