Correlation of ERG Expression and DNA Methylation Biomarkers with Adverse Clinicopathologic Features of Prostate Cancer

Ken Kron1,2, Liyang Liu1,2, Dominique Trudel3, Vaijayanti Pethe1, John Trachtenberg4, Neil Fleshner4, Bharati Bapat1,2, and Theodorus van der Kwast2,3

Abstract

Purpose: Fusion of the TMPRSS2 gene with the ERG oncogene and aberrant DNA methylation patterns are commonly found in prostate cancer. The aim of this study was to analyze the relationship between ERG expression, DNA methylation of three biomarkers, and clinicopathologic features of prostate cancer.

Experimental Design: Immunohistochemistry for ERG protein was conducted as a surrogate for TMPRSS2-ERG fusions. We analyzed methylation of CYP26A1, TBX15, and HOXD3 in 219 prostatectomy specimens by the quantitative MethyLight assay. DNA methylation was compared between ERG-positive and -negative cases and correlations of ERG and DNA methylation with clinicopathologic features were analyzed using \( \chi^2 \), Spearman correlation, logistic regression, and Cox regression.

Results: ERG expression varied according to Gleason pattern (almost absent in pattern II, highest in pattern III, and lower in pattern IV/V) and showed a strong positive correlation with methylation levels of CYP26A1, TBX15, and HOXD3 (Spearman \( P < 0.005 \)). TBX15 and HOXD3 methylation were significantly associated with pathologic stage, Gleason score, and Gleason pattern (\( P \leq 0.015 \)). In multivariate regression analysis, PSA, TBX15 high methylation, and HOXD3 high methylation were significantly associated with stage (\( P < 0.05 \)), whereas ERG expression was negatively correlated with Gleason score (\( P = 0.003 \)). In univariate time-to-recurrence analysis, a combination of HOXD3/TBX15 high methylation predicted recurrence in ERG-positive and -negative cases (\( P < 0.05 \)).

Conclusions: CYP26A1, TBX15, and HOXD3 are methylation markers of prostate cancer associated with ERG expression and clinicopathologic variables, suggesting that incorporation of these markers may be useful in a pre- and posttreatment clinical setting. Clin Cancer Res; 18(10); 1–9. © 2012 AACR.

Introduction

Prostate cancer is the most common male malignancy and the third leading cause of cancer-associated death for men in the developed world (1). The introduction of the prostate-specific antigen (PSA) test in the late 1980s has resulted in a marked increase in the diagnosis of prostate cancer but has not subsequently led to a dramatic reduction in death from prostate cancer (2). A large effort in searching for markers that may more accurately identify aggressive prostate cancer, however, has yet to yield a widely adopted clinical assay. In spite of the current lack of clinically applied prostate cancer biomarkers capable of increasing prognostic accuracy, there has been heavy focus on the development of molecular markers of a genetic, expression, and proteomic nature. For example, assessment of EZH2 protein in tissue samples correlates with poor prognosis (3), whereas circulating levels of TGFβ1 in the blood predicts biochemical recurrence (4). In addition, circulating tumor cells and circulating plasma DNA were recently shown to be correlated with prostate cancer grade, stage, and metastasis (5).

TMPRSS2-ETS gene family fusions were the first commonly occurring gene fusions discovered in epithelial malignancies (6). The most common of these fusions, TMPRSS2-ERG, occurs in approximately 50% of prostate cancer cases (7, 8). This fusion event most often arises from deletion of an interstitial fragment of chromosome 21, resulting in the androgen responsive promoter and 5’ end of TMPRSS2 driving the expression of the ERG oncogene (7). Recent work has shown that TMPRSS2-ERG is present in both low-grade and high-grade prostatic intraepithelial

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neoplasia (PIN), indicating that this alteration is an early event in prostate cancer development (9, 10). Indeed, mouse models of the TMPRSS2-ERG translocation develop PIN lesions, but fail to develop prostate cancer without concomitant activation of the phosphoinositide 3-kinase pathway (11). Conflicting results about the prognostic value of TMPRSS2 fusions have also arisen, with some reports claiming a positive association with clinicopathologic features such as disease stage and metastases (7) as well as disease recurrence (12), whereas others have found no association with these variables (13, 14).

