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Clin Cancer Res 2011;17:6563-6573. Published OnlineFirst August 30, 2011.

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**PTEN Protein Loss by Immunostaining: Analytic Validation and Prognostic Indicator for a High Risk Surgical Cohort of Prostate Cancer Patients**

Tamara L. Lotan¹, Bora Gurel¹, Siobhan Sutcliffe⁴, David Esopi², Wennuan Liu⁵, Jianfeng Xu⁵, Jessica L. Hicks¹, Ben H. Park², Elizabeth Humphreys³, Alan W. Partin³, Misop Han³, George J. Netto¹,²,³, William B. Isaacs²,³, and Angelo M. De Marzo¹,²,³

**Abstract**

**Purpose:** Analytically validated assays to interrogate biomarker status in clinical samples are crucial for personalized medicine. PTEN is a tumor suppressor commonly inactivated in prostate cancer that has been mechanistically linked to disease aggressiveness. Though deletion of PTEN, as detected by cumbersome FISH spot counting assays, is associated with poor prognosis, few studies have validated immunohistochemistry (IHC) assays to determine whether loss of PTEN protein is associated with unfavorable disease.

**Experimental Design:** PTEN IHC was validated by employing formalin fixed and paraffin-embedded isogenic human cell lines containing or lacking intact PTEN alleles. PTEN IHC was 100% sensitive and 97.8% specific for detecting genomic alterations in 58 additional cell lines. PTEN protein loss was then assessed on 376 prostate tumor samples, and PTEN FISH or high resolution single nucleotide polymorphism microarray analysis was done on a subset of these cases.

**Results:** PTEN protein loss, as assessed as a dichotomous IHC variable, was highly reproducible, correlated strongly with adverse pathologic features (e.g., Gleason score and pathologic stage), detected between 75% and 86% of cases with PTEN genomic loss, and was found at times in the absence of apparent genomic loss. In a cohort of 217 high risk surgically treated patients, PTEN protein loss was associated with decreased time to metastasis.

**Conclusion:** These studies validate a simple method to interrogate PTEN status in clinical specimens and support the utility of this test in future multicenter studies, clinical trials, and ultimately perhaps for routine clinical care.

**Introduction**

Prostate cancer is the most common solid organ tumor in American men and the second most common cause of cancer deaths (1). Despite improvements in early detection, we still lack molecular markers to effectively distinguish men with high risk disease from the indolent majority. Identification of molecular aberrations that contribute to the development of lethal disease has the additional benefit of providing specific targets for therapy. Work over the last decade has firmly established that loss of the PTEN tumor suppressor (phosphatase and tensin homolog on chromosome 10) is one of the most common somatic genetic aberrations in prostate cancer and is frequently associated with high risk disease (2–13). Despite these findings, however, prostate cancer specimens are not yet routinely interrogated for PTEN loss in any clinical setting.

The majority of previous work on PTEN loss in prostate cancer has focused on genomic deletions of the PTEN locus at 10q23. Such deletions, most commonly identified by FISH, occur in 10% to 70% of prostate cancer cases depending on the study population examined (2, 4, 6, 8, 10–15) and are associated with poor prognosis (13, 16–20). Interestingly, heterozygous PTEN deletions far outnumber homozygous deletions in prostate cancer and may also result in poor outcomes (11–13, 15–17, 20). There has been much debate about whether this may be explained by haploinsufficiency for PTEN or whether inactivation of the second allele in these cases has occurred but is not detectable by FISH (21, 22). Although a number of early studies suggested that the rate of PTEN mutation and epigenetic modification in prostate cancer was relatively high, it is now known that many of these initial estimates may have been falsely elevated due to the existence of a PTEN
**Translational Relevance**

*PTEN* is a tumor suppressor commonly inactivated in prostate cancer. Genomic deletions of *PTEN* are associated with poor prognosis, but are interrogated in tissue samples using relatively cumbersome FISH. We show that *PTEN* protein expression evaluated by immunohistochemistry (IHC), using a new commercially available monoclonal antibody, is simple to conduct and score after genetic validation and sensitively detects *PTEN* genomic loss. Furthermore, *PTEN* IHC detects additional cases of *PTEN* protein loss compared with genomic methods. Finally, loss of *PTEN* by IHC correlated with adverse pathologic features and decreased time to metastatic disease in a surgical cohort. This analytically validated assay may be used in additional studies to determine its efficacy as a clinical test, including as a prognostic biomarker in needle biopsies and as a predictive marker to identify patients who could benefit from emerging PI3K pathway-targeted therapies or who may be resistant to hormonal therapies.

