Differential Gene Expression in Benign Prostate Epithelium of Men with and without Prostate Cancer: Evidence for a Prostate Cancer Field Effect


Abstract

Background: Several malignancies are known to exhibit a “field effect,” whereby regions beyond tumor boundaries harbor histologic or molecular changes that are associated with cancer. We sought to determine if histologically benign prostate epithelium collected from men with prostate cancer exhibits features indicative of premalignancy or field effect.

Experimental Design: Prostate needle biopsies from 15 men with high-grade (Gleason 8-10) prostate cancer and 15 age- and body mass index-matched controls were identified from a biospecimen repository. Benign epithelia from each patient were isolated by laser capture microdissection. RNA was isolated, amplified, and used for microarray hybridization. Quantitative PCR was used to determine the expression of specific genes of interest. Alterations in protein expression were analyzed through immunohistochemistry.

Results: Overall patterns of gene expression in microdissected benign prostate-associated benign epithelium (BABE) and cancer-associated benign epithelium (CABE) were similar. Two genes previously associated with prostate cancer, PSMA and SSTR1, were significantly upregulated in the CABE group (false discovery rate <1%). Expression of other prostate cancer–associated genes, including ERG, HOXC4, HOXC5, and MME, were also increased in CABE by quantitative reverse transcription-PCR, although other genes commonly altered in prostate cancer were not different between the BABE and CABE samples. The expression of MME and PSMA proteins on immunohistochemistry coincided with their mRNA alterations.

Conclusion: Gene expression profiles between benign epithelia of patients with and without prostate cancer are very similar. However, these tissues exhibit differences in the expression levels of several genes previously associated with prostate cancer development or progression. These differences may comprise a field effect and represent early events in carcinogenesis.

Clin Cancer Res; 16(22); 5414–23. ©2010 AACR.

Prostate cancer is a multifocal disease, with 67% to 96% of radical prostatectomy specimens containing more than one focus of disease (1–7). Among several explanations, this multifocality suggests that factors that underlie the development of prostate cancer may affect the entire prostate or at least separate foci within the same prostate. Molecular changes occurring as a result of carcinogenic stimuli may be detected throughout the prostate, similar to other solid tumors, in what has been termed a “field effect” or “field cancerization” (8, 9). Histologic studies have shown changes in the nucleus of benign epithelia adjacent to cancer, which are not detectable by light microscopy (10). Additionally, several studies have provided evidence that histologically benign tissue adjacent to prostate cancer harbors molecular changes in both normal adjacent epithelium and stroma (11–16); however, these studies have key design issues that limit their interpretation.

The molecular characterization of normal prostate epithelium involves several important methodologic issues with substantial implications for data interpretation. First, many studies consider the histologically normal tissue either immediately adjacent to prostate cancer or within the cancerous prostate gland as the reference “normal” tissue, and this practice is questionable in light of potential field effect changes. Second, the tissue adjacent to cancer may contain changes that are intimately associated with carcinogenesis and lend insight into mechanisms of cancer initiation. Lastly, the ideal reference normal prostate tissue would originate from age-matched men with no evidence...
of prostate cancer, which, given the high prevalence of prostate cancer, would require, at minimum, a prostate biopsy to rule out occult cancer. Previous studies have been hampered by this lack of appropriate reference control tissues.

The objective of this study was to compare the transcriptomes from benign prostatic epithelia of men with high-grade prostate cancer with benign prostatic epithelia of age- and body mass index (BMI)–matched men who have undergone multiple negative prostate biopsies without evidence of prostate cancer. Differences in gene profiles may identify important pathways that are involved in the earliest stages of prostate cancer development. These results may provide targets for cancer prevention or biomarkers for diagnostics in early detection and improve our understanding of the early events in prostate carcinogenesis.

