Multiplex Biomarker Approach for Determining Risk of Prostate-Specific Antigen-Defined Recurrence of Prostate Cancer

Daniel R. Rhodes, Martin G. Sanda, Arie P. Otte, Arul M. Chinnaiyan, Mark A. Rubin

Background: **Molecular signatures in cancer tissue may be useful for diagnosis and are associated with survival. We used results from high-density tissue microarrays (TMAs) to define combinations of candidate biomarkers associated with the rate of prostate cancer progression after radical prostatectomy that could identify patients at high risk for recurrence.** ***Methods:*** **Fourteen candidate biomarkers for prostate cancer for which antibodies are available included hepsin, pim-1 kinase, E-cadherin (ECAD; cell adhesion molecule), α-methylacyl-coenzyme A racemase, and EZH2 (enhancer of zeste homolog 2, a transcriptional repressor). TMAs containing more than 2000 tumor samples from 259 patients who underwent radical prostatectomy for localized prostate cancer were studied with these antibodies. Immunohistochemistry results were evaluated in conjunction with clinical parameters associated with prostate cancer progression, including tumor stage, Gleason score, and prostate-specific antigen (PSA) level. Recurrence was defined as a postsurgery PSA level of more than 0.2 ng/mL. All statistical tests were two-sided.** ***Results:*** **Moderate or strong expression of EZH2 coupled with at most moderate expression of ECAD (i.e., a positive EZH2:ECAD status) was the biomarker combination that was most strongly associated with the recurrence of prostate cancer. EZH2:ECAD status was statistically significantly associated with prostate cancer recurrence in a training set of 103 patients (relative risk [RR] = 2.52, 95% confidence interval [CI] = 1.09 to 5.81; *P* = .021), in a validation set of 80 patients (RR = 3.72, 95% CI = 1.27 to 10.91; *P* = .009), and in the combined set of 183 patients (RR = 2.96, 95% CI = 1.56 to 5.61; *P*<.001). EZH2:ECAD status was statistically significantly associated with disease recurrence even after adjusting for clinical parameters, such as tumor stage, Gleason score, and PSA level (hazard ratio = 3.19, 95% CI = 1.50 to 6.77; *P* = .003).** ***Conclusion:*** **EZH2:ECAD status was statistically significantly associated with prostate cancer recurrence after radical prostatectomy and may be useful in defining a cohort of high-risk patients.** **[J Natl Cancer Inst 2003;95:661–8]**

The dilemma in managing patients with prostate cancer is that only a fraction of cases would lead to cancer-related death if left untreated but, because of the extremely high prevalence of prostate cancer, its mortality rate in men is second only to lung cancer. Consequently, there is a great need to accurately assess the risk of disease progression in patients with prostate cancer so that appropriate treatment options can be considered. Several clinical parameters including tumor stage, tumor grade as measured by the Gleason score, and the serum level of prostate-specific antigen (PSA) are typically used to assess the risk of disease progression at the time of diagnosis *(1).* However, with the adoption of population-based PSA screening, the majority of men in the United States who are diagnosed with prostate cancer are at low to intermediate risk for disease-specific mortality and will often die of comorbidities. A recent study *(2)* demonstrated that PSA screening may in fact lead to the overdiagnosis and overtreatment of patients with prostate cancer, suggesting that some patients who undergo radical prostatectomy might have lived out their lives without any symptoms of the disease.

Important clinical trials have begun to evaluate watchful-waiting protocols, in which the decision to have surgery is postponed until disease progression is observed, because currently the risk of waiting as opposed to having immediate surgery is not fully known *(3).* One important limitation to current watchful-waiting protocols lies in the subjective criteria used to select patients for waiting versus having surgery. If the likelihood of disease progression could be more accurately predicted at diagnosis, the success of such protocols would improve, allowing more men to remain on watchful-waiting protocols for clinically localized disease.

Although surgery may be unnecessary for some patients with clinically localized disease, others will require more aggressive treatment despite having localized disease. After radical prostatectomy, the disease recurs in an estimated 15%–30% of patients, suggesting that undetected disease may have spread beyond the prostate gland before surgery *(4,5).* Kattan et al. have developed useful nomograms that use pretreatment clinical and pathologic parameters to help evaluate the likelihood of disease-free survival after radical prostatectomy *(6)* or brachytherapy *(7)* for localized prostate cancer. However, these and other models have limitations as demonstrated by good but not excellent associations with outcome, as reviewed by Ross et al. *(8).* Therefore, given the limitations of current nomograms to accurately predict which patients have the greatest risk for developing aggressive prostate cancer, researchers have been identifying and characterizing biomarkers for prostate cancer to aid the pretreatment evaluation of patients with clinically localized prostate cancer.

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Recent advances in genomic and proteomic technologies suggest that molecular signatures of disease can be used for diagnosis *(9,10)*, to predict survival *(11,12)*, and to define molecular subtypes of the disease *(13)*. Complementary DNA (cDNA) microarrays have been used to characterize gene expression profiles for prostate cancer tissue, benign prostate disease tissue, and normal prostate tissue *(14–19)*. We reported *(20)* a meta-analysis of four microarray studies, identifying the genes that are most consistently overexpressed and underexpressed in prostate cancer tissues. In another study *(17)*, researchers devised a gene expression system that could predict the risk of recurrence after surgery; however, its importance was limited by the small number of patients evaluated (i.e., eight recurrences).

