High Levels of Phosphorylated Form of Akt-1 in Prostate Cancer and Non-Neoplastic Prostate Tissues Are Strong Predictors of Biochemical Recurrence

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ABSTRACT

Akt is a serine-threonine-kinase that phosphorylates proteins in several pathways regulating aspects of metabolism, apoptosis, and proliferation. Akt signaling promotes proliferation and increased cell survival and is thought to play an important role in prostate cancer progression. Tissue microarrays (640 patients) with triplicate cores of non-neoplastic prostate, BPH, and index tumor were immunostained with antibody to Phospho-Akt (Ser473), digitized, and quantified. The expression index (Intensity*Percentage) was used for statistical analysis. P-Akt-1 staining was found in both the non-neoplastic and cancer tissues, predominantly in cytoplasmic locations. High level P-Akt-1 is expressed almost exclusively in cancer. By Kaplan-Meier actuarial model, high expression of P-Akt-1 in prostate cancer was predictive of a higher probability of recurrence on univariate and multivariate analysis. Akt-1 expression was an independent prognostic indicator of biochemical recurrence-free survival when Gleason 6 and 7 patients were analyzed separately. Surprisingly, a high level of P-Akt-1 expression in non-neoplastic tissues is also an independent predictor of biochemical recurrence. This suggests that some patients might have an inherent predisposition to express a high level of P-Akt-1 and, therefore, to have an adverse prognosis. We conclude that P-Akt-1 is most likely involved in the progression of prostate cancer and is an excellent biomarker for biochemical recurrence.

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INTRODUCTION

Prognostic markers in prostate cancer are of great importance not only because of the mortality associated with prostate cancer but also because of the morbidity associated with current forms of therapy. Numerous ongoing trials are attempting to identify markers to discriminate between patients who require immediate therapeutic intervention and those who are candidates for watchful waiting observation. Although prostate cancer is the most common male cancer in the United States, we currently have few suitable prostate cancer biomarkers that distinguish between tumors with a high potential for recurrence and tumors that are not likely to recur.

Several studies have combined clinical and pathological parameters, as well as serum markers in an attempt to find predictive models. They have been relatively successful (1, 2). However, their results are limited in scope and depend heavily on markers of epithelial differentiation of cancer (Gleason score). The difficulty in predicting disease behavior is a particular problem in patients with a Gleason score of 7, a group that represents a large proportion of patients with prostate cancer. To address novel predictive markers for prostate cancer recurrence, we have focused on tissue microarray studies of cell survival pathways in prostate cancer. The most successful biomarker identified to date is the s-473 phosphorylated form of Akt.

Although the regulatory pathways in prostate cancer progression remain poorly understood, there is increasing evidence of a role for Akt in promoting cell survival in prostate cancer. Akt, which is also known as protein kinase B (PKB), consists of a family of highly conserved serine/threonine kinases including Akt1, Akt2, and Akt3 (3, 4). Akt/PKB can be expressed in both non-neoplastic and tumor tissues of a variety of origins including prostate (3). Increased Akt activity has been associated with prostate cancer progression and hormone independence in prostate cell lines (5, 6). Meanwhile, Akt activity may result in down-regulation of proapoptotic and inhibitor of cell cycle proteins such as PTEN and $P27^{Kip1}$ (6, 7). More interestingly, Akt expression may have clinical implications in prostate cancer, because increased Akt expression has been associated with higher Gleason grades 8–10 (8). Unfortunately, the clinicopathological significance of Akt in human prostate cancer remains largely unknown. In this regard, we examined the phospho-Akt (P-Akt-1) expression in 640 prostate cancer specimens obtained from radical prostatectomies and explored the possibility of P-Akt-1 expression as a novel prognostic indicator of prostate cancer recurrence and biochemical recurrence-free survival.

MATERIALS AND METHODS

Cohort Enrollment and Follow-up. As of March 2004 the Baylor Medical Informatics Core Specialized Program of Research Excellence in Prostate Cancer Database contained

information on 6,201 patients with BPH or cancer. More than 5,400 of these patients underwent radical prostatectomies at one of the Baylor College of Medicine affiliated institutions and willingly provided tissues (IRB H-1158). Of these patients, 1,291 were operated on by a single surgeon (P. S.) between 1983 and 1998 without any previous form of adjuvant therapy such as radiation or hormonal therapy. This study was approved by the Baylor Institutional Review Board (IRB H-11436).

Entry criteria for this retrospective cohort study to create a radical prostatectomy tissue array included the following: (1) no preoperative treatment, (2) operation performed by a single surgeon (P. S.) between 1983 and 1998, (3) radical prostatectomy specimen in the tissue bank, and (4) prostate cancer present in the surgical specimen and sufficiently large to be cored for microarrays. A total of 640 patients fulfilled the above-mentioned criteria, and specimens were cored to produce a large outcomes array.