Materials and Methods

Study design and tissue acquisition

Between May 2003 and July 2006, 367 patients undergoing prostate needle biopsy for suspicion of prostate cancer [elevated serum prostate-specific antigen (PSA) and/or abnormal digital rectal examination] consented to acquisition of four extra prostate biopsies for research studies, in addition to a 12-core biopsy regimen for standard pathologic examination. Patients who underwent previous hormonal therapy, chemotherapy, radiation therapy, treatment with 5-α-reductase inhibitors, or testosterone replacement were excluded. Each research biopsy specimen was immediately embedded in polyethylene glycol freezing media (Tissue-Tek OCT Compound, Sakura Finetek), placed in isopentane that was precooled in liquid nitrogen, and stored at −80°C. All protocols for tissue acquisition and processing were approved by the local Institutional Review Board. As part of this protocol, each patient underwent written informed consent for research tissue acquisition before prostate needle biopsy. Among the 367 patients, 135 (37%) were diagnosed with prostate cancer; of these patients, 21 (16%) were diagnosed with high-grade (Gleason 8-10) prostate cancer on biopsy. One hundred and eighty-nine patients (51%) had no evidence of cancer on prostate biopsy; 56 of these patients (30%) had a second or third set of biopsies that also showed no evidence of carcinoma. The remaining 43 patients (12%) had atypical small acinar proliferation (ASAP) or high-grade prostatic intraepithelial neoplasia (PIN) on prostate biopsy.

Target sample size for this study was set to 20 samples per group, which would yield a power of 80% to detect 2-fold differences in expression of genes with a SD of gene expression of 0.75 at a false discovery rate (FDR) set to 5%. In a previous study on the effect of tissue ischemia on prostatic epithelial gene expression (17), only 10% of genes had a SD of ≥0.75; therefore, power for the majority of genes would be higher than 80%. The achieved sample size was only 15 per group because several of the samples from men with high-grade cancer had little or no normal epithelia in the prostate biopsy specimen available for analysis. With this reduced sample size, the power to detect a 2-fold difference in expression of genes with SDs of 0.75, 0.50, and 0.25 was 65%, 81%, and ≥99%, respectively. Our study cohort was composed of 30 total patients. (a) Control group: benign prostate-associated benign epithelium (BABE) patients ($n = 15$). Fifteen patients were chosen from the 56 patients with multiple negative prostate biopsies. Men with multiple negative prostate biopsies were used to diminish the likelihood of false-negative results from a single biopsy session. (b) Cases: cancer-associated benign epithelium (CABE) patients ($n = 15$). Fifteen patients were chosen from the 21 patients with high-grade (Gleason sum 8-10) prostate cancer. Eight of these 15 men with high-grade cancer on biopsy subsequently underwent retropubic radical prostatectomy; seven had high-volume, bilateral, high-grade disease and one patient was downgraded to Gleason 4+3 on final pathology. To prevent potential bias from prostate cancer risk factors that may also affect prostate gene expression, patients were stratified based on age (5-year groups) and BMI (<30 or ≥30) and matched between two patient groups (CABE versus BABE).

Tissue processing and laser capture microdissection

Eight-micrometer sections of the frozen biopsy tissue from the 15 cases and 15 controls were placed on slides and stained with H&E. We used needle biopsy cores for these studies, as we have previously shown that surgical resection of the prostate gland can introduce significant alterations in the expression of a substantial number of genes (17). The consistency of this approach also eliminated methodologic differences in comparing tissues collected from various forms of surgical removal (e.g., organ-donor prostate tissue versus radical prostatectomy prostate tissue). A board-certified pathologist (B.S.K.) reviewed the slides to ensure that all cancer, PIN, ASAP, inflammation, and
atrophy were excluded from the captured areas. Approximately 2,000 cells from histologically benign epithelial glands were captured using laser capture microdissection (LCM). Representative pictures were saved before, during, and after dissection for pathologic confirmation.

Captured individual specimens were lysed, and RNA was isolated using the Arcturus PicoPure RNA Isolation kit (Molecular Devices) according to the manufacturer's protocol. Samples were treated with RNase-free DNase (Qiagen) to minimize DNA contamination. This RNA was converted to a cDNA library and amplified and amino-allyl labeled using the TransPlex WTA kit (Sigma-Aldrich Corp.), and its purity was confirmed by spectrophotometric analysis.

To provide a reference standard RNA for use on cDNA microarrays, we isolated total RNA from LNCaP, DU145, PC3, and CWR22 cell lines (American Type Culture Collection) growing at log phase in dye-free RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen). RNA was purified using Trizol (Invitrogen) following the manufacturer's protocol. This RNA was amplified using the TransPlex WTA kit following procedures used for the study.

