EUROPEAN JOURNAL OF CANCER 45 (2009) 694–704

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Prognostic significance of Akt, phospho-Akt and BAD expression in primary breast cancer

Yousif O. Al-Bazz[a], *James C.E. Underwood*[b], *Barry L. Brown*[a], *Pauline R.M. Dobson*[a,*]

[a]*Section of Endocrinology and Reproduction, University of Sheffield Medical School, Academic Unit of Endocrinology, Beech Hill Road, Sheffield S10 2RX, UK*

[b]*Academic Unit of Pathology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, UK*

ARTICLE INFO

Article history:
Received 20 August 2008
Received in revised form 13 November 2008
Accepted 18 November 2008
Available online 12 January 2009

Keywords:
Akt
Phospho-Akt
BAD
Immunohistochemistry
Breast cancer

ABSTRACT

Apoptotic markers in breast cancer are reported to have prognostic significance. The aim of this study was to assess the prognostic value of Akt, phospho-Akt and BAD expression in primary tumours from breast cancer patients. Expression of phospho-Akt did not correlate with menopausal status, nodal involvement or tumour size, although there was a significant correlation between phospho-Akt and oestrogen receptor status and tumour grade. No association was found between phospho-Akt and BAD. However, a significant correlation was found between Akt and BAD. Akt and phospho-Akt expression did not correlate with either disease-free survival (DFS) or overall survival (OS). Conversely, BAD immunostaining correlated significantly with increasing tumour size and with oestrogen receptor (ER) immunostaining in both frozen and paraffin sections. Expression of BAD appeared to be nucleolar in addition to its cytoplasmic and nuclear staining. Comparison of immunohistochemical staining on frozen sections and paraffin sections showed a reasonable concordance in Akt and BAD immunoreactivity. However, the results showed for the first time that strong BAD expression is related to a favourable prognosis but is not an independent prognostic factor. In conclusion, these results could provide the basis for understanding how Akt, phospho-Akt and BAD expression contributes to the prognosis of invasive breast cancer.

1. Introduction

Defective-programmed cell death mechanisms contribute significantly to the progression of breast cancer.[1] Activation of the phosphatidylinositol 3-kinase (PI3K)/serine–threonine kinase-Akt signalling pathway promotes cell survival partly by inhibiting apoptosis through phosphorylation of the pro-apoptotic protein BAD (Bcl-X_L/Bcl-2-associated death promoter homologue) and other proteins.[2–4] This pathway has been implicated in the survival mechanisms of a number of cell types[5–8]; therefore, it may be able to promote the survival of transformed cells under physiological conditions and may influence the behaviour of many tumours.[7,9] Akt is one of the critical regulators of apoptosis.[2,10,11]

Akt is a serine/threonine kinase, which is activated by phosphorylation at Ser473, leading to signals that either stimulate anti-apoptotic cellular responses or block apoptotic functions of the cell. It was identified as a retroviral oncogene, which has a similarity to protein kinases A and C. Studies have documented that active Akt could be an indicative of poor prognosis,[12,13] probably by promoting cell survival. Expression of Akt has been specifically associated with hormone-independent primary breast cancers.[14]

* *Corresponding author:* Tel.: +44 (0) 1142261411; fax: +44 (0) 1142713892.
E-mail address: p.dobson@sheffield.ac.uk (P.R.M. Dobson).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2008.11.044

BAD (Bcl-X_L/Bcl-2-associated death promoter homologue) is a unique Bcl-2 family,[15] which is involved in the control of the apoptotic process in cells. Both the phosphorylated and unphosphorylated forms of BAD localised to the cytoplasm[16,17] and the nuclei of cells.[18] Dephosphorylated BAD appears to be active and bound to BCL-2 family members at the mitochondria, whereas when phosphorylated on serine sites (Ser-112, -136, and -155), BAD is inactive and can be bound by 14-3-3.[19] Survival factors such as IL-3, insulin-like growth factor 1 (IGF-1) and nerve growth factor can inhibit the apoptotic activity of BAD by activating intracellular signalling pathways that result in the phosphorylation of BAD. The phosphatidylinositol 3-kinase (PI3K) pathway, including Akt,[20] p70S6K and mitochondrial-tethered protein kinase A (PKA), has been implicated in the phosphorylation of BAD. The importance of BAD was especially emphasised in apoptosis that occurs in the absence of growth factors,[21] much like the importance of BAD inactivation in response to E_2,[22] which is a well-known growth factor for breast cancer cells. Recently, pro-apoptotic BAD was observed as an independent prognostic variable for stages II and III colon cancer patients[23]; however, only limited data are available on the expression of BAD in human breast cancer.