Recent studies have suggested that alternative mechanisms of *PTEN* posttranscriptional down-regulation may play an important role in prostate cancer (24–28). To date, however, the relative frequency of *PTEN* inactivation by mechanisms other than genomic deletion in clinical prostate cancer specimens remains unclear (29).

For clinical settings, the evaluation of allelic loss of *PTEN* by FISH is cumbersome, requiring counting of the number of fluorescent signals relative to control signals in partially sectioned interphase cells. Thus, reliable detection of *PTEN* protein status by immunohistochemistry (IHC) in routinely processed clinical formalin-fixed and paraffin-embedded pathology specimens could prove highly useful for the implementation of *PTEN* status as a clinically relevant prognostic biomarker. Importantly, development of such an assay may also detect cases where *PTEN* inactivation occurs by mechanisms other than genomic deletion. As more therapies targeting various components of the PI3K signaling cascade become available, a robust assay to determine *PTEN* status will likely have an important role in clinical care. To address this need, we have developed and validated a simple and robust IHC assay to detect *PTEN* protein in paraffin-embedded specimens using a commercially available rabbit monoclonal antibody. Because our approach to analytical validation of *PTEN* staining can be ported easily to other laboratories, if our findings can be validated in additional large cohorts, this robust IHC assay may prove to be useful in a number of clinical arenas.

**Materials and Methods**

**Patient and tissue selection**

Ten tissue microarrays (TMA) were constructed from formalin-fixed paraffin-embedded prostate, lymph node or distant metastasis tissue collected from a total of 376 patients with prostate cancer who underwent radical prostatectomy or surgical resection of metastatic lesions at our institution. Between 1 and 4 (average = 3) 0.6 mm cores of tumor tissue for each patient were arrayed. For most patients, surrounding benign prostate tissue was included in the TMA as a control. Of these 376 patients, 56 patients were enrolled in an adjuvant trial of docetaxel treatment due to high risk pathologic features (30). An additional 217 patients were from a previously described cohort with biochemical recurrence following radical prostatectomy for clinically localized prostate cancer (31).

Finally, an additional group of 53 patients with concurrent high resolution single nucleotide polymorphism (SNP) microarray analysis for genomic copy number alterations in their prostatic carcinoma were also included. Detailed results of the genome-wide study from these patients will be reported elsewhere (J. Xu and colleagues; unpublished data). For these cases, a single representative histologic section that was adjacent to the tumor lesion harvested for genomic DNA was analyzed for *PTEN* protein loss.

**Cell culture and transfections**

Three different human cell lines in which *PTEN* alleles were subjected to targeted disruption using homologous recombination were used in this study as controls for IHC, including MCF-10A mammary epithelial cells (32; gift of K.E. Bachman), and the DLD-1 and HCT116 human colorectal cancer cell lines (33; gift of T. Waldman). Wild-type (*PTEN*+/−) heterozygous null (*PTEN*−/−), and homozygous null (*PTEN*−/−) cell variants were maintained as previously described (32, 33), harvested and subjected to formalin fixation and paraffin embedding as described (34). The human prostate cancer cell lines LNCaP, DU145, and PC-3 were acquired from the American Type Culture Collection. LNCaP cells harbor one deleted allele of *PTEN* and one mutated allele of *PTEN* and do not express *PTEN* protein (7). PC3 cells harbor a homozygous deletion of *PTEN* and do not express *PTEN* protein (7, 10). DU145 cells harbor an apparent coding SNP in *PTEN*, but are otherwise wild type and express *PTEN* protein (10). We also obtained 56 additional cell lines of the NCI-60 panel of cell lines from the Developmental Therapeutics Program at the NCI to evaluate *PTEN* staining. Cell lines were used to generate cell line microarrays (analogous to TAMs; ref. 35) using a recently updated method that will be described in detail elsewhere (D. Esopi and colleagues; unpublished data).

As an additional positive control, we obtained a molecular clone of *PTEN* that is driven by the CMV promoter (*PTEN Nm_000314 Human cDNA Clone, Origene) and transfected this construct into HCT116 *PTEN*−/− and DLD-1 *PTEN*−/− cells using Oligofectamine according to the manufacturer’s directions (Invitrogen). Mock or *PTEN* transfected cells were harvested after 48 hours and prepared for cell blocks as described above.

**Fluorescence in situ hybridization**

FISH was done on 2 TAMs (145 patients, 361 spots representing a spectrum of lesions including benign
prostate, prostatic intraepithelial neoplasia (PIN), primary tumors, and lymph node metastases) using the Vysis LSI PTEN (10q23)/CEP 10 Dual Color Probe (Abbott Molecular Inc.) according to the manufacturer’s directions. Briefly, a 4 μm paraffin section was baked at 56°C for 2 hours, then dewaxed and rehydrated using xylene and graded ethanol, respectively. The TMA sections were pretreated using Paraffin Pretreatment Reagent Kit III (Abbott Molecular Inc.). TMAs and the PTEN/CEP10 FISH probes were denatured at 94°C for 5 minutes and hybridized overnight at 37°C in a humid chamber (StatSpin ThermoBrite; IRIS Inc.).