Over the last few years, we have used tissue microarrays (TMAs) of clinical specimens to study the individual expression of potential protein biomarkers. We immunohistochemically characterized hepsin and pim-1 kinase proteins, which had been identified by cDNA microarray analysis, as being overexpressed in prostate cancer tissue *(16)*. Both proteins were highly expressed in prostate cancer tissue and, interestingly, both were inversely associated with recurrence-free survival. In addition, α-methylacyl-coenzyme A racemase (AMACR) was identified as a sensitive and specific tissue biomarker for prostate cancer *(21)*, and EZH2 (enhancer of zeste homolog 2, a transcriptional repressor) was identified as being overexpressed in metastatic prostate cancer *(22)*.

In this study, we used our TMA database to define combinations of biomarkers associated with disease progression in prostate cancer that, in conjunction with current clinical and pathologic parameters, could improve our ability to identify patients at high risk for disease recurrence after radical prostatectomy.

METHODS

Dataset

The original dataset contained the results of 38 immunohistochemical analyses. In total, 19 206 evaluations, consisting of a diagnosis and stain intensity, were available. For this study, the analysis was limited to a set of 6096 TMA specimens that were confirmed as primary prostate cancer. TMA samples from patients diagnosed with metastatic prostate cancer or from benign prostatic tissue were not included. Also, because some antibodies were tested on TMAs with specimens from fewer than 30 patients with prostate cancer, the dataset was further reduced to results from 14 antibodies that were detected on one or more of six unique TMAs that collectively contained more than 2000 prostate tumor specimens, representing tumors from 259 men with clinically localized prostate cancer.

Subjects, Clinical Data, and Prostate Sample Collection

Clinical samples were taken from the radical prostatectomy series at the University of Michigan, part of the University of Michigan Prostate Cancer Specialized Program of Research Excellence (S.P.O.R.E.) Tissue Core. Prostatectomy specimens used to construct the TMAs were obtained from 259 patients who underwent radical retropubic prostatectomy as the primary monotherapy (i.e., no adjuvant or neoadjuvant hormonal or radiation therapy) for clinically localized prostate cancer from January 1, 1995, through August 1, 2002, and who had sufficient cancer tissue available at prostatectomy for representative cancer tissue to be harvested for TMA construction after the standard clinical histopathologic assessment of the primary prostate tumor was completed. This study was approved by the Institutional Review Board at the University of Michigan Medical Center. Patients provided written informed consent according to the University of Michigan S.P.O.R.E. Clinical Core protocol as part of the ongoing molecular analysis of prostate cancer. Detailed clinical, pathology, and TMA data are maintained in a secure relational database as previously described *(23)*. Pertinent clinical information about these patients (clinical stage, pretreatment PSA level, tumor stage, surgical margin status, and Gleason score) was prospectively collected and stored in this database. Clinical post-prostatectomy follow-up data, including an annual patient assessment by clinic visit, phone, or mail contact to ascertain overall, cancer-specific, and recurrence-free survival (defined as a PSA level of 2 ng/mL or less), were also ascertained and stored prospectively in this database. All patients were advised to undergo a serum PSA test at least once a year. A PSA level of greater than 0.2 ng/mL is defined as biochemical evidence of disease recurrence or progression (i.e., a PSA-defined recurrence).

All 259 patients studied received radical prostatectomy to treat their localized disease. The median postsurgery follow-up was 57 months (range = 2.7–87.4 months), and the average age at surgery was 61 years (range = 39–80 years). More than 90% of the tumors had a Gleason score of 6 or 7 (Gleason score <6, 4%; Gleason score = 6, 38%; Gleason score = 7, 53%; Gleason score >7, 5%), 77% of tumors were organ-confined (stage pT2), 19% of tumors had signs of local invasion (stage pT3), and 4% of tumors had disease involving the urinary bladder (stage pT4). Among the 259 patients, 53 had a biochemical treatment failure. A detectable level of PSA after radical prostatectomy is indicative of surgical treatment failure or disease recurrence *(24)*. Median time to PSA-defined recurrence was 11.4 months (range = 1.4–60.3 months). Table 1 summarizes patient demographics and clinical and pathology variables.

Tumors were graded by the Gleason grading system *(25)*. To construct high-density TMAs, all glass specimen slides were re-examined to identify areas of prostate cancer. Because the majority of specimens contained multiple tumor foci, we selected areas corresponding to the primary and secondary Gleason patterns. To optimize the tissue transfer to the arrays, each lesion was tightly circled on the glass slide.

