Immuno-analysis of phospho-Akt in primary human breast cancers

Y.O. AL-BAZZ[1], B.L. BROWN[1], J.C.E. UNDERWOOD[2], R.L. STEWART[2,3] and P.R.M. DOBSON[1]

[1]Cell Signalling Group, Department of Human Metabolism, and [2]Academic Unit of Pathology, University of Sheffield Medical School, Sheffield S10 2RX, UK

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Abstract. The aim of this study was to investigate the expression of the activated (phosphorylated) form of Akt (Ser473) in primary breast cancer and to correlate the results with clinicopathological and prognostic variables for clinically relevant associations. Phospho-Akt expression was studied using immunoblot analysis in 49 invasive breast carcinomas (median follow-up time 55 months, range 7-74 months). We assessed the level of phospho-Akt in different types of primary breast cancers and compared the use of autoradiograph X-ray film with a PVDF-DAB-staining system. Twelve percent of the tumours had no phospho-Akt protein, 25% had low phospho-Akt expression, 51% had intermediate expression and 12% had high phospho-Akt expression. No relationship was observed between phospho-Akt and tumour grade, tumour size or nodal status. A significant relationship was demonstrated between phospho-Akt score and oestrogen receptor status (P=0.014). Univariate analysis demonstrated that intermediate levels of phospho-Akt in breast tumour tissue are associated with a lower probability of developing recurrences (P=0.035), while in multivariate analyses, none of the phospho-Akt levels appeared to be independent predictors of disease recurrence or death. In addition, it has been clearly established that a suitable composition of reagents and components such as PVDF membranes treated with DAB substrate will enable the performing of sensitive immuno-analyses.

Introduction

Breast cancer is the most common malignancy in women in Western countries and is a major cause of mortality and morbidity. Decisions for breast cancer patient management are based on prognostic and predictive factors, such as tumour grade, stage and steroid hormone receptor status. The ability to predict the clinical course of disease at the time of diagnosis is of considerable value in the management of all patients with breast cancer. The assessment of specific proteins involved in the control of cell death not only promises better separation of patients into prognostic groups so that the best treatment can be chosen, but also will further our understanding of the biology of breast cancer cells.

Intracellular signalling pathways are intimately involved in cancer progression and so could provide targets for therapeutic intervention. One such process is apoptosis (programmed cell death) which is characterized by a series of distinct morphological and biochemical alterations. Inappropriate apoptosis is implicated in many human diseases, and resistance to apoptotic stimuli and/or increased survival (anti-apoptotic) function are involved in several forms of cancer. Although much attention has been given to apoptosis, there has been less emphasis on the importance of survival mechanisms.

Akt protein is one of the critical regulators of survival (1-3). Akt (also known as protein kinase B) is a serine/threonine kinase downstream of PI3-OH kinase (PI3K) which is activated in response to growth-factor treatment and can influence the activity of a number of proteins including p70 S6 kinase, Bad and GSK3. Akt was identified as a retroviral oncogene (4), which has a similarity to protein kinases A and C (5). Akt activation (phosphorylation) leads to signals that either stimulate anti-apoptotic cellular responses or block apoptotic functions of the cell (6,7). Therefore, the significance of Akt expression and activation in human cancer is becoming increasingly evident (8-10). Active Akt indicates poor prognosis (11-13) in spite of a low proliferative state of the breast tumour, probably by promoting cell survival. Expression of Akt has been specifically associated with hormone-independent primary breast cancers (14). Oestrogen has been shown to exhibit growth-promoting properties in breast epithelial cells (15). Akt is activated in many breast cancer tumours (9,16-18), and its activation is induced by the oestrogen receptor (ER) via an interaction between ER and PI3K (19). Moreover, AKT-3 expression was selectively up-regulated in more advanced ER-negative primary human breast cancer (16), while others suggest that AKT-3 expression is not restricted to ER-negative breast cancer cells (20).

Based on all these observations, we examined whether Akt, found *in vitro* to play a prominent role in oncogenesis,

Correspondence to: Dr Pauline R.M. Dobson, Cell Signalling Group, Department of Human Metabolism, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, UK
E-mail: p.dobson@sheffield.ac.uk

Present address: [3]Biosciences Dept., School of Science and Technology, Nottingham Trent University, Nottigham NG11 8NS, UK

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showed the same significance in clinical material. We also investigated the relationship between ER and phospho-Akt for clinically relevant associations and determined its expression in different types of primary breast cancers.

