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

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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 fluorescence in situ hybridization (FISH) spot counting assays, is associated with poor prognosis, few studies have validated immunohistochemical (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 SNP microarray analysis was performed 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 pathological 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.

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

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 fluorescence in situ hybridization (FISH). We demonstrate that PTEN protein expression evaluated by immunohistochemistry (IHC), using a new commercially available monoclonal antibody, is simple to perform and score after genetic validation and sensitively detects *PTEN* genomic loss. Further, PTEN IHC detects additional cases of PTEN protein loss compared to genomic methods. Finally, loss of PTEN by IHC correlated with adverse pathological 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.

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 homologue 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 fluorescence in situ hybridization (FISH), occur in 10-70% 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). While 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* pseudogene (2, 3, 23). Recent studies have suggested that alternative mechanisms of PTEN post-transcriptional 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 (FFPE) 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. Since 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, WB Isaacs et al., manuscript in process). 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 immunohistochemistry, including MCF-10A mammary epithelial cells (32; gift of Kurtis E. Bachman), and the DLD-1 and HCT116 human

colorectal cancer cell lines (33; gift of Todd 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 (Manassas, VA). 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 wildtype 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 TMAs) (35) using a recently updated method that will be described in detail elsewhere (D. Esopi, S Yegnasubramanian, AM De Marzo, manuscript in process).

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; Rockville, MD) and transfected this construct into HCT116 *PTEN*$^{-/-}$ and DLD-1 *PTEN*$^{-/-}$ cells using Oligofectamine according to the manufacturer's directions (Invitrogen; Carlsbad, CA). Mock or *PTEN* transfected cells were harvested after 48 hours and prepared for cell blocks as described above.

Fluorescence in situ hybridization: Fluorescence in situ hybridization (FISH) was performed on two TMAs (145 patients, 361 spots representing a spectrum of lesions

including benign prostate, PIN, primary tumors and lymph node metastases) using the Vysis LSI PTEN (10q23) / CEP 10 Dual Color Probe (Abbott Molecular Inc.; Abbott Park, IL) according to the manufacturer's directions. Briefly, a 4 μm paraffin section was baked at 56°C for 2 hours, then de-waxed and rehydrated using xylene and graded ethanol, respectively. The TMA sections were pretreated using Paraffin Pretreatment Reagent Kit III (Abbott Molecular Inc.; Abbott Park, IL). TMAs and the PTEN/CEP10 FISH probes were co-denatured at 94°C for 5 min and hybridized overnight at 37°C in a humid chamber (StatSpin ThermoBrite; IRIS Inc, MA)

FISH scoring: FISH scoring was conducted using a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) and a 100x/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 performed by a pathologist (BG) 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 non-neoplastic normal-appearing prostate specimens contained on the same TMAs as the experimental specimens, and the mean and standard deviation (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 one 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 (Carlsbad, CA) rabbit polyclonal cat No 51-2400; Epitomics (Burlingame, CA) rabbit monoclonal clone Y184; Cascade BioSciences (Winchester, MA) mouse monoclonal clone 6H2.1; Santa Cruz Biotechnologies (Santa Cruz, CA) mouse monoclonal clone 28H6; and Cell Signaling Technology (Beverly, MA) rabbit monoclonal 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 performed 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. Non-specific 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, Beverly, MA) overnight at 4°C. A horseradish peroxidase–labeled polymer (PowerVision Poly-HRP anti-Rabbit IgG; Leica Microsystems, Bannockburn, IL) was then applied for 30 minutes at room temperature.

Signal detection was performed 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.

Interpretation of immunohistochemistry: PTEN protein was visually scored using a dichotomous scoring system by two urologic pathologists (TLL and AMD). 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 to 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 performed on an additional 53 prostate tumors for which *PTEN* copy number data was available from high resolution single nucleotide polymorphism (SNP) microarray analysis (Affymetrix 500K). Further details regarding the genome wide copy number analyses will be provided in a separate publication (J Xu, WB Isaacs et al., manuscript in process).

Statistical analysis: Pearson chi-squared 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 performed using SAS version 9.1.

