

Antibody-Based Profiling of the Phosphoinositide 3-Kinase Pathway in Clinical Prostate Cancer

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ABSTRACT

Purpose: As kinase inhibitors transition from the laboratory to patients, it is imperative to develop biomarkers that can be used in the clinic. The primary objectives are to identify patients most likely to benefit from molecularly targeted therapies and to document modulation of the drug target. Constitutive activation of the phosphoinositide 3-kinase (PI3K) pathway and its downstream effectors, as a result of PTEN loss or by other mechanisms, occurs in a high proportion of prostate cancers, making it an ideal template for the design of clinical trials involving PI3K pathway inhibitors. Prostate cancers also present unique organ-specific challenges, in that tumors are heterogeneous and diagnostic tissue is extremely limited.

Experimental Design: Working within these limitations, we have developed a set of immunohistochemical assays that define activation of the PI3K pathway in clinical samples.

Results and Conclusions: Using both univariate and multivariate analyses, we show that loss of PTEN is highly correlated with the activation of AKT, and this, in turn, is associated with the phosphorylation of S6, one of its main effectors. These three antibodies are potentially able to

fine a molecular signature of PTEN loss and/or AKT pathway activation in prostate cancer.

INTRODUCTION

Inactivation of the *PTEN* tumor suppressor gene is implicated in the development of both primary and metastatic prostate cancers. Conditional deletions of *PTEN* in the mouse prostate leads to cancer, and many human prostate cancer cell lines and xenografts have *PTEN* deletions or point mutations (1–5). The best characterized function of *PTEN* is as a lipid phosphatase that counteracts the growth- and survival-promoting effects of phosphoinositide 3-kinase (PI3K). PI3K is a lipid kinase that phosphorylates phosphatidylinositols at the 3-position (PtdIns[3,4,5]P₃), which subsequently recruit kinases such as AKT (a potent oncogenic survival factor), leading to a cascade of constitutive activation of downstream effectors (Fig. 1), including the mammalian target of rapamycin (mTOR; ref. 6–10). We have previously shown that *PTEN*-deficient prostate cancer cell lines and xenografts are more sensitive to the pharmacological inhibition of mTOR with the rapamycin analog, CCI-779, when compared with their wild-type counterparts (11, 12). We also demonstrated that the phosphorylation of S6 ribosomal protein (hereafter called S6) was a potential surrogate marker for both pathway activation and mTOR inhibition by drug.

Lessons learned from molecularly targeted therapies point to two key elements that will ultimately determine whether we can successfully translate these findings into the clinic (13–16). One, we need to identify subsets of patients who will most likely benefit from this class of drugs. The importance of this knowledge is exemplified by the recent experience with epidermal growth factor receptor inhibitors in lung cancer, in which knowing the mutation status of the epidermal growth factor receptor can enrich for patients most likely to benefit from these class of drugs (17, 18). Our present understanding suggests that this may depend on the level of signal activation of the pathway being targeted and the molecular lesion leading to target activation. Second, we need to measure the inhibition of the signaling pathway when using targeted therapy to guide dose selection and scheduling. Translating these goals to the clinic presents several challenges not encountered *in vitro*. Diagnostic needle core biopsies of the prostate invariably consist of small amounts of tissue. Also, unlike cancer cell lines, clinical samples of human prostate cancers are not homogeneous populations but consist, instead, of a mixture of both normal and tumor bearing cells. Clinical decisions with molecularly targeted agents are likely to require assessment of signaling pathway abnormalities with limited biopsy material.

With these goals in mind, we designed this study to pilot the detection of activated signaling pathways *in situ* on formalin-fixed, paraffin-embedded tissue, analogous to detecting Her-2/Neu amplification by immunohistochemistry in breast cancers. Because the target (mTOR) is a kinase, we examined the

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Note: Supplementary data for this article can be found at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org>).