At present, various chemiluminescent methods are alternatives to radioactive methods commonly employed in molecular biology and biochemical analysis (21,22). For the detection of phospho-Akt, polyvinylidene difluoride (PVDF) membranes were used. The transfer of protein from the interaction gel to PVDF, a hydrophobic membrane with a high protein immunostaining binding capacity and a great mechanical strength (23) with subsequent immunodetection, offers an easy and sensitive way to determine the relative amount of protein (24-26). For this reason various staining procedures have been developed, but obviously there is still a demand for a sensitive and compatible staining technique for blotted proteins. Therefore, we also compared the use of autoradiograph X-ray film and a PVDF-DAB-staining system.

Materials and methods

Tissue samples. Forty-nine frozen tissue samples form primary breast carcinoma patients were selected from a computer database in the Academic Unit of Pathology, University of Sheffield (1995-1996). The study was approved by the South Sheffield Research Ethics Committee. Detailed clinical and pathological information including menopausal status, age at diagnosis, location of primary tumour, time of local recurrence, time to metastases, metastatic sites, therapeutic interventions (surgical, chemotherapeutic, radiotherapeutic, and hormonal), cause of death, lymph node metastases and tumour size was recorded. Cases showing distant metastases at the time of diagnosis, a history of other malignancies or of radiation and/or chemotherapy prior to surgical resection were excluded. The primary samples consisted of 40 invasive ductal carcinomas of no special type, 3 invasive lobular carcinomas, 4 mucinous carcinomas, 1 tubular and 1 papillary carcinoma. The diagnosis of all the cases was based on a light microscopic examination using the conventional haematoxylin and eosin (H&E) stain. Grading of the ductal invasive carcinomas was performed by two independent pathologists according to the modified criteria of Bloom and Richardson described by Elston and Ellis (27) without knowledge of the clinical outcome of the patients.

Immunohistochemical staining for oestrogen and progesterone receptors. Frozen sections cut at 6 μm were fixed in acetone for 10 min. Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in methanol for 20 min. Non-specific staining was blocked with 1.5% normal horse serum diluted in PBS (pH 7.3) for 30-60 min at room temperature. The following primary antibodies were used: anti-human oestrogen receptor (ER, monoclonal mouse; Dako, Glostrup, Denmark; clone 1D5, code no. M7047) 1:100 and anti-human progesterone receptor (PgR, monoclonal; Dako; clone 1A6, code no. M3529) 1:10. The antibodies were diluted in PBS containing 1.5% normal horse serum. Biotinylated secondary antibodies (Vectastain ABC rabbit/mouse Elite; Vector Laboratories, Burlingame, CA) were used at dilutions of 1:200 to 1:400. Bound antibodies were visualized using the avidin-peroxidase complex (ABC) reagent, followed by 3,3-diaminobenzidine (DAB) (Vector Laboratories). The sections were washed, dehydrated, and mounted. The slides were counterstained with Gill's haematoxylin, and the oestrogen and progesterone receptor status was determined. The staining of ≥10% of tumour cell nuclei was regarded as positive. Negative controls were treated identically, omitting the incubation with the primary antibody.

Protein extraction from frozen tissue and assay. Frozen tissue samples were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate). The solution was then stored at 4˚C. Fresh 1 mM NaF and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Boehringer Mannheim, Lewis, East Sussex, UK) were added on the day of use. Tissue was weighed and then diced under liquid nitrogen into very small pieces using a sterile razor blade. Then the tissue was ground to a powder under liquid nitrogen in a pestle and mortar. These tissue powders were then allowed to thaw in lysis buffer (1 ml/g tissue) containing fresh protease inhibitors. The samples were left on ice for 30 min. The tissue was further disrupted by homogenising with a tissue grinder, Sonicator, for 15-30 sec, maintaining 4˚C throughout all procedures. The homogenate was transferred to Eppendorf tubes and centrifuged at l3000 x g for 10 min at 4˚C. The supernatant was transferred to fresh tubes, and a freeze-thaw cycle was added before re-centrifuging. This appeared to precipitate more cellular debris than directly centrifuging again, leading to a more clarified cell lysate. The supernatant was used immediately or stored at -20˚C. Protein concentration was determined using a Bio-Rad Protein Kit II (Bio-Rad Laboratories, Inc., Richmond, CA), based on the Bradford assay (28). Bovine serum albumin (BSA) was used for the standard curve.