Radical Prostatectomy Specimens. Radical prostatectomy specimens from these patients were processed using whole-mount slides according to procedures described previously (9). After surgery, the prostate specimens were sliced into 5 mm-thick tissue whole mounts. The tissue slices were then fixed in 10% neutral-buffered formalin and embedded in paraffin according to a routine procedure. A single pathologist (T. W.) performed the pathological analysis that included staging, pathological stage, margins, capsular penetration, seminal vesicle invasion, primary and secondary Gleason grades from biopsy and prostatectomy, lymph node status, tumor volume, and geographic location. The clinical and pathological data of patients who met the entry criteria were available for analysis in the Baylor Prostate Specialized Program of Research Excellence data bank. The clinical follow-up data include prostatic-specific antigen recurrence (defined as prostatic-specific antigen >0.4 ng or two consecutive rises), clinical metastasis, and death.

Tissue Microarray. Slides from all 640 of the radical prostatectomy specimens were reviewed and mapped. The tissue microarrays were built using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). The index tumor, defined as the largest and/or highest Gleason score tumor, was identified on the slide, and areas representative of the highest Gleason grade were circled. Areas of normal peripheral zone away from the tumor were also circled, as well as areas of BPH. Triplicate 0.6-mm cores were obtained from the circled areas of tumor, non-neoplastic peripheral zone and BPH, and transferred onto a recipient paraffin block. Sausage internal controls, which included up to 10 different types of tissues within each 0.6-mm control core, were also placed with the standard controls. A database was built for every block produced, including the coordinates of each core and the area and case of origin. The final tissue array set consisted of 15 blocks with 9 cores for every one of the 640 patients for a total of ~6,000 cores (large outcomes array).

Clinical Characteristics. Age of patients ranged from 37 to 80 with a mean of 62 and median of 63 years. The patients were followed postoperatively for an average of 42.08 ± 33.2 months (mean $\pm$ SD, median = 45.2, maximum = 167.74). Preoperative prostatic-specific antigen level was available in 603 prostate cancer cases and ranged from 0.3 to 100 ng/mL with a median of 7.2 ng/mL, and a SD of 10.99 ng/mL. Approximately 30% of the patients had a preoperative-prostatic-specific antigen level >10.5 ng/mL. Approximately 7% had a Gleason score <6; 85% had Gleason score of 6 or 7, and 8% had a higher Gleason score (8 to 10). Lymph node metastasis was found in 40 (6.4%) patients, and biochemical recurrence was seen in 120 patients (19.3%). Extracapsular extension was found in 44.5%; margins were positive in 15.3%, and seminal vesicle invasion had occurred in 12.4% of the patients.

Immunohistochemistry. Slides were stained with a rabbit polyclonal antibody to phospho-Akt (Ser473): (detects Akt only when phosphorylated at Ser473, and Akt2 and Akt3 only when phosphorylated at equivalent sites) using a standard avidin-streptavidin peroxidase method

Briefly, the slides were deparaffinized, rehydrated, and then heated in 10 mmol/L citrate buffer (pH 6.0) for 40 minutes using a steamer. The slides were blocked with 10% normal rabbit serum for 30 minutes. After washing in PBS, the slides were incubated with the rabbit anti-AKT-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, concentration 1:50) for 1 hour at room temperature. Then the secondary biotinylated antirabbit IgG was applied for 30 minutes followed by 30 minutes of incubation with a polymer (Envision plus, DAKO, Carpenteria, CA). After rinsing, slides were visualized by diaminobenzidine chromogen solution and counterstained with routine hematoxylin.

Assessment of Immunostaining. All of the stained slides were digitized using an automated slide scanner (Bacus Laboratories, Lombard, IL) to produce an image of every dot and also inform the dot coordinates on the slide. Each image was interpreted for immunoreactivity using a 0–3+ semiquantitation scoring system for both the intensity of stain and percentage of positive cells (labeling frequency percentage). For the intensity, the grading scale ranged from no detectable signal (0) to strong signal seen at low power (3). A moderate signal seen at low to intermediate power was designated 2, whereas 1 indicated a weak signal seen only at intermediate to high power. Labeling frequency was scored as 0 (0%), 1 (1% to 33%), 2 (34% to 66%), or 3 (67%–100%). Because of the triplicate nature of the arrays, three values were obtained for every measurement. To represent the intensity hot spot, the highest intensity value was used. The average of the three percentage values was used for analysis. The multiplicative index of intensity and labeling was considered for analysis. The multiplicative index was obtained by totaling the scores of intensity and percentage (*i.e.,* if the intensity score was 3 and the labeling index 2, the multiplicative index is 6). P-Akt-1 expression was defined as low level (multiplicative index: 0 to 6) or as high level (multiplicative index: >6).