To understand further the detection, extent and significance of Akt, phospho-Akt and BAD alterations in primary breast cancer, we examined immunohistochemically a series of primary breast cancer frozen samples and a series of paraffin samples for Akt, phospho-Akt and BAD proteins.

2. Materials and methods

2.1. Culture of breast cancer cell lines

To determine whether Akt/PKB and BAD are expressed in human breast cancer cells, we analysed three human breast cancer cell lines; two oestrogen-positive cell lines (MCF-7 and T47D) and one oestrogen-negative cell line (MDA-MB231). The cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS). Adherent cell cultures were harvested using 0.25% (v/v) of trypsin. The cells were then washed in phosphate-buffered saline (PBS) and were fixed with 1% cooled paraformaldehyde. The cells were mixed frequently and then centrifuged. The pellet was resuspended and fixed in 70% cooled methanol for 15 min. Cells were used directly or stored in solution for longer periods. Fixed cells (T47D, MCF-7 and MDA-MB231) were spun onto slides at 1500 rpm for 5 min. Fixed slides were either immunostained directly or the following day.

2.2. Patient population for fixed breast cancer samples

Primary breast cancer specimens were obtained from 106 patients from 1994 to 1997. The protocol was approved by the South Sheffield Research Ethics Committee. Clinical and pathological information obtained from the patients' medical records included 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. Six women had undergone a previous hysterectomy and their menopausal status was not assessable. None of the patients had received chemotherapy or radiotherapy before the study. After exclusion criteria had been applied, histological slides from suitable patients for the study were investigated. The histopathology of tumours was assessed on sections stained by the haematoxylin and eosin (H&E) method.

2.3. Patient population for frozen breast cancer samples

Primary breast cancer specimens obtained from 51 patients, were kept in a bank of tissue frozen in liquid nitrogen; 31 cases overlapped with patient material stored as wax sections. In this group, thirteen patients died from breast cancer, three patients were alive with recurrent cancer, and 35 patients were alive and free of disease. Similar clinical and pathological data were obtained as for fixed samples.

2.4. Immunohistochemical analysis

2.4.1. Cell lines

For immunohistochemical analysis, slides were incubated with primary anti-human antibody as follows: polyclonal rabbit anti-human BAD protein (SeroTec, Oxford, UK, Cat. No. AHP475) 1:200; Akt antibody (Ser473; Cell Signalling Technology, Beverly, MA, Cat. No. 9272) 1:25; 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, Glostrup, Denmark, clone 1A6, Code No. M3529) 1:20 prepared in PBS supplemented with 0.1% bovine serum albumin (BSA).

2.4.2. Paraffin sections

Five micron sections were deparaffinised with xylene and rehydrated in a series of ethanols. Endogenous peroxidase was blocked by 1% hydrogen peroxide in methanol. After washing in tap water, the slides were microwaved for antigen retrieval in 0.01 M tri-sodium citrate for 4 min on high power and 10 min on simmer power, then left at room temperature for 20 min. The sections were circumscribed with a PAP pen (Dako, Glostrup, Denmark, Cat. No. S2002) to contain the immunohistochemical reagents in contact with the tissue. Non-specific staining was blocked by incubating the slides with 1.5–2.5% normal horse/goat serum diluted in phosphate-buffered saline (PBS; pH 7.3). Excess serum was drained off before the addition of primary anti-human antibody as follows: polyclonal rabbit anti-human BAD protein (SeroTec, Oxford) 1:200; phospho-Akt antibody (Ser473; Cell Signalling Technology, Beverly, MA, Cat. No. 9277, IHC specific) 1:50, anti-human oestrogen receptor (ER; Monoclonal Mouse, Dako) 1:100, and anti-human progesterone receptor (PgR; Monoclonal Mouse) 1:20. The antisera were diluted in PBS containing 1.5% normal horse/goat serum. Slides were then incubated overnight at 4 °C in humid conditions. After washing with PBS, the slides were processed for the detection of positive immunohistochemical reaction by incubating them with biotinylated goat anti-rabbit or horse anti-mouse antibody, and then with a avidin-biotin peroxidase complex (Vectastain

ABC rabbit/mouse Elite, Vector Laboratories, Burlingame, CA, Cat. No. BA-1000 & BA-2000). The slides were stained with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA, Cat. No. SK-4100). After counterstaining with Gill's haematoxylin, the sections were cleared and mounted. For negative controls, the primary antibody was replaced with PBS containing 1% bovine serum albumin.

