A Multicenter Study Shows *PTEN* Deletion Is Strongly Associated With Seminal Vesicle Involvement and Extracapsular Extension in Localized Prostate Cancer

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BACKGROUND. Loss of the phosphatase and tensin homolog (*PTEN*) tumor suppressor gene is a promising marker of aggressive prostate cancer. Active surveillance and watchful waiting are increasingly recommended to patients with small tumors felt to be low risk, highlighting the difficulties of Gleason scoring in this setting. There is an urgent need for predictive biomarkers that can be rapidly deployed to aid in clinical decision-making. Our objectives were to assess the incidence and ability of *PTEN* alterations to predict aggressive disease in a multicenter study.

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METHODS. We used recently developed probes optimized for sensitivity and specificity in a four-color FISH deletion assay to study the Canary Retrospective multicenter Prostate Cancer Tissue Microarray (TMA). This TMA was constructed specifically for biomarker validation from radical prostatectomy specimens, and is accompanied by detailed clinical information with long-term follow-up.

RESULTS. In 612 prostate cancers, the overall rate of *PTEN* deletion was 112 (18.3%). Hemizygous *PTEN* losses were present in 55/612 (9.0%) of cancers, whereas homozygous *PTEN* deletion was observed in 57/612 (9.3%) of tumors. Significant associations were found between *PTEN* status and pathologic stage ($P < 0.0001$), seminal vesicle invasion ($P = 0.0008$), extracapsular extension ($P < 0.0001$), and Gleason score ($P = 0.0002$). In logistic regression analysis of clinical and pathological variables, *PTEN* deletion was significantly associated with extracapsular extension, seminal vesicle involvement, and higher Gleason score. In the 406 patients in which clinical information was available, *PTEN* homozygous ($P = 0.009$) deletion was associated with worse post-operative recurrence-free survival (number of events = 189), pre-operative prostate specific antigen (PSA) ($P < 0.001$), and pathologic stage ($P = 0.03$).

CONCLUSION. *PTEN* status assessed by FISH is an independent predictor for recurrence-free survival in multivariate models, as were seminal vesicle invasion, extracapsular extension, and Gleason score, and preoperative PSA. Furthermore, these data demonstrate that the assay can be readily introduced at first diagnosis in a cost effective manner analogous to the use of FISH for analysis of *HER2/neu* status in breast cancer. Combined with published research beginning 17 years ago, both the data and tools now exist to implement a *PTEN* assay in the clinic. *Prostate 75:1206–1215, 2015.*

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KEY WORDS: **active surveillance; Gleason score; biomarker; PI3K/PTEN/Akt pathway; fluorescence in situ hybridization; tissue array analysis**

INTRODUCTION

Screening, detection, and treatment of prostate cancer remain highly controversial [1,2] and PSA screening has led to the potential over-diagnosis and over treatment of low-risk disease [3,4]. Radical prostatectomy and radiation therapy are treatments that offer high cure rates; however, it is estimated that over 1,055 men need to be screened and 37 cancers detected to prevent one prostate cancer death 11 years later [5]. Recently, recommendations for screening have been narrowed [6], and active surveillance protocols are commonly recommended for men with low risk disease [7,8]. Prognostic biomarkers are needed to aid in clinical decision making for these men to more accurately distinguish between low risk and moderate to high risk prostate cancer.

Currently, the clinical tools available for making these treatment decisions include PSA level, number of positive core biopsies, percent of cores involved by tumor, and Gleason score. Various nomograms are used to facilitate clinical decision making using these data [9]. However, the Gleason score of the biopsy sample, which remains the most powerful prognostic marker, is inaccurate in a large percentage of patients especially when only a small volume tumor is sampled during biopsy. The vast majority of biopsies are scored as either Gleason 6 or 7 and yet up grading or down grading occurs in 14–51% and 9%, respectively when comparing the Gleason score of the biopsy to that found in the prostatectomy specimen [10–12]. Likewise, clinical stage poorly estimates final pathological stage [13], an important predictor of clinical outcome, second only to Gleason score [14]. There is a need for biomarkers that distinguish aggressive from indolent forms of prostate cancer. It is difficult to address the utility of prognostic markers for prostate cancer in a formal prospective study. While randomized studies remain the gold standard for diagnostic and therapeutic trials, several constraints preclude this. Follow-up times of 8–10 years are required to prospectively assess clinical outcomes and the need to evaluate promising biomarkers in a reasonable time frame drives translational studies of prostate cancer toward retrospective analysis of prostatectomy specimens. Active surveillance and watchful waiting protocols have focused attention on the difficulties in grading small tumors. Thus the widely used tools for risk assessment for active surveillance urgently require additional informative biomarkers to supplement Gleason scores [15]. As such, there is an absolute necessity to rigorously evaluate all emerging biomarkers to improve pre-treatment assessment of Gleason score and pathological stage so that urologists and patients can make well-informed treatment decisions at first diagnosis.