Results:

Validation of immunohistochemical assay:

We first validated our immunohistochemical (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, while isogenic lines which had undergone two rounds of somatic homologous recombination (resulting in disruption of both alleles of *PTEN* showed complete absence of PTEN protein by IHC (**Figure 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 ($PTEN^{-/-}$) (**Figure 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 (**Figure 1**). Taken together, these isogenic cell line experiments verify the specificity of the Cell Signaling Technologies rabbit monoclonal antibody as well as 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, since *PTEN* genomic status has been reported from these lines (**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 tissue microarrays (TMAs). PTEN protein was expressed in all (100%) cases of benign prostate tissue present on tissue microarrays. While 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 (**Figure 2**). In fact, virtually all cell types present (including endothelial cells, smooth muscle cells, inflammatory cells, and peripheral nerves) were 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 (**Figure 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. Figure 2C, left panel**). Cases considered negative for PTEN protein either showed a complete absence of PTEN staining or extremely weak intensity staining in >10% of cells. In the latter cases, however, the overall staining was always strikingly reduced as compared to 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 two pathologists (TLL and AMD), there was 95% (111/117 spots) agreement on the presence or absence of PTEN protein by IHC in malignant glands (kappa = 0.87 or "almost perfect" agreement). In this set, 26-27% (depending on the observer) of the spots showed PTEN loss in tumor cells.

A fraction of tumors arrayed on the TMAs showed prominent intra-tumoral heterogeneity for PTEN expression, with some spots positive for PTEN while other spots from the same index tumor were negative. Overall, intra-tumoral 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 while adjacent glands (or even adjacent cells within a malignant gland) were entirely negative (**Figure 2D and 2E**). 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 two 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 ($p<0.0001$, **Figure 3**). Overall, 41% (17/41) of pT3bN0 cases showed PTEN loss, compared to 34% (39/112) of pT3aN0 cases and 14% (6/44) of pT2N0 cases. Similarly, 45% (65/144) of Gleason 8-10 cases showed PTEN protein loss compared to 39% (61/155) of Gleason 7 cases and 20% (20/98) of Gleason 5-6 cases.

Consistent with the correlation with pathologic stage and grade, PTEN loss by IHC was also highly correlated with the tissue diagnosis (**Figure 3**). High grade prostatic intraepithelial neoplasia (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 (**Figure 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 one *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 one allele of *PTEN*. Of these cases, 86% (19/22) showed PTEN protein loss by immunohistochemistry. Of the cases with SNP microarray data and heterogeneous intra-tumoral PTEN immunohistochemical 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.

PTEN immunohistochemistry 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 immunohistochemistry, 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 (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 8-10. 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 ($p=0.03$, **Figure 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, loss of heterozygosity (LOH) at the *PTEN* locus was reported in 10-55% of primary tumors from surgical cohorts (2, 4-6, 8, 9). In more recent studies using FISH, loss of at least one *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 17-68% 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 three independent studies using different methods of detection (6, 15, 19). Despite the variations in reported rates of genomic *PTEN* loss, a nearly universal finding in the FISH studies is that loss of one *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. While homozygous loss of *PTEN* hovers around 0-10% of primaries, it is closer to 50% of metastatic or hormone-resistant cases (11-13, 15-17, 20).

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 two separate studies have found that *PTEN* genomic loss

is independently correlated with decreased time to biochemical recurrence (13, 16, 20). However, at least three separate studies performed 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 demonstrated higher hazard ratios for homozygous compared to hemizygous *PTEN* loss, the correlation between loss of only one 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. While 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 non-genomic 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* FISH 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 immunohistochemistry to detect PTEN protein levels is an obvious alternative, until recently, studies using this method have been impeded by the lack of reliable antibodies as well as 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 immunohistochemistry for the detection of PTEN loss in prostate cancer (10-12, 14-16, 39-42). While 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 was in preparation, a study evaluating commercially available PTEN antibodies for immunohistochemistry found that many antibodies resulted in nonspecific nucleolar 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 two studies have looked at the association between PTEN protein loss and disease progression and survival in prostate cancer, and while one 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 immunohistochemical assay for PTEN that would be simple enough to allow routine use in clinical pathology specimens. One advantage of our study is that we had access to a number of isogenic cell lines both with and without *PTEN* genomic deletion. Using these cell lines, we optimized a staining protocol for a recently available rabbit monoclonal antibody, wherein all lines with PTEN deletion showed a complete absence of PTEN protein. With our staining protocol, background benign prostatic

glands and stroma showed robust staining for PTEN. Because of the presence of an internal positive control in all samples, we were able to apply a simple dichotomous and highly reproducible scoring system for malignant glands, with cytoplasmic PTEN either present, or markedly decreased. Interestingly, Sangale et al. also applied a simple dichotomous scoring strategy in their approach to PTEN IHC scoring in clinical samples (43).

