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 determine 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 phosphatidylinositol-3,4,5-trisphosphate (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...
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 T1c), 27 were stage T2a, 22 were stage T2b, 23 were stage T2c, 36 were stage T3a, and 34 were stage T3b. Eighty four cases were organ confined (i.e., stage T1c), 27 were stage T2a, 22 were stage T2b, 23 were stage T2c, 36 were stage T3a, and 34 were stage T3b. The arrays were scored by one genitourinary pathologist (J. S.) in an independent manner, i.e., with no prior immunoreactivity in the benign glands) were included in the scoring protocol. The arrays were scored by one 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. 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 μmol/L for 2 hours), the mTOR inhibitor, CCI-779 (20 nmol/L for 18 hours), or the MEK inhibitor, UO126 (20 μmol/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.)

We performed antigen retrieval with a 10-μmol/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), FKHR1 (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.

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6 See Supplemental Data at http://clincancerres.aacrjournals.org.
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 ordinally scored data (Table 1). When fitting a logistic regression model where staining score was the outcome, we dichotomized the scored data (Table 1). When fitting a logistic regression model used Pearson correlation, which is appropriate for ordinally scaled data.

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 scaling. Therefore, we used the R function cmdscale to arrive at 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), we next examined their immunohistochemical staining profiles within the human prostate cancer tissue microarray. 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 multi-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Univariate analysis</th>
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<tr>
<td>Pearson correlation</td>
<td>P</td>
</tr>
<tr>
<td>PTEN</td>
<td></td>
</tr>
<tr>
<td>p-AKT</td>
<td>−0.46</td>
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<tr>
<td>p-S6</td>
<td>−0.16</td>
</tr>
<tr>
<td>p-FKHR</td>
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<td>p-ERK</td>
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<tr>
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<tr>
<td>p-S6</td>
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<tr>
<td>p-FKHR</td>
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<td>p-AKT</td>
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</tr>
<tr>
<td>p-ERK</td>
<td>0.16</td>
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* Internet address: http://cran.r-project.org/.

**Table 2 | Multivariate logistic regression analysis**

<table>
<thead>
<tr>
<th>Outcome and covariates</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P</th>
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<tr>
<td>PTEN</td>
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<tr>
<td>p-AKT</td>
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<td>0.14–0.58</td>
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<tr>
<td>p-S6</td>
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</tr>
<tr>
<td>p-FKHR</td>
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<td>0.51–2.00</td>
<td>0.97</td>
</tr>
<tr>
<td>p-AKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-S6</td>
<td>2.17</td>
<td>1.09–4.32</td>
<td>0.028</td>
</tr>
<tr>
<td>p-FKHR</td>
<td>0.98</td>
<td>0.49–1.95</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* 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.
Multidimensional 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...
ACKNOWLEDGMENTS

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REFERENCES


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