**Microarray hybridization and analysis**

Two micrograms of amplified amino-allyl cDNA from each were labeled with Cy3 fluorescent dye, and the reference standard was labeled with Cy5 fluorescent dye, both as described previously (18). Samples were then hybridized to Agilent 44K whole human genome expression oligonucleotide microarray slides (Agilent Technologies, Inc.) following the manufacturer's suggested protocols. Fluorescence array images were collected for both Cy3 and Cy5 using the Agilent DNA microarray fluorescent scanner G2565BA, and Agilent Feature Extraction software was used to grid, extract image intensities, and normalize data. Spots of poor quality, as determined by the software, were removed from further analysis. The microarray data for this experiment have been deposited in the Gene Expression Omnibus database (available at http://www.ncbi.nlm.nih.gov/geo) under accession number GSE24171.

To compare the overall expression patterns of all radical prostatectomy cancer samples with their patient-matched normal samples, the filtered log ratio measurements were analyzed by using the significance analysis of microarrays (SAM) procedure (http://www.stat.stanford.edu/~tibs/SAM; ref. 19). In this analysis, an unpaired two-sample t test was used to determine which genes were significantly differentially expressed between CABE and BABE, and initial determination of significantly altered genes was determined using FDR of 5% (q < 0.05). Before analysis, probes that had average signal below 300 or greater than 30% missing values were excluded from further analysis. Hierarchical clustering of the data revealed subtle clustering on date of microarray scan, potentially a result of covariates that could not be completely accounted for in the analysis. This effect reflected in the cluster analysis potentially diminished the number of significant genes between the CABE and BABE groups found in this study.

**Pathway analysis**

Further evaluation of the microarray data was performed using gene set enrichment analysis (GSEA; ref. 20) and Ingenuity Pathway Analysis. GSEA was used to compare expression differences between BABE and CABE in the prostate cancer molecular signature identified in a meta-analysis of microarray data on prostate cancer gene expression (21). Two separate gene sets based on this meta-analysis were created: one with 40 genes identified as increased in prostate cancer relative to benign tissue, and another with 39 genes decreased in prostate cancer (Supplementary Table S2). Genes differentially expressed between CABE and BABE with P < 0.05 were ranked based on their relative expression in CABE versus BABE. GSEA was then determined if there was significant relative enrichment in either CABE or BABE in the expression of the genes listed in each gene set. Significance was determined at FDR of 0.25 as recommended by GSEA User Guide (http://www.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html!Interpreting_GSEA).

Ingenuity Pathway Analysis (Ingenuity Systems; http://www.ingenuity.com) was used to determine if any biological functions or pathways were altered between the CABE and BABE groups. A data set containing the genes altered at P < 0.05 level between the CABE and BABE groups was uploaded into the application. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base. The functional analysis identified the biological functions that were most significant in the data set. The network analysis identified networks containing greatest number of affected molecules from the data set. Right-tailed Fisher's exact test was used to calculate the P values for both the functional analysis and the identification of pathways, with all those referenced in the text and in Supplementary Data having P < 0.05.

**Quantitative reverse transcription-PCR**

We used quantitative reverse transcription-PCR (qRT-PCR) to validate microarray results for selected genes. Primers specific for the genes of interest were designed using the web-based primer design service Primer3 provided by the Whitehead Institute for Biomedical Research (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are listed in Supplementary Table S1. We determined acceptable performance characteristics of the PCR primers using normal human prostate cDNA, Biolase Taq polymerase (Bioline, Inc.), and the GeneAmp PCR System 9700 (Applied Biosystems) as previously described (17). The whole transcriptome amplification (WTA)-amplified cDNA was used as a template for the reaction, and each patient sample was individually analyzed for each gene of interest. Relative quantification of gene expression by quantitative PCR (qPCR; 40 cycles of 60°C annealing, 72°C extension, and 95°C melting) was performed on a 7700 Sequence Detector using SYBR Green Master mix and gene-specific primers following the manufacturer's recommendations. Gene expression differences between the BABE and CABE groups were.
determined by Student’s t test using GraphPad Prism version 5.00 (GraphPad Software).