FISH scoring
FISH scoring was conducted using a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc.) and a 100×1.4 NA oil immersion Neofluar lens. Grayscale images were captured for presentation using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged.

FISH interpretation
FISH interpretation was done by a pathologist (B.G.) blinded to the IHC results. To establish normal cutoffs for scoring and to ensure that FISH signal truncation seen in tissue sections was not scored as a false positive, the PTEN/centromere 10 (CEP10) signals were tallied in 50 nonneoplastic normal-appearing prostate specimens contained on the same TMAs as the experimental specimens, and the mean and SD for PTEN/CEP10 ratios and percentage of epithelial cells containing 0 or 1 PTEN probe were calculated. After scoring 30 cells, a tumor was considered to have PTEN loss if the percentage of cells showing 0 or 1 PTEN probe signal was greater than 2 SD above the mean established from the normal tissue and the PTEN/CEP10 ratio was more than 2 SD below the mean established in normal tissue. A case that was represented by more than 1 tissue core on the TMA was considered to have PTEN loss if any TMA spot in the case showed PTEN loss.

Immunohistochemistry
A number of commercial antibodies were evaluated to identify those most highly sensitive and specific in IHC cell line staining experiments. These antibodies included the following: Zymed/Invitrogen rabbit polyclonal catalog No. 51-2400; Epitomics rabbit monoclonal clone Y184; Santa Cruz Biotechnologies mouse monoclonal clone 6H2.1; Cascade BioSciences mouse monoclonal clone 6H2.1; Santa Cruz Biotechnologies mouse monoclonal clone 28H6; and Cell Signaling Technology rabbit monoclonal clone D4.3. Using the PTEN positive and negative cell lines described above, we found that the rabbit monoclonal antibody from Cell Signaling Technology performed the best overall at the following conditions: Antigen unmasking was done by steaming in EDTA buffer (pH 8.0) for 45 minutes. Endogenous peroxidase activity was quenched by incubation with peroxidase block for 5 minutes at room temperature. Nonspecific binding was blocked by incubating in 1% bovine serum albumin in Tris-HCl pH 7.5 for 20 minutes at room temperature. Slides were incubated with a 1:100 dilution of rabbit monoclonal anti-PTEN antibody (clone D4.3, #9188, Cell Signaling Technologies) overnight at 4°C. A horseradish peroxidase-labeled polymer (PowerVision Poly-HRP anti-Rabbit IgG; Leica Microsystems) was then applied for 30 minutes at room temperature. Signal detection was done using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromagen. Slides were counterstained with hematoxylin, dehydrated, and mounted. In a number of experiments, we also evaluated another rabbit monoclonal antibody from Cell Signaling Technology (clone 138G6) and found it to perform similarly to the D4.3 clone.

FISH interpretation
PTEN protein was visually scored using a dichotomous scoring system by 2 urologic pathologists (T.L.L. and A.M.D.). IHC scoring was blinded with respect to FISH and SNP array results, pathologic stage, and final Gleason score at radical prostatectomy, as well as patient outcome. Using this system, each spot of tumor tissue was scored as negative or positive for PTEN protein by comparing staining in malignant glands with that of adjacent benign glands and/or stroma which provided an internal positive control within each tissue core. Staining was classified as negative if the intensity was markedly decreased or entirely negative across all tumor cells compared with the surrounding benign glands and/or stroma. A given spot was dropped from the analysis if these benign areas lacked PTEN staining (this occurred in <5 TMA spots total).

High resolution SNP microarray analysis
PTEN IHC was done on an additional 53 prostate tumors for which PTEN copy number data was available from high resolution SNP microarray analysis (Affymetrix 500 K). Further details regarding the genome wide copy number analyses will be provided in a separate publication (J. Xu and colleagues; unpublished data).

Statistical analysis
Pearson χ² and Fisher’s exact tests were used to determine the association of PTEN protein loss with pathologic variables and PTEN genomic loss. To investigate univariable associations between PTEN loss and time to distant metastasis and prostate cancer-specific death, Kaplan–Meier survival curves were calculated and compared by log-rank tests. Multivariable-adjusted associations were investigated using Cox proportional hazards regression; models included terms for pathologic stage, Gleason grade, preoperative PSA, and surgical margin status. Statistical analyses were done using SAS version 9.1.

