Prognostic Value of Akt-1 in Human Prostate Cancer: A Computerized Quantitative Assessment with Quantum Dot Technology

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Abstract Background: Akt/protein kinase B signaling pathway has been implicated in tumorigenesis and progression. Previous studies showed the predictive potential of p-Akt-1, but total Akt-1 could provide more reliable information. We used image deconvolution, nanotechnology (quantum dots), and image analysis to improve Akt-1 quantification.

Design: This tissue microarray study included 840 radical prostatectomy cases. Slides were incubated with primary antibody against nonphosphorylated Akt-1 (Akt-1) followed by biotinylated secondary antibody and then by Qdot655 streptavidin conjugate. Slides were imaged under fluorescence microscopy and spectral deconvolution (Nuance) and quantified using plug-in image analysis software. Average intensity of Akt-1 signal was measured and subject to statistical analysis. Multivariate analysis (Cox regression) was applied to assess the prognostic value of Akt-1 for biochemical recurrence and prostate cancer-specific death. Akt-1 expression was also examined for correlations with Ki-67 index and apoptotic index in our database.

Result: Akt-1 was inversely correlated with apoptotic index ($\rho = -0.203$; $P = 0.004$) but not with Ki-67 index. The correlation between Akt and p-Akt is significant but weak ($P = 0.0496$; $R^2 = 0.118$). On multivariate analysis Akt-1 was independently predictive of biochemical recurrence [hazard ratio, 2.863 (95% confidence interval, 1.127-7.271); $P = 0.0270$]. Akt-1 level is also predictive of prostate cancer-specific death ($P = 0.0376$).

Conclusion: High levels of Akt-1, assessed by quantum dots, deconvolution imaging, and image analysis, are associated with a higher risk of biochemical recurrence and prostate cancer-specific death.

Prostate cancer can grow slowly, with a relatively benign course, or advance more aggressively, leading to severe morbidity and early cancer death. Prostate cancer progression might be controlled by diverse biological processes, such as proliferation, apoptosis, signal transduction, androgen receptor signaling, cellular adhesion, and angiogenesis (1). Recently, phosphatidylinositol 3-kinase/Akt signal transduction has been suggested to be a major survival pathway that is central to the development and progression of human cancers including prostate cancer (2). Increased expression of Akt, in either phosphorylated or nonphosphorylated form, has been associated with a hormone-resistant phenotype, high Gleason scores, or poor prognosis in prostate cancer (3–9).

Previously, we showed that p-Akt-1 is an independent factor for the prediction of biochemical recurrence in human prostate cancer (7). However, there seems to have drawbacks to the use p-Akt-1. p-Akt-1 expression rate in prostate cancer was relatively low (<50%) as shown in our previous work (7) and only very high levels of p-Akt-1 were predictive of biochemical recurrence-free survival. Other studies also yielded similar or lower percentage of p-Akt-1 expression (10–14). It is likely that Akt-1 phosphorylation status has a fast turnover and that quick fixation might be necessarily to avoid degradation. Formalin has slow penetration rates; therefore, rapid fixation is not possible in large specimens from radical prostatectomy. Hence, for this study, we chose a total Akt-1 instead of p-Akt-1 antibody. The Akt-1 antibody in this study detects the total Akt-1 regardless of its phosphorylation status.

Precise measurement of Akt-1 expression would be key in providing reliable prognostic information as well as increasing reproducibility of the biomarker. Previous studies on Akt expression were based on a traditional scoring system that is semiquantitative in nature, which may result in poor reproducibility (15, 16). Moreover, human eyes are limited in determining quantitative differences of protein expression. Although a
Translational Relevance

Phosphatidylinositol 3-kinase/Akt signal transduction as a major survival pathway has been linked to the development and progression of cancer including prostate cancer. Previous studies suggested that Akt might have prognostic value. We have shown that p-Akt-1 is predictive of biochemical recurrence, but phosphoproteins are fixation dependent. Therefore, precise measurement of total Akt expression has the potential to be a more reliable prognostic biomarker. To measure the amount of Akt-1 expression in formalin-fixed, paraffin-embedded tissue, we employed a new methodology, which combines quantum dot-based immunofluorescence with computerized multispectral image deconvolution and image analysis, to improve on analytical variables. The objective was to produce continuous and objective quantitative data that could allow for a more accurate prediction of disease outcome and potentially increase reproducibility. Increased Akt-1 levels were predictive of both biochemical recurrence and prostate cancer-specific death. A novel biomarker analytical approach that integrates quantum dot technology, image deconvolution, image analysis, and new algorithms has been introduced here to conduct quantitative study of total Akt-1 protein expression in prostate cancer (15). The quantitative data were then analyzed statistically for biological correlates and prognostic significance in prostate cancer.