2.4.3. *Frozen sections*

Cryostat sections of the frozen breast tumours cut at 6 μm were collected on 3-aminopropyltriethoxysilane (APES)-coated slides, then put in a humidifying chamber at room temperature for 10 min. The slides were fixed in acetone and were air dried. Slides were incubated in 1% hydrogen peroxide (H_2O_2) in methanol to block the endogenous peroxidase activity. Non-specific antibody binding was blocked with PBS/2.5% normal goat serum. Test sections were subsequently incubated with anti-BAD protein 1:200, anti-oestrogen receptor at 1:100 or anti-progesterone receptor at 1:10 prepared in PBS supplemented with 0.1% BSA. Following this step, the rest of the staining protocol was similar to that for paraffin sections. In some of the cases, adjacent areas of normal breast epithelium were also available for assessment along with invasive carcinoma.

2.4.4. *Akt and phospho-Akt*

Tumour sections were cut at 5 or 6 μm from paraffin-embedded or frozen specimens, respectively. Sections were washed in tris-buffered saline (TBS) containing 0.1% Triton X-100 (TBST) and were treated with freshly made 1% H_2O_2 in TBS, then washed with TBS Triton, three. Non-specific binding sites were blocked with 3% BSA in TBST. Slides were incubated with the following primary antibodies: Akt antibody (Ser473; Cell Signalling Technology) 1:100 for frozen sections, and phospho-Akt antibody (pSer473; Cell Signalling Technology) 1:50 diluted in 3% BSA/TBST for paraffin sections. The sections were rinsed in TBST, three times for 10 min each before incubating the sections in biotinylated anti-rabbit secondary antibody diluted in 1% BSA/TBST. Sections were then washed in TBST, incubated in the ABC solution (Vector Laboratories), washed in TBST and then incubated in 3,3-diaminobenzidine (DAB) solution until staining was optimal. Sections were finally counterstained with haematoxylin, cleared and mounted.

2.5. *Immunohistochemical scoring*

All immunostaining was assessed in a blinded fashion without any knowledge of the clinical outcome or other clinicopathological data. Staining for Akt/PKB, phospho-Akt and BAD was visually classified into four groups: no staining present in any of the breast cancer cells (–), slight staining in some cells or in most of the cells (+), moderately strong staining (++) or strong staining present in almost all cells (+++). Classification was done by a pathologist who is experienced in breast pathology and in the assessment of immunohistochemical staining. To test the repeatability of classification, another investigator independently classified the same slides without the knowledge of the previous classification or other data.

2.6. *Statistical analysis*

For statistical analysis, groups stained – and + were combined and scored 0, and groups stained ++ and +++ were scored 1. Relationships among the variables were determined using a variety of statistical techniques. The 2×2 contingency tables were analysed using the χ^2 test. The Bonferroni test for multiple comparisons was used in identifying differences between pairs of means. The Kappa concordance test was used to measure the degree of concordance observed between two methods on the same data set.[24] Spearman's rank correlation coefficient was used to examine the degree of correlation between phospho-Akt or BAD and other immunohistochemical variables. The probability of disease-free survival (DFS) or overall survival (OS) was calculated using the Kaplan–Meier methods. The Cox regression model was used to identify which factors are jointly significant in their association with OS or DFS. A *P*-value less than 0.05 was considered significant.

3. Results

3.1. *Measurement of Akt/PKB & BAD protein levels in breast cancer cell lines*

The extent of Akt/PKB and BAD expression was determined by two independent pathologists as described in Section 2. As previously been reported,[25] Akt expression was high in oestrogen-positive cells (MCF-7 and T47D) (Fig. 1, A1 and A3), but highest in oestrogen-negative cells (MDA-MB231) (Fig. 1, A2). Overall, these findings were also consistent with the previous reports demonstrating cytoplasmic and nuclear localisation of Akt/PKB[26,27] in the cells. Expression of BAD in MCF-7, MDA-MB231 and T47D cells was high (Fig. 1, B1–B3).