Immunoblot analysis. Equal amounts of proteins (30 μg) were analysed by electrophoresis in 8% polyacrylamide gels with 0.1% sodium dodecyl sulphate (SDS) in buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 0.1 SDS. Thereafter, resolved proteins were transferred to PVDF membranes (BDH, Warwickshire, UK). The membranes were incubated for 1 h at room temperature in Tris-buffered saline (50 mM Tris-HCl/200 mM NaCl, pH 7.4) containing 5% BSA to block non-specific binding sites. Rabbit polyclonal phospho-Akt antibody (Ser473; Cell Signalling Technology, Beverly, MA; cat. no. 9271) 1:1000 dilution in TBS, was incubated with the membranes on a rotator overnight at 4˚C. The following morning the membranes were briefly rinsed twice in TBS-T, then washed once for 15 min and twice for 10 min at room temperature, to remove any unbound protein. The ABC technique was used for detection of phospho-Akt primary antibody which involved the addition of a biotinylated secondary antibody diluted at 5:100 in blocking buffer, for exactly 1 h on a rocking platform at room temperature, followed by a preformed avidin and biotinylated horseradish peroxidase complex (ABC) solution (Vector Laboratories, Inc.) by mixing equal amounts of reagents A and B in 10 ml

Table I. Relationship of phospho-Akt expression with patient and tumour characteristics.

Factors	Total (%)	Phospho-Akt immunoblot (staining scores)				P-value
		-	+	++	+++	
Age (years)						
≤50	12 (24.5)	2	2	7	1	0.790
>50	37 (75.5)	4	10	18	5	
ER-positive	29 (59)	4	7	18	0	0.014
ER-negative	20 (41)	2	5	7	6	
PgR-positive	36 (73)	4	11	17	4	0.440
PgR-negative	13 (27)	2	1	8	2	
Nottingham prognostic index						
<3	5 (10)	0	1	4	0	0.660
3-5	29 (59)	5	8	12	4	
>5	15 (31)	1	3	9	2	
Menopausal status						
Pre-menopause	6 (12)	1	1	4	0	0.580
Peri-menopause	3 (6)	1	0	1	1	
Post-menopause	40 (82)	4	11	20	5	
Node-positive	22 (45)	3	5	12	2	0.900
Node-negative	27 (55)	3	7	13	4	
Tumour size (mm)						
<10	4 (8)	0	1	2	1	0.106
11-20	15 (31)	3	6	5	1	
≥21	30 (61)	3	5	18	4	
Histological grade						
Grade I	7 (14.3)	0	1	6	0	0.210
Grade II	27 (55.0)	5	7	13	2	
Grade III	15 (30.7)	1	4	6	4	
Histological types						
IDC	40 (82)	6	9	19	6	0.310
Other	9 (18)	0	3	6	0	

Summary of the results, where direct side by side comparisons were made between the intensity of the immunostaining with invasive breast cancers. Statistical comparisons were performed for intensity by Pearson χ^2 test using a contingency table for coded values. Immuno-intensity scores for invasive breast cancer were evaluated from 49 frozen breast cancer tumours. Scoring of the intensity on an arbitrary 4-point scales: -, none; +, weak; ++, intermediate; +++, strong. IDC, invasive ductal carcinoma. Other, non-IDC tumours: lobular, 3 cases; mucinous, 4 cases; tubular, 1 case and papillary, 1 case.

TBST buffer (Tris-buffered saline, Tween-20). This was left to stand for 30 min before use. Membranes were then incubated with the ABC reagent for 30 min on a shaking platform at room temperature and rinsed twice in TBST for 10 min. Immuno-complexes were visualized by chemi-luminescence [Enhanced Chemiluminescence (ECL)] Western blotting detection reagents (Amersham Life Sciences) and exposure to X-ray film (Kodak, Amersham Life Sciences, UK). The darkness of slots on the developed film was quantitated by scanning the film with a densitometer (PDI Discovery System; Pharmacia Biotech., Hertfordshire, UK). All values were corrected for background. Biotin broad range protein molecular mass markers were used to determine the molecular masses, and Kaleidoscope polypeptide markers were used to monitor electrophoresis and Western transfer of proteins in the gel (Bio-Rad Laboratories, Inc.).

Detection of phospho-Akt on PVDF membranes using DAB staining. After ECL detection was carried out and film was taken, the same phospho-Akt membranes were incubated with DAB. The membranes were briefly rinsed twice in TBS and then washed once for 10 min to remove any excess chemiluminescence substrate from the PVDF membranes. The DAB Substrate Kit was prepared according to the manufacturer's instructions. Membranes were incubated with this substrate for 2-5 min on a shaking platform at room