Materials and Methods

Cohort and tissue microarrays. A total of 840 prostate cancer patients who had undergone radical prostatectomy between 1983 and 1998 were included in this study, which was approved by the Baylor College of Medicine Institutional Review Board (IRB H-11436). In some cases, individual written documentation of informed consent for subjects participating in the study has been lost. The institutional review board is aware of the loss and circumstances surrounding the loss and has granted permission for the use of the tissue and data. The patients did not receive any preoperative treatment. Radical prostatectomy specimens from these patients were processed for whole mount slides according to procedures described previously (17). Pathologic analysis included evaluation of staging, pathologic stage, margins, capsular penetration, seminal vesicle invasion (SVI), biopsy and prostatectomy primary and secondary Gleason grades, lymph node status, tumor volume, and geographic location. The clinical and pathologic data on patients who met the entry criteria were available for analysis in the Baylor Prostate SPORE data bank. The clinical follow-up data include prostate-specific antigen (PSA) recurrence (defined as PSA > 0.4 ng/mL on two consecutive measurements), clinical metastasis, and prostate cancer-specific deaths.

Sections from all 840 radical prostatectomy specimens were reviewed and mapped. The tissue microarrays were built using a manual tissue arrayer (Becker Instruments). The index tumor, defined as the largest and/or highest Gleason score, was identified on the slide, and areas representative of the highest Gleason grade were circled. A single 2 mm core was obtained from each tumor and transferred to a recipient paraffin block. Normal liver tissue cores were also placed in certain positions for array orientation purpose. The database was built for every block produced, including the coordinates of each core and the area and case of origin.

Quantum dot immunofluorescence. Sections were deparaffinized in xylene and rehydrated through decreasing concentrations of alcohol and PBS. Slides were then subjected to steam heat in 10 mmol/L citrate buffer (pH 6.0) for 20 min followed by cool-off for 30 min. We did not attempt fluorescence quenching, as it might affect protein expression levels. Slides were then incubated with primary antibody (mouse monoclonal antibody against Akt-1; Cell Signaling) for 60 min followed by incubation with biotinylated anti-mouse secondary antibody (Vector) for 30 min. Subsequently, a Qdot655-streptavidin conjugate (Invitrogen) was added with 30 min incubation. Slides were air-dried and Fluor mounting medium (Trevigen) and a coverslip were applied. Sections of formalin-fixed, paraffin-embedded human breast cancer MCF-7 cell line cell block were used as positive controls, and negative controls were sections immunostained as above but, instead of incubation with Akt-1, were incubated with normal mouse serum. We did not use quantum dot-conjugated primary or secondary antibody, as in our experience the systems were not stable and not sensitive enough in the detection of antigens.

Ki-67 labeling and terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Tissue microarray slides were immunostained with Ki-67 using an automated immunostainer (DAKO). The 5 mm sections were stained with polyclonal antibody Ki-67 (Santa Cruz Biotechnology). The proliferative index is defined as the percentage of Ki-67-positive cancer cells in total cancer cells in the “hotspot” fields at ×40 magnification. Each case had a total count of 2,000 nuclei.

The detection of DNA fragmentation in tissue microarray slides was subjected to the terminal deoxynucleotidyl transferase-mediated nick end labeling assay using TACS XI diaminobenzidine in situ apoptosis kit (Trevigen) following the manufacturer’s indications with minor modifications. Apoptotic bodies were counted under a light microscope (×400) selecting highest positive stain area for counting. Number of apoptotic bodies was counted per 2,000 tumor cells. Apoptotic bodies were dark brown rounded structure (Fig. 1). Apoptotic index was the percentage of positive cells in all cells counted. The detailed protocols used for Ki-67 labeling and terminal deoxynucleotidyl transferase-mediated nick end labeling assay were described previously (18).