3.2. *Expression of Akt/PKB and BAD in normal human breast tissue*

Expression patterns of the Akt/PKB and BAD in normal breast tissue have also been examined. The data revealed that the majority of normal breast tissues expressed Akt/PKB (Fig. 2A); therefore, these findings suggest that Akt/PKB may be expressed in normal tissue, tumour tissue or both. These data are consistent with previously published reports[28–30] indicating that both normal and tumour tissues express Akt/PKB. However, BAD immunostaining was not seen in the normal breast tissue (Fig. 2B).

3.3. *Association between Akt and BAD in frozen sections and their relationship to clinicopathological criteria*

Akt and BAD were detected by immunohistochemistry in 17/51 (33%) and 26/51 (51%) of the primary breast cancer specimens, respectively (data not shown), and a significant correlation was found between them ($P = 0.009$). The correlation between the expression of Akt, BAD and clinicopathological variables is clearly different for these two protein markers (Table 1). Expression of the Akt did not correlate with ER or PgR status. Conversely, BAD immunostaining displayed a significant correlation with increasing tumour size and age ($P < 0.05$) and a positive correlation with stronger ER immuno-

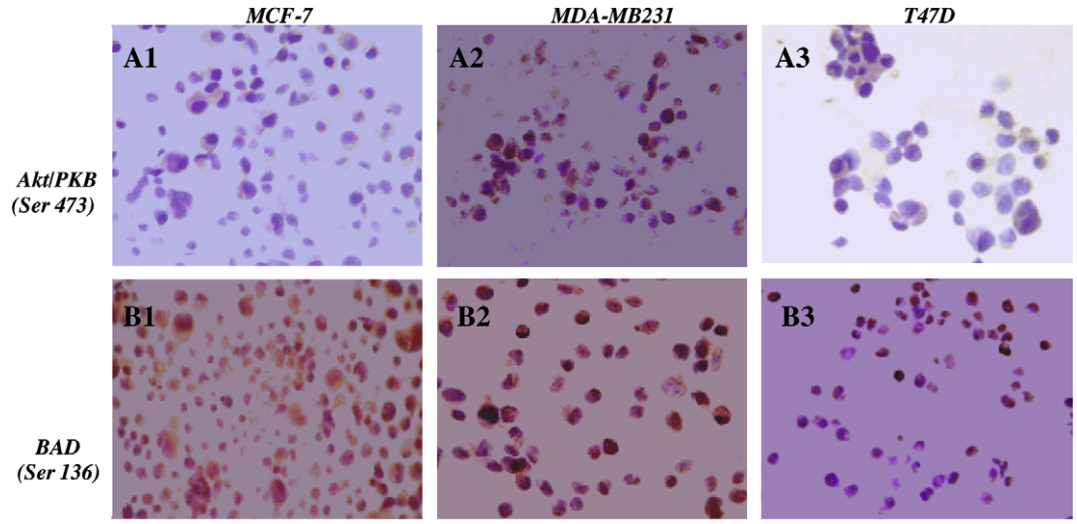

Fig. 1 – Expression of Akt/PKB (Ser 473) and BAD (Ser 136) in MCF-7, MDA-MB231 and T47D human breast cancer cells using immunohistochemical analysis shows cytoplasmic and nuclear expression of Akt/PKB and BAD antibodies. Fixed cytospin breast cancer cell lines (MCF-7, MDA-MB231 and T47D) were analysed using immunohistochemistry. Fixed cells were incubated with specific primary antibodies to Akt/PKB and BAD at 1:25 and 1:200 dilutions (respectively) and visualised under light microscopy. The cells show positive immunostaining for both Akt/PKB and BAD. Both cytoplasmic and nuclear immunoreactivity can be observed. These figures represent one of three experiments showing similar results.

staining ($r = 0.33$, $P = 0.017$). Neither BAD nor Akt expression correlated with menopausal status, histological grade, histological type or axillary lymph node status. Overall, 11 of 15 (73.3%) high grade tumours expressed low levels of Akt, whereas 9 of 15 (60%) high grade tumours expressed high levels of BAD. In contrast, 6 of 7 (85.7%) low grade cases expressed low levels of Akt, whereas 4 of 7 (57.1%) low grade cases expressed increased levels of BAD. In addition, 11 of 31 (35.5%) tumours $\geqslant$21 mm expressed Akt, whereas 20 of 31 (64.5%) of such tumours expressed BAD. Although the case numbers are modest, these findings could indicate that BAD protein may have potential to be an indicator for disease prognosis.