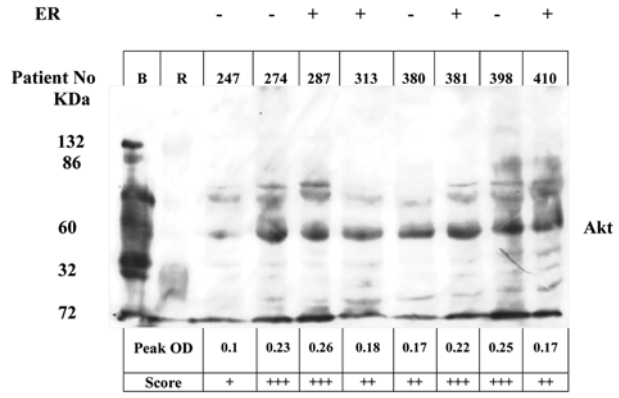

Figure 1. Immunoblot analysis of Akt protein in primary breast carcinoma patients. Lysates from 8 previously frozen breast tissues (30 μg per lane) were subjected to SDS-PAGE, transferred to PVDF membranes, incubated with Akt antibody and visualised by ECL. ER status is indicated at the top of the columns. The source of proteins was primary breast cancer [mucinous (287) and tubular (398)], and the remaining were IDC (247, 274, 313, 380, 381, 410). A lysate from one of the patients was included each time as a positive control. The figure represents one of two independent experiments showing similar results. PAGE, transferred to PVDF membranes and analysed by ECL. Note, lane R represents labelled protein standards of known molecular weight; lane B represents biotinylated markers.

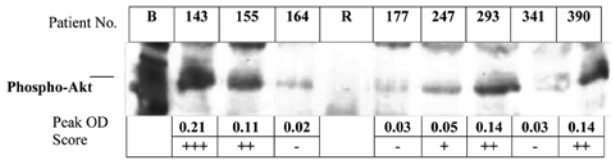

Figure 2. Immunoblot detection of phospho-Akt in primary breast tumours. Comparison of phospho-Akt protein activity in different patients with invasive breast carcinoma as assessed by Western blot analysis using an antibody to phospho-Akt (Ser473) on tumour cell extracts from 8 different patients [(lobular (164) the remaining were IDC (NOS)]. Thirty micrograms of tumour cell lysates from each patient was subjected to SDS-PAGE, transferred to PVDF membranes and analysed by ECL. Note, lane R represents labelled protein standards of known molecular weight; lane B represents biotinylated markers.

temperature to detect the bound antibodies, so that the sensitivity of staining could be compared between the PVDF-DAB stain and the autoradiograph X-ray film.

Scoring and statistical analysis. The strength of the association between phospho-Akt levels in lysates from tumours (treated as a coded variable) and other prognostic variables (patient or tumour characteristics treated as grouping variables) was tested with the χ^2 test (Table I). To study the possible relationship with clinicopathological factors, an alternative method for scoring relative intensities of immunostaining in invasive cancer was employed, which lends itself to statistical analysis (Pearson correlation). In this approach, a 4-point scale was used to score immuno-intensity: (0-0.03, none), (0.04-0.1, weak), (0.11-0.2, intermediate) or (>0.21, strong). As shown by Western blot analysis, levels of phospho-Akt protein differed in different tumours (Fig. 3), with an average level of 0.17 (0.02-0.44) optical density units (OD) in all tested cases. The probability of overall survival (OS) or disease-free survival (DFS) was calculated using Kaplan-Meier methods and examined by the log-rank test. The Cox regression model was used to identify which factors were jointly significant in their association with OS or DFS for both univariate and multivariate analyses. Relative hazard ratios were calculated and presented with 95% confidence intervals. For multivariate analysis, a basic model was introduced, including classical prognostic factors (age, menopausal status, tumour size, number of positive lymph nodes, ER status, PgR status and differentiation grade). A P-value <0.05 was considered significant. All statistical analyses were performed with SPSS Statistical Software.

Results

ER and PgR immunohistochemical staining. The status of ER and PgR was assessed on frozen sections with anti-oestrogen and anti-progesterone receptor antibodies. In this study, positive ER status was noted in 29 (59%) cases (Table I). Positive ER status was associated with grade II tumours (data not shown). The intensity of the bands obtained by densitometric analysis of the immunoblots revealed a statistically significant association between phospho-Akt scores and ER (P=0.014). No association was observed between PgR and phospho-Akt.

Expression of Akt in primary human breast cancers. The expression of Akt was assessed in frozen primary breast cancer tissues from 8 patients [6 patients with invasive ductal carcinoma (IDC), 1 with tubular and 1 with mucinous carcinoma]. Immunoblot analysis of the Akt protein in these eight tumours using a polyclonal antibody against total Akt1, Akt2 and Akt3 protein levels revealed the presence of Akt in all of the samples at the expected mass of ~60 kilodaltons (kDa) (Fig. 1). These data from primary tumours were in agreement with recent results of Zinda *et al* (20). However, our data cannot exclude the possibility that there are differences in the extent of the positivity of Akt protein expression. In addition, phospho-Akt protein expression was evaluated in breast cancer tissue from 49 patients. Fig. 2 shows that the phospho-Akt (Ser473) polyclonal antibody used recognized the human phospho-Akt protein with an apparent molecular mass of 60 kDa (29). All of these data were obtained by blotting onto PVDF and visualizing bands by ECL.