Statistics. Associations between clinical/pathological parameters and P-Akt-1 expressions were evaluated using Spearman correlation coefficient testing. For biochemical recurrence-free survival analysis, the end point was the biochemical recurrence of the cancer, defined as serum prostatic-specific antigen level >0.4 ng/mL on two successive measurements (Hybritech, Inc., San Diego, CA). Time to recurrence was defined as the time interval between the date of surgery and the date of identification of biochemical recurrence. The predictive value of P-Akt-1 for biochemical recurrence-free survival was evaluated using the Kaplan-Meier actuarial analysis and the log

rank test. Kaplan-Meier biochemical recurrence free survival curves were constructed for patients with low and very high levels of P-Akt-1 expression. The differences between the biochemical recurrence free survival curves of these groups were tested for statistical significance using the log-rank test. The cutoff points were identified through the detailed study of log-rank *P*s' behavior across the range of the P-Akt-1. The Cox univariate and multivariate proportional hazard models were used to determine the hazard ratios. In the multivariate analysis the model included lymph node status, Surgical Margins, seminal vesicle invasion, Gleason grade, extracapsular extension status, International Union Against Cancer, and preoperative prostatic-specific antigen levels. The hazard ratio and its 95% confidence interval were recorded for each marker. Preoperative markers were compared univariately and multivariately, adjusting for postoperative variables: lymph node status, Surgical Margins, seminal vesicle invasion, extracapsular extension status, and International Union Against Cancer using Cox proportional hazard models. Also, the Cox model was used to evaluate predictive value of P-Akt-1 in the presence of other preoperative information. *P*s < 0.05 were considered statistically significant in all of our analyses. Because ~10 log-rank tests were done to identify each cutoff point for the biochemical recurrence-free survival analysis, *P*s < 0.005 identify significant differences that can be generalized beyond the dataset used. All of the analyses were performed with statistical software SPSS 11.0 (SPSS Inc., Chicago, IL).

RESULTS

Expression of P-Akt-1 Is Greater in Prostate Cancer Than in Non-Neoplastic Prostate. P-Akt-1 staining was found in both non-neoplastic prostate and prostate cancer tissues, predominantly in cytoplasmic locations (Figs. 1 and 2). Nuclear staining was also observed in a few cases of primary prostate cancer. However, P-Akt-1 was expressed differentially in malignant and non-malignant prostate, with a significant difference of P-Akt-1 expression between cancer and noncancer. On visual quantitation, P-Akt-1 was expressed (either weakly or strongly) in 8.4% of non-neoplastic tissues and in 45.8% of the cancers, indicative of overexpression in cancer. Furthermore, high intensity levels of P-Akt-1 expression (>6) were seen in 7.1% of cancers as compared with 1.4% in non-neoplastic tissues. High levels of P-Akt-1 are almost exclusively expressed in cancer.

Correlation between P-Akt-1 Expression and Clinicopathological Variables. Continuous measure P-Akt-1 in prostate cancer index expression was correlated only with clinical staging of tumor ($\rho = 0.087$, $P = 0.0368$; Table 1). No correlation was found with Gleason score, extracapsular extension, surgical margin status, lymph node metastasis, preoperative-prostatic-specific antigen, or seminal vesicle status. The continuous measure P-Akt-1 in non-neoplastic prostatic tissues was positively correlated with lymph node status ($\rho = 0.088$, $P = 0.0357$). We also found an association between continuous measures of P-Akt-1 in non-neoplastic prostatic tissues and prostate cancer ($\rho = 0.131$, $P = 0.0024$).

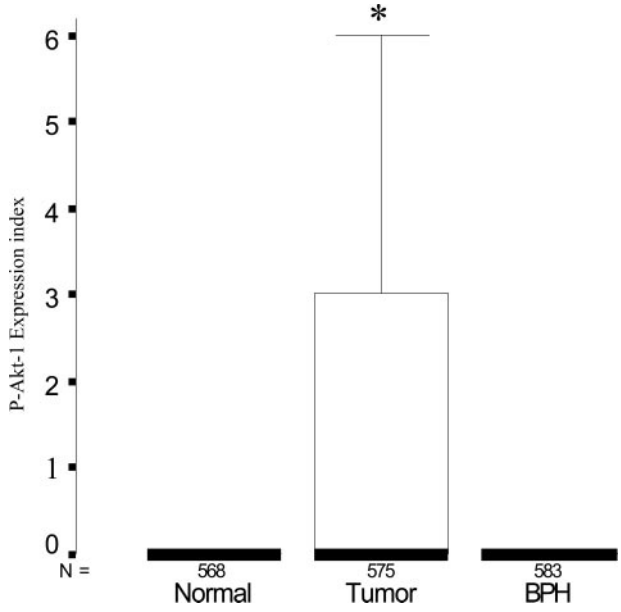


Fig. 1 The bar graph represents P-Akt-1 expression in non-neoplastic prostate and prostate cancer. The expression in prostate cancer is higher than both non-neoplastic prostate and BPH tissues; *bars*, ±SD.

P-Akt-1 Predicts Biochemical Recurrence-Free Survival. The expression index was inversely associated with the biochemical recurrence-free survival, *i.e.*, higher-level expression of P-Akt-1 was a significant predictor of earlier recurrence. After an extensive search for the optimal cutoffs, two groups were identified within the general population: no or low intensity of expression (index <6) with 529 patients and high intensity (index >6) with 41 patients. High expression of P-Akt-1 was predictive of a higher probability of recurrence on univariate analysis [$P = 0.0054$, hazard ratio 2.17(95% confidence interval, 1.26 to 3.73)]. The median biochemical recurrence-free survival time of high expressors was 96.6 months compared with 132.8 months for the low expressors. By multivariate analysis, P-Akt-1 expression was an independent prognostic indicator of biochemical recurrence-free survival [$P = 0.0001$; hazard ratio 3.44 (95% confidence interval, 1.83 to 6.43); Fig. 3]. Assuming all of the other clinicopathological parameters are identical, a patient with high intensity levels has almost twice as much risk of having earlier recurrence as one with no or low levels. These results indicate that prostate cancer patients with high-level expression of P-Akt-1 have a much shorter biochemical recurrence-free survival time.

