

The Biology Behind

More than a Marker. . . Phosphorylated Akt in Prostate Carcinoma

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INTRODUCTION

Advances in understanding carcinogenesis have fostered many ideas for improving cancer prognostication and treatment. However, development of these ideas has far outpaced the rate of clinical testing. The advent of tissue microarrays has accelerated analysis and reduced the expense of analysis of tumor specimens, which, in turn, will help relieve this bottleneck in translating biologically based hypotheses to clinical applications.

The excessive proliferation and reduced susceptibility to apoptosis of cancer cells are tied to changes in activity of several major signal transduction pathways. In principle, measurement of the activities of these pathways will provide a window on the state of precancerous or cancerous cells and yield prognostic and predictive information. Moreover, components of the same signal transduction pathways are logical targets for rational cancer therapeutics.

The study by Ayala *et al.* (1) in this issue of *Clinical Cancer Research* investigates the hypothesis that signaling through pathways encompassing the Akt family of protein kinases is associated with prostate cancer progression (1). This is reasonable because Akt kinases are intermediaries in signaling by growth and antiapoptotic regulators and because components of the pathways are direct targets for carcinogenic mutations (reviewed in refs. 2 and 3).

PHOSPHATIDYLINOSITOL 3'-KINASE AND PTEN

Akts are activated by second messengers produced by phosphatidylinositol 3'-kinases (PI3Ks). These lipid kinases are regulated by receptor tyrosine kinases or G protein-coupled receptors. The class 1a PI3Ks, which have 85-kDa SH2-containing subunits and 110-kDa catalytic subunits, are particularly important in normal growth regulation and cancer. p85 SH2 domains bind Tyr-phosphorylated YXXM motifs on growth

factor-activated receptor tyrosine kinases or receptor-activated adaptors. Once localized near the inner face of the plasma membrane by interaction with receptors or adaptors, the PI3Ks phosphorylate plasma membrane-associated phosphatidylinositols to produce derivatives phosphorylated at the 3' position. PI3Ks are also activated by receptor kinases through a second mechanism involving binding of p110 to Ras. PI3K-dependent production of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate is counterbalanced by the action of PTEN phosphatases (4). Loss of PTEN function often causes chronic up-regulation of Akt pathways. Although genetic studies demonstrate that Akts are among the most important proteins regulated by these second messengers, PI3K regulation of the cytoskeleton and cell motility through Rac and Cdc42 and other parallel outputs is also likely to be significant in the context of cancer (2).

Akt REGULATION

In humans, three genes encode Akt-1, Akt-2, and Akt-3. All three are coexpressed in many normal tissues and tumors (5). Akts have pleckstrin homology domains that recognize the phosphatidylinositol derivatives produced by PI3K and recruit Akt to the plasma membrane. Relocalization brings Akt into proximity with another pleckstrin homology protein kinase, PDK1, which phosphorylates and partly activates Akt at Thr³⁰⁸ in the activation loop of the protein kinase domain. Akt is further activated through phosphorylation at the COOH-terminal Ser⁴⁷³ by an activity called PDK2, which consists of integrin-linked kinase and/or other protein kinase(s) (6). Once activated, Akt can relocate to the nucleus for phosphorylation of nuclear substrates.

Akt OUTPUTS

Akt-regulated pathways enhance cell division and cell survival. Metabolic regulation through Akt and its targets is important for insulin and insulin-like growth factor I-coupled responses. Inhibition of glycogen synthase kinase-3 by Akt-dependent phosphorylation promotes accumulation of β -catenin, which forms complexes with T-cell factor/lymphoid enhancer factor transcription factors and transcriptionally up-regulates cyclin D1, Myc, and other positive growth regulators. Cyclin D1 is also inhibited by glycogen synthase kinase-3 through effects on stability and localization (7). Concomitantly, Akt phosphorylation of cyclin-dependent protein kinase inhibitors p21^{Cip1} and p27^{Kip1} interferes with negative growth regulation (3, 8).

Phosphorylation of Mdm2 by Akt enhances nuclear entry, which promotes ubiquitin-dependent proteolysis of p53, and impedes p53-dependent growth suppression and apoptosis (9). Akt directly forestalls apoptosis by phosphorylation of proapoptotic Bad and caspase-9, which are sequestered by interaction with 14-3-3 proteins (10). Akt facilitates nuclear factor (NF)- κ B signaling and contributes to the antiapoptotic effects of Akts (3, 11).

