

Immunohistochemical Demonstration of Phospho-Akt in High Gleason Grade Prostate Cancer¹

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ABSTRACT

Purpose: Whereas the early stage of prostate cancer is marked by excessive proliferation, in advanced stages of the disease, a decreased apoptotic death rate (increased cell survival) also contributes to net tumor growth. Altered regulation of the mitogen-activated protein kinase (MAPK)-regulated cell proliferation and Akt-regulated cell survival pathways are suspected causes. In this study, we wanted to determine: (a) whether the degree of Akt activation can be assessed by immunohistochemical staining of paraffin-embedded human prostate cancer biopsies with an antibody to phospho-Akt (Ser473); and (b) whether phospho-MAPK/Erk1/2 and phospho-Akt expression are altered in prostate cancer.

Experimental design: To examine the activation status of MAPK/Erk1/2 and Akt, archival paraffin-embedded sections from 74 cases of resected prostate cancer were immunostained with antibodies to phospho-MAPK/Erk1/2 (Thr202/Tyr204) and phospho-Akt (Ser473).

Results: The staining intensity for phospho-Akt was significantly greater in Gleason grades 8–10 (92% of such cases staining strongly) compared with prostatic intraepithelial neoplasia and all other grades of prostate cancer (only 10% of these cases staining strongly; $P \leq 0.001$). The staining intensity for phospho-MAPK/Erk, on the other hand, was significantly greater for normal, hyperplastic,

and prostatic intraepithelial neoplasia lesions but declined with disease progression, reaching its lowest level of expression in high Gleason grades 8–10 ($P < 0.0001$).

Conclusion: The activation state of the cell survival protein Akt can be analyzed in human prostate cancer by immunohistochemical staining of paraffin-embedded tissue with a phospho-specific Akt (Ser473) antibody. Advanced disease is accompanied by activation of Akt and inactivation of Erk.

INTRODUCTION

Prostate cancer causes more than 41,000 deaths annually in the United States and is the second leading cause of cancer deaths in men (1). Although prostate cancer is initially dependent on androgens for growth and, thus, is responsive to androgen ablation, progression to an androgen-insensitive state generally ensues (1). When this occurs, the prognosis is poor, because no systemic therapy is effective. Therefore, there is an urgent need for targeted nonhormonal treatment that inhibits prostatic cancer cells. In normal prostate epithelium, cell proliferation is balanced by an equal rate of apoptosis, such that there is neither involution nor overgrowth (1). In prostate cancer this balance is altered. Whereas the early stage of the disease is marked by excessive proliferation, in advanced stages of the disease, net growth of the tumor results from a decreased apoptotic death rate (cell survival) in addition to increased proliferation (1).

Activation of the PI3k³/serine-threonine kinase Akt signaling pathway promotes cell survival by inhibiting apoptosis through phosphorylation of the proapoptotic protein BAD and other proteins (2–5), whereas activation of the MAPK signaling pathway is accompanied by increased cellular proliferation (6, 7). *PTEN* is a tumor suppressor gene that is altered and inactive in many types of tumors, including prostate cancer (2, 3). Among its substrates are the lipid products of PI3k, phosphatidylinositol 3,4,-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate, which mediate the activation of Akt (4, 5). It was demonstrated recently by IHC that high Gleason-grade prostate cancer displays loss of tumor suppressor phosphatase *PTEN* (2). This suggests that increased activation of Akt in poorly differentiated prostatic carcinoma results from the loss of *PTEN*.