P-Akt-1 Predicts Biochemical Recurrence-Free Survival in Gleason 6 and 7 Patients. We examined patients with Gleason scores 6 and 7 separately (488 patients), because the majority of patients with prostate cancer fall in this subgroup and because of known limitations in our prognostic capacity in this group. After determining the best cutoff points (same as previous), we identified a group of 33 patients with high levels of P-Akt-1 (>6). The median biochemical recurrence-free survival time for these patients was 96 months compared with135.6 months for all of the others in the Gleason 6 and 7 subgroup. The difference in biochemical recurrence-free survival was sig-

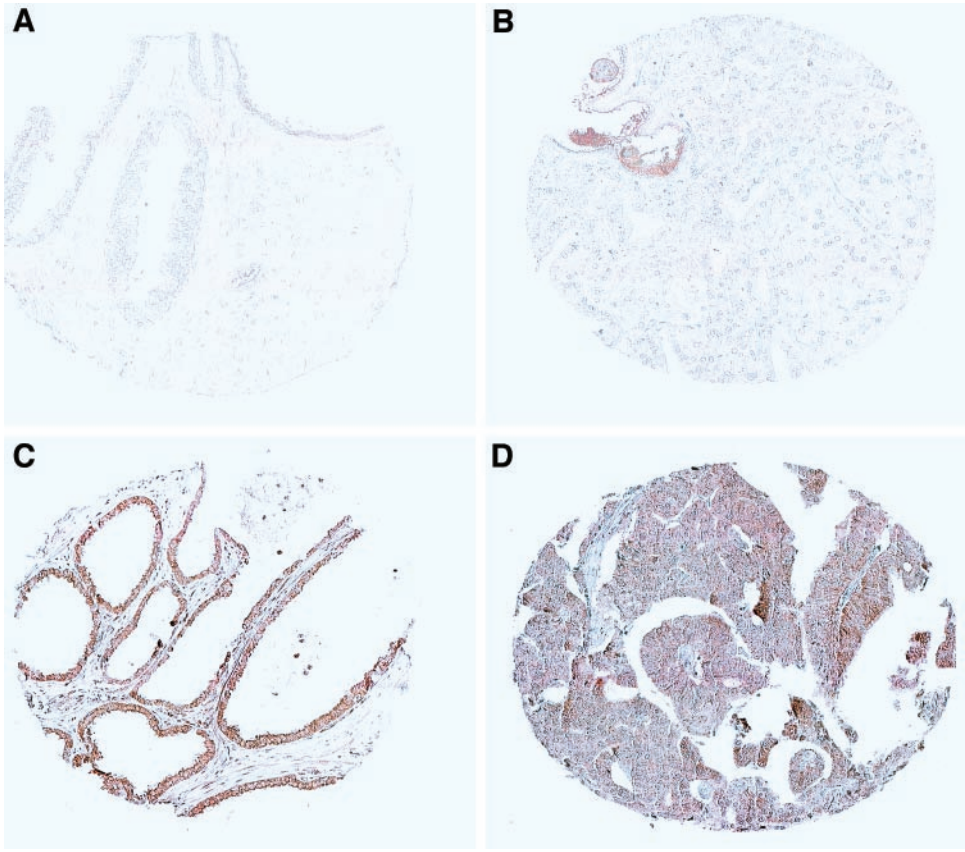


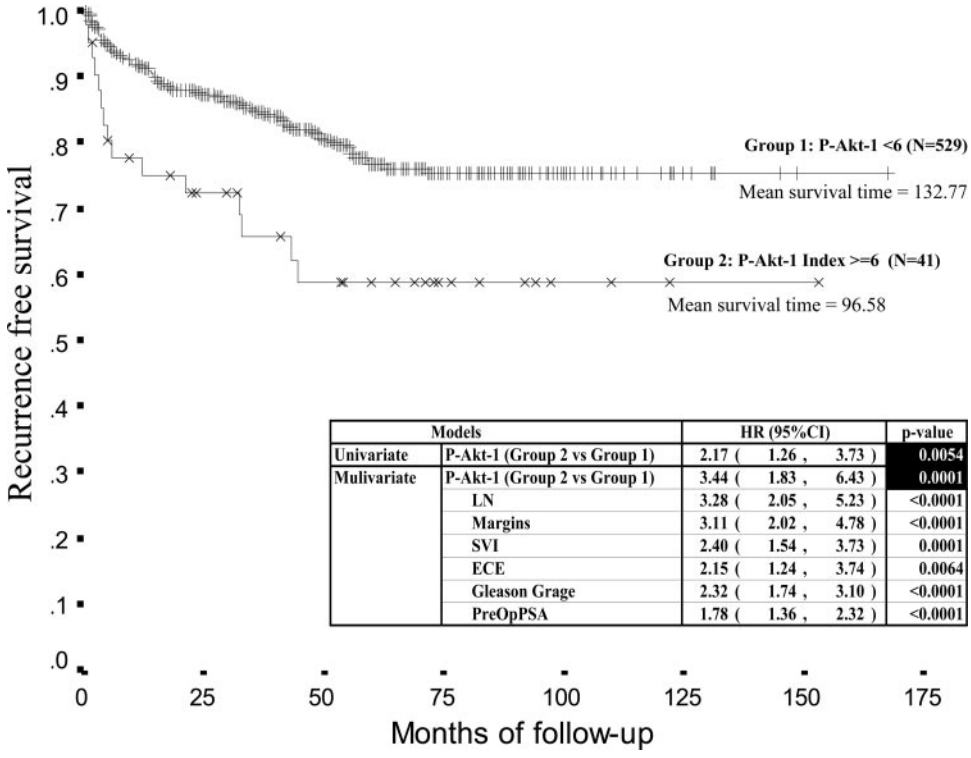
Fig. 2 P-Akt-1 expression in normal prostate and prostate cancer. The (*top figures*) show absent staining in normal (*left*) and prostate cancer (*right*). The (*bottom figures*) show high levels of expression in normal (*left*) and prostate cancer (*right*).

Table 1 Spearman's correlations

		P-Akt-1 tumor index	P-Akt-tumor index <6 *vs.* ≥6	P-Akt-normal index	P-Akt-1 normal index <6 *vs.* ≥6
PREPSA	Correlation coefficient	−0.045	−0.027	−0.021	0.043
	Sig. (2-tailed)	0.2853	0.5176	0.6173	0.3160
	N	561	561	554	554
AGE@RP	Correlation coefficient	0.041	0.002	0.048	0.002
	Sig. (2-tailed)	0.3279	0.9712	0.2569	0.9618
	N	576	576	570	570
UICC	Correlation coefficient	0.087	0.041	0.050	−0.004
	Sig. (2-tailed)	0.0368	0.3236	0.2358	0.9199
	N	576	576	570	570
LN	Correlation coefficient	0.048	0.040	0.088	0.034
	Sig. (2-tailed)	0.2523	0.3348	0.0357	0.4129
	N	577	577	571	571
ECE	Correlation coefficient	0.038	0.004	0.016	0.075
	Sig. (2-tailed)	0.3622	0.9193	0.6963	0.0715
	N	577	577	571	571
SVI	Correlation coefficient	0.076	0.074	0.006	0.047
	Sig. (2-tailed)	0.0691	0.0772	0.8919	0.2596
	N	577	577	571	571