DNA methylation of CpG dinucleotides is an epigenetic mechanism responsible for regulation of gene expression. Aberrant DNA methylation is a common anomaly in prostate cancer that results in increased methylation at the promoters of tumor suppressor genes (15), and global hypomethylation leading to genomic instability at later stages of prostate cancer (17). Some of the commonly reported hypermethylated genes in prostate cancer include GSTP1 (18), RASSF1A (19, 20), and adenomatous polyposis coli (APC; ref. 19). The former of these 3 genes is regarded as arising early in the development of prostate cancer, whereas the latter 2 have been reported to cluster more frequently in higher grade and later stage malignancy. We have previously reported hypermethylation of the homeobox gene HOXD3 and its association with aggressive prostate cancer features (21), which was initially discovered through a genome-wide methylation screen of low-grade and high-grade prostate cancer (22). Potential associations of TRX15 and CYP26A1 methylation with high-grade Gleason scores were similarly discovered through the same genome-wide DNA methylation screen.

A link between DNA methylation and ERG expression in prostate cancer has been reported for the prognostic marker PITX2 (23) and for global LINE-1 methylation status (24). Increased methylation of PITX2 and decreased LINE-1 methylation associate with biochemical recurrence (25) and metastatic disease (17), respectively. On the basis of the reports linking DNA methylation and TMPRSS2-ERG fusions, and given the prognostic potential of our previously discovered novel methylation markers CYP26A1, TRX15, and HOXD3 that were discovered through a genome-wide epigenetic screen of high-grade and low-grade prostate cancer (Supplementary Table S1), we assessed the association between ERG expression, DNA methylation, and clinicopathologic features of prostate cancer.

Materials and Methods

Patient cohort and pathology

Prostatectomy specimens from a total of 253 patients diagnosed with prostate cancer between 1998 and 2001 were included in this study. Table 1 lists the clinicopathologic characteristics of the cohort that was analyzed in this study. All samples, as well as clinical and pathologic follow-up data, were obtained according to protocols approved at the Research Ethics Board of Mount Sinai Hospital, Toronto, and the University Health Network, Toronto (ON, Canada).

Table 1. Cohort clinicopathologic characteristics

<table>
<thead>
<tr>
<th>Clinicopathologic characteristics</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gleason score</strong></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>87</td>
</tr>
<tr>
<td>7 (3 + 4)</td>
<td>79</td>
</tr>
<tr>
<td>7 (4 + 3)</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td><strong>Pathologic stage</strong></td>
<td>165</td>
</tr>
<tr>
<td>pT2</td>
<td>62</td>
</tr>
<tr>
<td>pT3a</td>
<td>21</td>
</tr>
<tr>
<td>pT3b</td>
<td>5</td>
</tr>
<tr>
<td><strong>Surgical margin status</strong></td>
<td>195</td>
</tr>
<tr>
<td>Negative</td>
<td>58</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td><strong>Average preoperative PSA (range)</strong></td>
<td>8.4 (0.1–45.8)</td>
</tr>
<tr>
<td><strong>Average follow-up time (range), y</strong></td>
<td>4.38 (0.17–9.48)</td>
</tr>
<tr>
<td><strong>Number of biochemical recurrences (%)</strong></td>
<td>76 (34.7)</td>
</tr>
<tr>
<td><strong>Median age (range), y</strong></td>
<td>62 (32–75)</td>
</tr>
<tr>
<td><strong>N</strong> = 204.</td>
<td></td>
</tr>
<tr>
<td><strong>N</strong> = 219.</td>
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</table>

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The complete set of hematoxylin and eosin (H&E)-stained slides of the 253 prostatectomy specimens were collected and reviewed by an expert pathologist (T. van der Kwast) to assign Gleason score (WHO/ISUP 2005 criteria), pathologic stage (tumor-node-metastasis), and surgical margin status. A subset of slides of the dominant (largest and/or highest grade) tumor was then selected to be representative of the Gleason score for the case.