Prostate cancer biomarker assays must perform well not only in prostatectomy specimens, but must

also be effective when only small amounts of tissue are available, as is the case in core needle biopsies at the time of initial diagnosis. Tissue microarrays (TMAs) place small samples of many cases on a single slide for rapid evaluation and validation of tissue biomarkers.

The phosphatase and tensin homolog (*PTEN*) is a tumor suppressor gene that can be deleted in patients with prostate cancer [16,17]. *PTEN* was initially studied in human prostate tumors using molecular techniques such as microsatellite analysis [18]. Molecular methods are not readily adaptable to the clinical laboratory, and immunohistochemistry (IHC) is a useful and cost-effective tool for biomarker analysis. IHC studies of PTEN protein were long hampered by the lack of a robust antibody [19]. Fluorescence in situ hybridization (FISH) has therefore been frequently used, and genomic deletions of *PTEN* have been reported in 20–30% of prostate carcinomas [20–22], and are associated with aggressive disease [23,24]. These well-annotated studies have indicated that loss of the *PTEN* gene independently predicts more aggressive disease and poorer outcomes in prostate cancer. However, virtually all of these cohorts were derived from surgical cases from a single institution, which may limit the generalizability of the study population with regards to patient ethnicity, disease severity, and type of practice. In addition, local treatment patterns and methods of follow-up also contribute to intrinsic biases of single-institution patient cohorts. The Canary Foundation Retrospective Prostate Tissue Microarray Resource [25] includes samples from 1,116 subjects treated for prostate cancer with radical prostatectomy between 1995 and 2004 from six participating institutions in the United States and Canada. These samples were ideal to evaluate the role of PTEN as a biomarker to help identify aggressive prostate cancer for implementation to supplement existing predictive tools. Using *PTEN* FISH probes optimized for sensitivity and specificity [26], our objectives were to confirm the ability of *PTEN* deletions to predict aggressive disease, and to determine an expected incidence of PTEN loss in a multicenter study. The accumulated clinical data, combined with newly available probes for FISH and new reagents for IHC published by others [19] open the door to implementation of PTEN assays in the clinical setting.

MATERIALS AND METHODS

Tissue Specimens and TMA Design

The Canary Foundation Retrospective Prostate Tissue Microarray Resource [25] is a retrospective prostate cancer TMA built with the collaboration of six academic medical centers: Stanford University, University of California, San Francisco, University of British Columbia, University of Washington, University of Texas Health Science Center at San Antonio, and Eastern Virginia Medical School. The TMAs contained cores from 1,116 patients undergoing radical prostatectomy between 1995 and 2004. For each case, three cores of cancer tissue were obtained from the highest grade cancer in the dominant tumor. In addition, one core of histologically benign prostate glandular tissue was obtained from the peripheral zone of each case yielding a total of four cores per case on the TMA. The TMA was constructed to assess biomarkers that provide prognostic information independent of clinical and pathological information. The AJCC pathologic staging system was used [27] with stages pT1 and pT2 being combined, as were stages pT3 and pT4. For practical purposes, the vast majority of cases were stages pT2 and pT3. The cases included samples from men with biochemically recurrent prostate cancer within 5 years of surgery and non-recurrent prostate cancer after 5 years of follow-up. In addition, non-recurrent cases censored prior to 5 years and with recurrence after 5 years were included to correct for spectrum bias [25]. Recurrent prostate cancer is defined by one of the following: A single serum PSA level >0.2 ng/ml more than 8 weeks after radical prostatectomy; salvage or secondary therapy after radical prostatectomy; clinical or radiologic evidence of metastatic disease after radical prostatectomy. Although lower thresholds for biochemical recurrence have been proposed [28], the lower bound of sensitivity for PSA testing at some sites during the study period was limited to 0.2 ng/ml. Non-recurrent prostate cancer was defined as disease with none of the indicators of recurrence for at least 5 years after radical prostatectomy. There was no central pathology review in this cohort. The prostatectomy specimens were therefore scored prior to the modification introduced by the International Society of Urological Pathology (ISUP) [29]. We oversampled recurrent cases of Gleason score $3+3=6$ and $3+4=7$ as well as non-recurrent cases with Gleason score $4+4=8$. While this strategy diminishes the prognostic significance of Gleason score, it improves power to discover biomarkers that provide prognostic information independent of Gleason score [25].