3.4. *Relationship between tumour phospho-Akt, BAD expression and patient/tumour characteristic in paraffin tissue samples*

Phospho-Akt (Ser473) expression in 106 paraffin-embedded primary breast carcinomas was scored and correlated with the established clinical and histological parameter of prognostic significance (Table 2). The coded phospho-Akt scores were correlated with similarly coded test-parameter results. In 78% of the samples, there was either no reactivity or very little expression, and it was scored negative; 22% showed overexpression. Of all the tumours evaluated by phospho-Akt (Ser473) antibody, 62 tumours (59%) were ER positive, 42 tumours (40%) were PgR positive, 32 tumours (30.5%) were ER positive and PgR positive, 30 tumours (28.6%) were ER positive and PgR negative, 10 tumours (9.5%) were ER negative and PgR positive and 33 tumours (31.4%) were ER negative and PgR negative. There was a significant correlation between phospho-Akt and ER ($P = 0.008$) and PgR ($P = 0.02$). A similar correlation was observed with the age of the patients with $P = 0.022$ and histological grade ($P = 0.00046$). No significant correlation was observed between phospho-Akt and menopausal status, nodal involvement or tumour size. No association was found between phospho-Akt and BAD.

Immunopositivity of BAD protein was found in 35% of the tumours (Fig. 3C and D), and 65% were either completely negative or showed only weak staining (Fig. 3A and B). No immunoreactivity was observed in non-neoplastic cells and no staining was noted in negative control tissue. Considering all the cases, BAD expression was associated with nodal status ($P = 0.03$). Low levels or negative BAD expression was observed in 12 of 18 (66.7%) grade I tumours, 30 of 57 (52.6%) grade II tumours and in 27 of 31 (87%) grade III tumours ($P = 0.005$, χ^2 test). BAD was also significantly associated with tumour size ($P = 0.03$) and age of the patients ($P = 0.024$). There was no significant association between the menopausal status and the expression of BAD protein.

3.5. *Intracellular localisation of Akt/PKB, phospho-Akt and BAD in invasive breast cancer cells*

Microscopy revealed that Akt and phospho-Akt were distributed homogeneously throughout the cytoplasm in most of the primary breast tumours, although nuclear immunostaining was also observed (Figs. 1, A1–A3 and 4). Intracellular localisation of endogenous BAD protein was also detected by immunohistochemistry. As shown in Fig. 1, B1–B3, BAD localised diffusely in the cytoplasm and nucleus; in some tumour cells, high magnification revealed BAD nucleolar staining (data not shown); several breast tumours exhibited intense immunostaining.

3.6. *Association between immunohistochemical results and survival data*

Akt/PKB expression in relation to DFS and OS in all patients and in subgroups of patients was not associated with any