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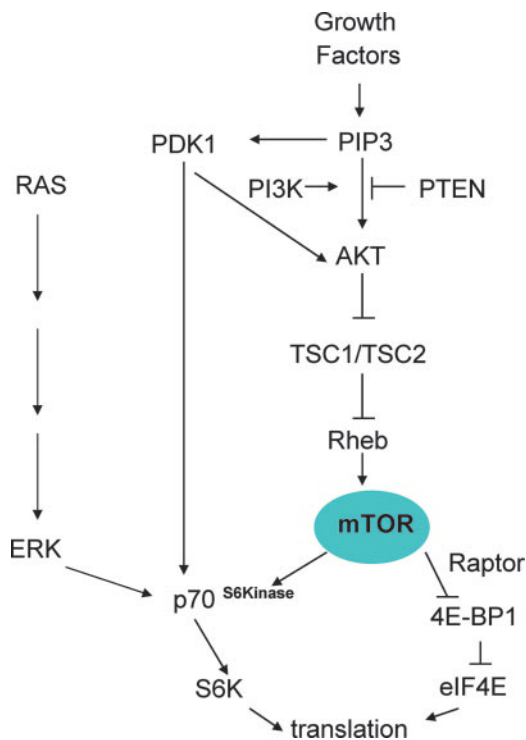


Fig. 1 Schematic demonstrating the components of the PI3K signaling pathway. PDK1, phosphoinositide-dependent kinase-1; PIP3, phosphatidylinositol-3,4,5-triphosphate; TSC1/TSC2, tuberous sclerosis 1/2; ERK, extracellular signal related kinase; Rheb, Ras homologue enriched in brain.

phosphorylation status of its downstream effectors by using activation-specific, phospho-specific antibodies. We showed that PTEN loss, when examined within the context of a tissue microarray, was highly correlated with AKT activation, and AKT activation was correlated with up-regulation of p-S6.

MATERIALS AND METHODS

Human Prostate Cancer Tissue Microarray

We constructed a tissue microarray from formalin-fixed, paraffin-embedded archival tissue blocks from 133 radical prostatectomies, done for prostate cancer at the University of California-Los Angeles (UCLA) Medical Center, following protocols set by the hospital institutional review board. The distribution of Gleason scores and pathologic tumor-node-metastasis (TNM) stage were as follows: 79 cases had Gleason score 5–6; 37 cases had Gleason score 7 and 9 cases had Gleason score 8–10. Eighty four cases were organ confined (*i.e.*, stage II), 34 were stage III, and 13 cases were stage IV. The array was designed so that three cores were taken from tumor-bearing tissue and one core from morphologically benign tissue (only areas containing normal prostate glands and stroma were sampled; atrophic and hyperplastic glands were not included in the array construction or in the evaluation) from the same patient. These cores were placed adjacent to each other on the tissue array, which enabled comparison of antibody staining pattern and intensity between benign and cancer tissue from the same patient.

In vitro Optimization of Phospho-Specific Antibodies

Cells (LNCaP, DU 145, HT 129) were grown in culture (DMEM supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin) and were exposed to vehicle, the PI3K inhibitor LY294002 (30 $\mu\text{mol/L}$ for 2 hours), the mTOR inhibitor, CCI-779 (20 nmol/L for 18 hours), or the MEK inhibitor, UO126 (20 $\mu\text{mol/L}$, preincubated for 3.5 hours, followed by stimulation with 100 ng/mL of epidermal growth factor for 20 minutes). Cell pellets were prepared, were formalin fixed, and were embedded in paraffin, then were sectioned for immunohistochemical staining with phospho-AKT, -S6, -FKHR and -ERK antibodies. (Detailed methodology pertaining to cell pellet preparation and associated immunohistochemical and immunofluorescence controls are supplied in the Supplemental Data.)⁶ We performed antigen retrieval with a 10-mmol/L sodium citrate buffer (pH 6.0) for 30 minutes in a pressure cooker. Peroxidase activity was quenched with 3% hydrogen peroxide in water. Sections were stained overnight at 4°C with monoclonal antibodies to PTEN at 1:300 (clone 6H2.1, Cascade Bioscience, Winchester, MA; this antibody recognizes a 99-amino-acid epitope at the COOH-terminus of PTEN) and polyclonal phosphorylation-specific antibodies to p-AKT (Ser 473); p-FKHR, which recognizes phosphorylated forms of the forkhead family of transcription factors FKHR (FOXO 1), FKHRL1 (FOXO 3a), and AFX (FOXO 4); p-S6 ribosomal protein (Ser 235/236); and p-ERK (Thr-202/Tyr-204) at 1:100 (Cell Signaling Technology, Beverly, MA). This was followed by application of biotinylated secondary antibodies (Vector) at 1:1000 dilution for 30 minutes and Avidin-biotin complex (Elite ABC; Vector). Negative control slides received normal mouse serum (DAKO) as the primary antibody. Diaminobenzidine tetrahydrochloride (DAB) was used as the enzyme substrate to visualize specific antibody localization for PTEN and p-AKT; Vector NovaRed (Vector) for p-FKHR, p-S6 and p-ERK. Slides were counterstained with Harris hematoxylin.