Perhaps one of the most important advantages of our study is that we were able to correlate PTEN protein expression with *PTEN* genomic status. We found that PTEN immunohistochemistry is highly sensitive for detection of *PTEN* genomic loss, detecting nearly 80% of cases with loss by FISH and over 80% of cases with loss by high resolution SNP array. Only three previous studies have validated their immunohistochemical assay in a similarly rigorous fashion. Yoshimoto et al 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 et al 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 et al 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 immunohistochemistry 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. Additionally, 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 suggests that in addition to genomic deletion, alternative mechanisms for PTEN inactivation likely exist. Interestingly, other authors have reported similar findings. Han et al reported that 35% (6/17) of their PTEN protein negative cases did not show genomic deletion by FISH and Verhagen et all 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 non-genomic 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 (2, 3, 11, 15, 23, 37, 44-46). More recent studies have documented only rare cases in which *PTEN* is inactivated by point mutations or small insertions or deletions (indels) (19, 28, 47). Additionally, the functional consequences of *PTEN* promoter methylation for PTEN expression are unclear since 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). Further, 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.

While several studies have shown an association between PTEN protein expression and the surrogate clinical endpoint of biochemical recurrence, only two prior studies have examined whether PTEN protein expression is associated with metastasis and death in prosate cancer. Halvorsen et al looked at PTEN protein expression in prostate tumors from 104 surgically treated patients and found that cytoplasmic PTEN expression was an independent predictor of locoregional recurrence in this cohort (14). McCall et al found that nuclear PTEN was independently associated with disease specific survival in a group of 68 tumors in TURP specimens (16). 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 didn't 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 prognosticator 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 perform than FISH, could easily be routinely performed on needle biopsy specimens and compared to 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).

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Figure Legends:

Figure 1: **PTEN protein expression by immunohistochemistry in isogenic cell line controls with and without somatic *PTEN* gene loss.** **(A)** Wildtype HCT116 and **(D)** DLD-1 colon cancer cells, and **(G)** MCF-10A breast epithelial cells show PTEN protein expression by immunohistochemistry, while the same cell lines with homozygous *PTEN* deletion by somatic homologous recombination (PTEN KO), show absent PTEN protein **(B, E, H)**. **(C, 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 wildtype and cells with homozygous *PTEN* deletion.

Figure 2: **PTEN protein expression by immunohistochemistry in human prostate specimens. (A)** Typical cancer case with uniform retained PTEN protein expression in all malignant glands. **(B)** Typical cancer case with PTEN protein loss in malignant glands, while adjacent benign glands (arrow) retain PTEN protein expression. **(C)** Some tumors showed only weak PTEN protein positivity (left panel) but were still readily distinguishable from cases with total PTEN protein loss (right panel). **(D)** Intra-tumoral heterogeneity for PTEN protein expression is evident in this tumor specimen, where malignant glands with PTEN loss and PTEN protein retention are intermingled. Note the presence of cells with and without PTEN expression within a single malignant gland (arrow). **(E)** High grade prostatic intraepithelial neoplasia (PIN) with PTEN loss in most of the involved gland, while PTEN protein is retained in a minority of luminal cells (arrow) and all basal cells (arrowhead). An adjacent benign gland expresses PTEN. **(F)** PTEN protein loss in lymph node metastasis of prostatic carcinoma. While the cytoplasm is negative, some glands show a small amount of staining at the apical plasma membrane (arrow), a finding of uncertain significance.

Figure 3: PTEN protein loss by immunohistochemistry 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 square test). **(B)** PTEN protein loss is more common in higher Gleason grade tumors (p=0.0001 by Pearson's chi square 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 square test).