Image deconvolution and quantification. An imaging system (software version 1.4.6, CSI-Nuance Multispectral Imaging System) was used for image procurement and analysis. Briefly, slides were observed under fluorescent microscope in a dark room. Positive signals were red and present within the cytoplasm (TRITC filter applied; Fig. 2). We took a representative image for each positive core by using ×20 objective lens. Negative cases were recorded with no pictures taken. A 500 ms exposure time was set for all cases before image cubes were obtained. An image cube is a stack of images taken at a different wavelength starting from 420 to 720 nm with 20 nm increment. Subsequently, autofluorescence reduction was done on images by using Real Component Analysis plug-in software. Clean images were then analyzed by running measurement
plug-in software. Before measuring the intensity of positive signal, an automated (default) threshold was set, which creates a pseudo-color image that highlights most, if not all, of the positive signals. The grossly “positive signals” consist of true positive and background stain. After deconvolution of the images, the background stain was filtered and only the true positive signals were shown on the images (Fig. 3A and B). Analysis yielded quantitative data of Akt-1 signal: the numeric data of the average intensity of region of interest. The number of regions of interest differed from case to case depending on the distribution and variation of signal. Each case was calculated for its mean value of the signal intensity of all regions of interest for statistical analysis. The output of the computerized measurement produced a continuous data ranging from 21 to 158. We did not identify any measurement data less than 21.

**Statistics.** Correlations between clinicopathologic parameters and Akt-1 were evaluated using Spearman correlation coefficient testing. For survival analysis, the endpoint was the biochemical recurrence of the cancer, defined as serum PSA level higher than 0.4 ng/mL on two successive measurements. Time to recurrence was defined as the interval between the date of surgery and the date of identification of biochemical recurrence. The predictive value of Akt-1 for recurrence-free survival and prostate cancer-specific death was evaluated using the Kaplan-Meier actuarial analysis and the log-rank test. Kaplan-Meier survival curves were constructed for patients with low and high levels of Akt-1.
expression. Actual cutoff value of Akt-1 expression was determined by the most significant P value that was calculated by using the follow-up data of biochemical recurrence and prostate cancer-specific death. The Cox univariate and multivariate proportional hazard models were used to determine the hazard ratios. Cox multivariate analysis was applied to assess the prognostic value of Akt-1 against clinical stage, preoperative PSA (Pre-PSA), extracapsular extension (ECE), SVI, margins, and Gleason grade. The hazard ratio and its 95% confidence interval were recorded for each marker. Akt-1 expression was also tested for correlations with some other biomarkers in our database.

Results

Clinical and pathologic characteristics. Patients' age ranged from 38 to 81 years, with a mean 62 years. Patients were postoperatively followed-up for an average of 61 months ranging from 1 to 132 months. Pre-PSA level ranged from 1 to 82 ng/mL with a mean of 11 ng/mL. Lymph node metastasis was found in 7% patients, and biochemical recurrence was seen in 20.5% patients. ECE was present in 28% of the patients. Positive surgical margin was seen in 4% of the patients, whereas SVI was found in 5% of the patients. Thirty-eight percent of the cases had a Gleason score of <7, 37% of them had a Gleason score of 7, and 25% of the patients had a Gleason score of >7. There were 13 patients died from prostate cancer.

Akt-1 expression and computerized image analysis. Due to repeated sectioning of the array, we had significant tissue core loss. Furthermore, antigen retrieval also contributed to tissue damage and/or core loss. These cores were thus disqualified and excluded from the analysis. In total, 73 cases classified as negative, whereas 327 cases were positive and imaged. The output data obtained by Nuance Imaging System included the numeric value of the measurement of Akt-1 signal, that is, the average mean intensity. This parameter represented the expression level of Akt-1 and was used for further analysis.