Scoring Protocols

PTEN. PTEN staining was scored according to a previously established scale of 0 to 2, which has been shown to be highly consistent (19–25). Tumor cells are graded as 2 if their staining intensity is equal to that of the adjacent benign cells, as 1 if their staining was diminished relative to the benign cells, and as 0 if staining intensity is undetectable in the tumor cells and is present in the benign cells. Only cores that contained tumor and that were technically interpretable (*e.g.*, immunoreactivity in the benign glands) were included in the scoring protocol. The arrays were scored by one genitourinary pathologist (G. V. T) on two occasions. In addition, 20% of the cases were randomly picked and scored by a second genitourinary pathologist (J. S.) in an independent manner, *i.e.*, with no prior knowledge of the original results. Both inter-rater and intra-rater agreement were >90%.

Phosphorylation-Specific Antibodies. For scoring purposes, we included only membranous or cytoplasmic staining for p-AKT and only cytoplasmic staining for p-FKHR and p-S6.

⁶ See Supplemental Data at <http://clincancerres.aacrjournals.org>.

Table 1 Univariate analysis

	Pearson correlation	P
PTEN		
p-AKT	-0.46	0.00000002
p-S6	-0.16	0.07
p-FKHR	-0.09	0.27
p-ERK	-0.027	0.76
p-AKT		
p-S6	0.21	0.01
p-FKHR	-0.09	0.31
p-ERK	-0.07	0.42
p-S6		
p-FKHR	0.04	0.59
p-ERK	0.16	0.09

For p-AKT, p-S6, and p-FKHR, we scored the staining according to a scale of 0 to 2 (0 = no staining; 1 = mild intensity of cytoplasmic staining; and 2 = strong cytoplasmic staining). For p-AKT and p-FKHR, staining of 1 and 2 were considered positive; for p-S6, staining of 2 was considered positive, as published previously (26). For p-ERK, tumors that focally contained >5% positive nuclear staining were considered positive, as reported previously (26, 27). The agreement between the pathologists (see PTEN scoring protocol), as well as for the same pathologist on independent reviews, was 85% for p-AKT and p-S6K, 80% for p-FKHR, and 90% for p-ERK.

Statistical Analysis of Immunohistochemical Scoring.

When studying the correlations between the staining scores, we used Pearson correlation, which is appropriate for ordinal data (Table 1). When fitting a logistic regression model where a staining score was the outcome, we dichotomized the staining score (e.g., 0 versus 1, 2), i.e., no expression versus at least some expression. The computation of the Pearson correlations and the logistic regression analysis were all done with the R software (<http://cran.r-project.org/>; ref. 28).⁷

We then fitted two multivariate logistic regression models (Table 2). First, we regressed PTEN status on p-AKT, p-FKHR, and p-S6. Second, we regressed p-AKT status on the p-FKHR family and p-S6K.