**Tissue microarray construction and immunohistochemistry**

A range from 3 to 13 cores were taken from each of the 253 cases to have representation of each primary, secondary, and when possible, tertiary Gleason pattern present within the case as well as benign glandular tissue adjacent to the tumor. This yielded a total of 1,490 cores within 7 tissue microarray (TMA) blocks. Five-micrometer serial sections of the tumor. This yielded a total of 1,490 cores within 7 tissue microarray (TMA) blocks. Five-micrometer serial sections of each microarray were used for H&E verification of tumor and normal tissue.

**Immunohistochemistry**

Immunostaining of the TMAs for ERG was conducted as follows: deparaffinized 4-μm sections were dehydrated, blocked in 0.6% hydrogen peroxide in methanol for 20 minutes and processed for antigen retrieval in EDTA (pH 9.0) for 30 minutes in a microwave, followed by 30 minutes of cooling in EDTA buffer. Sections were then blocked in 1% horse serum followed by an overnight incubation with the ERG-mAb mouse monoclonal antibody (Biocare Medical; clone 9Fy), diluted 1:300 at room temperature. The immunostaining was developed by the Polymer-HRP Immunohistochemistry Kit (Biogenex) according to manufacturer's instructions. Next, sections were counterstained in hematoxylin for 1 minute, dehydrated, cleared, and mounted. Immunostained slides were scanned with the Aperio system at objective × 20, facilitating the scoring of the individual TMA cores. ERG staining was evaluated on the basis of percentage of epithelial cells staining positive and the intensity of staining relative to an internal control (endothelial cells with positive staining). Cores with faint or negative endothelial cell staining were excluded from analysis. Intensity was graded on a scale of 0 to 3 with 0 representing no staining: 1, faint positivity; 2, intensity equal to internal control; and 3, intensity greater than internal control. ERG expression was then separated into binary values for positive and negative expression. Those cores with an intensity of 1 or more in greater than 10% of cells were considered positive whereas a score of 0 or staining in ≤10% of cells were considered negative. We considered a case positive for ERG expression if any of the arrayed cores from that case displayed positive ERG immunohistochemistry as described earlier.

**DNA extraction, sodium bisulfite modification, and the MethyLight assay**

Extraction of DNA, bisulfite modification, and MethyLight analysis were conducted as previously described (21). Briefly, tumor areas were marked on H&E slides and the area was outlined on 10-μm sections of tissue after superimposing. The areas were scraped with a scalpel into a 1.5 mL microfuge tube and DNA was extracted by the Qiagen DNA Mini Kit (Qiagen) with a modified protocol (21). Sodium bisulfite modification was carried out by the EZ DNA Methylation Gold Kit (Zymo Research Corporation) following the manufacturer’s protocol and DNA was eluted to a final concentration of 20 ng/μL.

The MethyLight quantitative PCR assay was conducted using the TaqMan Gold Buffer A pack (Life Technologies). All benign tissue and tumor foci were analyzed in duplicate. Primers and probe sequences for CYP26A1 and TBX15 are as follows: CYP26A1 forward, 5′-TTG TAG AGA AGA CTA CGT CAA ACA TCC TCT ACG-3′; CYP26A1 reverse, 5′-AAA ACC TTC CGT CAA ACA TCC TCT ACG-3′; CYP26A1 probe, 5′-FAM-ACG CCC ACG AGC TAC CCC CCT TAC-3′BHQ1; TBX15 forward, 5′-GCC GTT TTG TAA GTAT ATT TGT TGC G-3′; TBX15 reverse, 5′-ACT CCG AAT AAA ACA AAA ACT AAA TCC CG-3′; and TBX15 probe, 5′-FAM-CAA ATA ACG CCG CCG AAC GCC T-3′BHQ1.

Primers and probe sequences for HOXD3 have been described previously (21). The control Alu-C4 reaction was used to assess both DNA quantity and quality of bisulfite modification (26). An Alu-C4 Ct ≥ 22 was used as a threshold for poor quality DNA/incomplete modification and were not included in the analyses. Two hundred and nineteen cases were of sufficient quality and quantity, 212 of which overlapped with the 253 used for ERG immunohistochemistry. A percentage of methylation value (percentage of methylated reference; PMR) was obtained from averaging duplicate runs. PMR duplicate values with a difference greater than 10% were repeated. When multiple Gleason patterns/foci were analyzed for the same case, an average of all foci was assigned to obtain an overall PMR for that case.