Immunohistochemistry

Standard biotin–avidin complex immunohistochemistry was used to evaluate the following biomarkers: E-cadherin (ECAD) *(26)*, activated in prostate cancer (AIPC) *(27)*, pim-1 kinase *(16)*, hepsin *(16)*, AMACR *(21)*, and EZH2 *(22)*. Additionally, eight other biomarkers were tested but failed to demonstrate significant associations at the univariate level and therefore are not further discussed. Protein expression was scored as follows: negative = 1, weak = 2, moderate = 3, or strong = 4.

TMA Construction, Digital Image Capture, and Analysis

TMAs were assembled with the manual or the newly developed automated tissue arrayer (Beecher Instruments, Sun Prairie, WI), as previously described *(28)*. Tissue cores from the

Table 1. Patient demographics for the training and validation tissue microarray sets*

Variable	Training set	Validation set	Complete set
No. of patients	103	97	259
Median age, y (range)	59 (43–80)	62 (39–78)	61 (39–80)
Median pretreatment PSA level, ng/mL (range)	6.7 (0.5–43.3)	6.3 (0.4–85.7)	6.3 (0.4–85.7)
DRE, No. (%)			
Negative	63 (62)	55 (57)	157 (61)
Positive	40 (39)	42 (43)	102 (40)
Gleason score category, No. (%)			
≤6	39 (38)	40 (42)	109 (42)
3 + 4 = 7	52 (51)	39 (40)	110 (43)
4 + 3 = 7	8 (8)	15 (16)	30 (12)
≥8	4 (4)	3 (3)	10 (4)
Pathologic stage, No. (%)			
pT2	83 (81)	69 (71)	199 (77)
pT3	16 (16)	24 (25)	49 (19)
pT4	4 (4)	3 (3)	10 (4)
Surgical margin status, No. (%)			
Negative	74 (72)	68 (70)	187 (72)
Positive	29 (28)	29 (30)	72 (28)
Median maximum tumor diameter, cm (range)	1.4 (0.1–3.6)	1.5 (0.3–3.5)	1.4 (0.1–3.6)
Median gland weight, g (range)	47 (26–128)	45 (22–169)	47 (22–169)
PSA-defined recurrence, No. (%)†			
No	80 (78)	78 (80)	205 (79)
Yes	23 (22)	18 (19)	53 (21)

*PSA = prostate-specific antigen; DRE = digital rectal examination.

†PSA-defined recurrence = biochemical treatment failure after prostatectomy for clinically localized prostate cancer defined at a PSA level greater than 2 ng/mL.

tightly circled areas (as described above) were targeted for transfer to the recipient array blocks. Up to six replicate tissue cores were sampled from each circled area. To construct a TMA, tissue cores with a 0.6-mm diameter were spaced at 0.8 mm from core center to core center. After TMA construction, 4-μm sections were cut, and the initial slide was stained with hematoxylin and eosin to verify the histologic diagnosis. Hematoxylin and eosin images of the TMA were acquired with the BLISS microscope imaging workstation (Bacus Laboratories, Inc., Lombard, IL). Protein expression was evaluated in a blinded manner with an Internet-based TMA presentation tool, the TMA Profiler (University of Michigan, Ann Arbor, MI), by one dedicated genitourinary pathologist (M. A. Rubin). In addition, all TMA samples were histologically classified (i.e., benign, atrophy, high-grade prostatic intraepithelial neoplasia, or prostate cancer). The targeted tissue may not be the tissue that was actually transferred; therefore, verification is required at each step. All data entered into the TMA Profiler were stored in a relational database.

Data Analysis

Data tables were exported from a Microsoft Access database, formatted, and then loaded into SPSS (SPSS Inc., Chicago, IL) for analysis. When a tumor was represented by multiple tissue cores, median stain intensities were taken. Median stain values were also evaluated as dichotomized variables, defined as negative for absent or weak staining (score = 1 or 2, respectively) and positive for moderate or high staining (score = 3 or 4, respectively). This approach has been previously determined to be reliable and reproducible *(16,21)*. Immunohistochemistry results were evaluated in conjunction with clinical parameters associated with prostate cancer progression, including tumor stage, Gleason score, and PSA level. The log-rank test was used to evaluate statistical significance of disease-free survival by the Kaplan–Meier method for univariate analysis. The Wald test was used for Cox proportional hazards regression analysis. The data were tested and met the assumptions for using the Cox test. In multivariable model building, all variables were first included; variables were then removed in a stepwise fashion if they did not have an independent statistical significance of $P<.10$. This statistical significance level was selected to allow for optimal discovery in the initial phases of this analysis. To form the testing or training set and validation set, patients were randomly assigned before analysis; all patients were from the same clinical cohort treated over the same time periods. These sets are similar to each other and to the entire cohort of 259 patients, as shown in Table 1, with nearly identical tumor sizes and rates of PSA-defined recurrence. In the initial training set analysis, all specimens were evaluated regardless of the number of tumor tissue cores obtained from each patient in the TMA; however, in the validation experiments, analysis was limited to those patients with more than two representative cores in the TMA. All statistical tests were two-sided.