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Akt negatively regulates three members of the FoxO subfamily of forkhead transcription factors, FoxO1, FoxO-3a, and FoxO4, by impeding their nuclear localization (reviewed in ref. 12). FoxO transcription factors promote insulin-dependent glucose production and differentiation of some cell lineages. These transcription factors promote cell cycle arrest, in part, through induction of the cyclin-dependent kinase inhibitor p27^{Kip1}. Negative regulation of p27^{Kip1} by Akt-dependent inhibition of FoxOs is reinforced by Akt-dependent phosphorylation and, indirectly, through NF- κ B. In complexes with Smads, which are activated by transforming growth factor β , FoxOs also enhance transcription of the cyclin-dependent kinase inhibitor p21^{Cip1} (13). Finally, FoxO proteins promote apoptosis through transcriptional induction of FAS ligand, tumor necrosis factor-related apoptosis-inducing ligand, and BIM (12).

Another major arm of Akt output pathways works through the protein kinase mammalian target of rapamycin [mTOR (reviewed in ref. 14)]. Mammalian target of rapamycin is a member of the phosphoinositide kinase-like kinase family, a group of variant protein kinases that are mainly involved in DNA damage responses and recombination. Akt activates mTOR, possibly by direct phosphorylation. Akt phosphorylates and negatively regulates TSC2/tuberin of the tuberous sclerosis complex (15), which itself inhibits the G protein RHEB that enhances mTOR signaling (14).

Mammalian target of rapamycin activation enhances translational initiation directly by phosphorylation-dependent inactivation of 4E-BP1 proteins that interfere with translational initiation by eIF4E (14, 15). Mammalian target of rapamycin (mTOR) stimulates ribosomal S6 p70 kinases. These kinases stimulate translation of a subset of mRNAs associated with cell growth, including some that encode components of the translational apparatus. Mammalian target of rapamycin pathways are implicated positively in cell size control and may destabilize p27 Kip1 (14, 15).

Akt AND CANCER

PI3K and Akt pathways are often dysregulated in cancer. Akt was originally discovered in mutated form as a viral oncogene, and early work on Akt-2 identified amplifications in ovarian carcinoma (16). PIKE-A, which enhances activation of Akt, is amplified in glioblastoma (17). Akt is commonly activated by excessive accumulation of phosphatidylinositol 3,4,5-trisphosphate resulting from loss of functional PTEN. Cowden and other hamartoma syndromes are often caused by germ-line PTEN mutations. In sporadic cancers, both genetic (10q23 deletion/loss of heterozygosity) and epigenetic mechanisms contribute to loss of PTEN expression (reviewed in 4). Amplification of the receptor kinase HER2/Neu results in cross-activation of HER-3, which couples strongly to PI3K through six p85 binding sites. This is important for oncogenic activity of HER2 (18). Direct or indirect targets regulated through Akt and linked to carcinogenesis include β -catenin, cyclin D1, and 4E-BP1s. Mutations of the tuberous sclerosis complex, which antagonizes mTOR, promote tumor formation. Conversely, Akt-dependent negative regulation of antiapoptotic genes and suppression of antiproliferative and differentiative effects of FoxO proteins are consonant with carcinogenic activity of Akt. Translocations

involving *FOXO1*, *FOXO2*, and *FOXO4* have been identified in cancer (reviewed in 12).

Taken together, these findings suggest that quantifying signaling throughput in the PI3K to Akt pathways would provide important prognostic information and help distinguish clinically important subsets of cancers. Other findings link these pathways specifically to prostate cancer. PTEN inactivation or loss of heterozygosity is common in prostate cancers, especially metastatic carcinoma (4, 19), and targeted deletion of PTEN in mouse prostate activates Akt and induces prostate carcinoma (20). In a xenograft model for progression of the androgen-dependent (or androgen-sensitive) LNCaP cell line to androgen independence, Akt activity (but not expression) was elevated and correlated with Ser⁴⁷³ phosphorylation (21). Introduction of constitutively activated Akt into these cells permitted androgen-independent growth. Progression from normal prostate epithelium to prostatic intraepithelial neoplasia or carcinoma is associated with elevated Akt phosphorylation (22, 23). Interestingly, mitogen-activated protein kinase activation monitored with phospho-extracellular signal-regulated kinase (ERK) antibodies was enhanced in prostatic intraepithelial neoplasia in these studies but reduced in carcinoma.