In this paper, with phospho-specific antibodies we demonstrate by IHC that advanced prostate cancer is accompanied by the expression of the activated (phosphorylated) form of Akt and decreased expression of activated MAPK/Erk1/2. These results may provide the molecular basis for the observed activation of a cell survival pathway that has been reported to

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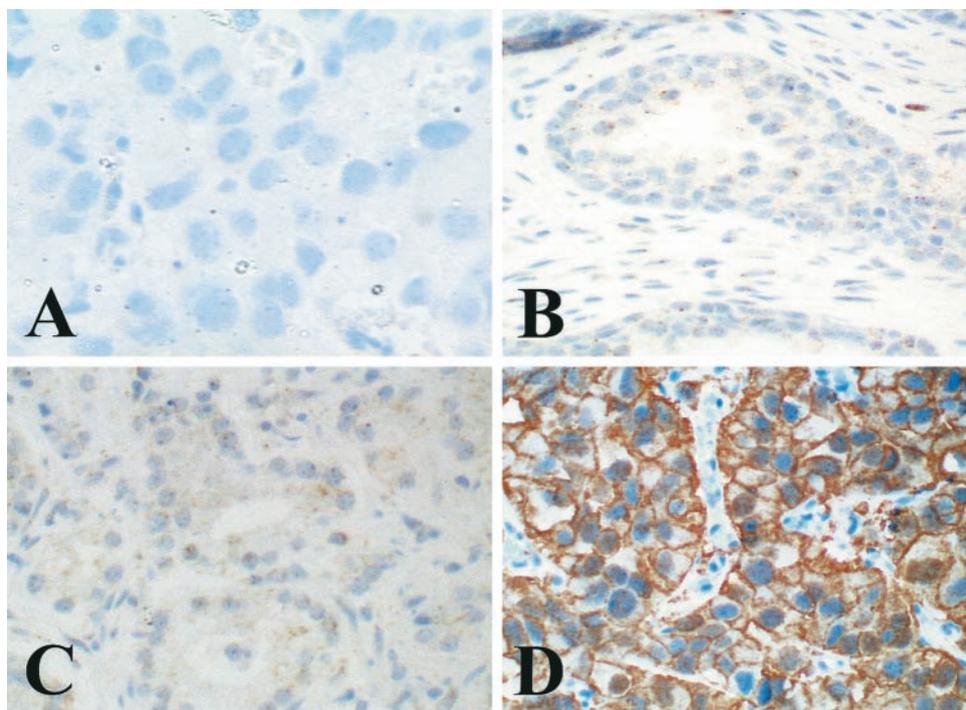
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³ The abbreviations used are: PI3k, phosphatidylinositol 3'-kinase; MAPK, mitogen-activated protein kinase; IHC, immunohistochemistry; PIN, prostatic intraepithelial neoplasia; Erk, extracellular-regulated kinase; TBS-T, tris-buffered saline-tween.

Fig. 1 Immunoperoxidase staining for phospho-Akt using a phospho-specific Akt (Ser 473) antibody. *A*, negative control. *B*, PIN showing weak cytoplasmic staining for phospho-Akt. *C*, well-differentiated adenocarcinoma showing weak cytoplasmic staining. *D*, poorly differentiated carcinoma showing strong membrane staining for phospho-Akt ($\times 40$, original magnification).



contribute significantly to the progression of prostate cancer growth (1).

MATERIALS AND METHODS

Primary Antibodies. Rabbit polyclonal phospho-Akt (Ser 473; Cell Signaling Technology, Beverly, MA, Cat. No. 9277, IHC specific) was used at a 1:50 dilution; rabbit polyclonal phospho-p44/42 MAP kinase (Thr 202/Tyr 204; Cell Signaling Technology; IHC-specific) at a 1:50 dilution; rabbit polyclonal Akt (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:50 dilution; and rabbit polyclonal Erk1 (Santa Cruz Biotechnology) at a 1:100 dilution.

Analysis of Human Tissues. A total of 74 formalin-fixed, paraffin-embedded human primary prostate cancer specimens were studied from the archival files of Audie Murphy Veterans Medical Center. Fifty-three samples were obtained from radical prostatectomies, and 22 samples were obtained from transurethral resections. H&E-stained slides were reviewed for Gleason score. In a majority of the cases, adjacent areas of normal prostatic epithelium, benign prostatic hyperplasia, and PIN were also available for review along with infiltrating carcinoma.