**TMPRSS2:ERG fusion PCR**

The WTA-amplified cDNA was used as a template for PCR to examine for evidence of TMPRSS2 and ERG gene fusion using primers to the first exon of TMPRSS2 (T1) and the fourth exon of ERG (E4; Supplementary Data). Total RNA from VCap cells was converted to cDNA using SuperScript II (Invitrogen) according to the manufacturer’s protocol. The WTA-amplified cDNA was also generated from VCap cells, and both total VCap cDNA and WTA-amplified VCap cDNA were used as positive controls. Biolase Taq enzyme (Bioline) was used for the reaction according to the manufacturer’s specifications with 10 pmol/L of each primer. Samples were denatured at 94°C for 3 minutes, followed by 35 cycles of replication (94°C for 45 seconds, 57°C for 45 seconds, and 72°C for 1.5 minutes) and final extension at 72°C for 10 minutes. Samples were analyzed by 1.5% agarose gel electrophoresis.

**Immunohistochemistry**

Five-micrometer sections of prostate biopsy tissue from a random subset of cases and controls were used for immunohistochemical analysis of PSMA and CD10 expression. Four cases and four controls were used for immunohistochemistry. Target Retrieval Solution pH 9 EDTA (DAKO) was used for antigen retrieval, first for 20 minutes in a vegetable steamer, followed by a 20-minute cooldown at room temperature. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution for 8 minutes. Sections were then blocked for 10 minutes in 15% swine serum, and primary antibodies were applied for 1 hour at room temperature. Both PSMA (DAKO) and CD10 (Zymed) were used at a final dilution of 1:200. This antibody dilution is within the linear range of the antibody concentration curve. Sections were incubated for 30 minutes with Envision secondary reagent (DAKO) and developed with two applications of 3,3′-diaminobenzidine–horseradish peroxidase substrate and chromagen for 4 minutes each. Slides were counterstained with hematoxylin (DAKO), dehydrated, and coverslipped.

The scoring was performed on a four-tiered scale. Both staining intensity and the percentage of positive glands included in the overall score for each case. The study pathologist (B.S.K.) was blinded to cancer status while performing the scoring. The scores from two slides from the same block that were stained on separate days were averaged. In addition, if multiple blocks existed from the same patient, the average score was calculated across the blocks. Mean immunohistochemical scores between the groups were then compared using Student’s t test.

**Results**

**Patient demographics and clinical characteristics**

The characteristics of the two populations used in this study are given in Table 1. The two groups were similar in age, BMI, and family history of prostate cancer. Serum PSA levels were significantly higher in cancer, and the two groups also differed in terms of race, with four African Americans and one Pacific Islander in the CABE group, whereas all BABE patients were Caucasian. One control patient was removed from the study as he developed prostate cancer during the period of data analysis.

**Gene expression profiles from benign prostate epithelium differ depending on cancer status**

BABE and CABE samples had similar overall gene expression profiles. Table 2 gives the distribution of differences in gene expression between CABE and BABE specimens for 17,933 cDNAs. Expression differences in the table are categorized symmetrically above and below zero in units of log2, which are also shown as relative expression on a linear scale. In approximately 98% of transcripts, the relative expression of normal epithelium between BABE and CABE specimens was between 0.67 and 1.49. However, there were several transcripts with greater expression differences.

Of the 17,933 array probes with expression above background levels and with at least a 2-fold difference from the mean in at least two samples, three genes (FOLH1, PSMAL, and SSTR1) were significantly different between the two groups after correcting for multiple comparisons using the SAM algorithm (q < 0.01). Although no other genes were significantly different by q value, many genes differed by t test (P ≤ 0.05) and had differences >1.5-fold between the two groups (Fig. 1). These included several genes previously implicated in prostate carcinogenesis, including ERG, CD10/MME, and HOXC4 (22–24), as have FOLH1 and SSTR1 (25–31). The array data were not significantly different when only Caucasian subjects were included in the analysis (Supplementary Fig. S3). For reference, we have included the races for the individual CABE patients in Supplementary Table S4.