Fluorescence In situ Hybridization

The *PTEN* Del TECT FISH utilizes a four-color probe combination as described [26]. Probes were supplied by CymoGenDx LLC (New Windsor, NY) as follows: centromeric copy control probe-CYMO-Red;

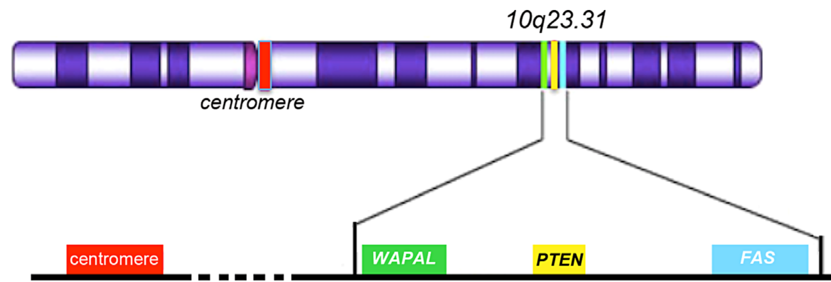


Fig. 1. Schematic diagram of chromosome 10 showing genomic locations and respective positions of the four-color FISH probe used. The relative probe length and color are shown on the linear map at the bottom ofthefigure by the length of the rectangle.

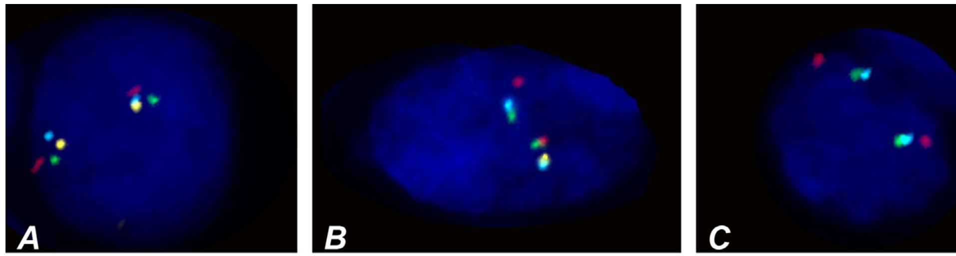
WAPAL – CYMO-Green; *PTEN* – CYMO-Orange; and *FAS* – CYMO-Aqua (Fig. 1). The two probes *WAPAL* and *FAS* on either side of *PTEN* provide information about the size of larger deletions and also allow recognition of artifactual losses of *PTEN* due to histologic sectioning. Artifacts in assessing *PTEN* loss can arise when histologic sectioning cuts away the *PTEN* locus in cells in the section while leaving the centromere in place. The latter is a result of the long distance between the centromere and the *PTEN* locus on chromosome 10. Loss of all three probes distal to the centromere in a small fraction of cells was regarded as artifact, whereas consistent loss of a single copy of *PTEN* in >50% of cells was scored hemizygous deletion. We have shown previously that use of the probes bracketing *PTEN* improves the fidelity of assessments of *PTEN* loss [26]. FISH analysis was performed using 5 μm TMA sections stained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) in areas selected by the pathologist using an immediately adjacent section stained with hematoxylin and eosin. *PTEN* copy number was evaluated by counting spots for all four probes using SemRock filters appropriate for the excitation and emission spectra of each dye in 50–100 non-overlapping, intact, interphase nuclei per tumor TMA core. For each case, two cores were scored based on the overall quality of FISH hybridization. In cases where different clonal deletions were present, all three cores were analyzed. Hemizygous (single copy) *PTEN* loss was assigned when >50% of nuclei exhibited clonal loss of *PTEN* and adjacent probes. Homozygous deletion was defined by a simultaneous lack of both *PTEN* locus signals in 30% of scored nuclei [30].