Results

Evaluation of Clinical and Pathology Parameters

Before investigating the prognostic value of the profiled markers, the importance of known clinical and pathologic parameters was examined to demonstrate that this dataset is representative and to determine the need for improved risk stratification among this cohort. Clinical stage was dichotomized by results of the digital rectal examination into palpable and nonpalpable groups. Pathologic stage was simplified to two classes, pT2 (organ-confined) and pT3 (extraprostatic extension and/or seminal vesicle invasion). Gleason score was categorized into groups with a score of less than 7, 3 + 4 = 7, 4 + 3 = 7, and more than 7, to take into account the relative amount of Gleason pattern 4, which can be quite different between tissues with a Gleason score of 7, depending on the primary pattern *(29)*. The natural logarithm of the preoperative level of PSA (ln[PSA]) was used. By univariate Cox proportional hazards analysis, ln[PSA], Gleason score categories, surgical margin status, pathologic stage, and maximum tumor diameter were statistically significantly associated with PSA treatment failure (all $P<.001$), whereas age and clinical stage were marginally associated with PSA treatment failure ($P = .096$ and $P = .197$, respectively), and gland weight was not statistically significantly associated with PSA treatment failure ($P = .859$) (Table 2). In a multivariable Cox proportional hazards model, ln[PSA] ($P<.001$), pathologic stage ($P<.001$), and surgical margin status ($P = .003$) were independently statistically significantly associated with PSA-defined recurrence (Table 2). These findings are consistent with a recent analysis of the entire patient cohort *(30)*, although Gleason score had only marginally independent statistically significant association ($P = .145$), which was not surprising because the majority of tumors had a Gleason score of 6 or 7.

Table 2. Univariate and multivariable Cox hazards analysis of clinical and pathology parameters for 259 patients with prostate cancer*

Variable	Univariate analysis		Multivariable analysis	
	HR (95% CI)	*P* value†	HR (95% CI)	*P* value†
Clinical stage		.197		
ln[PSA]	2.413 (1.689 to 3.448)	<.001	2.142 (1.460 to 3.144)	<.001
Gleason category	2.017 (1.499 to 2.712)	<.001		
Age	1.033 (0.994 to 1.073)	.096		
SM	3.306 (2.046 to 5.341)	<.001	2.273 (1.326 to 3.897)	.003
Pathologic stage	4.315 (2.461 to 7.565)	<.001	3.330 (1.877 to 5.906)	<.001
Gland weight		.859		
Maximum tumor diameter	2.048 (1.382 to 3.034)	<.001		

*HR = hazard ratio; CI = confidence interval; ln[PSA] = natural logarithm of the pretreatment prostate-specific antigen level (ng/mL); SM = surgical margin status.

†All statistical tests were two-sided (Cox hazards analysis).

Molecular Marker Analysis

The following six markers that we had previously studied on more than two TMAs were first evaluated for their association with prostate cancer progression: ECAD *(26)*, AIPC *(27)*, pim-1 kinase *(16)*, hepsin *(16)*, AMACR *(21)*, and EZH2 *(22).* Because single tumors were represented by multiple tissue cores on the TMAs, median stain intensities were used to represent the overall expression of a marker and were evaluated as continuous variables (staining intensity values 1–4) by Cox proportional hazards models (Table 3). Dichotomized stain intensities were also evaluated by the Kaplan–Meier method with the log-rank test, optimizing the absent or present cutoff levels for each antibody. By univariate Cox proportional hazards analysis, only decreased expression of pim-1 kinase ($P = .036$), as reported in our previous study *(16)*, was statistically significantly associated with PSA-defined recurrence-free survival. By Kaplan–Meier analysis, positive expression of EZH2, defined as stain intensity of 3 or 4 (i.e., moderate or strong), was associated with PSA-defined recurrence ($P = .062$) (Table 3). To investigate the possibility that combining multiple markers could generate an improved prognostic model, multivariable Cox hazards analysis was performed. Because markers were evaluated with different combinations of TMAs, multivariable analyses were limited to sets of TMAs that were profiled with the same markers. The first analysis was performed on a set of three TMAs, representing 108 patients with prostate cancer, each with multiple replicate tissue cores. These three TMAs were profiled with the same three markers: EZH2, ECAD, and AMACR. Median stain intensity values were used in the analysis, but the optimized dichotomized values were used with EZH2.To fit a multivariable Cox hazards model, all markers were first included in the model and then removed in a stepwise fashion when they failed to account for a statistically significant difference in the risk of recurrence (i.e., they had a *P* value <.10). Because of occasional tissue sample variability across TMAs, the sample set varied slightly in each step of the analysis. In the final model, which represented 103 of the 108 patients, two markers were independently statistically significantly associated with recurrence: EZH2 ($P = .058$) and ECAD ($P = .038$) (data not shown).

Table 3. Univariate Cox hazards analysis and dichotomized log-rank analysis of biomarkers*

Biomarker	No.	Univariate analysis		Optimized dichotomous†		
		Wald‡	*P* value§	Cutoff‖	Log-rank	*P* value§
ECAD	100	1.798	.180	NA	NA	NA
AIPC	42	0.004	.951	max ⩾4	1.110	.292
pim-1 kinase	180	4.384	.036#	NA	NA	NA
Hepsin	80	0.255	.663	NA	NA	NA
AMACR	258	0.058	.810	max ⩾2	0.680	.408
EZH2	116	0.285	.593	max ⩾3	3.500	.062

*ECAD = E-cadherin; AIPC = activated in prostate cancer; AMACR = α-methylacyl-coenzyme A racemase; NA = not available; max = maximum.