**Pathway analysis**

Although there were few statistical differences in expression by microarray analysis, specific pathway analysis may provide insight into potential global gene expression differences. GSEA analysis was performed, specifically

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**Table 1. Patient demographics**

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Control</th>
<th>Cancer</th>
<th>P</th>
</tr>
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<tr>
<td>64.8</td>
<td>67</td>
<td>0.36</td>
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<tr>
<td>BMI</td>
<td>28</td>
<td>26.8</td>
<td>0.37</td>
</tr>
<tr>
<td>Mean PSA (ng/mL)</td>
<td>10.6</td>
<td>18.4</td>
<td>0.04</td>
</tr>
<tr>
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<td>0</td>
<td>3</td>
<td>0.11</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>15</td>
<td>10</td>
<td>0.05</td>
</tr>
<tr>
<td>African-American</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pacific Islander</td>
<td>0</td>
<td>1</td>
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</table>
examining the subset of genes found to be overexpressed and underexpressed in prostate cancer in a recent meta-analysis (21). This analysis found significant enrichment of the prostate cancer overexpressed genes in the CABE group, whereas it found significant enrichment of the prostate cancer underexpressed genes in the BABE group (Supplementary Tables S2 and S3). Further analysis of this gene set using Ingenuity Pathways analysis found enrichment of cancer-associated genes and pathways in the group of differentially expressed genes with enrichment of cancer-associated genes and pathways in the prostate cancer underexpressed genes in the BABE group (3-fold) in benign epithelium acquired from prostate glands known to contain prostate adenocarcinoma compared with surrounding neoplastic epithelia or debris. We also determined that CD10/MME, whose expression is decreased in high-grade PIN and prostate cancer (8-fold), and HOXC4 (18-fold), HOXC5 (8-fold), and FOLH1 (3-fold) in benign epithelium acquired from prostate glands known to contain prostate adenocarcinoma (CABE) compared with benign epithelium from prostates harboring high-grade malignant cells (Fig. 1). Using qRT-PCR, we confirmed the significantly increased expression of ERG (7-fold), HOXC4 (18-fold), HOXC5 (8-fold), and FOLH1 (3-fold) in benign epithelium acquired from prostate glands known to contain prostate adenocarcinoma (CABE) compared with benign epithelium from prostates in which no cancer was detected on multiple biopsies (BABE). We also determined that CD10/MME, whose expression is decreased in high-grade PIN and prostate cancer (24), was also decreased 4-fold in BABE relative to CABE. We also determined that CD10/MME, whose expression is decreased in high-grade PIN and prostate cancer (24), was also decreased 4-fold in BABE relative to CABE. Thus, we evaluated several genes whose expression has been strongly increased in prostate cancer, including α-methylacyl CoA racemase (AMACR), hepsin (HPN), fatty acid synthase (FASN), myosin VI (MYO 6), SPOCK, TPDS2, and EZH2. Quantitative measurements of these transcripts by qRT-PCR determined that none of these genes were significantly different in BABE versus CABE (Fig. 2B), suggesting that adjacent cancer contamination was unlikely.

**Confirmation of cancer-associated genes that are differentially expressed in benign epithelium**

Microarray-based analysis of gene expression in BABE and CABE samples shown in Fig. 1 suggests that molecular pathways associated with invasive prostate cancer were also altered in benign epithelium from prostates harboring high-grade malignant cells (Fig. 1). Using qRT-PCR, we confirmed the significantly increased expression of ERG (7-fold), HOXC4 (18-fold), HOXC5 (8-fold), and FOLH1 (3-fold) in benign epithelium acquired from prostate glands known to contain prostate adenocarcinoma (CABE) compared with benign epithelium from prostates in which no cancer was detected on multiple biopsies (BABE). We also determined that CD10/MME, whose expression is decreased in high-grade PIN and prostate cancer (24), was also decreased 4-fold in CABE relative to BABE samples by qRT-PCR (P < 0.05 for all genes; Fig. 2A).