Akt-1 expression varied from case to case. The quantitative data from image analysis showed that Akt-1 had a variable level of expression with a range of 21 to 158 (mean ± SD, 54 ± 28; median, 63). The percentage of cases expressing Akt-1 (81.75%) was much higher than the rate (45.8%) of p-Akt-1 identified previously (7). We are aware that some cases identified as "negative" might actually express some Akt-1 but were not appreciable by naked eyes or the computer (that is, <21).

Correlations between Akt-1 and biological and clinicopathologic parameters. Spearman's nonparametric correlations were tested between Akt-1 expression and a group of clinical and pathologic parameters. The results indicated that increased expression of Akt-1 was an indicator of aggressive features of prostate cancer as Akt-1 was correlated weakly with Pre-PSA (r = 0.106;

<table>
<thead>
<tr>
<th>Akt-1 expression vs</th>
<th>Correlation coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-PSA</td>
<td>0.106</td>
<td>0.037</td>
</tr>
<tr>
<td>Age</td>
<td>0.113</td>
<td>0.023</td>
</tr>
<tr>
<td>UICC</td>
<td>0.04</td>
<td>0.421</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>0.130</td>
<td>0.009</td>
</tr>
<tr>
<td>ECE</td>
<td>0.148</td>
<td>0.003</td>
</tr>
<tr>
<td>SVI</td>
<td>0.093</td>
<td>0.063</td>
</tr>
<tr>
<td>Margins</td>
<td>0.023</td>
<td>0.649</td>
</tr>
<tr>
<td>Gleason score</td>
<td>0.142</td>
<td>0.004</td>
</tr>
<tr>
<td>Ki-67</td>
<td>0.108</td>
<td>0.086</td>
</tr>
<tr>
<td>Terminal deoxynucleotidyl transferase-mediated nick end labeling index</td>
<td>−0.203</td>
<td>0.004</td>
</tr>
</tbody>
</table>

NOTE: P < 0.05 is considered as statistically significant. There was a negative correlation between apoptotic index and Akt-1 expression, which means the higher the Akt-1 expression, the less the apoptotic activities in prostate cancer. Other parameters had a positive correlation with Akt-1, except UICC, SVI, and Ki-67. Abbreviation: UICC, Union Internationale Contre le Cancer.
Akt-1 seems to promote survival by suppressing apoptosis as high level of Akt-1 expression was correlated with decreased apoptotic index determined by terminal deoxynucleotidyl transferase-mediated nick end labeling ($\rho = -0.203; P = 0.004$). Akt-1 was not correlated with proliferating marker Ki-67 index (Table 1).

**Predictive value of Akt-1 in biochemical recurrence.** After extensive search for an optimal cutoff by using minimum $P$ value method, a value of 50 was found to be statistically significant for categorizing patients as high expression group (Akt-1 $\geq 50$; 294 cases) or low expression group (Akt-1 $<50$; 103 cases). Kaplan-Meier survival analysis showed that the recurrence-free survival was significant higher in patients with low level of Akt-1 than that in those with high level of Akt-1 ($P < 0.0001$; Fig. 4A). Univariate analysis showed that patients with high level of Akt-1 had approximately 5-fold increase of risk for biochemical recurrence compared with those with low level of Akt-1 [hazard ratio, 5.65 (95% confidence interval, 2.29-13.98); $P = 0.0002$; Table 2]. More significantly, Akt-1 was an independent indicator for biochemical recurrence; that is, when clinical and pathologic conditions were assumed identical, patients with high level of Akt-1 expression had nearly three times of risk for biochemical recurrence compared with those with low level of Akt-1 [hazard ratio, 2.86 (95% confidence interval, 1.13-7.27); $P = 0.027$; Table 2]. In this model, Gleason, Pre-PSA, SVI, lymph node status, and margins were also independently significant. However, ECE was dropped out of the model when assessed against Akt-1.