MARGINS	Correlation coefficient	−0.034	−0.022	0.033	−0.007
	Sig. (2-tailed)	0.4101	0.5933	0.4313	0.8699
	N	577	577	571	571
GGTOT	Correlation coefficient	0.063	−0.015	−0.007	−0.004
	Sig. (2-tailed)	0.1333	0.7137	0.8624	0.9164
	N	577	577	571	571

NOTE. Only UICC has weak but significant correlation with P-Akt-1 expression in prostate cancer.

Abbreviations: PREPSA, preoperative prostate-specific antigen; AGE@RP, age at radical prostatectomy; UICC, International Union Against Cancer; LN, lymph node; ECE, extracapsular extension; SVI, seminal vesicle invasion; MARGINS, surgical margins; GTOT, gleason grade



Models		HR (95%CI)	p-value
Univariate	P-Akt-1 (Group 2 vs Group 1)	2.17 (1.26 , 3.73)	0.0054
Mulivariate	P-Akt-1 (Group 2 vs Group 1)	3.44 (1.83 , 6.43)	0.0001
	LN	3.28 (2.05 , 5.23)	<0.0001
	Margins	3.11 (2.02 , 4.78)	<0.0001
	SVI	2.40 (1.54 , 3.73)	0.0001
	ECE	2.15 (1.24 , 3.74)	0.0064
	Gleason Grage	2.32 (1.74 , 3.10)	<0.0001
	PreOpPSA	1.78 (1.36 , 2.32)	<0.0001

Fig. 3 High expression of P-Akt-1 in prostatic-specific antigen predicts decreased biochemical recurrence-free survival. Patients with high-level P-Akt-1 expression in the prostate cancer have a median biochemical recurrence-free survival of 96.58 months as compared with 132.77 months for others. The patients with high levels of P-Akt-1 expression have 3.44 times the risk (hazard ratio, *HR*) of developing biochemical recurrence as compared with patients with moderate, low or no expression.

nificant on univariate [$P = 0.0044$, hazard ratio 2.43 (95% confidence interval 1.32 to 4.47)] and multivariate analysis [$P = 0.0012$, hazard ratio 3.31 (95% confidence interval, 1.60 to 6.82)] (Fig. 4). Assuming all of the other clinicopathological variables are identical, a Gleason 6 or 7 patient with P-Akt-1 index >122 has two and three times the risk of having earlier recurrence than one with low levels.