Results
Validation of IHC assay
We first validated our IHC assay analytically for PTEN protein loss using formalin fixed paraffin-embedded...
cell blocks created from 3 distinct isogenic human cell lines with and without PTEN deletion. In wild-type HCT-116, MCF-10A, and DLD-1 cells, a variable degree of cytoplasmic and nuclear PTEN protein immunostaining was evident, whereas isogenic lines which had undergone 2 rounds of somatic homologous recombination (resulting in disruption of both alleles of PTEN) showed complete absence of PTEN protein by IHC (Fig. 1). In MCF-10A cells that were heterozygous for PTEN disruption (PTEN\(^{+/-}\)), there were intermediate levels of staining between wild-type (PTEN\(^{+/+}\)) and homozygous mutant variants (PTEN\(^{-/-}\); Fig. 1). In contrast, PTEN\(^{-/-}\) HCT-116 or DLD-1 cells transiently transfected with an expression vector containing a cDNA encoding full-length human PTEN showed a number of cells with greatly increased PTEN protein by IHC (Fig. 1). Taken together, these isogenic cell line experiments verify the specificity of the Cell Signaling Technologies rabbit monoclonal antibody and our IHC protocol. For additional validation, we also stained cell pellets that were obtained from PC3, LNCaP, and DU145 human prostate cancer cell lines that have known PTEN genomic status. As expected, both PC3 cells and LNCaP were completely negative for staining, whereas DU145 cells showed moderate levels of both cytoplasmic and nuclear staining for PTEN (not shown). To further assess the performance of the assay we next examined 55 of the remaining cell lines from the NCI-60 cell line panel, because PTEN genomic status has been reported from these lines (Supplementary Table S1; 36, 37). The sensitivity for IHC staining to identify cell lines with predicted oncogenic alterations in PTEN was 100% (13 of 13 lines with mutant PTEN showed loss of IHC staining), with a specificity of 97.8% (44 of 45 cell lines without PTEN mutation did not show PTEN protein loss by IHC).

**PTEN protein scoring in human prostate tissues**

PTEN protein loss by IHC was scored on paraffin-embedded prostate tissues from a total of 376 patients on 10 TMAs. PTEN protein was expressed in all (100%) cases of benign prostate tissue present on TMAs. Although there was some variability in staining intensity, PTEN protein was diffusely expressed in the cytoplasm and nucleus of luminal and basal epithelial cells, as well as in the surrounding stromal tissue (Fig. 2). In fact, virtually all cell types present (including endothelial cells, smooth muscle cells, inflammatory cells, and peripheral nerves) were

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**Figure 1.** PTEN protein expression by IHC in isogenic cell line controls with and without somatic PTEN gene loss. A, wild-type HCT116 and (D) DLD-1 colon cancer cells, and (G) MCF-10A breast epithelial cells, show PTEN protein expression by IHC, whereas the same cell lines with homozygous PTEN deletion by somatic homologous recombination (PTEN KO) show absent PTEN protein (B, E, H). C and F, HCT116 and DLD-1 cells with PTEN deletion transiently transfected with CMV-PTEN show high PTEN protein expression in a subset of cells. I, MCF10A cells with hemizygous deletion of PTEN show levels of PTEN immunostaining intermediate between wild type and cells with homozygous PTEN deletion.
positive for PTEN staining. This pattern of staining was also found on 53 standard tissue slides (see below).

To score PTEN protein expression in prostatic carcinoma, we devised a binary scoring system wherein malignant glands were scored as positive or negative for cytoplasmic PTEN protein relative to the internal control of surrounding benign glands and/or stroma (Fig. 2). We considered cases in which the majority (>90%) of cells retained easily discernible levels of PTEN staining to be positive for PTEN protein (e.g., Fig. 2C, left panel). Cases considered negative for PTEN protein either showed a complete absence of PTEN staining or extremely weak intensity staining in more than 10% of cells. In the latter cases, however, the overall staining was always strikingly reduced as compared with surrounding tissue, particularly in nuclei which appeared blue only without discernible brown staining. Interobserver reproducibility for this simple scoring system was high. On an array with 117 tumor spots scored independently in a blinded fashion by 2 pathologists (T.L.L. and A.M.D.), there was 95% (111/117 spots) agreement on the presence or absence of PTEN protein by IHC in malignant glands (k = 0.87 or "almost perfect" agreement). In this set, 26% to 27% (depending on the observer) of the spots showed PTEN loss in tumor cells.