†Optimized dichotomous = biomarker was optimized for the best categorical cutoff.

‡Wald statistic *(31).*

§All statistical tests were two-sided (Cox hazards and Kaplan–Meier log-rank test).

‖Cutoff values are maximal staining intensity scores.

#Hazard ratio = 0.622 (95% confidence interval = 0.402 to 0.963).

EZH2 and ECAD Training Set

It is interesting that the association between ECAD and recurrence improved from the univariate analysis ($P = .18$) to the multivariable analysis when EZH2 was included in the model ($P = .038$), suggesting that the status of ECAD may be related to the status of EZH2. To investigate this possibility, a Kaplan–Meier analysis, stratified by the level of EZH2 expression, was performed to test the association of ECAD with recurrence. Decreased ECAD expression, defined as a median staining intensity score of less than 4, occurred with approximately equal frequency in the EZH2-negative and EZH2-positive groups but was only marginally associated with recurrence in the EZH2-positive group ($P = .11$). A staining intensity of 3 or 4 was considered positive for EZH2. An interaction term, referred to as EZH2:ECAD status, was derived and defined as positive only if EZH2 expression was positive (a median staining intensity score of 3 or 4) and if ECAD expression was decreased (median staining intensity score of <4) (Fig. 1). By Kaplan–Meier analysis, EZH2:ECAD status was statistically significantly associated with disease recurrence (relative risk [RR] = 2.52, 95% confidence interval [CI] = 1.09 to 5.81; $P = .021$) (Fig. 2, A). Because the expression cutoffs of EZH2 and ECAD were defined to optimize their association with disease recurrence in this set of patients, these 103 patients were defined as the training set.

Validation of EZH2:ECAD Status

To validate the prognostic importance of the molecular signatures of EZH2 and ECAD, two more TMAs were stained and

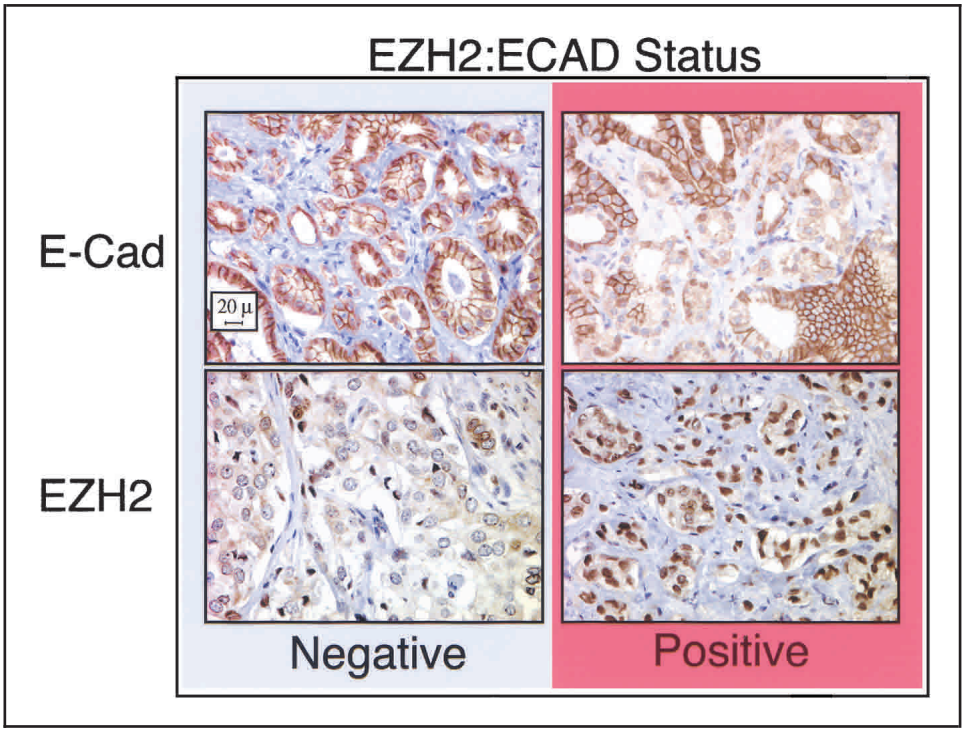
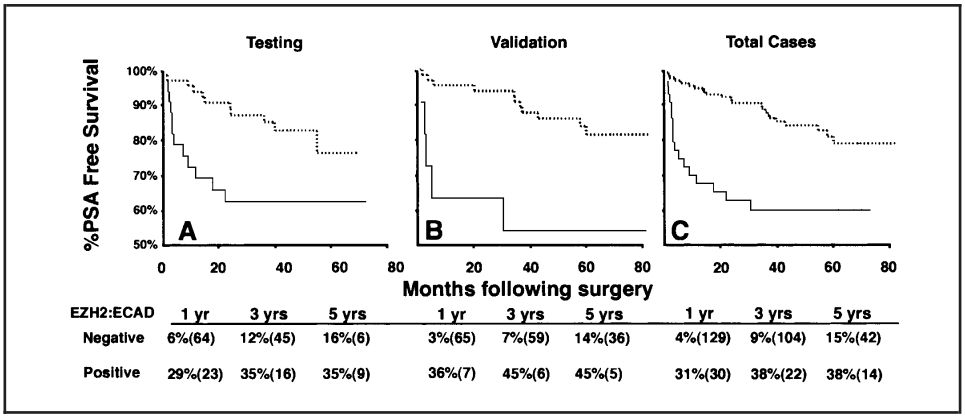