Figure 4: **PTEN protein expression by immunohistochemistry is highly correlated with *PTEN* genomic loss by FISH.** In Case A, PTEN protein is highly expressed in malignant glands, corresponding to a normal *PTEN*/CEP10 ratio by FISH, with retention of *PTEN* (red) and CEP10 (green) FISH signals in malignant cells. In Case B, PTEN protein is markedly decreased in malignant glands, with a corresponding *PTEN*/CEP10 ratio of 0.18. Malignant cells show homozygous loss of the *PTEN* (red) signal in malignant cells, with retention of the CEP10 (green signal).

Figure 5: **PTEN protein loss by immunohistochemistry 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 immunohistochemistry (p= 0.03). **(B)** The Kaplan-Meier curve for disease-specific survival shows a non-significant decrease in prostate-cancer specific survival in patients with PTEN protein loss (p = 0.06).

FIGURE 1

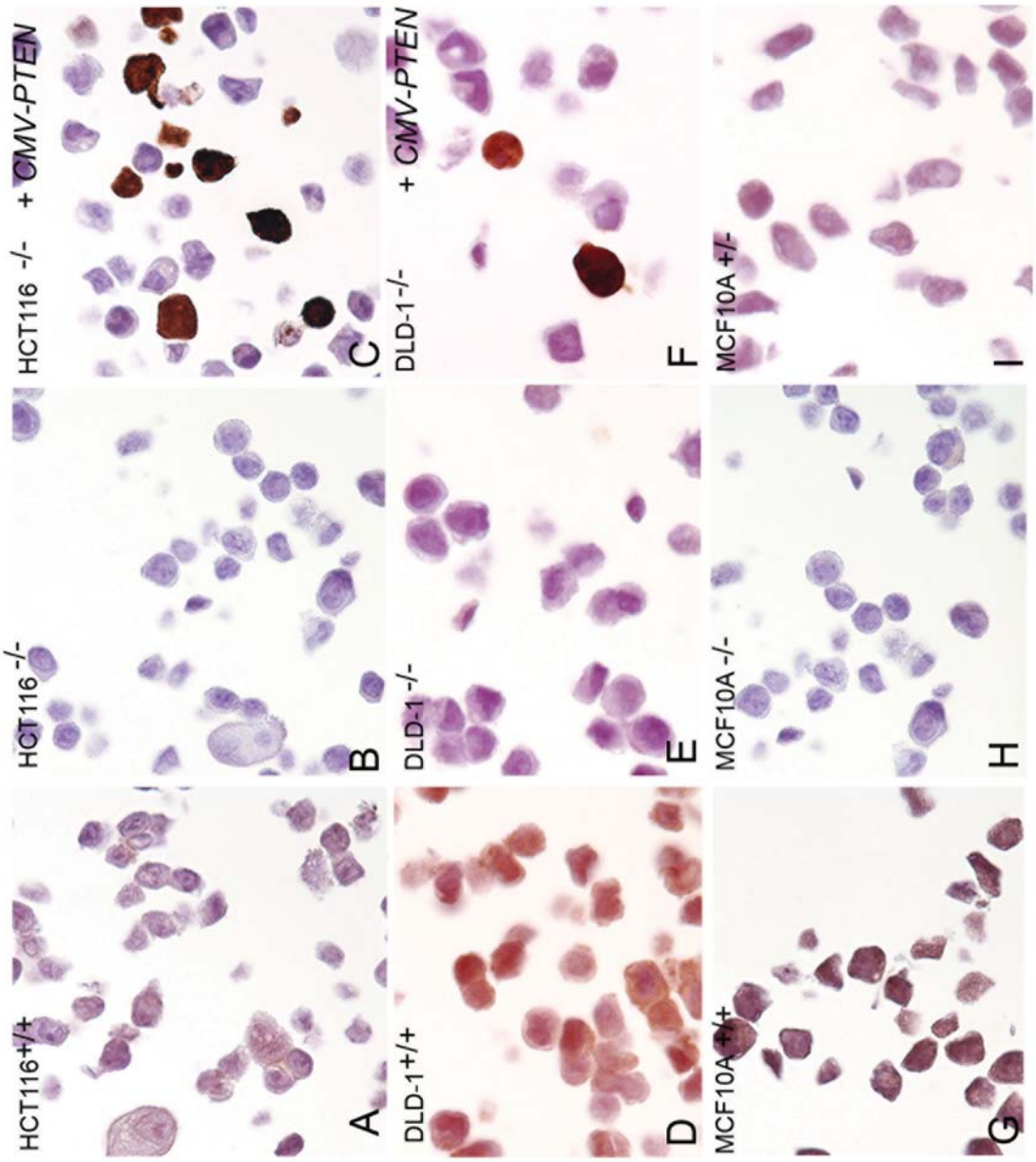
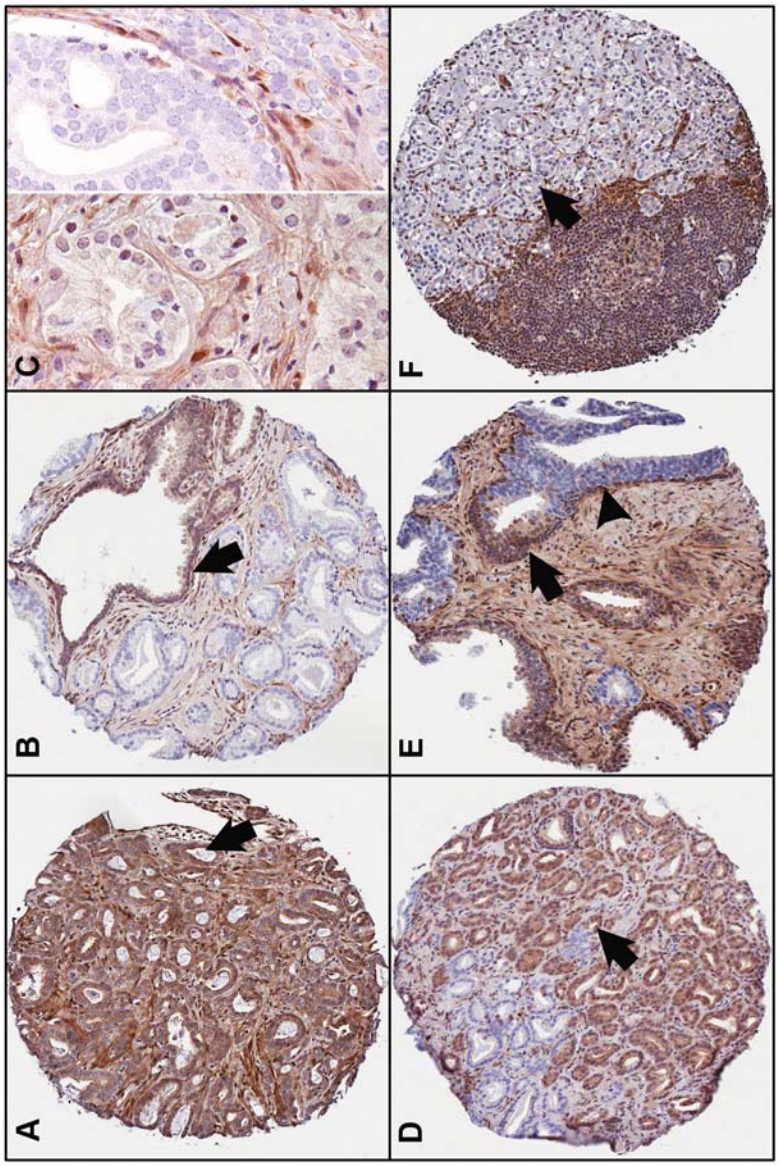


FIGURE 2



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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