Relationships between clinicopathological factors and phospho-Akt level. The relationships observed between tumour phospho-Akt levels and clinicopathological factors are summarized in Table I. Twelve percent of the tumours exhibited no phospho-Akt protein, 25% had low phospho-Akt expression, 51% had intermediate expression and 12% had high phospho-Akt expression. The majority of patients (55%) were diagnosed with grade II tumours and the same percent were node-positive. Twelve patients were dead from breast cancer, 3 were alive with recurrent cancer, and 34 patients were alive and free of the disease. Follow-up data were available on all patients. The time range of follow-up was from 7 to 74 months, with a median follow-up time of 55 months. Six patients were pre-menopausal, 3 were peri-menopausal, and 40 were post-menopausal. There was no significant trend in the percentage of pre-, peri-, or post-

Table II. Univariate Cox analysis of clinicopathological factors for survival in breast cancer patients.

Factors	Disease-free survival			Overall Survival		
	P-value (Univariate)	RR	95% CI for RR	P-value (Univariate)	RR	95% CI for RR
Phospho-Akt scoring	0.4570	0.88	0.64-1.22	0.320	0.85	0.62-1.17
ER[a] status	0.0010	0.35	0.17-0.73	<0.001	0.38	0.18-0.80
PgR status	0.0409	0.20	0.10-0.38	0.067	0.19	0.09-0.36
Tumour size[b] (mm)	0.1850	1.35	0.87-2.11	0.190	1.34	0.86-2.10
Histological grade	0.8100	0.94	0.55-1.58	0.490	0.84	0.49-1.40
Lymph node status[c]	0.0190	0.21	0.11-0.39	0.030	0.19	0.11-0.35
NPI[d]	0.0910	0.16	0.09-0.29	0.290	1.35	0.77-2.37
Menopausal status	0.5300	0.87	0.56-1.35	0.470	0.85	0.55-1.32

[a]Oestrogen receptor status was defined using an immunohistochemical method, cut-off 10% of reactive cells. [b]Tumour size according to TNM system. [c]Nodal status according to TNM system. [d]Nottingham prognostic index: three prognostic groups according to the NPI value. ER, oestrogen receptor; PgR, progesterone receptor; CI, confidence interval; RR, relative risk.

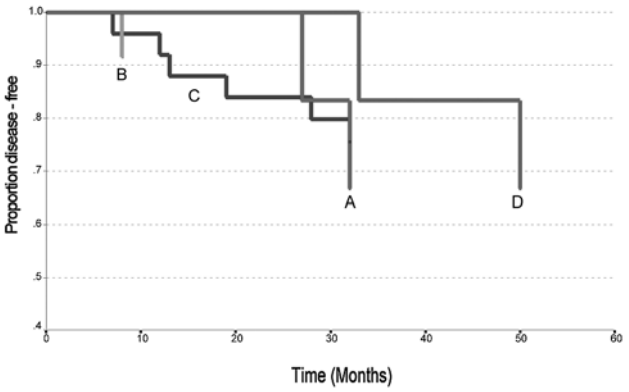

Figure 3. Relationship between phospho-Akt and recurrence in patients with primary breast cancer. Disease-free survival of 49 breast carcinoma patients was assessed in relation to the expression level (negative, weak, intermediate, or strong) of phospho-Akt protein in the extracts of primary breast tumours as estimated by life-table methods (Gehan's generalized Wilcoxon test). Group A represents patients with phospho-Akt-negative tumours, group B with positive (+) phospho-Akt tumours, group C with positive (++) phospho-Akt tumours and group D with positive (+++) phospho-Akt tumours.

menopausal patients whose breast tumours possessed different levels of phospho-Akt protein. In agreement with a previous study (11), a moderate, although statistically significant, correlation was found between Akt and phospho-Akt staining (P=0.039). The only other statistically significant association was between phospho-Akt and the oestrogen receptor (P=0.014). Positive ER was also found significantly in grade II tumours (P<0.05). In contrast there was no association noted with PgR, nodal status and tumour size. A non-significant trend was observed towards a positive correlation of tumour sizes >20 mm and phospho-Akt alteration (P=0.123). Histological grade was assessed in all tumours. Moderate levels of phospho-Akt activity were noted in stages I, II and III (85.7, 48.1 and 40%, respectively), while high levels of phospho-Akt kinase activity were not detected in grade I tumours, and were observed in 7.4 and 26.7% of grades II and III tumours, respectively. It is thus possible that within the class of grade II breast tumours, phospho-Akt ratios may have a prognostic significance.