Phospho-Akt AND PROSTATE CANCER

In this issue of *Clinical Cancer Research*, Ayala *et al.* (1) compare Akt expression and phosphorylation at Ser⁴⁷³ in normal and cancer tissue from 640 patients (1). Phospho-Akt-1 staining was greatly enriched in cancer (45.8% versus 8.4%), and high intensity immunostaining was even more selective for neoplasia. Immunoreactive phospho-Akt (P-Akt)-1 was correlated with staging of prostate cancer, but with not other clinical/pathological parameters. A range of cutoff points was used to determine whether P-Akt-1 is associated with recurrence. With the optimal cutoff, P-Akt-1 was associated with a significantly earlier biochemical recurrence, marked by elevated serum levels of prostate-specific antigen. Outcome of patients with Gleason scores of 6 and 7, a major patient group for which prognosis is difficult, was linked to P-Akt status. The finding that P-Akt in *nonneoplastic* prostate tissue predicts recurrence suggests the influence of genetic or regulatory changes in the local milieu and will be of great interest for further investigation.

Another recent publication (24) addresses similar questions. Earlier, this group had reported an association between increased P-Akt Ser⁴⁷³ and reduced phospho-ERK with poor differentiation of prostate cancer (23). In the new study of a group of 60 patients, strong P-Akt immunostaining was significantly more common in the subset of patients with biochemical recurrence. Phospho-ERK was uninformative alone but had improved specificity in combination with low P-Akt in predicting better outcome.

PHOSPHOPROTEOMICS

Because protein phosphorylation regulates the activity of many proteins involved in cancer, phosphorylation-specific antibodies should have many practical clinical applications (25). Phospho-epitopes, as direct markers of many forms of protein regulation, have the advantage of marking active or inactive forms of proteins. However, there have been concerns about the

potential lability of these epitopes. Although positive findings in many studies using phospho-antibodies indicate that this is not a fatal problem, it is possible that small changes in tissue fixation procedures (*e.g.*, inclusion of phosphatase inhibitors) would improve the usefulness of such markers. A second technical issue, which is a concern for all antibody-based measurements, is the specificity of the antibodies under the assay conditions used. For tissue microarray analysis, inclusion of control cores from paraffin-embedded pellets of cells expressing wild-type proteins, in phosphorylated and nonphosphorylated states, and mutated forms that lack the phosphorylation sites would help assure specificity under the actual assay conditions.

Several other clinical studies have also indicated the potential of phosphorylation-dependent markers. Among these are studies on HER2/Neu/ErbB2, showing that phosphorylation of the receptor marks patients with poor prognosis (26), and trends toward association with response to Herceptin/trastuzumab (27). Patients with P-Akt-positive non-small-cell lung cancers who are treated with the epidermal growth factor receptor inhibitor ZD-1839 (Iressa/gefitinib) have longer time to progression and higher response rates (28). Activated colony-stimulating factor-1 receptor is associated with poorer prognosis of ovarian carcinoma patients (29), and high ratios of phosphorylated 46- and 52-kDa Shc isoforms relative to expression of the inhibitory 66-kDa Shc protein foreshadow recurrence (30).

PATHWAY-TARGETED THERAPIES

Genotypic and phenotypic profiling of tumors will be important for rational use of pathway-targeted anticancer drugs because these drugs will be most useful for tumors where the pathways are highly active. For example, STI-571 (Gleevec/imatinib) is efficacious for chronic myelogenous leukemia, with activated Abl, and gastrointestinal stromal tumor, with activated Kit. Herceptin is most effective for breast cancers with high expression of HER2. Besides Akt itself, a number of other components of the PI3K signaling system are regulated by protein phosphorylation. A detailed analysis of these components may yield important information about relative activation of subpathways (TOR, FoxO, NF- κ B, and so forth) that are also subject to regulation through other inputs.

PI3K activators (receptor kinases and Ras), the PI3K itself, and various downstream branches are targets for drugs in pre-clinical development and clinical use (31). Antagonists for several receptor kinases and for Ras proteins are in clinical use or in clinical trials. In model systems, wortmannin and LY294002 block PI3K, rapamycin and orthologs (some of which are in clinical use as immunosuppressants) target mTOR, and inhibitors for the various protein kinases are under development. Eventual production of drugs targeting these multiple levels will make it possible to tune the treatment to the cancer, and such drugs, when used in combination, may reduce selection of resistant clones. For example, tumors that lack PTEN may be treated with PI3K inhibitors, whereas amplification or mutational activation of Akt may call for direct inhibition of Akt and downstream mediators.