A fraction of tumors arrayed on the TMAs showed prominent intratumoral heterogeneity for PTEN expression, with some spots positive for PTEN whereas other spots from the same index tumor were negative. Overall, intratumoral heterogeneity in PTEN expression was observed in 9% (20/220 cases) of primary tumors. Occasionally, this heterogeneity of PTEN protein expression was seen even within a given TMA spot, with some malignant glands expressing PTEN whereas adjacent glands (or even adjacent cells within a malignant gland) were entirely negative (Fig. 2D and E). For the purposes of this study, a case was scored as PTEN negative if any tumor spot from that case showed greater than 10% of tumor cells with markedly decreased PTEN protein. Similarly, a total of 53 prostatic tumors were scored for PTEN expression using single tumor sections on standard histologic slides rather than TMAs, and 11% (6/53) of these cases showed 2 clear populations of tumor cells where one population had PTEN loss and the other did not. In this setting, as above, a case with PTEN protein loss in greater than 10% of the sectioned tumor was considered to have PTEN loss for the purposes of data analysis.

**Association of PTEN protein loss with pathologic variables and tissue type**

Overall, PTEN protein was scored by IHC in 397 tissue samples collected from 376 individual patients. Pathologic stage data was available on 263 patients (66%) and Gleason grade was available on all tissues. In these samples, PTEN protein loss by IHC was highly correlated with
increased pathologic stage ($P = 0.003$) and Gleason grade ($P < 0.0001$, Fig. 3). Overall, 41% (17/41) of pT3bN0 cases showed PTEN loss, compared with 34% (39/112) of pT3aN0 cases and 14% (6/44) of pT2N0 cases. Similarly, 45% (65/144) of Gleason 8 to 10 cases showed PTEN protein loss compared with 39% (61/155) of Gleason 7 cases and 20% (20/98) of Gleason 5 to 6 cases.

Consistent with the correlation with pathologic stage and grade, PTEN loss by IHC was also highly correlated with the tissue diagnosis (Fig. 3). High grade PIN showed the lowest rate of PTEN loss (12% or 3/25 cases). In contrast, pelvic lymph node metastases and distant metastases showed the highest rates of PTEN loss (46% or 20/43 cases and 57% or 4/7 cases, respectively). Primary prostatic tumors showed an intermediate rate of PTEN loss when considered as a group (38% or 119/308).

**Sensitivity of PTEN IHC for detecting PTEN genomic loss**

To determine whether the PTEN IHC assay was sensitive or specific for detecting PTEN allelic loss in clinical prostate cancer specimens, we evaluated PTEN protein by IHC in a series of prostate tumors for which we also had available PTEN genomic status by concurrent FISH (66 cases, of which 56 were from the high risk group enrolled in an adjuvant trial of docetaxel) or high resolution copy number SNP microarray analysis (53 cases). For the cases with concurrent PTEN FISH data, presence or absence of PTEN protein was highly correlated with presence or absence of PTEN loss of heterozygosity (Fig. 4, Table 1, $P = 0.0044$ by Fisher’s exact test). Of 66 primary prostate tumors with available PTEN FISH data, 24 (36%) had loss of at least 1 PTEN allele. Of these, 75% (18/24) had loss of PTEN protein by IHC, indicating that PTEN IHC is sensitive for the detection of genomic PTEN loss. For the cases with concurrent SNP microarray copy number analysis, PTEN IHC was also highly correlated with PTEN genomic status (Table 2, $P = 0.00026$ by Fisher’s exact test). Of 53 cases with SNP microarray data, 41% (22 cases) showed loss of at least 1 allele of PTEN. Of these cases, 86% (19/22) showed PTEN protein loss by IHC. Of the cases with SNP microarray data and heterogeneous intratumoral PTEN IHC staining, 33% (2/6 cases) showed no evidence of genomic PTEN loss, 50% (3/6 cases) showed loss of one PTEN allele, and 17% (1/6) showed homozygous loss of both PTEN alleles.

![Figure 3. PTEN protein loss by IHC is highly correlated with prostate cancer pathologic stage and grade. A, PTEN protein is more frequent in higher pathologic stage tumors ($P = 0.003$ by Pearson’s $\chi^2$ test). B, PTEN protein loss is more common in higher Gleason grade tumors ($P = 0.0001$ by Pearson’s $\chi^2$ test). C, PTEN protein loss is least common in benign prostate tissues and PIN and most common in metastatic prostate tumors ($P = 0.001$ by Pearson’s $\chi^2$ test).]
PTEN IHC was as sensitive for the detection of cases with loss of one PTEN allele (heterozygous deletion) as it was for the detection of cases with loss of both alleles (homozygous deletion; 87% or 13/15 vs. 86% or 6/7, respectively, Table 3). Importantly, of cases with PTEN protein loss by IHC, 45% (15/33) and 37% (11/30) did not have any PTEN genomic loss detectable by FISH or SNP microarray, respectively.