As several of these gene alterations correspond to those previously reported in cancer, one possible confounder of these findings would be contamination of laser-captured CABE with surrounding neoplastic epithelia or debris. Thus, we evaluated several genes whose expression has been strongly increased in prostate cancer, including α-methylacyl CoA racemase (AMACR), hepsin (HPN), fatty acid synthase (FASN), myosin VI (MYO 6), SPOCK, TPDS2, and EZH2. Quantitative measurements of these transcripts by qRT-PCR determined that none of these genes were significantly different in BABE versus CABE (Fig. 2B), suggesting that adjacent cancer contamination was unlikely.

**Immunohistochemistry**

There was a significant difference in protein expression of PSMA and CD10 in BABE compared with CABE (Fig. 3; Table 3). The mean immunohistochemical staining intensity score for PSMA expression in BABE was 0.88 ± 0.69, whereas it was 1.98 ± 1.04 in CABE (P = 0.003). Conversely, the mean immunohistochemical staining intensity score for CD10 was 2.88 ± 0.35 in CABE and 1.43 ± 1.12 in BABE (P = 0.003). PSMA and CD10 proteins were localized at the luminal aspect of the prostate epithelium, consistent with previous reports (27, 32), and confirm the specific reactivity of the antibody clones. These immunohistochemical results confirm the transcript gene expression data, which show an increased PSMA and decreased CD10 expression in CABE compared with BABE.

**TMPRSS2:ERG fusion analysis**

Recent studies have shown that a fusion between the TMPRSS2 gene and the ERG gene is a frequent event in prostate cancer, and that this recombination event may account for the increased expression of ERG in prostate cancer. This fusion is thought to occur early in prostate carcinogenesis, as it is found in precancerous lesions such as PIN. We investigated if the TMPRSS2:ERG recombination event was responsible for the elevated levels of ERG in the CABE group through PCR analysis. We found evidence of the gene fusion in one of the samples, which also was the sample with highest ERG expression on microarray and qPCR analysis (Supplementary Fig. S2). This specific sample was re-reviewed by two pathologists.

<table>
<thead>
<tr>
<th>Absolute difference (CABE/benign log2)</th>
<th>Relative difference (CABE/benign ratio)</th>
<th>Total</th>
<th>%</th>
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<tr>
<td>&gt;+1.58</td>
<td>&gt;3.0</td>
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<td>0.01</td>
</tr>
<tr>
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<td>3</td>
<td>0.02</td>
</tr>
<tr>
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<td>2.00-2.49</td>
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<tr>
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<td>1.50-1.99</td>
<td>60</td>
<td>0.33</td>
</tr>
<tr>
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<td>8,529</td>
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<tr>
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<td>9,266</td>
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<td>7</td>
<td>0.04</td>
</tr>
<tr>
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<tr>
<td>&lt;−1.58</td>
<td>&lt;-0.33</td>
<td>3</td>
<td>0.02</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>17,933</td>
<td>100</td>
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</table>

Table 2. Distribution of differences in expression of 17,933 transcripts comparing cancer-associated normal epithelium (CABE) with benign-associated normal epithelium.
(B.S.K. and L.D.T.) and was found to contain no histologic evidence of cancer or PIN.

**Discussion**

In this study, examining gene expression in histologically normal epithelium from those with and without biopsy-proven prostate cancer, we found that overall expression patterns were similar, but distinct differences in specific genes were apparent between the two groups. We also showed that these significant transcript differences were associated with biologically plausible pathways of prostate cancer carcinogenesis, suggestive of the existence of a field effect.

Our study differs from prior studies using microarray expression data to compare normal tissue from prostatectomy specimens with normal prostate tissue obtained from organ donors (12, 33) or prostate needle biopsy tissue from healthy controls with low risk of prostate cancer (34). These other studies each found >1,000 gene differences between their respective equivalent CABE and BABE groups, and there are several potential reasons for our disparate findings. First, we used prostate biopsy specimens that were processed in exactly the same method, namely, immediately freezing the specimen within seconds of retrieval by needle biopsy. We have previously found that surgical manipulation and tissue ischemia have an effect on gene expression profiles in prostate tissue (17), and the other studies used surgical samples and/or organ donor tissues with variable handling and processing. Second, we used LCM to examine changes that are found in a specific histologic cell type, as opposed to using homogenized whole prostate tissue with variable stromal-to-epithelial ratios as in the other studies. For example, differences in stromal gene expression or small, unrecognized foci of prostate cancer may have contributed to the large differences in gene expression seen in prior studies. Importantly, prostate gene expression may be affected by age, race, and BMI, all of which affect prostate cancer risk. In all prior studies, age was significantly different between cancer and control groups, and race and BMI were not provided. Imbalance in these factors, most notably age, may account for some of the gene expression differences in the previous studies, and we stratified for these factors a priori in our study design. On the other hand, the elevated PSA in our control group may have minimized the expression differences in our study, as we did not determine the source of elevated PSA (benign prostatic hyperplasia, inflammation, or occult cancer). Despite this, as stated previously, the risk of occult cancer in our control patients given their prior negative biopsies is low.