**Statistical analyses**

For χ2, regression, and survival analyses described later, the continuous PMR variable was separated into high methylation and low methylation based on a third quartile threshold as previously described (21). Univariate disease-free survival was assessed using the Kaplan-Meier curve and log-rank tests. Cox proportional hazards regression analysis and likelihood ratio tests were used for multivariate disease-free survival. The cohort size in the Cox models was reduced to 195 patients as pretreatment PSA data were not available for all cases. A bootstrap sampling of 1,000 cases was further conducted in the final model to verify robustness. Each factor was coded as a binary variable (with the exception of PSA and age) to keep the number of parameters in the multivariate model ≤7 (approximately 10 events per variable), which reduces the likelihood of fitting noise into the model (27). Stage was coded as organ confined (pT2) or locally advanced (pT3/pT4), surgical margin status as negative or positive, Gleason score as ≤7 versus 8 and above, ERG expression as present versus absent, and methylation as low methylation or high methylation. Age and PSA were included as linear variables.
The association of DNA methylation (as a continuous PMR variable) with clinicopathologic features and ERG immunohistochemistry was assessed using the Spearman method for correlation. Independent effects of each significant variable from Spearman correlation analysis were further verified using logistic regression. Data were again coded as binary as described earlier for Cox regression, with the exception that Gleason score was dichotomized into $\leq 3$ and $> 3$. The proportion of ERG positivity distributed by pathologic stage is shown in C. $P$ values represent $\chi^2$ analyses.

Results

ERG immunohistochemical findings

The monoclonal ERG antibody used in this study has previously been shown to accurately reflect the presence of TMPRSS2-ERG fusions in prostate cancer (28), and we observed an ERG nuclear staining pattern (Supplementary Fig. S1) similar to immunohistochemical results previously reported for similar antibodies (29).

Of the 253 cases that were represented on the TMA, 8 did not yield information due to a lack of internal control endothelial cell immunohistochemical staining. Positive ERG protein expression was observed in 125 of 245 (51.0%) of cases, consistent with prior reports of the frequency of TMPRSS2-ERG fusions (8, 30). We analyzed ERG expression comparing Gleason patterns 2, 3, 4, 5, and benign tissue (Fig. 1A). We combined high-grade patterns 4 and 5 into one category as there were only 11 specimens available in the pattern 5 category (4 of 11 positive; 36.4%).

Of the 194 tumor-adjacent benign specimens analyzed, only 1 was positive for ERG expression. In addition, ERG expression was only present in 3 of 51 (5.6%) of pattern 2 specimens, whereas it was highly enriched in pattern 3 (56.9%) and somewhat reduced in pattern 4 and 5 (43.0%) compared with pattern 3. Overall, the distribution of ERG expression was significantly different across the well differentiated to poorly differentiated tumor spectrum ($\chi^2$ $P$ value $= 1.14 \times 10^{-10}$). The difference between pattern 4 and pattern 3 expression was also statistically significant (Fig. 1A; $\chi^2$ $P$ value $= 0.011$).

With regard to its association with Gleason score, ERG expression was found more frequently in low- and intermediate-grade (Gleason score 5, 6, and 7) prostate cancer, whereas Gleason score 8 to 10 prostate cancer had a lower frequency of positive expression (Fig. 1B). The distribution of ERG expression according to Gleason score was significantly different ($\chi^2$ $P$ value $= 0.004$). With respect to pathologic stage, we found a significant increase in ERG positivity when comparing organ confined pT2 to locally advanced pT3/pT4 prostate cancer (Fig. 1C; $\chi^2$ $P$ value $= 0.005$).

We next examined the relationship between ERG expression and biochemical recurrence within this patient cohort. Univariate Kaplan–Meier/log-rank analysis showed no association between ERG expression and disease-free survival ($P$ value $= 0.367$; Supplementary Fig. S2A). Clinicopathologic variables including PSA, Gleason score, pathologic stage, and surgical margin status were significantly associated with biochemical recurrence (Supplementary Fig. S3). We also conducted multivariate Cox regression analysis including clinicopathologic variables PSA, Gleason score, pathologic stage, surgical margin status, and age (Supplementary Table S2). Again, ERG was not significant in predicting recurrence ($P = 0.103$), whereas stage, Gleason score, and positive surgical margin status remained significant predictors ($P < 0.01$).