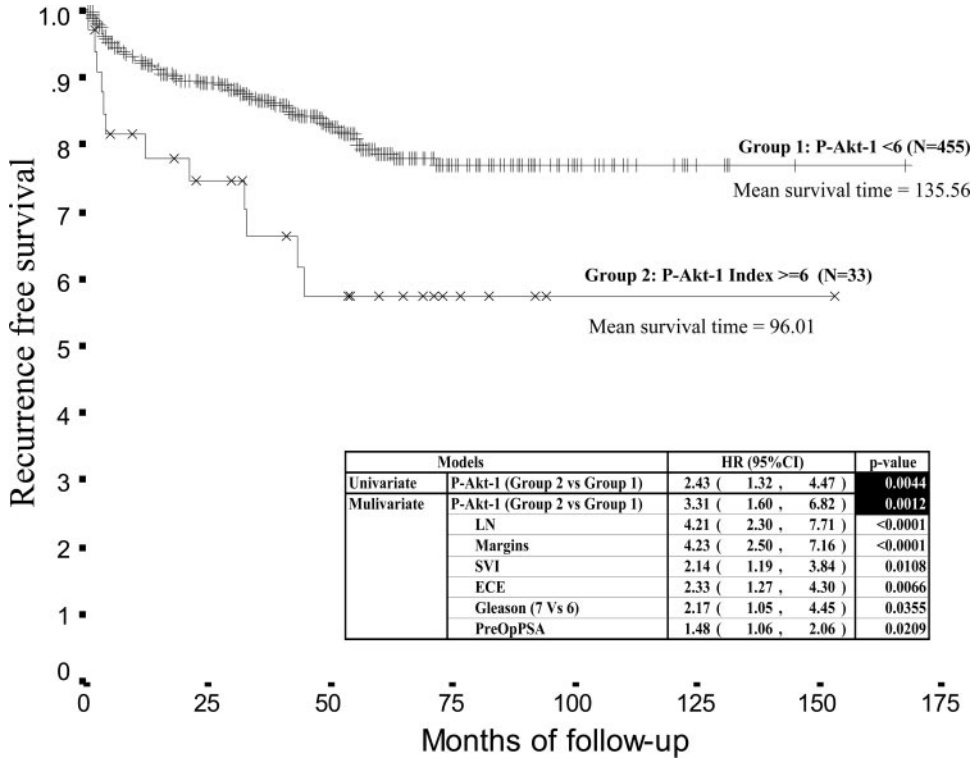
High Levels of P-Akt-1 in Non-Neoplastic Prostatic Tissues Predict Biochemical Recurrence. The biochemical recurrence-free survival curves of P-Akt-1 expression in non-neoplastic prostatic tissues were plotted by Kaplan-Meier actuarial model. Surprisingly, a high level of P-Akt-1 expression in non-neoplastic tissues was also an independent predictor of biochemical recurrence. There was a significant difference in biochemical recurrence-free survival between cases with no expression or low expression of P-Akt-1 in the non-neoplastic tissues (median biochemical recurrence-free survival 52.7 months) and those with high expression (index >6; median biochemical recurrence-free survival, 52.7 months). Patients with no or low indices had a better biochemical recurrence-free survival than those with high index. These results were significant on univariate [$P = 0.0435$, hazard ratio 2.80 (95% confidence interval, 1.03 to 7.61)] and multivariate analysis [$P = 0.0385$, hazard ratio 2.94 (95% confidence interval, 1.06 to 8.18)]. Significantly, of the 8 patients who had a high expression index in the non-neoplastic tissues, only 2 also had high expression index in the prostate cancer, whereas 6 had low levels of P-Akt-1 expression (Fig. 5).

DISCUSSION

Akt/PKB has been shown to be involved in a number of proliferative, metabolic, and antiapoptotic pathways that are dependent on phosphatidylinositol 3′-kinase signaling to be activated (4). Activated Akt has been suggested to regulate the/a number of intracellular targets involved in prostate cancer progression and hormone independence (10). *In vitro* studies have found that elevated Akt activity protects the prostate cancer cell line LNCaP from tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (5). The antiapoptotic Akt appears to interact with pro-apoptotic proteins such as $p27^{Kip1}$, MMAC/PTEN, BAD, and Caspase-9. Indeed, inactivation of BAD and Caspase-9 by Akt may suppress the normal apoptotic activity (11), whereas Akt activation may enhance prostate cancer progression by diminishing $p27^{Kip1}$ expression (6). Acute expression of MMCP/PTEN via an adenoviral construct has been found to result in a dose-dependent and specific inhibition of Akt/PKB activation (7).