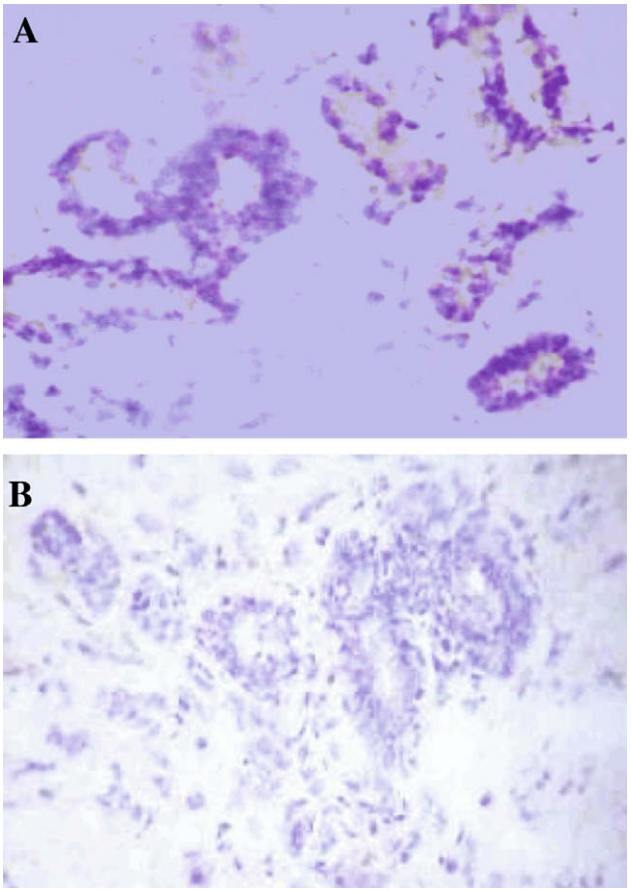

Fig. 2 – Immunohistochemical analyses of Akt/PKB and BAD expressions in normal breast tissue. Expression patterns of Akt/PKB and BAD in normal breast tissue: (A) most of the mammary epithelial cells exhibit positive immunostaining with Akt/PKB polyclonal antibody and (B) shows negative BAD immunostaining.

difference in clinical outcome (log-rank test, $P > 0.05$; Table 3). Again in the phospho-Akt patients group, univariate analysis revealed no statistical difference in DFS (log-rank test, $P > 0.05$) between phospho-Akt negative and phospho-Akt positive tumours (Table 4). In contrast, DFS and OS of patients were significantly better for those with moderate or strong BAD tumour expression ($P = 0.0032$ Fig. 5i and $P = 0.0103$ Fig. 5ii, respectively) as estimated by the Kaplan–Meier method. Multivariate analysis by Cox regression analysis following adjustment for nodal and ER status did not show any difference in clinical outcome (data not shown). Although BAD protein expression was not a significant factor in multivariate analysis, BAD might be a useful predictor for prognosis as it is significantly correlated with ER expression and DFS.

3.7. *Comparison of immunohistochemistry analysis*

The frequency of expression of Akt/PKB and BAD in fixed breast cancer tissue was comparable to the frequency of Akt/PKB and BAD expression in frozen sections determined by immunohistochemical analysis for 31 patients with primary breast tumours. To determine whether the protein expression by immunohistochemistry for frozen samples correlated with the expression by IHC for paraffin, Kappa concordance statistic for Akt/PKB and BAD was calculated (Tables 5 and 6). The concordance between immunohistochemistry frozen and immunohistochemistry paraffin for Akt/PKB and BAD was statistically significant. Comparison of immunohistochemical staining for frozen samples with that for paraffin samples showed a good agreement between the two methods: Akt/PKB and BAD expression was observed in 74% and 77% of the cases, respectively. However, as evidenced by the Spearman correlation coefficients, the BAD correlation (0.6) between methods was considerably stronger than the Akt/PKB correlation. Positive staining for Akt/PKB in paraffin was found in 13 of 31 (42%) of breast carcinoma, while 17 of them exhibited an elevated Akt/PKB and 14 cases without Akt/PKB in frozen specimens (Table 5). BAD staining of tumour cells was observed in 31 cases including 19 with and 12 without detected by immunohistochemistry analysis for the frozen sections.

4. Discussion

We have observed that overexpression of BAD protein was detectable in over a third (35%) of primary breast carcinomas in paraffin-embedded tissue and in half of the frozen tissue samples. A positive association was noted between BAD and ER immunoreactivity. Oestrogen receptor expression is one of the most important factors in determining the prognosis for women with primary breast cancer.[31] High apoptosis and proliferation rates in breast tumours are related to oestrogen receptor status.[32] BAD expression was significantly associated with tumour size, but not with lymph node status in frozen samples; absence of BAD expression was significantly correlated with lymph node metastasis in paraffin breast samples. In addition, the results showed that absence of BAD expression was significantly associated with higher grade tumours. The results also indicated a significant relationship between BAD expression and patients' age.

In univariate analysis, BAD expression was a statistically significant indicator of longer disease-free survival of the patients, although in multivariate analysis it did not retain its prognostic value. In the series of paraffin cases, BAD expression was also related to improved survival in patients; this could be explained by the fact that BAD is a death regulator and, therefore, its expression could be a good sign, while its lack is an indication of poor prognosis. These data suggest that evaluation of BAD could be a promising prognostic indicator in invasive breast carcinoma. The mechanism by which BAD is related to the biological pathology of mammary tumours is not yet resolved, but it is postulated that increased amounts of BAD may permanently activate a signal for tumour cell death. This may have important clinical implications in the management of patients with breast cancer. Our findings on BAD protein add new data to the emerging picture of the association between and longer survival of breast cancer patients. It should be noted that, apart from lymph node involvement, many of the parameters classically associated with poor prognosis were not significantly related to clinical outcome in this study. The reason for this is not

Table 1 – Relationship of Akt/PKB and BAD immunohistochemical expression in 51 patients with primary breast cancer.