Although the genes found in this study require further investigation and validation, these results have clear clinical implications and applications. The existence of these alterations in gene expression before histologic change suggests that they may be the earliest events on the pathway to overt prostatic malignancy. As such, they provide insight into mechanisms of prostate cancer development.
Fig. 2. qPCR of prostate cancer genes in BABE versus CABE patients. qRT-PCR was used to examine the expression of (A) prostate cancer genes that have altered expression in CABE versus BABE patients based on the microarray data and (B) prostate cancer–associated genes not altered between the two groups on microarray. Cycle threshold, denoted on the Y axis, is detected cycle number relative to housekeeping gene RPL13A(dCT). The data are expressed in the graphs as $-1dCT$ to visually reflect the differences in expression between the two groups; thus, in this format, lower mean level corresponds to decreased gene expression.
and serve as potential targets for early intervention or prevention of prostate cancer. They may also serve as molecular markers for men who are at higher risk for prostate cancer diagnosis (e.g., family history) or occult prostate cancer, and may allow for patient stratification in terms of repeat biopsy or follow-up. Lastly, the existence of a field effect has implications for focal therapy for prostate cancer (e.g., high-intensity focused ultrasound and cryotherapy), as treatment of the index tumor alone will potentially fail to extirpate preneoplastic epithelia.

Several genes that were found to be differentially expressed between CABE and BABE have a noteworthy biology, including \( \text{ERG} \), a gene that has been extensively investigated since the recent discovery of genetic translocations between \( \text{TMPRSS2} \) and members of the \( \text{ETS} \) gene family, resulting in \( \text{ERG} \) overexpression in prostate cancer (22, 35–39). It is interesting that \( \text{ERG} \) expression was upregulated in benign epithelial cells from patients with prostate cancer in the absence of the common \( \text{TMPRSS2} \) translocation, suggesting that other mechanisms are at work to increase \( \text{ERG} \) expression in these cells. Our findings suggest that \( \text{ERG} \) overexpression occurs diffusely in prostatic epithelia prone to carcinogenesis and may be one of the earliest changes on the path to overt cancer. Another interesting finding is the increase in \( \text{PSMA} \) expression in the CABE specimens. \( \text{PSMA} \) functions as a folate hydrolase, potentially increasing folate availability (40). Experimental evidence has shown that folate may increase the proliferative ability of prostate cancer cells through \( \text{PSMA} \) (41), and recently, oral folic acid supplementation in the setting of a randomized colorectal cancer clinical trial was associated with an increased risk of prostate cancer diagnosis (42). It is conceivable that overexpression of \( \text{PSMA} \) by normal epithelial cells may provide an early growth advantage through increased folate availability and propensity toward malignancy. \( \text{HOXC4} \) and \( \text{HOXCS} \), members of the \( \text{HOX} \) gene family, were also upregulated and are found to be increased in malignant prostate cell lines, primary prostate tumors, as well as lymph node metastasis (23). Although the mechanism of tumorigenesis in response to alterations in \( \text{HOX} \) gene expression is unclear, \( \text{HOXC4} \) overexpression has been linked to increased androgen receptor activity \textit{in vitro} (23). Of interest about the Ingenuity analysis is that \( \text{FOLH1} \) and \( \text{HOXC4} \), two genes identified as increased in CABE samples, are increased in response to estradiol in prostate and breast cancer cell lines, respectively (Supplementary Fig. S1). Selective estrogen receptor modulators have shown benefit as potential chemoprotective agents for prostate cancer in phase 2 studies, and effects on these two genes may have a role in this function. Lastly, \( \text{MME/CD10} \) was found to be decreased in the CABE group, similar to its decreased