Univariate and multivariate analyses of clinicopathological factors for survival data in breast cancer. To determine whether the status of phospho-Akt is associated with clinical outcome, disease-free survival (DFS) and overall survival (OS) of patients with or without phospho-Akt expression were examined (Table II). Univariate survival analyses were performed in the whole group and in subgroups of patients. In the whole series of cases, ER status was predictive for OS (60 vs. 51.7%); 6-year OS for 12 ER-negative vs. 15 ER-positive tumours (P=0.004). The other variables associated with OS were nodal status and PgR status. As for recurrence, ER and lymph node status among the other factors showed a significant univariate prognosis. In the group of node-negative cases, the only variables associated with DFS and OS were ER and intermediate Nottingham Prognostic Index (NPI).

The Kaplan-Meier disease-free survival curve as a function of phospho-Akt positivity is shown in Fig. 3. The degree of phospho-Akt positivity did not offer a selective advantage against recurrence or survival, as would be predicted by the hypothesis that phospho-Akt has anti-metastatic properties. In addition, there was no statistically significant difference between phospho-Akt-positive and -negative populations with respect to the prognostic markers analysed. Additional statistical analyses were performed using subgroups of phospho-Akt positivity, to compare immunoblot results with survival data. For a phospho-Akt score of 0 there was a trend toward longer OS as compared with scores 1, 2 and 3 (P=0.15, 0.059 and 0.07, respectively). Significant differences were observed between scores 2 and 3 (P=0.029), but there was no difference between scores 1 and 2. However, levels 0-3 also showed a trend in prognosis to longer overall survival, again only at P<0.05. Despite score 2 for phospho-Akt being associated with a longer DFS as compared with score 3 (P=0.027), there was no significant difference in terms of DFS

Table III. Cox regression analyses.

	Disease-free survival		Overall Survival	
Phospho-Akt score	RHR (95% CI)	P-value	RHR (95% CI)	P-value
Negative	0.990 (0.95-1.03)	0.690	0.980 (0.93-1.040)	0.55
Weak	1.014 (0.98-1.05)	0.420	1.008 (0.97-1.050)	0.70
Intermediate	0.980 (0.96-0.99)	0.035	0.980 (0.95-1.006)	0.13
Strong	1.002 (0.93-1.08)	0.950	0.990 (0.92-1.080)	0.99

Analysis of time to recurrence for patients grouped according to tumour phospho-Akt. RHR, relative hazard rate; CI, confidence interval.