Factors	Total $n = 51$[a]	Positive Akt/PKB staining (%)	P-Value	Positive BAD staining (%)	P-Value
Age (years)					
⩽50	12	4 (33.3)	0.54	3 (25)	0.03
>50	39	13 (35)		23 (59)	
ER positive	29	8 (27.6)	0.25	19 (65.5)	0.017
ER negative	22	9 (40.9)		7 (31.8)	
Menopausal status					
Pre-menopause	6	3 (50)	0.3	1 (16.7)	0.063
Post-menopause	42	14 (33.3)		24 (57.1)	
Node positive	23	7 (30.4)	0.64	12 (52.2)	0.87
Node negative	28	10 (35.7)		14 (50)	
Tumour size					
<2 cm	20	6 (30)	0.66	6 (30)	0.016
>2 cm	31	11 (35.5)		20 (64.5)	
Histological grade					
Grade I	7	1 (14.3)	0.31	4 (57.1)	0.59
Grade II	29	12 (41.4)		16 (55.2)	
Grade III	15	4 (26.7)		6 (40)	

This table summarises 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 the Pearson χ^2 using contingency table for coded values. Immunointensity scores for invasive breast cancer were evaluated from 51 breast cancer frozen tumours, scoring of the intensity on an arbitrary two-point scales as: negative = none and one plus and positive = two plus and three plus.

a IDC, invasive ductal carcinoma; other, non-IDC tumours (lobular [3 cases], mucinous [4 cases], tubular [one case] and papillary [one case]).

Table 2 – Relationship of phospho-Akt, BAD immunohistochemical expression and clinicopathological variables in 106 breast cancer patients.

Factors	Total $n = 106$[a]	Positive phospho-Akt staining (%)	P-Value	Positive BAD staining (%)	
Age (years)					
⩽50	23	9 (39.1)	0.022	6 (26.1)	0.024
>50	83	14 (16.9)		31 (37.3)	
ER positive	62	19 (30.6)	0.008	28 (45.2)	0.0085
ER negative	44	4 (9.1)		9 (20.5)	
PgR[b] positive	42	14 (33.3)	0.02	16 (38.1)	0.61
PgR negative	63	9 (14.3)		21 (33.3)	
Menopausal status					
Pre-menopause	23	8 (34.8)	0.07	9 (39.1)	0.66
Post-menopause	70	13 (17.1)		26 (34.2)	
Node positive	32	9 (28.1)	0.29	16 (50)	0.015
Node negative	74	14 (18.9)		21 (28.4)	
Tumour size					
<2 cm	70	17 (24.3)	0.28	20 (28.6)	0.03
>2 cm	36	6 (16.7)		17 (47.2)	
Histological grade					
Grade I	18	10 (55.6)	0.00046	6 (33.3)	0.005
Grade II	57	10 (17.5)		27 (47.4)	
Grade III	31	3 (9.7)		4 (12.9)	

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a IDC, invasive ductal carcinoma; other, non-IDC tumours are lobular 10 cases, mucinous five cases, tubular eight cases, papillary two cases, and one case medullary.

b For progesterone status, there was one missing datum.