FIGURE 3

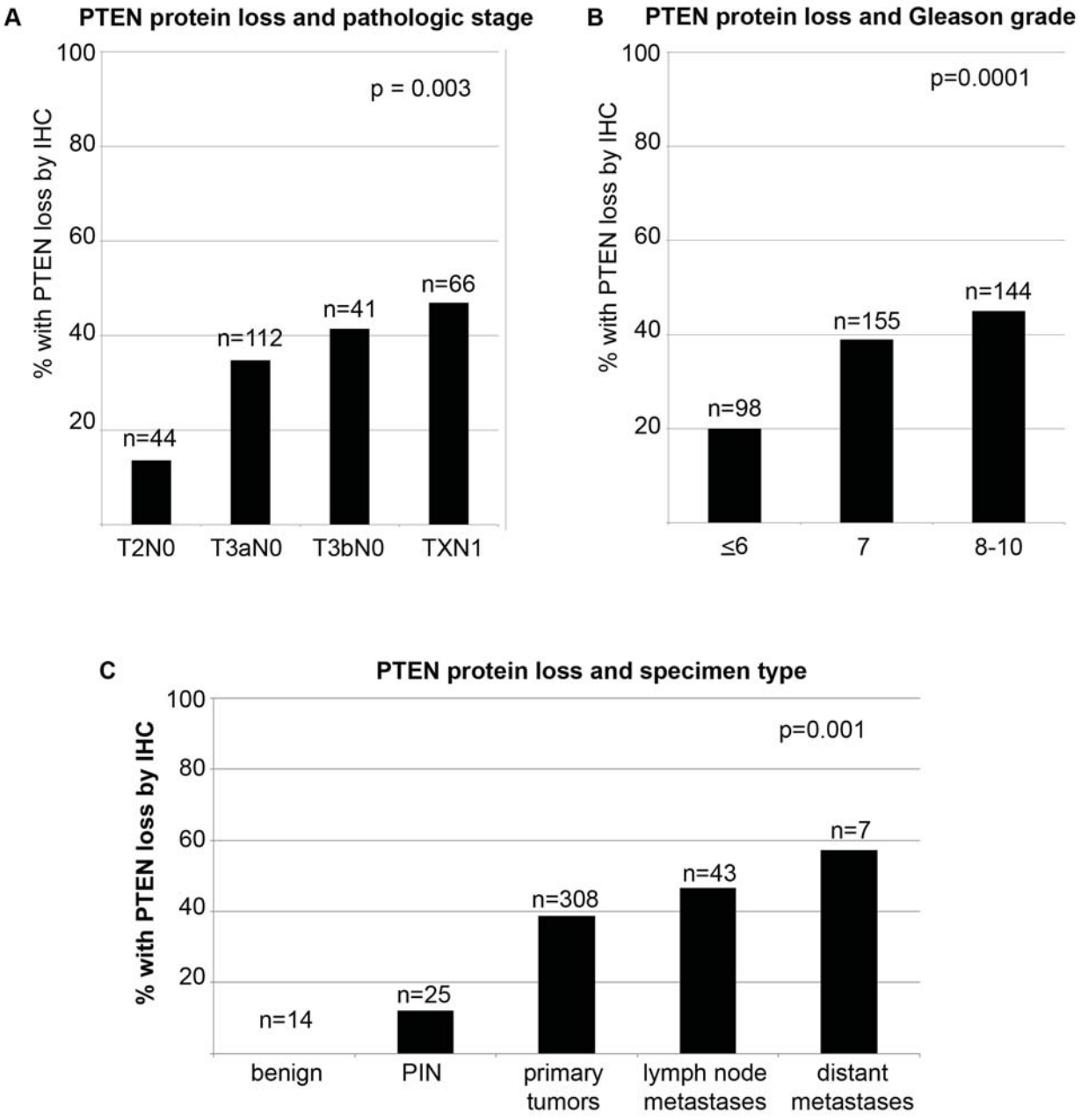


FIGURE 4