To depict the relationship between the variables, we used the R function cmd scale to arrive at a two-dimensional classic multidimensional scaling plot. The aim of the multidimensional scaling is to represent dissimilarities [here, 1 - the squared Pearson correlation (r)] between points (here, biomarker staining scores) well by choosing a two-dimensional configuration of points that minimizes a "stress" function (29).

RESULTS

Defining the Loss of PTEN Protein Expression *In vivo* and Correlation with Signaling Pathway Up-Regulation. Having validated the performance characteristics of these antibodies in defined *in vitro* cell line systems (See Supplemental Data Section),⁶ we next examined their immunohistochemical staining profiles within the human prostate cancer tissue mi-

croarray. First, we defined the frequency of PTEN loss as measured by protein expression with an antibody to the COOH-terminus. [The COOH-terminal tail region contains PDZ domain binding sequences and multiple phosphorylation sites. We and others have shown that mutations in the COOH-terminal tail of PTEN results in a loss of protein stability as well as a decrease in membrane affinity (30–32). Loss of PTEN staining with this antibody should reflect impaired PTEN activity]. Because some cancers may have missense mutations in PTEN that do not alter protein expression, we recognize that this approach may underestimate the true frequency of PTEN loss and function. Of note, immunohistochemical assays will identify loss of protein expression in tumors with homozygous deletions, nonsense mutations, certain internal deletions, promoter methylation, and, finally, posttranscriptional modifications. Complete loss of PTEN staining was seen in 29% of tumors (score = 0; $n = 37$); decreased staining compared with benign in 11% (Score = 1; $n = 14$), and staining intensity in tumor equivalent to that in benign glands in 60% (score = 2; $n = 78$; Fig. 2A). This is in keeping with previous reports of absent PTEN staining, done on conventional tissue sections as well as tissue microarrays (33, 34).

PTEN loss was significantly inversely correlated with activation of p-AKT ($r = -0.46$, $P = 0.00000002$) but not p-ERK (Fig. 2B). Activation of p-AKT (35) was significantly correlated with S6 phosphorylation ($r = 0.21$, $P = 0.01$) but not with p-FKHR. Both AKT and S6 phosphorylation did not correlate with p-ERK, a target of the mitogen-activated protein kinase (MAPK) pathway (Table 1).

We next examined PTEN loss within a multivariate logistic regression model, with PTEN as the outcome and p-AKT, p-FKHR, and p-S6 as covariates (Table 2). A strong independent association between PTEN and p-AKT was identified ($P = 0.0005$), with the odds of a case being PTEN positive when p-AKT is positive at only 0.28 [95% confidence interval (CI), 0.14–0.58] that of a p-AKT negative case. Fitting the model for p-AKT and including p-FKHR and p-S6 as covariates demonstrated a significant independent association between AKT and p-S6 activation ($P = 0.028$). The odds of a case being p-AKT positive when p-S6 is phosphorylated is 2.17 times (95% CI, 1.09–4.32) that of a case with p-S6 negative and AKT (positive versus negative). The association between PTEN loss and p-S6 activation almost reached statistical significance ($P = 0.065$).

We then asked whether our data might be used to uncover relationships between these relationships without prior knowledge of the connectivity between these proteins with multidimensional

Table 2 Multivariate logistic regression analysis

Outcome and covariates	Odds ratio	95% CI	P
PTEN			
p-AKT	0.28*	0.14–0.58	0.0005
p-S6	0.5	0.30–1.04	0.065
p-FKHR	0.99	0.51–2.00	0.97
p-AKT			
p-S6	2.17	1.09–4.32	0.028
p-FKHR	0.98	0.49–1.95	0.95

* Odds of a tumor being PTEN deficient if it is p-AKT positive are 3.57 times (1/0.28) greater than if it is p-AKT negative.

⁷ Internet address: <http://cran.r-project.org/>.