<table>
<thead>
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<th>Table 3. PSMA and CD10 immunohistochemical scores</th>
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<td>Mean immunohistochemical score ± SD</td>
</tr>
<tr>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>( \text{PSMA} )</td>
</tr>
<tr>
<td>( \text{CD10} )</td>
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expression in prostate cancer (24, 43, 44). Potential mechanisms by which MME contributes to tumorigenesis include interactions with heat shock proteins (45) and Akt activity (46), and loss of MME expression portends a higher risk of biochemical recurrence (47).

Potential limitations deserve mention. One limitation of this study is the lack of confirmation that our BABE control group did not indeed harbor occult prostate cancer despite multiple negative biopsies. Although such a confirmatory study is not possible, studies suggest that the likelihood of a false-negative biopsy decreases with multiple negative biopsies (48, 49), and the probability of finding a high-grade or high-volume prostate cancer is unlikely (49). Second, our findings certainly may be due to a field effect caused by the tumor microenvironment rather than due to changes that initiate tumor formation. To address this important issue, one would need to compare biopsies from men who would later develop cancer to biopsies from men who never developed cancer, an ongoing research pursuit. Furthermore, we are not certain that detected transcript differences yield corresponding protein level changes in all genes or, more importantly, altered function, although we were able to show altered protein levels for PSMA and MME by immunohistochemistry, which corresponded with their changes at the mRNA transcript level. Due to the limited number of patients in our tissue bank with high-grade prostate cancer and the fact that some of these patients did not have normal epithelium on the biopsy tissue, our sample size was another limitation. This may increase the potential for false negatives, and thus, it is possible that more genes are differentially expressed between the BABE and CABE groups. Consequently, the power to detect differences in gene expression ≤1.5-fold was modest; thus, some biologically important differences of gene expression may not have been detected. Another limitation is that we only characterized changes that are detectable in the normal epithelium associated with high-grade prostate cancer; further studies are needed to determine if these findings can be generalized to all patients with prostate cancer, in particular those with lower Gleason grades. Lastly, gene expression signatures in epithelium may be influenced by or as a result of changes in the associated stroma, and we did not interrogate the associated prostatic stroma for molecular alterations as has been reported by others (50).

Several additional strengths of our study also merit review. Our controls were age and BMI matched in an attempt to minimize the effects of variables that have a known effect on prostate cancer risk, and thus possibly normal prostate epithelial gene expression. Although there was some racial disparity between our cases and controls, in a separate analysis, we confirmed that our gene expression findings are unchanged when comparing only Caucasians in CABE versus BABE specimens (Supplementary Fig. S3). In addition, although the use of biopsies does not totally rule out occult cancer, it does eliminate the potential confounding effects of surgical manipulation and ischemia on gene expression (17). We used laser-captured microdissected tissue to minimize the cancer and stromal contamination, as well as the potential effects of inflammation and atrophy on our prostatic epithelial gene profiles. Lastly, through qPCR, we were also able to show that the observed alterations in gene expression were not likely to be the result of cancer contamination.

In conclusion, we have shown that there are gene expression differences between histologically normal prostatic epithelium in men with prostate cancer when compared with normal prostatic epithelium from men without prostate cancer. Several genes that we identified have been associated with prostate cancer in prior studies, although the exact mechanisms of cancer induction by these genes remain to be determined. One of these genes is ERG, whose elevated expression in these normal epithelia from men with prostate cancer does not seem to be due to the TMPRSS2:ERG fusion described in a majority of prostate cancers. Further studies are needed to determine if these expression changes precede the appearance of frank malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

DK65083 (D.W. Lin), Pacific Northwest Prostate Cancer SPORE grant CA97186 (D.W. Lin and P.S. Nelson), and Fred Hutchinson Cancer Research Center grant P30 CA015704.

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Received 01/31/2010; revised 09/16/2010; accepted 09/23/2010; published OnlineFirst 10/08/2010.

References

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*Clin Cancer Res* 2010;16:5414-5423. Published OnlineFirst October 8, 2010.

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