CONCLUSIONS

The studies of Ayala *et al.* (1) and Kreisberg *et al.* (24) pave the way for improvements in prostate cancer prognostication. Assuming that studies on independent test sets confirm their conclusions, it is likely that evaluation of Akt activity will prove useful for clinicians faced with the difficult treatment decisions necessary for patients with intermediate grades of prostate cancer. A more global look at functional parameters in these tumors through phosphoproteomic analysis or other means, ideally in conjunction with analyses that identify the mechanisms driving pathway activation, will further expand the repertoire of clinical analytical tools and their utility.

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REFERENCES

1. Ayala G, Thompson T, Yang G, et al. High levels of phosphorylated form of Akt-1 in prostate cancer and nonneoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin Cancer Res* 2004;10:6572–8.
2. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489–501.
3. Fresno Vara JA, Casado E, de Castro J, et al. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 2004;30:193–204.
4. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol* 2004;22:2954–63.
5. Zinda MJ, Johnson MA, Paul JD, et al. AKT-1, -2, and -3 are expressed in both normal and tumor tissues of the lung, breast, prostate, and colon. *Clin Cancer Res* 2001;7:2475–9.
6. Bellacosa A, Chan TO, Ahmed NN, et al. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* 1998;17:313–25.
7. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998;12:3499–511.
8. Zhou BP, Liao Y, Xia W, et al. Cytoplasmic localization of p21^{Cip1}/waf1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol* 2001;3:245–52.
9. Zhou BP, Liao Y, Xia W, et al. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol* 2001;3:973–82.
10. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science (Wash DC)* 1997;278:687–9.
11. Mayo MW, Madrid LV, Westerheide SD, et al. PTEN blocks tumor necrosis factor-induced NF-kappaB-dependent transcription by inhibiting the transactivation potential of the p65 subunit. *J Biol Chem* 2002;277:11116–25.
12. Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 2004;117:421–6.
13. Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117:211–23.
14. Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer* 2004;4:335–48.
15. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 2002;4:648–57.

16. Cheng JQ, Godwin AK, Bellacosa A, et al. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci USA* 1992;89:9267–71.
17. Ahn JY, Hu Y, Kroll TG, Allard P, Ye K. PIKE-A is amplified in human cancers and prevents apoptosis by up-regulating Akt. *Proc Natl Acad Sci USA* 2004;101:6993–8.
18. Holbro T, Beerli RR, Maurer F, et al. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci USA* 2003;100:8933–8.
19. Suzuki H, Freije D, Nusskern DR, et al. Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res* 1998;58:204–9.
20. Wang S, Gao J, Lei Q, et al. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 2003;4:209–21.
21. Graff JR, Konicek BW, McNulty AM, et al. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27^{Kip1} expression. *J Biol Chem* 2000;275:24500–5.
22. Paweletz CP, Charboneau L, Bichsel VE, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* 2001;20:1981–9.
23. Malik SN, Brattain M, Ghosh PM, et al. Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer. *Clin Cancer Res* 2002;8:1168–71.
24. Kreisberg JI, Malik SN, Prihoda TJ, et al. Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer Res* 2004;64:5232–6.
25. Stern DF. Phosphoproteomics. *Exp Mol Pathol* 2001;70:327–31.
26. Thor AD, Liu S, Edgerton S, et al. Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu): a study of incidence and correlation with outcome in breast cancer. *J Clin Oncol* 2000;18:3230–9.
27. Hudelist G, Kostler W, Czerwenka K, Kubista E, Singer CF. Predicting the clinical course of breast cancer patients undergoing trastuzumab-based therapy: an outlook. *Methods Find Exp Clin Pharmacol* 2004;26:201–10.
28. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* (Bethesda) 2004;96:1133–41.
29. Flick MB, Sapi E, Perrotta PL, et al. Recognition of activated CSF-1 receptor in breast carcinomas by a tyrosine 723 phosphospecific antibody. *Oncogene* 1997;14:2553–61.
30. Davol PA, Bagdasaryan R, Elfenbein GJ, Maizel AL, Frackelton AR Jr. Shc proteins are strong, independent prognostic markers for both node-negative and node-positive primary breast cancer. *Cancer Res* 2003;63:6772–83.
31. Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* 2003;4:257–62.