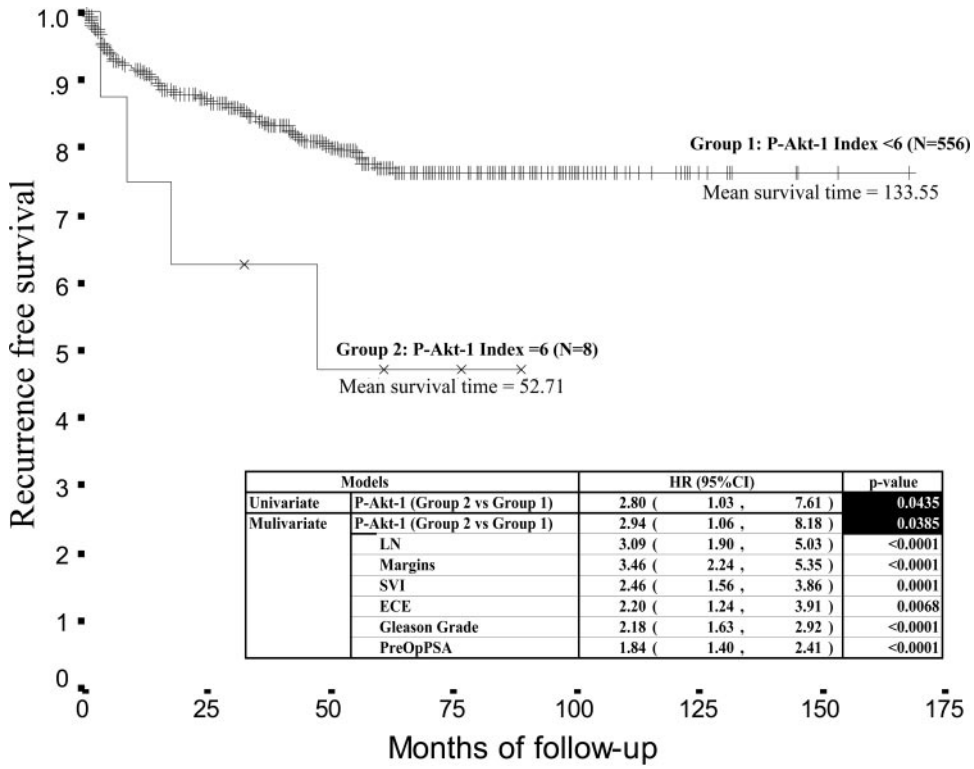
Although the clinical application of P-Akt-1 in human cancers remains very limited, preliminary studies have demonstrated its promising clinicopathological implications. Akt has been found to predict a worse outcome among hormonally treated breast cancer patients (12). Also evaluation of phosphatidylinositol 3′-kinase activation by Akt phosphorylation might be a prognostic marker for response to radiation in head and neck cancer (13).

P-Akt-1 expression has been found to be significantly stronger in association with high Gleason grades 8 to 10 than with prostatic intraepithelial neoplasia and all of the other grades of prostate cancer (8). Our data show that P-Akt-1 was predominantly expressed in prostate cancer, with >98% of patients having no expression in their non-neoplastic tissues. Furthermore, high levels of P-Akt-1 expression were found almost exclusively in prostate cancer. Because of the clinical significance associated with the latter finding, we believe that P-Akt-1 can become a significant therapeutic target. Anti P-Akt-1 therapy would not only target areas



Models		HR (95%CI)	p-value
Univariate	P-Akt-1 (Group 2 vs Group 1)	2.43 (1.32 , 4.47)	0.0044
Mulivariate	P-Akt-1 (Group 2 vs Group 1)	3.31 (1.60 , 6.82)	0.0012
	LN	4.21 (2.30 , 7.71)	<0.0001
	Margins	4.23 (2.50 , 7.16)	<0.0001
	SVI	2.14 (1.19 , 3.84)	0.0108
	ECE	2.33 (1.27 , 4.30)	0.0066
	Gleason (7 Vs 6)	2.17 (1.05 , 4.45)	0.0355
	PreOpPSA	1.48 (1.06 , 2.06)	0.0209

Fig. 4 Differences in prostatic-specific antigen recurrence in Gleason 6–7 patients exclusively. Patients with high-level P-Akt-1 the prostate cancer expression have a median biochemical recurrence-free survival of 96.01 months as compared with 135.56 months for others. The patients with high levels of P-Akt-1 expression have 3.31 times the risk (hazard ratio, *HR*) of developing biochemical recurrence as compared with patients with moderate, low, or no expression.



Models		HR (95%CI)	p-value
Univariate	P-Akt-1 (Group 2 vs Group 1)	2.80 (1.03 , 7.61)	0.0435
Mulivariate	P-Akt-1 (Group 2 vs Group 1)	2.94 (1.06 , 8.18)	0.0385
	LN	3.09 (1.90 , 5.03)	<0.0001
	Margins	3.46 (2.24 , 5.35)	<0.0001
	SVI	2.46 (1.56 , 3.86)	0.0001
	ECE	2.20 (1.24 , 3.91)	0.0068
	Gleason Grade	2.18 (1.63 , 2.92)	<0.0001
	PreOpPSA	1.84 (1.40 , 2.41)	<0.0001

Fig. 5 P-Akt-1 in non-neoplastic prostate also predicts biochemical survival. Note that high expression of P-Akt-1 predicts decreased biochemical recurrence-free survival. Patients with high-level P-Akt-1 expression the non-neoplastic prostate have a median biochemical recurrence-free survival of 52.71 months as compared with 133.55 months for others. The patients with high levels of P-Akt-1 expression have 2.94 times the risk (hazard ration, *HR*) of developing biochemical recurrence as compared with patients with moderate, low, or no expression.

with cancer, but would be most effective in those patients with increased risk of recurrence or progression.