Fig. 1. Enhancer of zeste homolog 2 (EZH2) and E-cadherin (ECAD and E-cad) staining of prostate cancer tissue. **Left:** Combination of strong ECAD staining and/or negative-to-weak EZH2 staining is an example of negative EZH2:ECAD status. **Right:** Combination of decreased ECAD staining and moderate-to-strong EZH2 staining is an example of positive EZH2:ECAD status. Scale bar = 20 μm.



EZH2:ECAD	1 yr	3 yrs	5 yrs	1 yr	3 yrs	5 yrs	1 yr	3 yrs	5 yrs
Negative	6%(64)	12%(45)	16%(6)	3%(65)	7%(59)	14%(36)	4%(129)	9%(104)	15%(42)
Positive	29%(23)	35%(16)	35%(9)	36%(7)	45%(6)	45%(5)	31%(30)	38%(22)	38%(14)

Fig. 2. Kaplan–Meier analyses of enhancer of zeste homolog 2:E-cadherin (EZH2:ECAD) status and prostate-specific antigen-defined recurrence-free survival after radical prostatectomy for clinically localized prostate cancer. Three patient populations were analyzed. The training set contained 103 patients (**A**), the validation set contained 80 patients (**B**), and the combined set contained 183 patients (**C**). Dashed lines = EZH2:ECAD-negative patients; solid lines = EZH2:ECAD-positive patients. The combination of EZH2 and ECAD was statistically significantly associated with prostate cancer progression in the training (development) set of 103 patients (relative risk [RR] = 2.52, 95% confidence interval [CI] = 1.09 to 5.81; P = .021), in the validation set of 80 patients (RR = 3.72, 95% CI = 1.27 to 10.91; P = .009), and in the combined set of 183 patients (RR = 2.96, 95% CI = 1.56 to 5.61; P<.001). The cumulative percentage of patients with prostate-specific antigen-defined recurrence and the number of patients at risk (in parentheses) is presented for each study set at years 1, 3, and 5 for EZH2:ECAD-negative and EZH2:ECAD-positive patients. All statistical tests were two sided.

evaluated. These two TMAs contained more than 700 prostate samples from 97 patients with prostate cancer. The experiments and analysis were performed as described above, except that a patient's sample was excluded from the validation experiment if a tumor was not represented by more than two tissue cores, because a minimum of three cores was optimal for outcome studies *(32).* Of the 97 patients, 80 satisfied this criterion for both EZH2 and ECAD. The expression cutoffs defined for the training set were applied to this validation set. EZH2 was again found to be marginally associated with disease recurrence, albeit with marginal statistical significance (P = .16), whereas decreased ECAD expression was more statistically significantly associated with disease recurrence (P = .002) in the validation set than in the original dataset or training set. Importantly, EZH2:ECAD status was statistically significantly associated with recurrence in this validation set (RR = 3.72, 95% CI = 1.27 to 10.91; P = .009) (Fig. 2, B), although the fraction of specimens with the EZH2:ECAD profile was 13% in the validation set compared with 31% in the training set. We believe that this observation resulted from higher staining intensity of the ECAD antibody in the validation experiments, leading to fewer tissues with decreased expression and, thus, fewer tissues being evaluated as having a positive EZH2:ECAD status.

To further define the accuracy of the interaction term and to study its relationship with clinical and pathologic variables, we next combined the training and validation sets into a combined set of 183 patients with 44 instances of PSA-defined disease recurrence. In the combined set, EZH2:ECAD status was highly statistically significantly associated with disease recurrence (RR = 2.96, 95% CI = 1.56 to 5.61; P<.001). To date, 38% of the EZH2:ECAD-positive patients and 15% of the EZH2:ECAD-negative patients have experienced a PSA-defined recurrence. Disease-free survival rates for patients based on their EZH2:ECAD status at 1, 3, and 5 years are presented in Fig. 2, C. At 5 years

(60 months), the PSA-defined survival rates were 15% for the EZH2:ECAD-negative patients at risk and 38% for the EZH2:ECAD-positive patients at risk.