Statistical Methodologies

Summary statistics of PTEN deletion status and other patient characteristics were provided in frequencies and percentages. Fisher's exact test was used to assess correlation between PTEN deletion status and other characteristics. Pre-surgery PSA was summarized using mean, standard deviation, and range. Comparison between PTEN deletion status groups with respect to pre-surgery PSA was performed using the Kruskal–Wallis test. A logistic regression model was used to assess correlation between PTEN deletion status and other clinical factors. A Cox model was used to assess the effects of PTEN status (homozygous deletion, hemizygous deletion, vs. undeleted for PTEN), and other patient characteristics of recurrence-free survival (RFS), where an event was defined as clinical recurrence, salvage treatment, metastasis, or death due to prostate cancer. A backward elimination procedure was used to identify final multivariate models. Factors of interest may be forced into the final model to account for their effects even if not significant. All tests were two-sided and *P*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC).

Study Population

PTEN gene status was studied in 3,150 cancer cores derived from 1,116 cases and controls. FISH results were obtained from 641 cases encompassing 1,160 tissue cores in total. Of the 409 cases excluded for technical reasons, 70 were due to inadequate tumor tissue, and 339 could not be analyzed because poor tissue digestion prevented adequate hybridization. There was no apparent bias in the distribution of technical failures across the six different study sites in the cohort. Tissue cores can hybridize with variable efficiencies on a TMA slide due to differences in aging and fixation effects from the tissue in the donor blocks. Unfortunately, it was not possible to optimize pretreatment digestion times for all cores as only one TMA slide was available for FISH. This meant that the proportion of successfully hybridized cores (61%) was lower than is usually reported. Of the 641 cases successfully studied by FISH, 29 cases were excluded from further analysis because the clinical information was inadequate resulting in 612 analytical cases.



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PTEN status	Signal configuration (Centromere; WAPAL PTEN FAS)
PTEN Intact by FISH (two copies)	
Hemizygous PTEN deletion (one copy)	
Homozygous PTEN deletion (no copies)	

Fig. 2. **A**: Representative signal pattern observed when the PTEN gene is intact and two copies ofthe gene and allchromosome 10 probes are present as two copies. **B**: Nuclear signal pattern observed for PTEN hemizygous deletions. **C**: Homozygous PTEN deletion (both copies lost). **D**: Scoring schemaused to classify FISH signals present in interphase nuclei based on the colored labels used for each probe. The schema only shows examples with simple interstitial deletions affecting the PTEN gene (yellow spot loss) only. In some tumors, larger deletions extending from WAPAL (green) to FAS (blue) were detected. In addition, five tumors with loss arising as a monsomy of chromosome 10 were detected.

PTEN Deletion Analysis

Homozygous or heterozygous *PTEN* deletion was seen in 112 (18.3%) of 612 tumor samples. Hemizygous *PTEN* deletion accounted for 55 of the 612 (9.0%) adenocarcinomas, whilst homozygous *PTEN* deletion was found in 57 of the 612 (9.3%) tumors. The counting scheme and representative images of undeleted, hemizygous, and homozygous deletions are shown in Figure 2. Of the 55 tumors with a homozygous deletion, 16 had interstitial deletions involving *PTEN* alone, with both flanking genes (*WAPAL* and *FAS*) being retained. The remaining 39 homozygous losses had larger deletions on one chromosome, with the majority extending in a telomeric direction, so that the *FAS* gene was more commonly deleted than *WAPAL*. The distribution of deletion size in the hemizygously deleted tumors was similar to the homozygous deletions, but 4/57 tumors had *PTEN* loss as part of a monosomy 10.

PTEN Deletion Correlated With Gleason Score

PTEN deletion status correlated very strongly with increasing Gleason score ($P = 0.0002$) (Table I). Furthermore, *PTEN* status (undeleted, hemizygous deletion, and homozygous deletion) showed a step-wise correlation with Gleason score. Undeleted *PTEN* was more commonly observed in Gleason score 6 tumors, while homozygous deletion was more common in Gleason score 8 cancers. For example, homozygous deletions were found in 18% (11/62) Gleason score >8 cancers, while only 3% (8/243) of Gleason <6 cases had a homozygous *PTEN* deletion. The Gleason 7 tumors fell between these extremes with 12% (26/225) of 3+4 tumors having a homozygous deletion and 16% (12/76) of 4+3 having homozygous deletions. For the hemizygous *PTEN* deletions, there was no apparent relationship between Gleason score and presence of a *PTEN* loss.