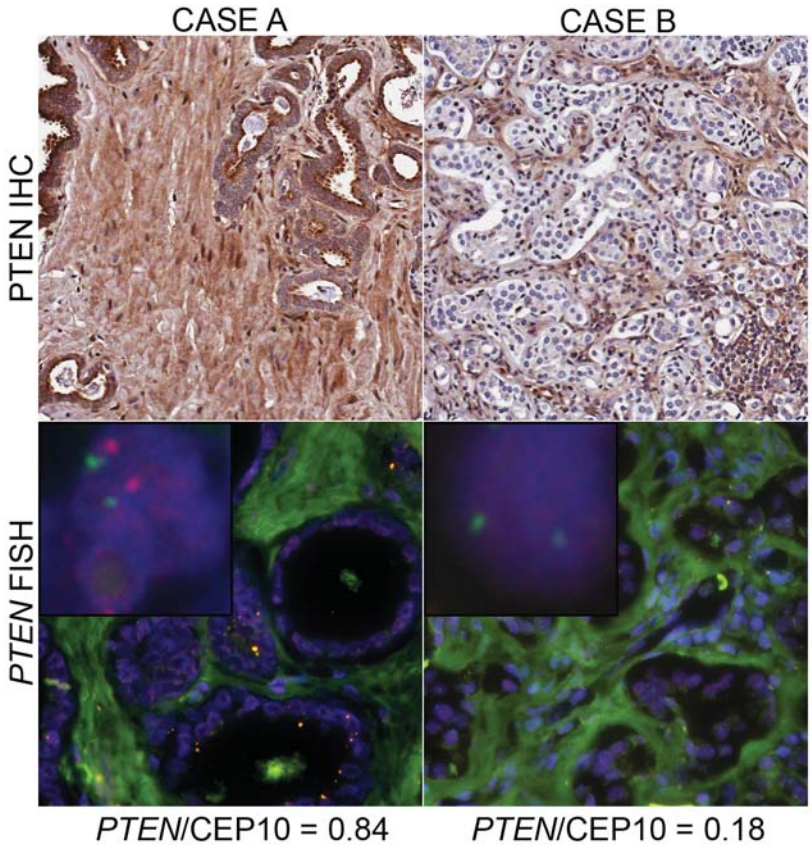
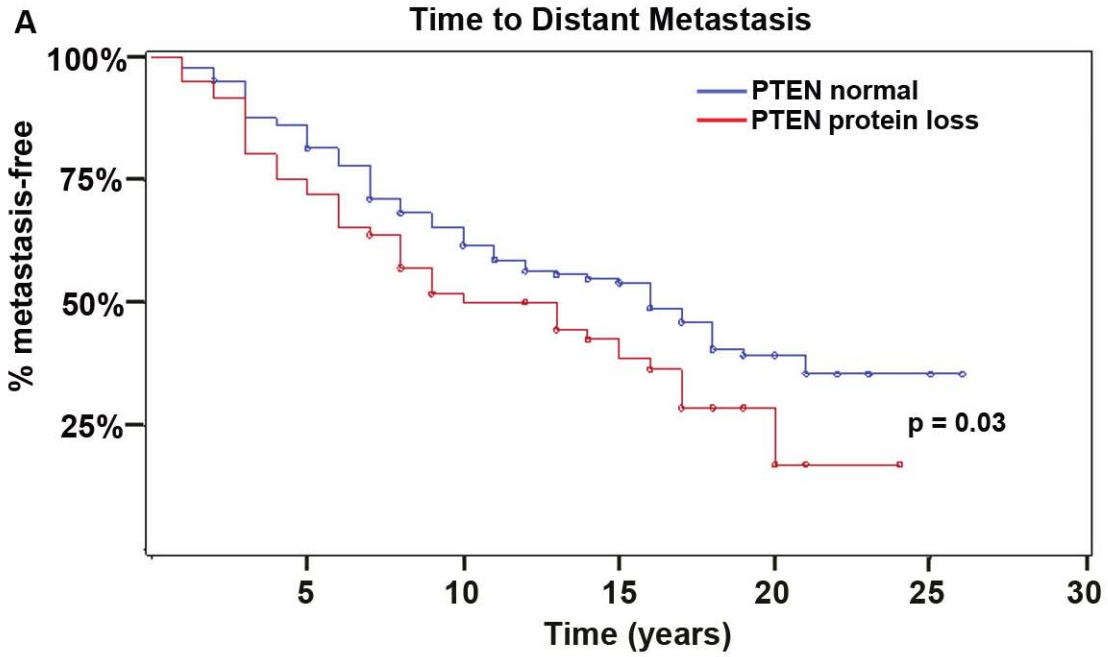