EZH2:ECAD Status and Loss of Differentiation

To better understand the role of increased expression of EZH2 coupled with decreased expression of ECAD in prostate cancer, we evaluated the association of this molecular profile with known clinical parameters. EZH2:ECAD status was statistically significantly associated with the dichotomized Gleason score category 1 (<7 and 3 + 4 = 7) versus category 2 (4 + 3 = 7 and >7) ($P = .004$ by χ^2 test). For example, 13 (46%) of the 28 patients with a Gleason score of 4 + 3 = 7 or 8 had a positive EZH2:ECAD status, whereas 11 (21%) of 53 with a Gleason score of 6 and 20 (32%) of 63 with a Gleason score of 3 + 4 had a positive status. EZH2:ECAD status was not statistically significantly associated with pathologic stage or with a preoperative level of PSA greater than 10 ng/mL.

Combining EZH2:ECAD With Known Prognostic Parameters

We next examined the prognostic value of EZH2:ECAD status independent of known clinical and pathologic parameters. In a multivariable Cox hazards model including EZH2:ECAD status with the clinical and pathologic variables considered previously, ln[PSA] (hazard ratio [HR] = 2.62, 95% CI = 1.61 to 4.26; $P<.001$), EZH2:ECAD status (HR = 3.19, 95% CI = 1.50 to 6.77; $P = .003$), surgical margin status (HR = 2.30, 95% CI = 1.19 to 4.42; $P = .013$), and pathologic stage (HR = 2.55, 95% CI = 1.19 to 5.45; $P = .016$) were statistically significantly associated with recurrence, suggesting that EZH2:ECAD status adds considerably to a predictive model (Table 4). Gleason score was not statistically significantly associated with recurrence in this model. Surgical margin status and pathologic stage demonstrated clinical utility, but they are postsurgical parameters not necessarily intrinsic to the disease. EZH2:ECAD status most likely reflects an underlying biologic state existing before a tumor has invaded or been excised and examined. To test this hypothesis, we determined whether EZH2:ECAD status was associated with disease recurrence in patients with organ-confined cancer (pT2) and negative surgical margins, suggesting successful surgery for apparent localized disease. In this focused set of 101 patients, EZH2:ECAD status was still statistically significantly associated with recurrence ($P = .013$); six (27%) of the 22 patients with organ-confined prostate cancers and a positive EZH2:ECAD status experienced PSA-defined disease recurrence, whereas only eight (10%) of the 79 such patients with a negative EZH2:ECAD status experienced disease recurrence. Although the number of disease recurrences in this set is relatively small and the risk stratification by EZH2:ECAD is not perfect, this result serves to highlight that even patients at low risk for recurrence can be further stratified by molecular markers and that EZH2:ECAD status may be associated with the potential for metastasis when the cancer is still confined to the organ. It is worth noting that traditional Gleason score, Gleason score categories, or a Gleason score of greater than 7 did not statistically significantly stratify the risk of this focused set of patients.

Table 4. Multivariable model for determining the risk of disease recurrence*

Variable	HR* (95% CI)	P‡
ln[PSA]	2.62 (1.61 to 4.26)	<.001
EZH2:ECAD	3.19 (1.50 to 6.77)	.003
SM	2.30 (1.19 to 4.42)	.013
pT	2.55 (1.19 to 5.45)	.016

*HR = hazard ratio; CI = confidence interval; ln[PSA] = natural logarithm of the pretreatment prostate-specific antigen level (ng/mL); EZH2:ECAD status = interaction term for enhancer of zeste homolog 2 (EZH2) and E-cadherin (ECAD) staining intensity; SM = surgical margin status; pT = pathology tumor stage (tumor–node–metastasis system).

‡Cox regression analysis. All statistical tests were two-sided.

Watchful Waiting and EZH2:ECAD Status

Another potential use of EZH2:ECAD status is to select patients for watchful-waiting protocols instead of localized treatment. Current protocols use clinical stage, biopsy Gleason score, and serum PSA levels to select patients for watchful waiting *(3)*. We hypothesized that a cohort of patients with seemingly indolent disease as defined by these parameters may have a positive EZH2:ECAD status and, thus, have an increased risk for disease progression. To test this hypothesis, patients were stratified by Gleason score, and EZH2:ECAD status was found to be statistically significantly associated with disease recurrence in patients with a Gleason score of 6 (RR = 5.11, 95% CI = 1.06 to 24.57; $P = .017$) or 7 (RR = 2.68, 95% CI 1.23 to 5.75; $P = .008$).

Discussion

TMA Approach

We have used TMA technology to validate several biomarkers in independent studies *(16,21,26,27,32)*. However, because of the heterogeneity of prostate cancer, it is widely believed that more than one molecular marker would be required to develop an optimal molecular nomogram to act as an adjunct to clinically useful nomograms *(1,6)*. To test this hypothesis, we analyzed multiple molecular markers in conjunction with clinical and pathologic parameters. We then validated the markers and tested the most promising candidate markers on a larger sample. Finally, we analyzed the markers in conjunction with clinical and pathologic parameters.