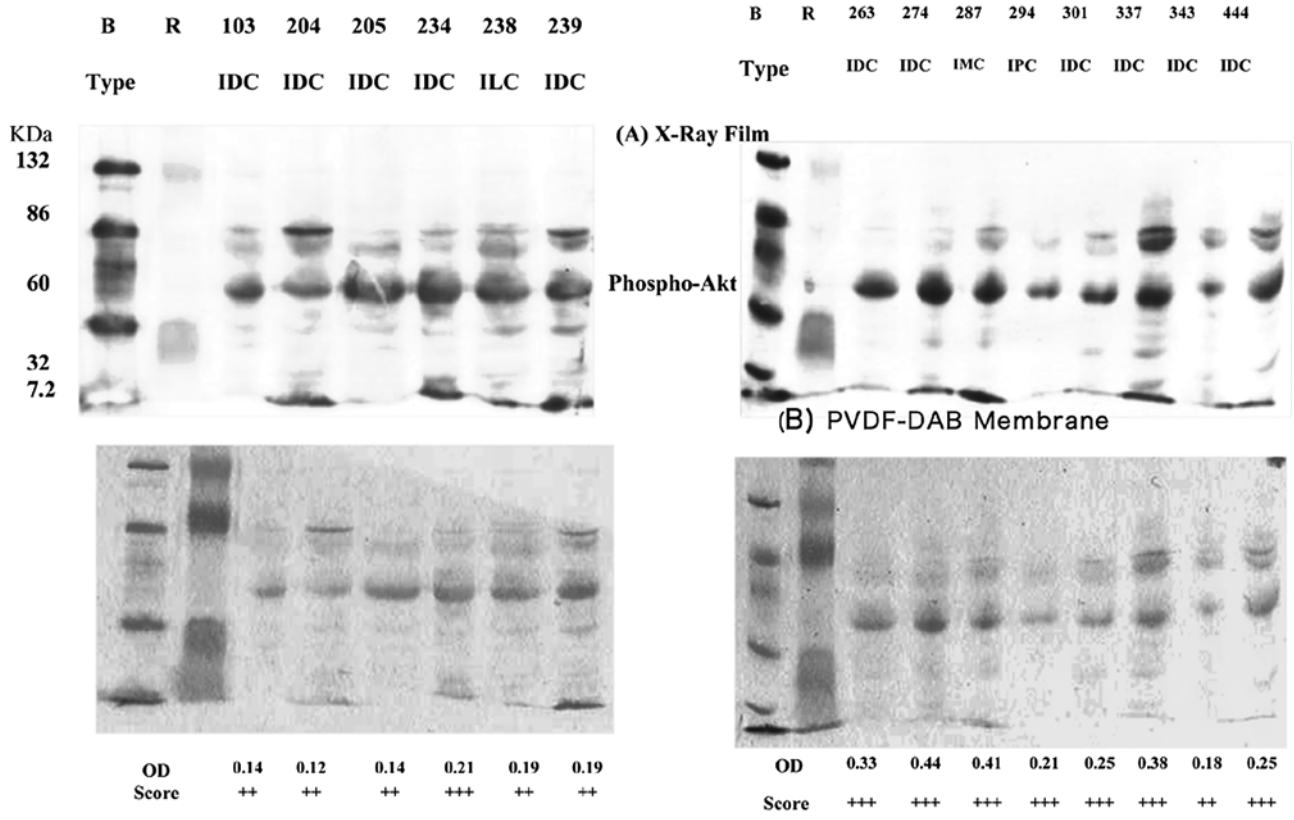

Figure 4. Determination of phospho-Akt (Ser473) in human breast cancer specimens and comparison of two different staining procedures. Immunoblot analysis of phospho-Akt (Ser473) in 49 breast cancer tumour lysates was conducted. (A) Proteins detected by the ECL method using the autoradiograph X-ray film detection method with expression levels of phospho-Akt quantified by a densitometer. Then the PVDF membrane was treated with ABC reagent and incubated in DAB substrate for 5-10 min. The PVDF-DAB stained membrane is shown in B. The positions of pre-stained molecular weight markers are shown in kilodaltons (kDa). IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IPC, invasive papillary carcinoma; IMC, invasive mucinous carcinoma; ITC, invasive tubular carcinoma. Note, lane R represents labelled protein standards of known molecular weight; lane B represents biotinylated markers.

between scores 0, 1, 2, or 3 for phospho-Akt. These results were confirmed by the Cox analysis model which showed that increasing levels of tumour phospho-Akt were insignificantly related to recurrence (P=0.46). However, a statistically significant association of intermediate phospho-Akt levels with disease-free survival was observed [(log-rank test for trend: χ^2=4.43, df=1, P=0.035) and trends towards overall survival (log-rank test for trend: χ^2=2.38, df=1, P=0.13)] (Table III). Patients with low and intermediate levels of phospho-Akt protein in their primary breast tumours had similar periods of disease-free survival (Table I). Thus, in univariate analysis, phospho-Akt had no significant positivity in predicting a higher risk of developing metastasis. Multivariate stepwise Cox regression analyses of the survival data were performed to compare the statistical power of the studied parameters. Analyses were conducted with respect to all studied parameters for those patients for whom complete data were available. In this analysis, the parameters that were independently and essentially of prognostic significance were ER status for DFS and OS (P<0.001 for both), PgR status for DFS and OS (0.0026 and 0.004, respectively) and the depth of invasion. Although phospho-Akt was not a significant

factor in multivariate analysis, phospho-Akt might be a useful predictor for prognosis as it significantly correlated with ER.

Comparison between DAB staining and autoradiograph X-ray film. DAB substrate was used after ECL detection had been performed on PVDF membranes, so that the sensitivity of staining could be compared between colorimetric and autoradiographic techniques. Fig. 4 demonstrates the detection of phospho-Akt and the usefulness of the PVDF-DAB method. Comparison between the two different methods of detection (PVDF-DAB and X-ray film) for the same amount of protein on the same PVDF membrane showed that, although the intensity of the background stain was different on the membranes, the immunodetection sensitivity levels were similar.