IHC. Sections were heated to 60°C, and rehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 for 20 min in a 95% water bath. Slides were allowed to cool for another 20 min, followed by sequential rinsing in PBS and 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, Tween 20 (0.1%; TBS-T). Endogenous peroxidase activity was quenched by incubation in TBS-T containing 3% hydrogen peroxide. Each incubation step was carried out at room temperature and was followed by three sequential washes

Table 1 Relationship between phospho-Akt staining intensity vs. Gleason score

Weak staining had scores of 0 and 1+. Strong staining intensity had scores of 2+ and 3+. The staining intensity for phospho-Akt is significantly greater in Gleason grade 8–10 compared with PIN, and all other grades of cancer ($P \leq 0.001$).

Gleason score	Number of cases	Weak staining intensity	Strong staining intensity
PIN	51	51	0
2–4	9	8	1
5–6	26	21	4
7	14	9	5
8–10	25	2	23

(5 min each) in TBS-T. Sections were incubated in primary antibody diluted in TBS-T containing 1% ovalbumin and 1 mg/ml sodium azide (12 h) followed by incubations with biotinylated secondary antibody for 15 min, peroxidase-labeled streptavidin for 15 min (LSAB-2; Dako Corp., Carpinteria, CA), and diaminobenzidine and hydrogen peroxide chromogen substrate (Dako Corp.) along with 3,3'-diaminobenzidine enhancer (Signet) for 10 min. Slides were counterstained with hematoxylin and mounted. The negative controls were incubated with nonimmune rabbit IgG in place of primary antibody.

One representative slide per case was evaluated with the above antibodies. The proportion of carcinoma and PIN staining, and the intensity of staining seen in different areas of the same slide were analyzed according to criteria described previously in the literature (8). The intensity is designated as 0 when no tumor cells stain, 1+ when 10–20% of cells stain (weak), 2+