Marker Selection

The markers statistically significantly associated with disease recurrence in this study—EZH2, ECAD, and pim-1 kinase—were originally evaluated for different purposes. EZH2 is overexpressed in metastatic prostate cancer samples *(16)*. ECAD is a tumor suppressor gene in multiple cancer types, but its role in prostate cancer progression has been debated *(26,33,34)*. pim-1 kinase is overexpressed in prostate cancer compared with benign prostate tissue and, unexpectedly, has been shown to be inversely associated with disease recurrence *(16)*. Although EZH2:ECAD status is statistically significantly associated with disease progression, addition of more markers to this combination may strengthen the association. It is also interesting to note that other markers previously found to be associated with an outcome in multivariable models were not included in the final best-fit model. One possible explanation for this observation is that some of these molecular markers may strongly interact with each other and, thus, this lack of statistical independence would prevent these markers from being included in the final best-fit model.

Biology of EZH2 and ECAD

EZH2 is a transcriptional repressor and member of the polycomb group proteins that are involved in silencing in *Drosophila* and vertebrates. Biochemical analysis indicates that polycomb group proteins belong to at least two multimeric complexes, the polycomb repressive complex 1 (PRC1) and embryonic ectoderm development–enhancer of zeste (EED–EZH) complexes *(35,36).* These complexes are thought to silence genes by acting at the level of chromatin structure. EZH2 is highly expressed and active in early embryogenesis, but its expression decreases as cells differentiate.

Recent work from our group *(16,22)* has demonstrated that EZH2 is highly overexpressed in metastatic hormone-refractory prostate cancer, as determined by cDNA and TMA analyses. EZH2 is also overexpressed in localized prostate cancers with a higher risk of disease recurrence after radical prostatectomy. These findings are consistent with the association of EZH2:ECAD status with prostate cancer progression.

In prostate carcinoma, ECAD has been implicated as a possible prognostic indicator because of its decreased expression in higher grade tumors *(37)* and its association with poor outcome *(38)*, but the exact mechanism for decreased ECAD expression is controversial. In normal prostate tissue, secretory epithelial cells express high levels of ECAD *(37).* The ECAD gene is located at 16q22 and is rarely mutated *(39,40).* Other mechanisms for regulation of ECAD beside mutations include DNA methylation *(41)* and post-translational changes *(41).* Thus, it is interesting that decreased ECAD expression is observed in both EZH2-positive and EZH2-negative tumors, suggesting that it may be a common independent tumorigenic event. Loss of ECAD expression has been suggested as a key event in the cascade leading to metastatic potential. Our data suggest that this ECAD-associated metastatic advantage may exist only if the cells express high levels of EZH2, thus, possibly indicating that an early state of differentiation and the loss of cell adhesion are necessary for metastasis. EZH2:ECAD status also was highly associated with disease recurrence for patients with organ-confined prostate cancer that has negative surgical margins. This observation may suggest that EZH2:ECAD status promotes micrometastasis. However, we also recognize that these findings may represent an epiphenomenon.

Clinical Utility

The clinical heterogeneity of prostate cancer has challenged researchers to improve the accuracy of methods for predicting disease progression, so that more appropriate treatment strategies can be chosen. EZH2:ECAD status has potential utility in a number of clinical scenarios. It is important to identify patients likely to experience disease recurrence after radical prostatectomy so that this cohort of high-risk patients can be offered adjuvant therapy. These patients are most likely to benefit from adjuvant therapy, and clinical trials are more likely to yield valuable results if they target appropriate patient populations. Using only clinical and/or pathologic variables, we found that surgical margin status, pathologic stage (pT2 versus pT3), and ln[PSA] were independently associated with disease recurrence, consistent with previous reports *(1,4–8).* Importantly, when EZH2:ECAD status was included in our multivariable model, it was found to be independently associated with disease recurrence.

EZH2:ECAD status may also have clinical utility at diagnosis. Although we have evaluated EZH2:ECAD status only in radical prostatectomy specimens, we presume that these markers could be accurately measured in biopsy samples. The tissue cores of the TMAs are smaller than specimens obtained at a biopsy examination for prostate cancer, suggesting that biopsy tissue could be used to evaluate the EZH2:ECAD status of individual patients. At diagnosis, it is clinically important to determine the risk of disease progression. Our results suggest that EZH2:ECAD status is highly statistically significantly associated with disease recurrence after radical prostatectomy, suggesting that EZH2:ECAD-positive tumors require more aggressive treatment. EZH2:ECAD-negative status may be a valuable selection criteria for watchful-waiting protocols, aiding in the definition of low-risk disease. Both hypotheses will require additional prospective studies.

In conclusion, a positive EZH2:ECAD status confers an increased risk of disease recurrence after radical prostatectomy, independent of clinical and pathologic parameters. This status is associated with Gleason score but also stratifies the risk of disease recurrence within Gleason score categories, suggesting that EZH2:ECAD status may more accurately define the course of the disease. Thus, EZH2:ECAD status may be valuable in determining the risk of prostate cancer progression and, thus, aid in treatment selection. Although future studies may yield additional molecular markers that are closely associated with prostate cancer progression, this study illustrates the feasibility of using TMAs to identify and validate multiple markers that have clinical utility and underlying biologic meaning.

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NOTES

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