Associations between DNA methylation and clinicopathologic variables

First, we analyzed the differences of average PMR values of CYP26A1, TRX15, and HOXD3 between all tumor foci and adjacent benign specimens. PMR values were significantly greater in cancer specimens than in tumor adjacent
benign specimens for CYP26A1, TBX15, and HOXD3 (Mann–Whitney U-test, P values = 1.96 × 10^{-42}, 2.10 × 10^{-45}, and 9.01 × 10^{-35}, respectively). Next, we tested the difference in the proportion of high methylation specimens between Gleason patterns for CYP26A1, TBX15, and HOXD3. CYP26A1 high methylation was not associated with Gleason pattern (χ² P value = 0.997), whereas TBX15 and HOXD3 high methylation were strongly associated with this variable (Fig. 2A, χ² P values = 1.41 × 10^{-4} and 5.64 × 10^{-20}, respectively). In particular, TBX15 methylation significantly increased when comparing pattern 3 versus pattern 4/5 (χ² P value = 0.003). As previously reported (20), the proportion of HOXD3 high methylation specimens also increased from pattern 2 to 3 and from pattern 3 to 4 (χ² P values = 0.001 and 2.46 × 10^{-4}, respectively).

Next, we tested the association between PMR values for each gene and the clinicopathologic variables including Gleason score, pathologic stage, and preoperative PSA levels. We also analyzed the association between PMR and age (as methylation of certain loci often increases with age), between PMRs for each gene (Table 2). For Gleason score, the data were separated into Gleason score 4/5 cancers, Gleason score 6 cancers, Gleason score 7 (3 + 4), Gleason score 7 (4 + 3) and high-grade (GS ≥ 8) cancers. Both TBX15 and HOXD3 were significantly associated with Gleason score and pathologic stage (Table 2). Similarly, TBX15 and HOXD3 high methylation were strongly associated with locally advanced cancers (Spearman P values = 1.14 × 10^{-10} and 5.32 × 10^{-5}, respectively), whereas TBX15 was also associated with preoperative PSA values. CYP26A1 was not associated with Gleason score, stage, or PSA, whereas age was not associated with methylation of any gene.

Next, we examined the relationship between disease-free survival (biochemical recurrence) and high methylation of

Table 2. Spearman correlation values of clinicopathologic data, ERG status, and methylation markers

<table>
<thead>
<tr>
<th></th>
<th>Gleason score</th>
<th>PSA</th>
<th>Age</th>
<th>ERG status</th>
<th>CYP26A1 PMR</th>
<th>TBX15 PMR</th>
<th>HOXD3 PMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage Coefficient</td>
<td>0.361</td>
<td>0.225</td>
<td>0.008</td>
<td>0.172</td>
<td>0.114</td>
<td>0.292</td>
<td>0.270</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.012</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gleason score Coefficient</td>
<td>0.215</td>
<td>0.126</td>
<td>−0.142</td>
<td>0.025</td>
<td>0.164</td>
<td>0.349</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.002</td>
<td>0.062</td>
<td>0.038</td>
<td>0.717</td>
<td>0.015</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>PSA Coefficient</td>
<td>0.003</td>
<td>−0.082</td>
<td>0.093</td>
<td>0.171</td>
<td>0.015</td>
<td>0.323</td>
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</tr>
<tr>
<td>P value</td>
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<td>0.255</td>
<td>0.191</td>
<td>0.015</td>
<td>0.015</td>
<td>0.323</td>
<td></td>
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<tr>
<td>Age Coefficient</td>
<td>−0.104</td>
<td>0.036</td>
<td>0.032</td>
<td>0.012</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P value</td>
<td>0.130</td>
<td>0.593</td>
<td>0.637</td>
<td>0.862</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>ERG status Coefficient</td>
<td>0.194</td>
<td>0.352</td>
<td>0.260</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.459</td>
<td>0.192</td>
<td>0.004</td>
<td>0.402</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>CYP26A1 PMR Coefficient</td>
<td>0.459</td>
<td>0.192</td>
<td>0.004</td>
<td>0.402</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>P value</td>
<td>0.459</td>
<td>0.192</td>
<td>0.004</td>
<td>0.402</td>
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<td>0.192</td>
<td>0.004</td>
<td>0.402</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>P value</td>
<td>0.459</td>
<td>0.192</td>
<td>0.004</td>
<td>0.402</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
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</tbody>
</table>