Moreover, we have found P-Akt-1 to be one of the best biomarkers for biochemical recurrence in prostate cancer in our patient cohort. P-Akt-1 is a very strong and versatile marker, with multiple potential uses in varied populations. P-Akt-1 was found to be an independent prognostic indicator of biochemical recurrence-free survival. High levels of P-Akt-1 are associated with earlier recurrence, clearly indicating that P-Akt-1 is associated with aggressiveness and disease progression in prostate cancer. Multivariate Cox models suggest that P-Akt-1 index [hazard ratio = 3.0 (95% confidence interval, 1.7 to 5.2), $P = 0.0001$] is a better postoperative marker than prostatic-specific antigen [hazard ratio = 2.0 (95% confidence interval, 1.3 to 3.0), $P = 0.0015$], and BxGG [hazard ratio = 1.6 (95% confidence interval, 1.1 to 2.3), $P = 0.0230$], when information on stage, lymph node metastasis, extracapsular extension, seminal vesicle invasion, and margins is already known. The absence of significant correlations with clinicopathological variables, other than with clinical staging, indicates that P-Akt-1 is most likely measuring aspects of disease not currently quantifiable.

In addition we have shown that P-Akt-1 potentially could be used in the pretherapy setting at the time of diagnosis. P-Akt-1 was compared with preoperative markers currently used in practice, preoperative prostatic-specific antigen and biopsy Gleason Grade, in its ability to identify high-risk patients. First, patients were grouped into high P-Akt-1 ($\geq$6) and low P-Akt-1 ($<$6) categories, high prostatic-specific antigen($>$10) and low prostatic-specific antigen ($\leq$10) categories, and into high Bx Gleason ($>$6) and low Bx Gleason grade ($\leq$6) categories. Univariate analysis showed that individually, prostatic-specific antigen grouping is the best marker [hazard ratio = 4.2 (95% confidence interval 2.8 to 6.2)], whereas P-Akt-1 and Bx Gleason grade were equal in their ability to distinguish between high-risk and low-risk patients for recurrence [respectively, hazard ratio = 2.2 (95% confidence interval, 1.3 to 3.7) and hazard ratio = 2.4 (95% confidence interval, 1.7 to 3.5)]. However, these three preoperative markers carry different information and should be used together. Of 2 patients with identical prostatic-specific antigen levels and Bx Gleason grades, a patient with P-Akt-1 index $>$6 has more than 3 times the risk of having earlier recurrence than one with lower P-Akt-1 level [hazard ratio = 3.1 (95% confidence interval, 1.6 to 5.7), $P = 0.0005$]. In conjunction with prostatic-specific antigen and Gleason, P-Akt-1 could be used to determine which patients might actually benefit from adjuvant therapy. P-Akt-1 may also be used after radical prostatectomy, when our data indicate that it might outperform other clinicopathological markers currently in use.

Subsequently we found that P-Akt-1 can independently predict biochemical recurrence in the subgroup of patients with Gleason score 6 or 7. Because most of the patients with prostate cancer today are in this Gleason range and because of the limitations of the Gleason system in predicting biochemical recurrence-free survival in these patients, we believe that P-Akt-1 could become an extremely useful biomarker for prostate cancer.

Finally we found that high levels of P-Akt-1 expression in non-neoplastic tissues apart from the cancer were also predictive for biochemical recurrence independently with hazard ratios similar to those found for prostate cancer. Most patients with high levels of expression in the prostate cancer also had high levels of expression in the non-neoplastic tissues. However, some patients had high levels only in the non-neoplastic tissues, yet still had decreased biochemical recurrence-free survival. This underlines the need for the study of P-Akt-1 in non-neoplastic prostate tissues. To our knowledge, this is the only biomarker of which the presence in the non-neoplastic tissues away from the cancer is predictive of biochemical recurrence. This finding suggests that some patients might have an inherent genetic predisposition to express high level of P-Akt-1 and, therefore, have an adverse prognosis. It is also possible that P-Akt-1 could be used in random prostate biopsies to discriminate among clinically high-risk patients (*i.e.*, prostatic-specific antigen $>$ 4, free prostatic-specific antigen $<$ 15, biopsy negative) in whom no cancer has been detected.

Although the data presented are very promising, more research is needed to ascertain the validity of P-Akt-1 as a prognostic marker in prostate cancer. Additional studies will help validate its potential for universal use. We are currently involved in a multi-institutional prospective study that will help determine the validity of P-Akt-1 as a biomarker in prostate cancer. These studies will not be limited to radical prostatectomy specimens but will also include biopsies at the time of diagnosis.

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