Discussion

Previous studies (30,31) have shown that Akt is expressed in breast cancer cell lines, and its blockade inhibits growth of these cells (32). In addition, Akt may be overexpressed in breast cancers, and phosphorylated Akt has been detected in some breast cancers (7,33,34). To examine whether phospho-Akt expression in human primary breast cancer is related to other potential prognostic variables, assays in 49 human breast carcinoma specimens were performed, including 40 ductal invasive adenocarcinomas, 4 mucinous carcinomas, 3 lobular adenocarcinomas and 2 mixed tumours. Lysates from tumour specimens were incubated with an Akt antibody which recognizes all three isoforms of Akt. The results revealed an elevated level of Akt in all specimens examined (Fig. 1); however, Akt expression was not significantly changed in comparison to Akt activation (Ser473 phosphorylation). To further demonstrate phospho-Akt activation in breast cancer, Western blot analysis of tumour lysates with phospho-Ser473 antibody was also performed. Phospho-Akt was expressed in 88% of primary breast tumours with the highest frequency in invasive ductal carcinoma (NOS). Akt regulates many of the processes associated with metastatic progression as well as inhibiting apoptotis (34,35). In the present study, overexpression of activated phospho-Akt, observed in only 12% of breast cancer specimens examined, was alone insufficient to drive breast tumour growth, suggesting that additional factors must contribute to the ability of breast cells to form and grow tumours.

Western blot analysis revealed that 72% of the cases (18 of 25) with intermediate phospho-Akt level showed ER positivity. A significant correlation was found between Akt activation and ER status. These findings suggest that Akt expression and activity correlate with a potentially important prognostic marker for breast cancer. Moreover, activation of PI3K by ER resulted in activation of Akt (14). This may play an important role in pathogenesis of human breast cancer (12), and could contribute to ligand-independent breast cancer cell growth.

During the past decade, a series of publications from different laboratories have consistently reported the survival role for the PI3K/Akt pathway in cancer cells (32,36,37) and a significant role in the pathogenesis of tumours (8,13,17,38). Although several human carcinoma studies have shown an association between Akt activity levels and patient survival and/or prognosis (11,39-42), the present study is, to our knowledge, the first attempt to control for all other prognostic factors that may obscure the true role of phospho-Akt expression in patient survival. In this retrospective breast carcinoma population, it was demonstrated that primary breast cancer is accompanied by expression of phosphorylated Akt. Additionally, the present findings on phospho-Akt add new data to the emerging picture of the relationship between breast tumour tissue levels of phospho-Akt and patient survival, and demonstrated intermediate levels of phospho-Akt in breast tumour tissue are associated with a lower probability of developing recurrences and of experiencing an early death. Also, in multivariate analyses, which also included classical prognostic parameters, none of the levels of phospho-Akt appeared to be independent predictors of recurrence and death.

In this study, as previously reported (11), no significant correlation between phospho-Akt and nodal status was observed, whereas a trend between phospho-Akt levels and tumour size was detected. In agreement with others (38), no significant correlation was observed between the histological differentiation of tumours and phospho-Akt levels. Notably, the intensity of phospho-Akt was often higher in grade II invasive breast cancers.

These observations and the central role played by Akt in several growth factor-activated signal transduction pathways, suggest that Akt and phospho-Akt may be promising molecular targets for therapeutic intervention in this malignancy. The main consequence of Akt activation is obviously not directly coupled to increased metastatic behaviour, but this oncogene may need to be accompanied by additional modulating genetic events to exert its full effect in progressive systemic disease. The relative levels of phospho-Akt in primary breast tumours were not regulated in discernible patterns that would suggest commonalities in the mechanisms that control the expression of this protein. Thus, for the most part, phospho-Akt is probably independently regulated. This observation suggests that the genetic and environmental factors that account for dysregulation of programmed cell death pathways in breast cancer are likely to be multifactorial. This could be ascribed to the fact that there are indeed several different molecules whose interplay is important for regulating the apoptotic pathway explained for example by the fact that Akt expression is oestrogen-regulated.

A simple, yet less expensive technique employing staining with DAB, is described for visualization of proteins transferred to PVDF. Two simple protocols for detecting phospho-Akt protein on PVDF membranes were performed, one involving ECL-based detection and the other a colorimetric readout using a DAB staining procedure modified for use with PVDF membranes. This modified protocol provides a convenient immunoblotting method that permits detection of phospho-Akt. The PVDF-DAB staining procedure has a similar sensitivity to the commonly used ECL method and thus provides an alternative to radiochemical detection. By allowing this amount of data to be obtained from a single membrane, the ECL and PVDF-DAB Western methods have the potential to markedly streamline the work involved for

researchers who wish to compare the expression of proteins within biological samples.

In conclusion, this study demonstrates the relevance of phospho-Akt expression *in vivo* in breast carcinomas and reveals a possible influence on its expression, which may further improve the definition of disease outcome.

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