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

Shazli N. Malik, Michael Brattain, Paramita M. Ghosh, Dean A. Troyer, Thomas Prihoda, Roble Bedolla, and Jeffrey I. Kreisberg²

Departments of Surgery [S. N. M., P. M. G., R. B., J. I. K.], Pathology [D. A. T., T. P.], University of Texas Health Science Center, San Antonio, Texas 78229; Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263 [M. B.]; and South Texas Veterans Health Care System, Audie Murphy Veterans Administration Hospital, San Antonio, Texas 78229 [J. I. K.]

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 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 3,4,5-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

¹ 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.
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 (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 dianaminobenzidine 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+...
**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 < 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).

**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-

---

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

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Number of cases</th>
<th>Weak staining intensity</th>
<th>Strong staining intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>60</td>
<td>17</td>
<td>43</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>53</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>PIN</td>
<td>51</td>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>2–4</td>
<td>9</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>5–6</td>
<td>26</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>8–10</td>
<td>25</td>
<td>21</td>
<td>4</td>
</tr>
</tbody>
</table>

---

**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 χ² analyses with Kappa and McNemar statistics in contingency tables for agreement and disagreement of specific comparisons (9). Normality of residuals was assessed for phospho-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).
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 = 0.03) and normal (P = 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 differ-
tiated 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 detec-
tion 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 andro-
gen independence, and growth of the tumor becomes dependent
on activation of cell survival pathways as well as cell prolifera-
tion 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
antia apoptotic pathway in androgen-independent prostate cancer
as well as explain the enhanced proliferation observed in ad-
vanced prostate cancers. In human prostate cancer, the tumor
suppressor phosphatase PTEN is mutated and inactive (2, 3, 10,
11). This phosphatase normally negatively regulates compo-
ents 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 pro-
gression 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 in-
creasing Gleason score. Studies by Zimmerman and Moeling
(13) may explain our observations of high phospho-Akt expres-
sion 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 de-
creased levels of phospho-MAPK/Erk. Understanding the mech-
anisms of prostate tumor growth could prove critical to devel-
op new effective therapies for prostate cancer.

REFERENCES

1. Denmeade, S. R., Xiaohui, S. L., and Isaacs, J. T. Role of pro-
grammed (apoptotic) cell death during the progression and therapy for
2. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and
Sellers, R. Loss of PTEN expression in paraffin-embedded primary
prostate cancer correlates with high Gleason score and advanced stage.
3. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A.,
Sanghera, J., and Dedhar, S. Inhibition of integrin-linked kinase (ILK)
suppresses activation of protein kinase B/Akt and induces cell cycle
arrest and apoptosis of PTEN mutant prostate cancer cells. Proc Natl
4. Kandel, E. S., and Hay, N. The regulation and activities of the
5. Burgerring, B. M. T., and Coffer, P. Protein kinase B (c-Akt) in
phosphatidylinositol 3-OH kinase signal transduction. Nature (Lond.),
6. Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B.,
Lammers, R., Ullrich, A., Skolnik, E. Y., and Bar-Sagi, D. The SH2 and
SH3 domains containing proteins GRB2 links receptor tyrosine kinases
7. Halberg, B., and Rayter, S. I., Downward, J. Interaction of Ras
and Raf in intact mammalian cells upon extracellular stimulation. J. Biol.
8. Allerd, D. C., Clark, F. M., Elledge, R., Fuqua, S. A. W., Brown,
R. W., Channess, G. C., Osborne, A. K., and McGuire, W. L. Associ-
ation of p53 protein expression with tumor cell proliferation rate and
10. Graff, J. R., Konicek, B. W., McNulty, A. M., Wang, Z., Houck, K.,
Allen, S., Paul, J. D., Hb sau, A., Goode, R. G., Sandusky, G. E.,
Vessella, R. L., and Neubauer, B. L. Increased Akt activity contributes
to prostate cancer progression by dramatically accelerating prostate
tumor growth and diminishing p27kip1 expression. J. Biol. Chem.,
11. Paweletz, C. P., Charboneau, L., Bischel, V. E., Simone, N. L.,
Chen, T., Gillespie, J. W., Emmert-Buck, M. R., Roth, M. J., Petricoin,
E. F., III, and Liotta, L. A. Reverse phase protein microarrays which
capture disease progression show activation of pro-survival pathways at
12. Gioeli, D., Mandell, J. W., Petroni, G. R., Frierson, H. F., and
Weber, M. J. Activation of mitogen associated protein kinase b associated
of Raf by Akt (Protein kinase B). Science (Wash. DC), 286: 1741–1744,
1999.
Immunohistochemical Demonstration of Phospho-Akt in High Gleason Grade Prostate Cancer

Shazli N. Malik, Michael Brattain, Paramita M. Ghosh, et al.