**Predictive value of Akt-1 in prostate cancer-specific death.** Kaplan-Meier Survival was also used to analyze the predictive value of Akt-1 level in prostate cancer-specific deaths. We found the value of 54.27 was the most significant cutoff to predict prostate cancer-specific deaths ($P = 0.0376$). There were 13 prostate cancer-specific deaths among 275 patients with Akt-1 level more than 54.27, whereas no prostate cancer-specific deaths were reported among 124 patients with Akt-1 level no

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**Table 2.** Akt-1 and other clinicopathologic parameters were tested by univariate and multivariate analyses

<table>
<thead>
<tr>
<th>Models</th>
<th>Variables</th>
<th>Exp (B), hazard ratio (95% confidence interval)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate</td>
<td>Akt-1</td>
<td>5.65 (2.29-13.98)</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Akt-1</td>
<td>2.863 (1.13-7.27)</td>
<td>0.0270</td>
</tr>
<tr>
<td></td>
<td>Pre-PSA</td>
<td>1.542 (1.08-2.20)</td>
<td>0.0172</td>
</tr>
<tr>
<td></td>
<td>Lymph node metastasis</td>
<td>2.192 (1.19-4.04)</td>
<td>0.0120</td>
</tr>
<tr>
<td></td>
<td>Margins</td>
<td>2.208 (1.28-53.82)</td>
<td>0.0047</td>
</tr>
<tr>
<td></td>
<td>SVI</td>
<td>2.09 (1.16-3.76)</td>
<td>0.0140</td>
</tr>
<tr>
<td></td>
<td>Gleason</td>
<td>2.916 (1.95-4.36)</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>ECE</td>
<td>1.568 (0.83-2.99)</td>
<td>0.1669</td>
</tr>
</tbody>
</table>

NOTE: Akt-1 was a significant parameter to predict the biochemical recurrence by univariate and multivariate analyses.
Akt-regulated pathways enhance cell division and survival, whereas inhibition of Akt pathway suppresses prostate cancer cell proliferation and induced the G1 cell cycle arrest (9, 19). Our data confirm this, as Akt-1 expression was adversely correlated with apoptotic index. This seems plausible because prostate cancer may acquire Akt signaling via antiapoptotic mechanisms such as nuclear factor-κB activation (20), functional loss of PTEN (21), or down-regulation of p27 (22). The pro-survival role of Akt activities was further shown in several clinical studies. For instance, increased levels of Akt and p-Akt expression are associated with high Gleason grade and worse prognosis in prostate cancer (5–7). Here, we also showed that high levels of total Akt-1 were associated with more aggressive features of disease, as patients with high levels of total Akt-1 were identified with high PSA level, high risk of lymph node metastasis and ECE, and high Gleason score.

The active form of Akt appears to be the phosphorylated form of the protein. However, because of the limitations of using phosphoproteins as stable biomarkers in specimens from radical prostatectomy, we chose total Akt-1 as our potential biomarker. On multivariate analysis revealed that high level of Akt-1 had nearly three times of the increased risk for biochemical recurrence than those with low level of Akt-1. More significant, high level of Akt-1 predicts prostate cancer-specific death. Taken together, high level of Akt-1 is not only an indicator of more aggressive/advanced disease but also predicts biochemical recurrence and high probability of prostate cancer-specific death. A biological rationale for the significant total Akt-1 levels in prostate cancer can be found in Akt regulation mechanisms. Previous studies done at Baylor College of Medicine show that SRC3 increases not only the activity of Akt through phosphorylation but also the total levels of Akt by direct transcriptional mechanisms (2). Therefore, total Akt and p-Akt regulation have a single upstream modulator.

This study also attempts to improve on preanalytical and analytical considerations with the purpose of improving the ability to predict and to make the test reproducible across laboratories. Preanalytical validation of this biomarker was conducted using breast cancer cell lines (MCF-7) with known expression of Akt-1. Although imperfect, this could serve as standardized threshold for other populations in subsequent studies. To our best knowledge, this is the first report on quantum dot-based computerized quantification of Akt-1 expression in a large tissue microarray of human prostate cancer. We believe that the quantum dot-based immunofluorescence study combined with image deconvolution and computerized image analysis could become a usable approach for Akt-1 quantification that results in objective data and improves reproducibility.

In conclusion, our data suggest that up-regulation of Akt pathway may enhance survival and accelerate prostate cancer progression via an antiapoptotic mechanism. Total levels of Akt-1 measured using a combinatorial method may become a novel prognostic biomarker in human prostate cancer. Further studies are needed to validate these results and methodology.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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