TABLE I. Association of *PTEN* Deletion Status With Clinical Parameters of Progression

	PTEN deletion status								
	Undeleted		Hemi-deletion		Homo-deletion		All		
	N	%	N	%	N	%	N	%	*P*-value*
Margin									
Missing	100	20.00	5	9.09	12	21.05	117	19.12	
Positive	156	31.20	19	34.55	14	24.56	189	30.88	0.60
Negative	244	48.80	31	56.36	31	54.39	306	50.00	
Pathology stage									
Missing	115	23.00	12	21.82	8	14.04	135	22.06	
pT1/pT2	284	56.80	26	47.27	21	36.84	331	54.08	<0.0001
pT3/pT4	101	20.20	17	30.91	28	49.12	146	23.86	
Seminal vesicle invasion									
Missing	9	1.80	0	0	0	0	9	1.47	
No	468	93.60	49	89.09	47	82.46	564	92.16	0.0008
Yes	23	4.60	6	10.91	10	17.54	39	6.37	
Extra-capsular invasion									
Missing	6	1.20	0	0	0	0	6	0.98	
No	380	76.00	38	69.09	28	49.12	446	72.88	<0.0001
Yes	114	22.80	17	30.91	29	50.88	160	26.14	
Gleason score									
Missing	5	1.00	1	1.82	0	0	6	0.98	
≤6	216	43.20	19	34.55	8	14.04	243	39.71	0.0002
3 + 4	178	35.60	21	38.18	26	45.61	225	36.76	
4 + 3	58	11.60	6	10.91	12	21.05	76	12.42	
≥8	43	8.60	8	14.55	11	19.30	62	10.13	
Total	500	100.00	55	100.00	57	100.00	612	100.00	

*Fisher's exact test.

Association of *PTEN* Deletion With Parameters of Aggressive Disease

PTEN deletion was significantly associated with higher pathologic stage, presence of seminal vesicle invasion, extracapsular extension, and increased Gleason score (Table I). Each of these adverse pathological findings increased in cases with homozygous deletion compared to hemizygous deletion, suggesting a gene dosage effect. However, there was no association between surgical margin involvement and the presence of *PTEN* deletion. A multivariate Cox proportional hazard model was used to assess whether *PTEN* status, and clinical and pathological variables predicted survival after radical prostatectomy. A backward elimination procedure was used to identify significant factors in the final model. In 406 evaluable patients, there were 189 events and *PTEN* homozygous deletion was strongly associated with post-operative RFS (homozygous $P = 0.009$, HR 1.64) as were pre-operative PSA and seminal vesicle invasion ($P < 0.0001$ for both; Table II). Neither *PTEN* status nor the clinicopathological variables correlated with the development of metastatic disease or prostate cancer death; however, there were only 30 events among the 612 evaluable patients (data not shown).

PTEN Deletion Correlated With Pathology Stage, Extracapsular Extension, and Seminal Vesicle Invasion

PTEN deletion status showed a highly significant correlation with pathologic stage ($P < 0.0001$). For the stage pT3/pT4 tumors, 19% (28/146) had homozygous deletions compared to only 6% (21/331) of stage pT1/pT2 tumors. This effect was less pronounced for the hemizygous deletions with 12% (17/146) stage pT3/pT4 tumors showing deletions and 8% (26/331) of stage pT1/pT2. Both extracapsular extension (pT3a) and seminal vesicle invasion (pT3b) are associated with a high risk of recurrence after radical prostatectomy. The presence of a *PTEN* deletion correlated strongly with seminal vesicle invasion ($P = 0.0008$), with homozygous deletion having the strongest predictive value. Seminal vesicle invasion was present in 18% (10/57) of tumors with a

homozygous deletion compared to only 5% (23/491) of tumors without a *PTEN* deletion. Similarly, extracapsular extension correlated strongly with *PTEN* deletion ($P < 0.0001$). Extracapsular extension was present in 51% (29/57) of tumors with homozygous *PTEN* deletion, compared to only 23% (114/494) of tumors without *PTEN* deletion. For the 55 tumors with a hemizygous *PTEN* deletion, this effect was still present although less marked, with 6/55 (11%) having seminal vesicle invasion and 17/55 (31%) with extracapsular extension. A logistic regression model confirmed that tumors with homozygous *PTEN* deletions had a significantly higher probability of having extracapsular extension, seminal vesicle invasion, and higher Gleason scores compared to undeleted *PTEN* (Table III). However, for these clinical features, tumors with hemizygous deletions did not show significant difference in comparison to tumors without a *PTEN* deletion.