FIGURE 5



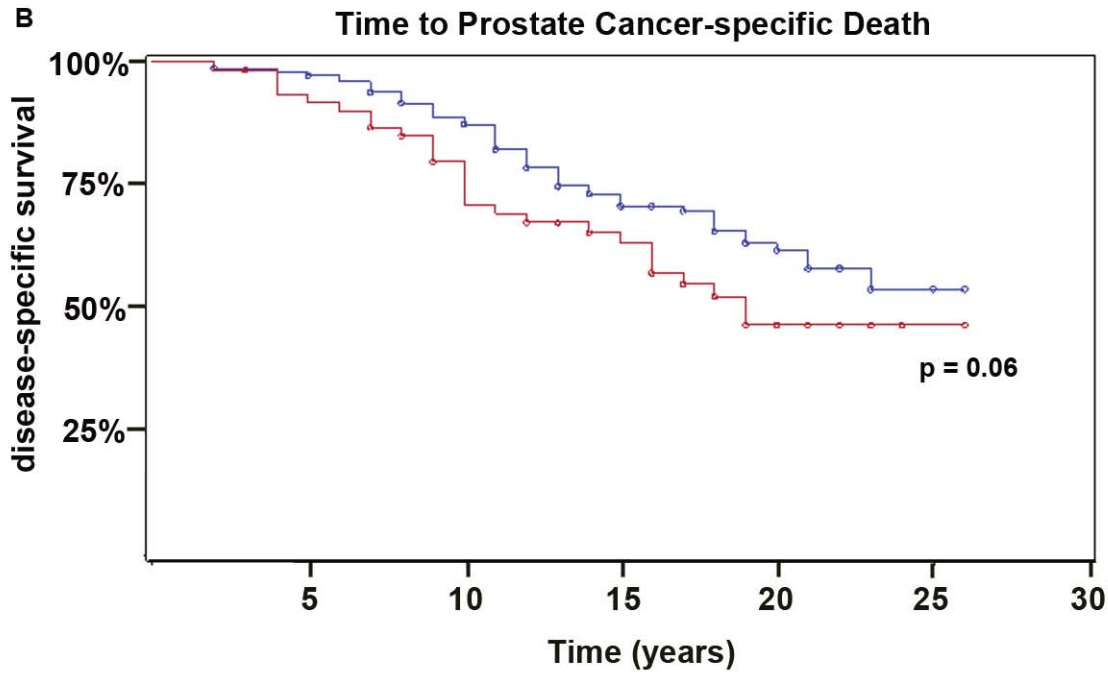


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Table 1: Correlation between PTEN protein loss detected by IHC and *PTEN* genomic loss detected by FISH.

PTEN genomic loss by FISH	PTEN protein by IHC	
	Present	Absent
Normal	**27**	**15**
Deleted	**6**	**18**

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.

	***PTEN* genomic loss by SNP array**	
PTEN protein by IHC	Present	Absent
No change	**20**	**11**
Heterozygous	**2**	**13**
Homozygous	**1**	**6**

P=0.000026 by Fisher's exact test

Table 3: Hazard ratios (HRs) and 95% confidence intervals (CIs) of distant metastasis and disease-specific death by PTEN loss and clinic-pathologic characteristics

Variable		number (percent)	Unadjusted HR (95% CI) of distant metastasis	Multivariable-adjusted HR (95% CI) of distant metastasis	Unadjusted HR (95% CI) of disease-specific death	Multivariable-adjusted HR (95% CI) of disease-specific death
PTEN protein	present	62 (29)	1	1	1	1
	absent	155 (71)	1.49 (1.02-2.16)	1.13 (0.77-1.67)	1.54 (0.97-2.44)	1.18 (0.72-1.92)
Gleason score	6	49 (22)		1		1
	7	110 (51)		1.85 (.99-3.47)		3.53 (1.36-9.17)
	8 to 10	58 (27)		4.51 (2.27-8.96)		10.8 (3.97-29.1)
Pathologic stage	pT2	61 (28)		1		1
	pT3a	82 (38)		1.63 (0.72-3.71)		1.35 (0.46-3.99)
	pT3b	74 (34)		2.68 (1.10-6.48)		2.42 (0.77-7.63)
Lymph nodes	negative	159 (73)		1		1
	positive	58 (27)		5.54 (2.34-13.1)		4.03 (1.35-12.1)
Surgical margins	negative	139 (64)		1		1
	positive	78 (36)		0.77 (0.52-1.14)		1.01 (0.62-1.66)
Biochemical recurrence		217 (100)				
Local recurrence	negative	158(75)				
	positive	52 (25)				
Distant metastasis	negative	84 (40)				
	positive	124 (60)				
Disease-specific death	negative	128 (62)				
	positive	79 (38)				