Correlation of PTEN protein loss with clinical outcome
A subset of the RRP specimens are part of a previously described retrospectively studied high risk surgical cohort of patients who underwent radical prostatectomy (RRP) by a single surgeon at the Johns Hopkins Hospital between 1986 and 1996 and subsequently developed biochemical recurrence (Ref. 31; Table 3). Of note, none received neoadjuvant chemotherapy or hormonal therapy prior to development of metastatic disease (31). Median patient follow-up was 16 years and tissue was present for TMA construction from 217 of the original 304 cases in this cohort. At RRP, 22% (49/217) had a Gleason score (GS) of 6 or less, 51% (110/217) had GS of 7 and 27% (58/217) had a GS of 8 to 10. 83% (181/217) had extracapsular extension, 34% (74/217) had seminal vesicle involvement and 27% (58/217) had lymph node metastases. 36% (78/217) had positive surgical margins. By definition in this cohort, 100% (217/217) of patients had biochemical recurrence during the follow-up period, with 25% (52/210) showing evidence of local recurrence and 60% (124/208) with distant metastases during follow-up. 38% (79/207) of patients died of prostate cancer during the study period. In Kaplan–Meier analysis, loss of PTEN immunostaining was significantly associated with decreased time to metastasis ($P = 0.03$, Fig. 5). There was a correlation between PTEN protein loss and decreased time to prostate-specific death, although this did not reach statistical significance ($P = 0.06$). In multivariable Cox regression analysis, PTEN protein was not associated with time to metastasis or prostate cancer death (Table 3).

Discussion
PTEN genomic loss was first identified as a molecular aberration common in prostate cancer nearly 15 years ago (2, 37). In early studies using microsatellite analysis, LOH at the PTEN locus was reported in 10% to 55% of primary tumors from surgical cohorts (2, 4–6, 8, 9). In more recent studies using FISH, loss of at least 1 PTEN allele has been reported in as few as 17% of patients with tumors incidentally discovered on transurethral resection (TURP), however PTEN allelic loss is present in 17% to 68% of primary tumors from surgical cohorts and up to 77% of hormone-resistant primaries discovered on TURP (11–13, 15–17, 20, 38). In general, PTEN loss is more common in prostate cancer metastases than in primary tumors, with rates of loss reported near 50% in 3 independent studies.

Table 1. Correlation between PTEN protein loss detected by IHC and PTEN genomic loss detected by FISH

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<tr>
<th>PTEN genomic loss by FISH</th>
<th>PTEN protein by IHC</th>
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<tr>
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<td></td>
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<td>Deleted</td>
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NOTE: $P = 0.0044$ by Fisher’s exact test.

Table 2. Correlation between PTEN protein loss detected by IHC and PTEN genomic copy number change detected by SNP array

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<th>PTEN genomic loss by SNP array</th>
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NOTE: $P = 0.00026$ by Fisher’s exact test.
the variations in reported rates of genomic PTEN loss, using different methods of detection (6, 15, 19). Despite the nearly universal finding in the FISH studies is that loss of 1 PTEN allele is significantly more frequent than loss of both PTEN alleles in surgical cohorts. However, rates of homozygous loss also vary by the cohort examined. Although homozygous loss of PTEN alleles in surgical cohorts. However, rates of homozygous loss are much more frequent than homozygous deletions. Although several studies have shown higher HR for hemizygous cases of prostate cancer with PTEN inactivation strongly suggests that alternative epigenetic mechanisms which are not detected by FISH. Although there is some evidence for PTEN haploinsufficiency in the mouse, data to support this hypothesis are lacking in humans (21, 22). Thus the correlation between loss of a single PTEN allele and features of aggressive disease in prostate cancer strongly suggests that alternative epigenetic or perhaps nongenomic mechanisms of PTEN inactivation play an important role in prostate cancer progression (29).

Overall, the wide range in reported frequency of PTEN genomic loss in prostate cancer likely reflects the close association of PTEN loss with high risk pathologic features and possibly an association with androgen-insensitive disease. Thus, the frequency of PTEN loss is higher in cohorts enriched for aggressive disease, with increased pathologic stage and Gleason grade (18). Despite the close association with pathologic variables, at least 2 separate studies have found that PTEN genomic loss is independently correlated with decreased time to biochemical recurrence (13, 16, 20). However, at least 3 separate studies done in TURP and surgical cohorts have not found an association between PTEN genomic loss and survival (16–18).

Interestingly, the association between PTEN genomic loss and biochemical recurrence has most commonly been documented for hemizygous deletions, likely because they are much more frequent than homozygous deletions. Although several studies have shown higher HR for homozygous compared with hemizygous PTEN loss, the correlation between loss of only 1 allele of PTEN and decreased time to biochemical recurrence remains significant (13, 16, 20). This suggests that either PTEN is a haploinsufficient gene, or in cases of hemizygous loss, the second allele is commonly inactivated by additional mechanisms which are not detected by FISH. Although there is some evidence for PTEN haploinsufficiency in the mouse, data to support this hypothesis are lacking in humans (21, 22). Thus the correlation between loss of a single PTEN allele and features of aggressive disease in prostate cancer strongly suggests that alternative epigenetic or perhaps nongenomic mechanisms of PTEN inactivation play an important role in prostate cancer progression (29). This also suggests that FISH may be systematically underestimating the frequency of PTEN loss in prostate cancer.