DISCUSSION

The clinical dilemma facing urologists is how to treat newly diagnosed low and intermediate risk prostate cancer. Treatment options include active surveillance, prostatectomy, hormonal therapy, and radiation therapy. Consequently, there is an intensive search for biomarkers to help distinguish the more aggressive from less aggressive tumors. The search for useful biomarkers in the blood has been slow and difficult [31] and over detection of indolent disease remains a significant problem for prostate cancer [32]. A sample of tissue itself, therefore, remains a mainstay to determine the prognosis of disease. Existing methods for measuring biomarkers in tissue include RNA expression arrays [33], DNA analysis, immunohistochemistry (IHC), and fluorescence in situ hybridization (FISH). An advantage of FISH is that it can be applied to small amounts of tumor in 18 gauge biopsies and fits well into existing work flows, consuming minimal amounts of tissue.

The *PTEN* gene and protein have shown promise in identifying aggressive prostate cancer. Loss of PTEN protein function is strongly associated with key properties of the aggressive cancer phenotype such as cell survival, proliferation, migration, adhesion, and invasion [34]. Our findings agree with other studies showing that prostate cancers with *PTEN* gene deletions have shorter recurrence free survival [35,36]. Moreover, homozygous *PTEN* deletion in tumors is strongly associated with castrate resistant disease, metastasis [23], and prostate cancer specific death [20–22]. Both *PTEN* deletions and the presence of the prostate cancer specific gene fusion, most notably *TMPRSS2:ERG*, have been associated with worse outcome in prostate cancer patients. However, the role of *TMPRSS2:ERG* fusions as a primary determinant of prognosis remains unclear [37–39]. The use of TMAs has many advantages in evaluating the performance of a biomarker in large studies such as this. While IHC is typically used to evaluate biomarker expression in TMAs, we successfully queried TMAs constructed at multiple sites using FISH. In the past, there have been concerns that *PTEN* FISH could be deployed in prostate core biopsies bearing small amounts of tumor if it cannot be successfully utilized in TMAs which similarly have small amounts of tissue.

While this study and the cumulative results of previous PTEN studies [20–24] are not strictly comparable to prospectively obtained biopsies, a thoughtfully designed retrospective study of prostatectomy specimens may offer more accurate results than a prospective study of prostate biopsies. Examination of prostatectomies remains the most accurate assessment of the pathologic stage of prostate cancer. Studies based solely on clinical staging remain hampered by lack of accurate pathologic staging. In the near future, improved imaging methods may fill this gap, but for the time being, pathologic staging based on prostatectomy remains a gold standard for staging. Furthermore, the centerpiece of risk assessment for prostate cancer remains Gleason score. Upgrading at radical

TABLE II. Multivariate Cox Proportional Hazard Model for Recurrence-Free Survival (RFS)

Factor	Comparison	Hazard ratio	95% hazard ratio confidence limits		Pairwise *P*-value	Overall *P*-value
Pre-op PSA	1 unit increase	1.04	1.02	1.05	<0.0001	
PTEN	Homo vs. no deletion	1.64	1.13	2.37	0.009	0.02
	Hemi vs. no deletion	1.28	0.84	1.95	0.25	
Seminal vesicle invasion	Yes. vs. No	2.31	1.53	3.48	<0.0001	
Gleason score	3 + 4 vs. ≤6	1.54	1.12	2.11	0.008	<0.0001
	4 + 3 vs. ≤6	2.34	1.59	3.45	<0.0001	
	≥8 vs. ≤6	2.31	1.52	3.53	<0.0001	

TABLE III. Logistic Regression Model Correlating PTEN With ECE, SV, and Gleason Score

Endpoint	Parameter	Comparison	Odds ratio	95% LCL	95% UCL	Pairwise *P*-value	Overall *P*-value
Extra-capsular invasion (yes, no)	PTEN	Homo vs. no del	3.45	1.97	6.06	<0.0001	<0.0001
		Hemi vs. no del	1.49	0.79	2.70	0.20	
	PTEN	Any del vs. no del	2.32	1.51	3.57	<0.0001	
Seminal vesicle invasion (yes, no)	PTEN	Homo vs. no del	4.33	1.87	9.43	0.0003	0.002
		Hemi vs. no del	2.49	0.89	6.07	0.06	
	PTEN	Any del vs. no del	3.39	1.70	6.62	0.0004	
Gleason ($\leq$6, 7, $\geq$8)	PTEN	Homo vs. no del	3.37	1.83	6.19	<0.0001	<0.0001
		Hemi vs. no del	1.54	0.82	2.89	0.23	
	PTEN	Any del vs. no del	2.32	1.55	3.48	<0.0001	

prostatectomy still occurs in 26–50% of prostatectomies as compared to biopsy Gleason score [40]. Thus, challenges in grading of biopsies include interobserver variability, particularly in assessing small tumors in biopsies, and the upgrading which occurs when prostatectomy specimens are examined.