DNA Methylation and ERG Expression in Prostate Cancer

![Figure 2. Proportion of high methylation (HM) cases for CYP26A1, TBX15, and HOXD3 separated by (A) Gleason pattern and (B) ERG expression status. P values obtained from χ² analysis.](https://www.aacrjournals.org/doi/abs/10.1158/1078-0432.CCR-11-2901)
CYP26A1, TBX15, and HOXD3. Univariate log-rank analysis of CYP26A1 and HOXD3 high methylation showed no significant association with disease-free survival (Supplementary Fig. S2; P values = 0.581 and 0.067, respectively), whereas TBX15 high methylation was significantly associated with biochemical recurrence (P = 0.003). A combination of TBX15 and HOXD3 high methylation (having neither gene highly methylated versus one, the other, or both) proved to be the most significant predictor of biochemical recurrence when considering the methylation data (P = 0.002). Using multivariate Cox regression analysis, however, none of the methylation markers were independently associated with biochemical recurrence (Supplementary Tables S3–S6; P values = 0.740, 0.424, 0.431, and 0.170 for CYP26A1, TBX15, HOXD3, and TBX15/HOXD3 combination, respectively).

**Correlation of ERG overexpression and DNA methylation with clinicopathologic variables**

We discovered strong correlations between methylation of each gene with each other (Table 2, all Spearman P values <0.01). Furthermore, methylation of these genes was also correlated with ERG, particularly HOXD3 and TBX15 (Fig. 2B and Spearman analysis; Spearman P values = 1.28 × 10^{-4} and 1.35 × 10^{-7}, respectively).

In the regression model, PSAs and TBX15 high methylation were the most significant variables (P = 0.010), whereas HOXD3 was also significant (P = 0.026) and ERG trended toward significance (P = 0.071). In the regression model for Gleason score, PSA (P = 0.020), ERG expression (P = 0.003), and HOXD3 high methylation (P = 2.47 × 10^{-4}) were significant. Of note, ERG expression was negatively associated with Gleason score.

Next, we tested the value of each methylation marker in predicting biochemical recurrence when stratified by ERG expression status as the correlation between ERG expression and methylation suggested methylation may be particularly relevant in ERG-positive cases. CYP26A1 was not a prognostic indicator regardless of ERG expression status (P > 0.6). HOXD3 high methylation trended toward significant in ERG-positive cases (P = 0.083), whereas in ERG-negative cases the difference was not significant (P = 0.213). For TBX15, however, high methylation was significant in ERG-positive cases (Figure 3; P = 0.01), whereas in ERG-negative cases there was a similar but nonsignificant trend (P = 0.066). For the HOXD3/TBX15 combination, high methylation was significant in both ERG-positive and ERG-negative cases, whereas there was an interesting time-dependent trend whereby ERG-negative cases with TBX15/HOXD3 high methylation had early recurrence and ERG-positive cases with TBX15/HOXD3 high methylation trended toward later biochemical recurrence (2 years; Supplementary Fig. S4).

Finally, we assessed the independent contributions of ERG alongside each methylation marker in predicting biochemical recurrence using a Cox proportional hazards model that included clinicopathologic variables Gleason score, pathologic stage, preoperative PSA, surgical margin status, and age. None of the methylation markers by themselves were significant predictors nor was ERG expression (P > 0.05). In a model that included the HOXD3/TBX15 combination and ERG expression, however, both were significant predictors in multivariate analyses, although upon log-ratio testing and bootstrap validation the P values did not meet the significance threshold (Table 4). Interestingly, ERG expression was a protective factor, whereas HOXD3/TBX15 high methylation was a poor prognostic indicator as expected. Taken together, these results suggest that the prognostic value of these methylation markers may in part depend on ERG expression and vice versa.