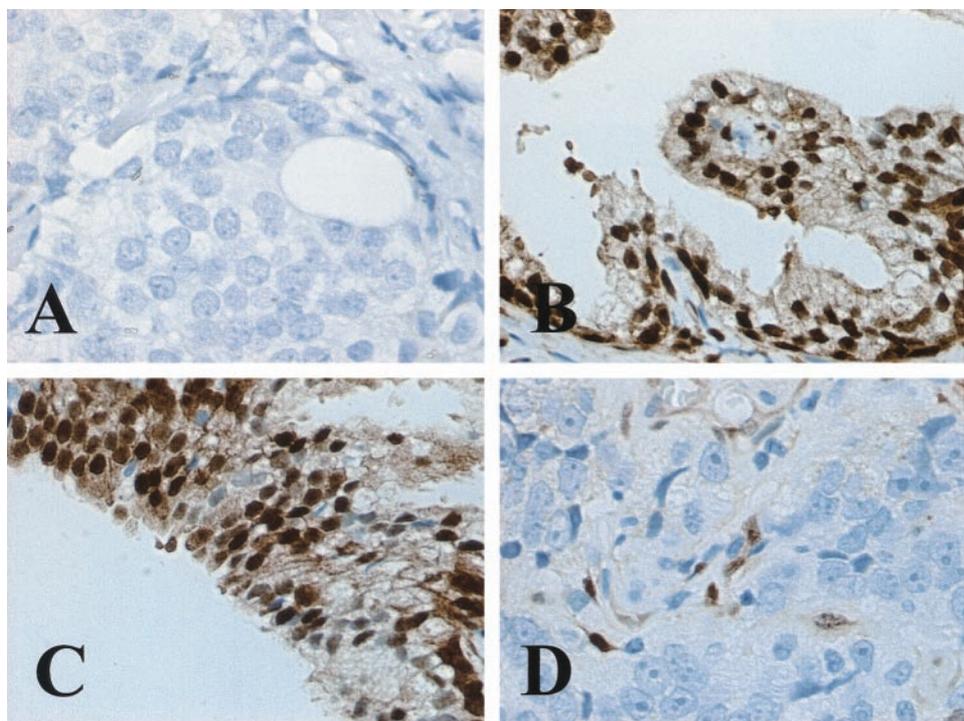


Fig. 2 Immunoperoxidase staining for phospho-Erk1/2 using a phospho-specific MAPK/Erk1/2 (Thr202/Tyr204) antibody. A, negative control. B, hyperplasia showing strong nuclear staining for phospho-MAPK/Erk1/2. C, PIN showing strong nuclear staining for phospho-MAPK/Erk1/2. D, poorly differentiated carcinoma showing weak staining for phospho-MAPK/Erk1/2. Note positive staining in vessels-serves as intrinsic positive control ($\times 40$, original magnification).

Table 2 Relationship between phospho-MAPK/Erk1/2 staining intensity vs. Gleason score

Weak staining had scores of 0 and 1+ and strong staining intensity is 2+ and 3+. The staining intensity for phospho-MAPK/Erk1/2 is significantly greater in normal, hyperplasia, and PIN versus all grades of cancer ($P < 0.0001$). PIN was significantly greater than normal ($P \leq 0.001$) and hyperplasia ($P \leq 0.03$).

Gleason score	Number of cases	Weak staining intensity	Strong staining intensity
Normal	60	17	43
Hyperplasia	53	20	33
PIN	51	3	48
2-4	9	3	6
5-6	26	17	9
7	14	13	1
8-10	25	21	4

when 20–50% of cells stain (moderate), and 3+ when $>50\%$ of cells stain (strong).

Imaging. Digital images for photomicroscopy were acquired with a Cool Snap camera from Nikon. Minor adjustments in the captured images were performed identically and in parallel for the images presented using Adobe PhotoShop 5.5. Composite images were made using Microsoft PowerPoint and printed on a Phaser 780 plus printer (Tetronix Co., Westborough, MA).

Statistics. For statistical analyses, groups scored 0 and 1+ were combined (“weak staining”) as were groups scored 2+ and 3+ (“strong staining”). Statistical analysis was performed by using χ^2 analyses with Kappa and McNemar statistics in contingency tables for agreement and disagreement of specific comparisons (9). Normality of residuals was assessed for phos-

pho-Akt and phospho-MAPK Erk levels each analyzed separately to assure valid analyses. The analyses were performed using a statistical analysis system on a PC-compatible computer with SAS 6.12 software (SAS Institute, Cary, NC).

RESULTS

Increased Expression of Phospho-Akt (Ser473) in Paraffin-embedded Poorly Differentiated Prostate Cancer.

The PI3k dependent serine threonine kinase Akt (also known as protein kinase B) has been implicated in mediating cell survival in various prostate cancer cells (2, 3, 10, 11). Therefore, we examined human prostate cancer tissues by IHC to determine whether the expression of the activated (phosphorylated) form of the cell survival protein Akt correlated with prostate cancer differentiation. Ninety percent of PIN, well to moderately differentiated adenocarcinomas, and Gleason score 7 carcinomas were either completely negative or showed only weak staining (intensity score of 0 to 1+) for phospho-Akt ($P < 0.001$; Fig. 1; Table 1). Phospho-Akt staining intensity progressed as the disease progressed with strongest staining observed in the highest Gleason scores. That is, $>90\%$ of poorly differentiated adenocarcinomas (Gleason score 8–10) exhibited strong staining for phospho-Akt (intensity score of 2 to 3+), ($P \leq 0.001$; Fig. 1; Table 1). Interestingly, the staining appeared to be localized to the membrane where Akt has been shown to be active (4, 5). Total Akt levels were expressed in all of the tissues with no change in the degree of expression during disease progression.