Improved antibodies have led to the use of IHC in the evaluation of PTEN expression as a surrogate for *PTEN* deletions and point mutations, and IHC fits the work-flow of diagnostic pathology and can be readily deployed at modest cost [13]. However, interphase FISH analyses can be performed on less than 100 tumor cells, so it is now feasible to obtain clinically useful genetic information such as *PTEN* status by applying FISH to tumor tissue in needle core biopsies. There is timeliness, therefore, in assessing the applicability of *PTEN* FISH analysis to prostate cancer risk assessment at first diagnosis. Sampling issues and heterogeneity of PTEN loss within a tumor may diminish the negative predictive value of PTEN assays [41]. However, the loss of PTEN has independent predictive value, and can be assayed alone or in combination with other emerging biomarkers such as TMPRSS-ERG that may add additional information. Using HER-2 status in breast cancer as a model [42], the availability of both IHC and FISH assays for PTEN status offers the prospect of implementing a widely studied biomarker. Two recent studies using prostate cancer needle biopsies [19,43] have shown a strong association between PTEN loss, as determined by immunohistochemistry, and poor outcome. Collectively, these recent data using needle core biopsies and the findings of this present manuscript draw attention to the value of PTEN as a predictive biomarker for intermediate risk prostate cancer and suggest a possible clinical workflow for assessing PTEN status. For example, initial analyses of PTEN expression could be carried out using immunohistochemistry with established methods [13,19]. Regions of tumor or suspicious areas in the biopsy that are PTEN weak or otherwise indeterminate by IHC could then readily be studied by FISH using the refined probes we describe.

PTEN deletion is a clinically meaningful finding, and we suggest that a combination of IHC and FISH can detect PTEN deletions if they are present in the sampled tissue. The upgrading that frequently occurs from biopsy to prostatectomy exemplifies the limitations of current clinical decision making tools deployed at the time of biopsy. Our analysis of small core samples of tumor tissue on the TMA suggest that clinically meaningful assessments of PTEN status and prognosis can be made in the context of biopsies. This finding, coupled with studies demonstrating the prognostic value of PTEN expression by IHC on biopsies suggests that PTEN testing of biopsy samples could be a useful adjunct for patients with low and intermediate risk prostate cancer in making therapeutic decisions. However, additional studies will be necessary to determine the best use of PTEN IHC, PTEN FISH, or a step-wise assessment of both in patients newly diagnosed with low and intermediate risk prostate cancer who are deciding between treatment and active surveillance. Furthermore, the false negative rate for PTEN status, particularly in the biopsy setting, has not been determined definitively and will require additional study.

Proper cancer staging is also critical in clinical decision-making in early stage prostate cancer. For example, it is well known that extracapsular extension or seminal vesicle invasion increases the risk of recurrence [44,45]. The prostate biopsy rarely gives information about extracapsular extension or seminal vesicle invasion because the sample rarely includes

those areas for evaluation. Our study demonstrates an association with *PTEN* loss, particularly in cases with homozygous deletion, and extracapsular extension, and seminal vesicle invasion. Therefore, the association of *PTEN* loss with pathological up-staging, and the consequential increased risk provide additional information that could be useful in clinical decision making.

This large multicenter retrospective TMA analysis of radical prostatectomy specimens shows that homozygous *PTEN* deletion is associated with higher stage, higher Gleason score, and a higher incidence of both extraprostatic extension, and seminal vesicle invasion. In addition, homozygous deletion of *PTEN* is associated with shorter RFS in men after radical prostatectomy. Our findings, suggest that *PTEN* deletion testing of biopsies could provide an important additional tool to assist urologists and patients making treatment decisions when faced with low and intermediate risk prostate cancer. Given the strong associations loss of *PTEN* by FISH and IHC, future studies will be needed to define optimal workflows using these methods to best define prognosis.

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