**Discussion**

The discovery of frequent TMPRSS2-ETS fusions (in particular TMPRSS2-ERG) and their biologic implications has led to a greater understanding of the development and progression of prostate cancer. Here, we have used ERG immunohistochemistry as a surrogate marker for TMPRSS2-

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**Table 3. Logistic regression model for pathologic stage and Gleason score**

<table>
<thead>
<tr>
<th>Pathologic stagea</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>Gleason scoreb</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PSA</td>
<td>1.080 (1.019–1.146)</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>ERG</td>
<td>1.828 (0.950–3.519)</td>
<td>0.071</td>
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<tr>
<td></td>
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<td>TBX15 HM</td>
<td>2.518 (1.252–5.066)</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>HOXD3 HM</td>
<td>2.211 (1.098–4.450)</td>
<td>0.026</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>1.066 (1.010–1.125)</td>
<td>0.020</td>
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<tr>
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<td></td>
<td></td>
<td>0.288 (0.129–0.646)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.472 (0.649–3.341)</td>
<td>0.355</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.443 (1.970–10.021)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; HM, high methylation.

aConcordance index for model = 0.739.
bConcordance index for model = 0.724.
ERG fusion expression, assessed the prognostic implications of ERG expression, and the association between ERG expression and DNA methylation markers with clinicopathologic features in prostate cancer.

Our results show that ERG expression is virtually absent from benign glandular epithelium adjacent to tumor, and is present at a very low frequency in pattern 2 transition zone cancers. ERG was predominantly observed in moderate to poorly differentiated carcinomas (Gleason patterns 3 and 4), in particular within Gleason scores 6 and 7. These results are consistent with published data (10, 31, 32). We also observed an intriguing decreased proportion of ERG expression in Gleason pattern 4 tumors when compared with Gleason pattern 3, similar to recent data reported by Darnel and colleagues (33). This difference seemed to be driven largely by pattern 3 tumors of Gleason score 6 origin, as 64% of these specimens were positive for ERG expression as compared with 52% for pattern 3 within Gleason score 7 cases, although this comparison was not significant \((P = 0.113)\). The difference observed in ERG expression stratified by Gleason pattern has interesting implications. First, it suggests that cancers of the transition zone and peripheral zone may have different biologic origins, which is expected given existing data showing different expression profiles of normal transition and peripheral zones as well as differential steroid effects on growth and gene expression (34, 35).

Second, it is possible that peripheral zone cancers with a TMPRSS2-ERG fusion are less likely to become poorly differentiated, which may be the result of activation of select transcriptional pathways by the ERG protein. Further studies are required to assess underlying biologic pathways.

Conversely, we did observe a strong positive association between ERG expression and pathologic stage, which has also previously been reported (7). This suggests that while ERG may not have an effect on genes involved in differentiation, it does activate the expression of genes that promote cell motility and invasion (36, 37). Despite the correlation with pathologic stage, our data indicates ERG expression does not predict biochemical recurrence after prostatectomy. This is in contrast to early reports about a positive association between TMPRSS2-ERG and disease recurrence (12) and consistent with recent reports that have failed to find any prognostic significance in fusion gene-positive cancers (14, 38).

Intriguingly, we observed significant correlations between ERG-positive cancers and methylation of CYP26A1, TBX15, and the previously published methylation marker HOXD3. This relationship is not a ubiquitous one, however, as we did not observe a correlation between ERG expression and the previously reported prognostic methylation marker APC (data not shown) in the same series of prostate cancer specimens. Also, the correlations between TBX15/HOXD3 methylation and ERG expression were much stronger than that for the cancer specific methylation marker CYP26A1. The functional mechanism that leads to the relationship between methylation of these specific genes and ERG expression is unknown and needs further investigation. One possibility is that ERG drives the expression of DNA methyltransferases such as DNMT3A and DNMT3B, which subsequently leads to de novo or increased methylation at specific gene loci. Indeed, expression of epigenetic silencing enzymes HDAC1 and EZH2 have been shown to significantly coexist with high ERG expression (in the case of HDAC1) or to be caused specifically by TMPRSS2-ERG (in the case of EZH2; refs. 39, 40).