Decreased Expression of Phospho-MAPK/Erk1/2 in Paraffin-embedded Poorly Differentiated Prostate Cancer.

In contrast to the PI3k/Akt signaling pathway, the MAPK signaling pathway is well recognized for mediating cell prolifera-

tion. As a measure of MAPK activation, we used an antibody that recognized phosphorylated MAPK/Erk1/2. More than 75% of normal, hyperplastic, and PIN displayed strong staining (2+ to 3+) for phospho-MAPK/Erk1/2 (Fig. 2; Table 2). Phospho-MAPK/Erk1/2 staining was significantly greater in PIN *versus* hyperplasia ($P \leq 0.03$) and normal ($P \leq 0.001$). The intensity of staining decreased as the disease progressed to carcinoma, with only 27% of the tumor cells showing strong staining for phospho-MAPK/Erk1/2 ($P < 0.0001$). The weakest staining was observed in poorly differentiated cancers (Fig. 2; Table 2). The staining appeared to be localized to the nucleus. Total Erk levels were expressed in all of the tissues with no change in the degree of expression during disease progression.

DISCUSSION

Immunohistochemical examination of paraffin-embedded human prostate cancer showed that 92% of the poorly differentiated adenocarcinomas of the prostate stained strongly for phospho-Akt in a membrane location. In all other grades of prostate cancer as well as in PIN, only 10% stained for phospho-Akt. On the other hand, >75% of normal, hyperplastic, and PIN lesions showed a high level of expression of phospho-MAPK/Erk1/2 that significantly decreased in adenocarcinoma.

This is the first report of the immunohistochemical detection of phospho-(active) Akt using a phospho-specific antibody in paraffin-embedded human prostate cancer. Similar to our observations, Paweletz *et al.* (11) showed by reverse-phase protein microarrays that cancer progression was associated with increased phosphorylation of Akt and suppression of apoptotic pathways as measured using antibodies to cleaved caspase 7 and poly(ADP-ribose) polymerase.

Advanced prostate cancer is often accompanied by androgen independence, and growth of the tumor becomes dependent on activation of cell survival pathways as well as cell proliferation pathways. Graff *et al.* (10) showed that Akt activation was markedly increased in an androgen-independent LNCaP cell line that was isolated from LNCaP xenografts. In addition to increased Akt activation, there was increased phosphorylation and inactivation of the proapoptotic protein BAD, a target protein of Akt, and decreased expression of the cyclin inhibitor, p27kip1 (10). These results would explain the emergence of an antiapoptotic pathway in androgen-independent prostate cancer as well as explain the enhanced proliferation observed in advanced prostate cancers. In human prostate cancer, the tumor suppressor phosphatase PTEN is mutated and inactive (2, 3, 10, 11). This phosphatase normally negatively regulates components of the PI3k pathway such as the cell survival protein Akt. Loss of PTEN activity is accompanied by increased expression of the activated form of Akt and activation of cell survival pathways. Similar to our findings by IHC, Paweletz *et al.* (11) demonstrated in protein microarrays that prostate tumor progression is accompanied by increased expression of phospho-Akt. Importantly, this coincided with suppression of apoptosis. Also similar to our findings, they showed that expression of phospho-Erk was suppressed with progression of disease. These findings are in contrast to the IHC studies by Gioeli *et al.* (12)

who showed increased expression of phospho-MAPK with increasing Gleason score. Studies by Zimmerman and Moeling (13) may explain our observations of high phospho-Akt expression accompanied by low levels of phospho-MAPK/Erk; namely, they showed that phospho-Akt inactivates Raf by direct phosphorylation on Ser259, resulting in inhibition of the Raf-MEK-Erk signaling pathway. In conclusion, we show by IHC on paraffin-embedded tissue that progression of prostate cancer is accompanied by increased levels of phospho-Akt and decreased levels of phospho-MAPK/Erk. Understanding the mechanisms of prostate tumor growth could prove critical to developing new effective therapies for prostate cancer.

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