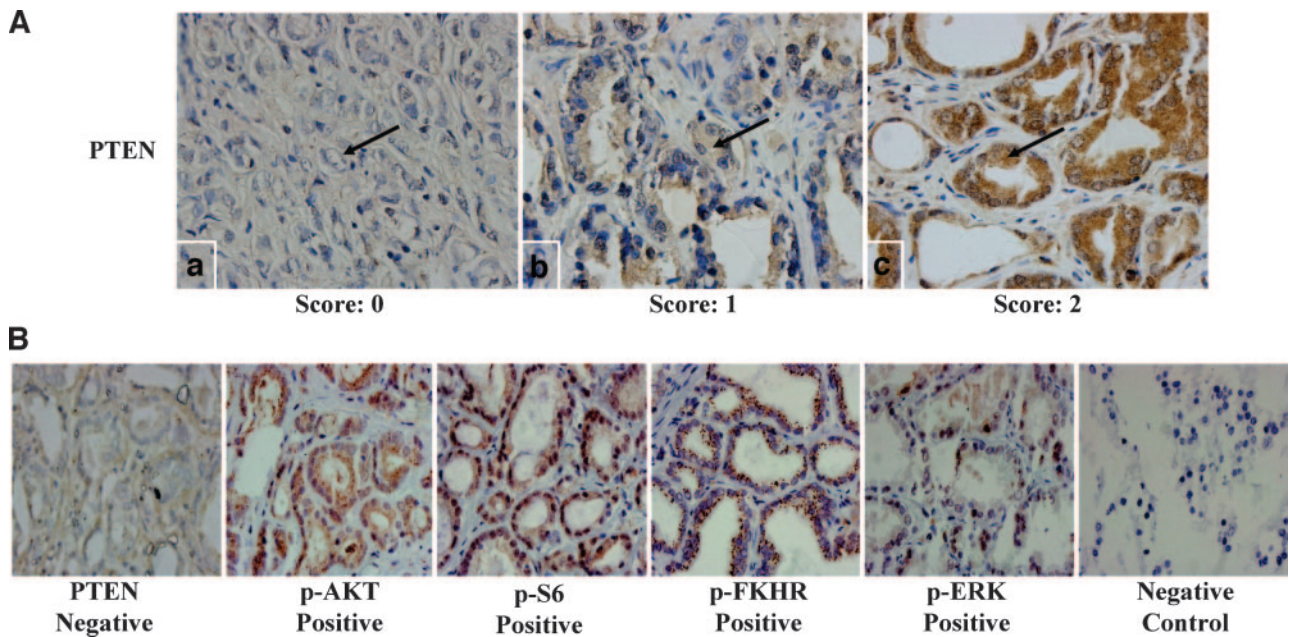


Fig. 2 PTEN scoring criteria defined. **A**, range of PTEN immunostaining. In our series, PTEN protein expression was found to be absent in 29% of tumors (*a*, score = 0), decreased compared with benign glands in 11% (*b*, score = 1), and equivalent to benign glands in 60% of tumors (*c*, score = 2). *Arrows*, staining characteristics. **B**, PI3K pathway activation in a patient with loss of PTEN expression. Serial sections from the same tumor show loss of PTEN expression results in the phosphorylation of p-AKT, p-S6, and p-FKHR. In this case, p-ERK is also active.

dimensional scaling analysis, a form of principal component analysis. Multidimensional scaling is an unsupervised data analysis method that allows examination of potential relationships between variables without assuming previous knowledge of their interaction. Using this approach, we plotted the signaling molecules in two dimensions, and the distance between two molecules provided a measure of their interrelatedness (36). This plot is a graphic representation of the correlations found in Table 1. Loss of PTEN protein expression and AKT activation are closely related, as shown by the short distance between them. In contrast, p-ERK is distant from PTEN and p-AKT (Fig. 3).