The possibility that PTEN IHC may fail to detect some cases of prostate cancer with PTEN inactivation strongly argues for the need for an alternative assay to detect PTEN loss. Although IHC to detect PTEN protein levels is an obvious alternative, until recently, studies using this method have been impeded by the lack of reliable antibodies and by a paucity of dependable genetic controls. To our knowledge, there are at least 10 prior studies in the literature looking at the utility of PTEN IHC for the detection of PTEN loss in prostate cancer (10–12, 14–16, 39–42). Although some found an association between PTEN protein loss and Gleason grade, stage, or biochemical recurrence, many did not. Some of this variation may be due to antibody performance. While this manuscript

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<tr>
<td></td>
<td>pT3b</td>
<td>74 (34)</td>
<td>2.68 (1.10–6.48)</td>
<td>2.42 (0.77–7.63)</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Negative</td>
<td>159 (73)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>58 (27)</td>
<td>5.54 (2.34–13.1)</td>
<td>4.03 (1.35–12.1)</td>
<td></td>
</tr>
<tr>
<td>Surgical margins</td>
<td>Negative</td>
<td>139 (64)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>78 (36)</td>
<td>0.77 (0.52–1.14)</td>
<td>1.01 (0.62–1.66)</td>
<td></td>
</tr>
<tr>
<td>Biochemical recurrence</td>
<td>Negative</td>
<td>217 (100)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>52 (25)</td>
<td>5.54 (2.34–13.1)</td>
<td>4.03 (1.35–12.1)</td>
<td></td>
</tr>
<tr>
<td>Local recurrence</td>
<td>Negative</td>
<td>158 (75)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>52 (25)</td>
<td>5.54 (2.34–13.1)</td>
<td>4.03 (1.35–12.1)</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>Negative</td>
<td>84 (40)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>124 (60)</td>
<td>7.63 (3.99–15.1)</td>
<td>8.96 (4.03–21.1)</td>
<td></td>
</tr>
<tr>
<td>Disease-specific death</td>
<td>Negative</td>
<td>128 (62)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>79 (38)</td>
<td>0.77 (0.52–1.14)</td>
<td>1.01 (0.62–1.66)</td>
<td></td>
</tr>
</tbody>
</table>
PTEN Protein Loss in Prostate Cancer

Figure 5. PTEN protein loss by IHC is associated with poor clinical outcomes in a surgical cohort of high risk prostate cancer patients. A, the Kaplan–Meier curve shows a significant decrease in metastasis-free survival for patients with PTEN protein loss by IHC ($P = 0.03$). B, the Kaplan–Meier curve for disease-specific survival shows a nonsignificant decrease in prostate-cancer-specific survival in patients with PTEN protein loss ($P = 0.06$).

was in preparation, a study evaluating commercially available PTEN antibodies for IHC found that many antibodies resulted in nonspecific nuclear staining in cell lines with known PTEN genomic loss (43). Importantly, this study independently corroborated our findings that recently available rabbit monoclonal antibodies to PTEN perform much more reliably than older clones or polyclonal antibodies. In addition to problems with older antibodies, earlier studies also employed widely variable and often complex scoring systems for PTEN protein, and did not account for interobserver variability in scoring. Only 2 studies have looked at the association between PTEN protein loss and disease progression and survival in prostate cancer, and whereas 1 study scored cytoplasmic staining intensity and extent, the other found an association only with nuclear PTEN staining (14, 16).

Given the wide variation of methodology and results in the literature, we set out to develop an IHC assay for PTEN that would be simple enough to allow routine use in clinical pathology specimens. One advantage of our study is that we were able to correlate PTEN protein expression with PTEN genomic status. We found that PTEN IHC is highly sensitive for detection of PTEN genomic loss, detecting nearly 80% of cases with loss by FISH and more than 80% of cases with loss by high resolution SNP array. Only 3 previous studies have validated their IHC assay in a similarly rigorous fashion. Yoshimoto and colleagues reported PTEN levels as a product of the intensity and percentage of cytoplasmic and/or nuclear staining and although they did not provide data for each individual case, they found an overall correlation between PTEN protein levels as a continuous variable and PTEN genomic status (12). Verhagen and colleagues employed a similar scoring system and found that 66% (10/15) of cases with PTEN deletion by FISH showed PTEN protein loss (11). Recently, Han and colleagues did a similar study where they scored cytoplasmic staining intensity on a 0-2+ scale and correlated with FISH results (15). They reported that they detected 52% of cases with PTEN loss by FISH using IHC if only cases with 0+ immunostaining were considered to be truly PTEN protein negative.