Alternatively, decreased histone acetylation via HDAC1 and/or increased H3K27 methylation via EZH2, and crosstalk between epigenetic modifying machinery might facilitate DNA methylation (41). Either of these mechanisms may also explain the positive correlation between each of our methylation markers, as overexpression of DNMTs or other epigenetic silencing machinery could lead to a predisposition for methylation of a specific set of gene loci.

It is now becoming increasingly clear that ERG-positive cancers represent a biologically distinct subclass of prostate cancer at the epigenetic and transcriptome level (23, 24, 42). Our data indicates that these differences may be exploited to identify aggressive prostate cancer and offer better treatment decisions. ERG expression and DNA methylation of TBX15 and HOXD3 have a clinical potential in both a preoperative and postoperative setting. Our logistic regression analysis revealed that HOXD3 and TBX15 high methylation independently associate with pathologic stage, whereas HOXD3 methylation was strongly associated with Gleason score and

### Table 4. Multivariate Cox proportional hazards model for disease-free survival

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>P value LR test</th>
<th>Bootstrap P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical margins</td>
<td>2.432 (1.475–4.008)</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Pathologic stage</td>
<td>3.212 (1.902–5.426)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Gleason score</td>
<td>2.369 (1.082–5.187)</td>
<td>0.031</td>
<td>0.032</td>
<td>0.040</td>
</tr>
<tr>
<td>Age</td>
<td>1.017 (0.980–1.055)</td>
<td>0.367</td>
<td>N/A</td>
<td>0.405</td>
</tr>
<tr>
<td>PSA</td>
<td>1.035 (0.996–1.075)</td>
<td>0.080</td>
<td>0.076</td>
<td>0.098</td>
</tr>
<tr>
<td>CYP26A1/TBX15/HOXD3 HM</td>
<td>1.712 (1.015–2.885)</td>
<td>0.044</td>
<td>0.056</td>
<td>0.087</td>
</tr>
<tr>
<td>ERG</td>
<td>0.560 (0.325–0.963)</td>
<td>0.036</td>
<td>0.033</td>
<td>0.061</td>
</tr>
</tbody>
</table>

**Abbreviations:** CI, confidence interval; HM, high methylation.

*P* value from log ratio test; age not included in final model.
ERG expression was negatively associated with Gleason score. Thus, detection of ERG expression (or alternatively TMPRSS2-ERG fusion RNA) combined with DNA methylation detection in urine specimens following digital rectal exam may function simultaneously as a diagnostic and prognostic test which gives information about the likelihood of high-grade and locally advanced cancer. It may also be useful in a postbiopsy, pretreatment setting to more accurately predict advanced cancer, perhaps in combination with already existing nomograms (43). In addition, the time-to-recurrence predictive capabilities of HOXD3 and TBX15 high methylation seems to differ according to ERG expression status, in particular as it predicts late biochemical recurrence in ERG-positive cases and early biochemical recurrence in ERG-negative cases. Furthermore, our results suggest that ERG expression may play a protective role in prostate cancer, similar to other studies which have found more favorable outcomes for ERG-positive prostate cancer (44, 45).

These results bring to light an important caveat in biomarker discovery—that is, the prognostic capabilities or pathologic correlations of any given marker may depend on other genetic/epigenetic factors. As TMPRSS2-ERG fusion is considered an early event in prostate cancer development that leads to activation of specific pathways, the functional and clinical significance of either concomitant or downstream epigenetic events may differ according to presence/absence of this fusion.

In conclusion, we have discovered a novel diagnostic and potential prognostic role for CY26A1 and TBX15, respectively. We also show that methylation of these genes, as well as HOXD3, are correlated with ERG expression and that ERG expression, TBX15 methylation, and HOXD3 methylation may be useful in a pretreatment model for grade and stage prediction. Finally, we show that the prognostic abilities of HOXD3 methylation, TBX15 methylation, and ERG expression appear to be codependent. Further definition of molecular subtypes in prostate cancer through combined epigenetic, genetic, and expression profiling may better classify patients into distinct categories for prognosis and individualized treatment regimens.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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