DISCUSSION

Using prostate cancer tissue microarrays, we have successfully shown that this pathway can be interrogated *in vivo* with immunohistochemical assays. We have identified a subset of patients who exhibit complete loss of PTEN protein expression. Loss of PTEN protein expression was significantly correlated with the activation of AKT. Activation of AKT was, in turn, associated with the phosphorylation of S6, in keeping with what is known of the relationships between these signaling molecules (10, 37–40). Of course, other pathways can also lead to S6 activation, and, therefore, p-S6 alone should not be interpreted as sufficient evidence for PI3K pathway abnormality. In terms of PTEN biology, it has been shown in genetic systems (*i.e.*, conditional knockouts) that the dosage of PTEN loss correlates with murine prostate cancer progression (1, 2). In our current series, PTEN loss, when examined by Pearson and Multivariate Analysis, is significantly associated with phosphorylation of AKT. Conceptually, it may be that PTEN score 1 may reflect decreased (but not absent) gene activity.

The lack of correlation between p-AKT and p-FKHR requires further examination, especially in light of previously published work from our institution showing a significant association in glioblastomas (26). When we were developing protocols to optimize the phospho-specific antibody immunohistochemical assays, we noted that the staining intensity of p-FKHR was less intense when compared with p-AKT or p-S6. This variation in staining characteristics, when transferred to human samples of different fixation and processing methodologies may

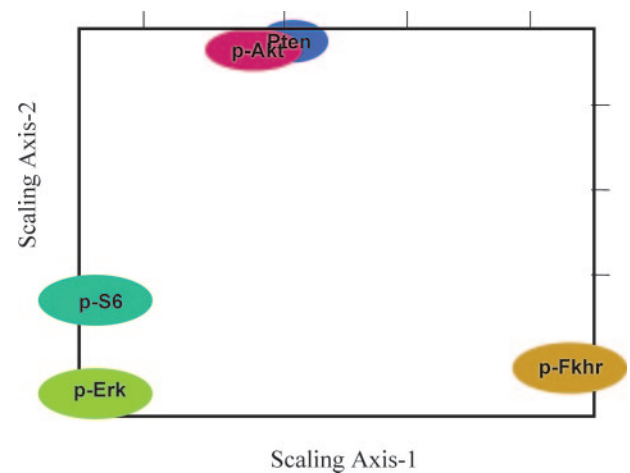


Fig. 3 Demonstration of the interrelationships between signaling molecules *in vivo*. Multidimensional scaling analysis shows that PTEN protein and AKT phosphorylation are tightly linked, confirming what is known of their *in vitro* relationship.

lessen the likelihood of consistent, reproducible results. Of note, p-FKHR scored the lowest in the intra- and interobserver correlation percentiles, which suggests that subtle differences in staining intensity may lead to variations in the scoring protocol. p-ERK is not correlated with any molecules in this pathway, consistent with *in vitro* data that places the MAPK pathway parallel to, rather than epistatic to, the PI3K cascade.

We did not see any correlation between PTEN status (or any of the other markers) and Gleason score or the pathologic stage. In contrast, two other studies have found associations between loss of PTEN protein expression or increased activation of AKT with higher Gleason scores and advanced stage (33, 41). One potential explanation is that our data set may be underpowered to address this question, because it is populated by lower Gleason grades and pathologic stages. In future studies, it will be important to correlate PI3K pathway activation with clinical outcome.

From a clinical standpoint, it will be important to determine whether the molecular aberrations seen in the tissue microarray cores can be reproduced in the context of a clinical trial in which core needle biopsies might be used to guide treatment decisions with a targeted agent. Ultimately, these questions can only be accurately answered by examining the staining patterns in matched biopsy and radical prostatectomy specimens from the same patients, as we have done previously with other biomarkers, such as p27 (42). Indeed, these results form the basis of a multicenter, neoadjuvant clinical trial of an mTOR inhibitor in men with newly diagnosed, high-risk prostate cancer. Ultimately, it will be of interest to compare data such as this, obtained by immunohistochemical assays, with genomic data obtained by PTEN gene sequencing or loss of heterozygosity analysis from clinical material. At present, such techniques require that samples contain a high fraction of tumor cells (*versus* normal) or isolation of pure populations of tumor cells by laser capture microdissection, thereby limiting their current use in the clinic.

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