One of the most intriguing findings in the current study was that 45% and 37% of tumors with PTEN protein loss did not show genomic deletions detectable by FISH or high resolution SNP microarray, respectively. Although it is possible that FISH may not detect some small deletions in PTEN, the similar data obtained from the high resolution SNP microarray suggests that this is a less likely explanation for our findings. In addition, we found that PTEN IHC was as sensitive for the detection of hemizygous loss by SNP array as it was for the detection of homozygous loss. Although even high resolution SNP microarrays can miss very small deletions depending on a number of factors, this data strongly suggest that in addition to genomic deletion, alternative mechanisms for PTEN inactivation likely exist. Interestingly, other authors have reported similar findings. Han and colleagues reported that 35% (6/17) of their PTEN protein negative cases did not show genomic deletion by FISH and Verhagen and colleagues reported that 33% (5/15) cases without PTEN protein showed no evidence of deletions by FISH (11, 15).

The frequency with which PTEN is inactivated by mutations, epigenetic modifications and/or nongenomic means remains unclear in prostate cancer. Although early studies reported a high rate of mutations and methylation in the PTEN promoter region, it is likely that some of these estimates were falsely elevated because of detection of a PTEN pseudogene that harbors a high rate of such changes.
More recent studies have documented only rare cases in which PTEN is inactivated by point mutations or small insertions or deletions (indels; refs. 19, 28, 47). In addition, the functional consequences of PTEN promoter methylation for PTEN expression are unclear because recent findings that the PTEN promoter is shared with that of a p53-target gene (KILLIN) which may be a tumor suppressor in its own right (48). Recent studies have elucidated the role of microRNAs and pseudogene deletion in the regulation of PTEN protein levels (26, 27). Furthermore, a separate study identified chromosomal translocations in MAGI-2, a membrane-associated guanylate kinase known to bind and stabilize PTEN protein (28, 49). Overall, these data strongly suggest that PTEN inactivation in prostate cancer occurs through a number of mechanisms, many of which have yet to be described. This, in turn, highlights the importance of an assay that will detect PTEN inactivation occurring via multiple mechanisms.

Although several studies have shown an association between PTEN protein expression and the surrogate clinical endpoint of biochemical recurrence, only 2 prior studies have examined whether PTEN protein expression is associated with metastasis and death in prostate cancer. Halvorsen and colleagues looked at PTEN protein expression in prostate tumors from 104 surgically treated patients and found that cytoplasmic PTEN expression was an independent predictor of death (although this did not reach statistical significance). In our cohort of 217 patients with biochemical recurrence, we found that PTEN protein expression, as a single variable, was a significant predictor of decreased time to metastasis and correlated with decreased time to prostate cancer-specific death (although this did not reach statistical significance). Given that this cohort was significantly enriched for high risk pathologic features and that PTEN protein loss is highly correlated with such features, it is likely that PTEN protein expression as a single variable prognostic factor will perform even better in more typical surgical cohorts.

Ultimately, the IHC test for PTEN protein expression described herein will most likely be of use in the setting of prostate tumors diagnosed on needle biopsy for a number of reasons. First, this simple test, which is easier and cheaper to conduct than FISH, could easily be routinely done on needle biopsy specimens and compared with FISH, will likely identify additional cases lacking PTEN protein. Second, although we found PTEN protein loss was highly correlated with tumor grade and pathologic stage in radical prostatectomy cases (and thus not independently associated with clinical outcome), in the setting of needle biopsies, pathologic tumor stage is unknown and tumor grade is routinely underestimated in approximately 20% of cases. In this way, as a prognostic biomarker, loss of PTEN protein in a needle biopsy specimen may be useful for the identification of presumed low risk prostate cancer patients (such those on active surveillance) that are prone to disease progression and hence require treatment. Third, as a predictive biomarker, loss of PTEN may prove useful for the selection of appropriate patients for treatment with emerging PI 3-Kinase pathway-targeted therapies, a number of which are currently in clinical trials for prostate cancer. Finally, PTEN loss may also serve as a biomarker of hormonal therapy resistance in advanced prostate cancer (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was funded in part by the NIH/NCI Prostate SPORE P50CA82386 and the Patrick C. Walsh Prostate Cancer Research Fund of which A.M. De Marzo is the Peter J. Sharp Scholar.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 13, 2011; revised July 27, 2011; accepted August 7, 2011; published OnlineFirst August 30, 2011.
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