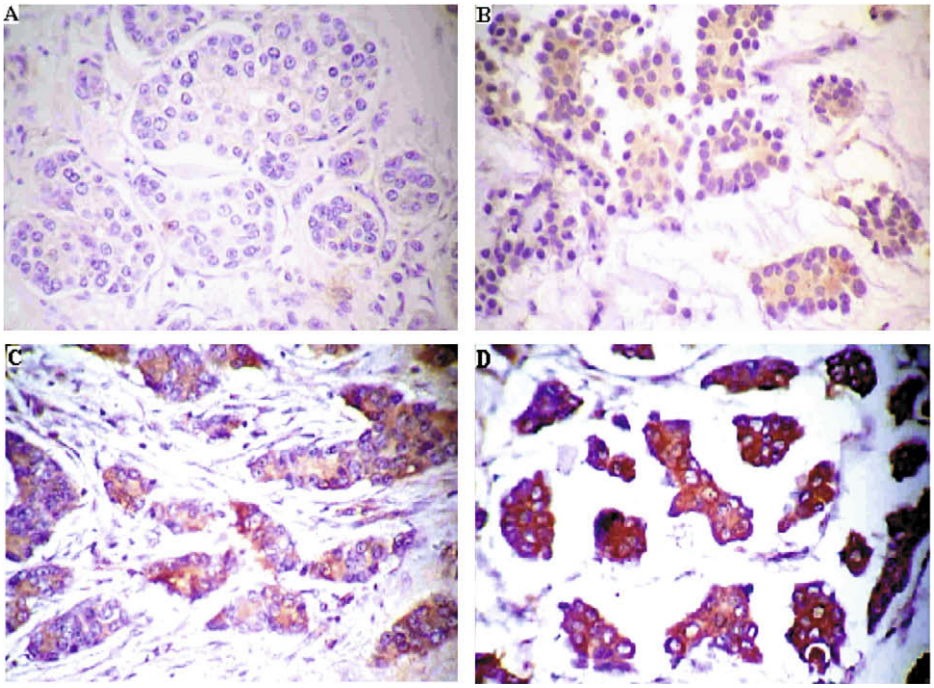

Fig. 3 – Immunohistochemical staining of BAD in paraffin-embedded human breast tumours using anti-BAD antibody. Representative immunohistochemical staining of BAD paraffin fixed specimens. Image A: negative control (lack of BAD staining in invasive ductal carcinoma of breast); image B: invasive breast tissue that was graded as BAD negative because it showed weak staining; image C: invasive ductal carcinoma showing moderate cytoplasmic expression for BAD and image D: invasive ductal carcinoma showing strong staining for BAD.

readily apparent and, although the study population is relatively small, no selection bias is immediately obvious. However, the importance of BAD and its identification as a prognostic parameter in breast cancer patients has been emphasised.

Akt has been demonstrated to be one of the major survival proteins in many cell types.[33] However, in contrast to our findings with BAD, and despite studies *in vitro* suggesting a crucial involvement of Akt in breast cancer, this study revealed that Akt was not associated with any other prognostic markers, despite a positive correlation between Akt and BAD. In good agreement with other studies,[34,35] we found that both Akt and p-Akt did not provide any new information regarding the survival of the patients. However, the results in this work are inconsistent with the other observations that correlate high Akt with negative oestrogen status.[14,34] Although up-regulated Akt-3 expression has been implicated in the progression of human breast cancer, Zinda et al.[28] showed that there was no particular relationship between hormone receptor status and Akt-3. Variations in the Akt results could be due to the type of antibodies used, differences in the patient population and the total number of patients studied. In addition, the previous reports[36,37] and the present study demonstrated that Akt/PKB is expressed in both primary human breast cancers and normal tissues (Fig. 2). Together, these data suggest that there may be no difference in the expression of Akt/PKB in normal and tumour tissues, and that its expression is not restricted to tumourigenic cells or ER negative breast cancer. Therefore, and in agreement with the previous study,[28] the data in this work suggest that Akt patterns are not dramatically involved in invasive breast cancer progression.

In agreement with our findings, Akt had previously been shown to be located in the cytoplasm and nucleus in several cases.[38,39] In contrast, although it has been shown that BAD phosphorylation is accompanied by translocation of BAD from mitochondria to the cytosol,[17,40] others revealed that as we have demonstrated, in primary human tumours, BAD in addition to being distributed between the cytoplasm and the nucleus[18,41] is also located in the nucleoli, suggesting that it is possible that activated endogenous BAD may even translocate to nucleoli.

Oestrogens are believed to modulate the expression of Akt, phospho-Akt and downstream anti-apoptotic signalling through activation of PI3K.[42,25,43–46] Consistent with the previous reports,[14] an oestrogen/phospho-Akt relationship has been observed. However, no significant association of phospho-Akt with nodal status or tumour size has been found, but a correlation with PgR status was observed. A higher prevalence of weak staining was found in moderately differentiated carcinomas than in poorly differentiated carcinomas in fixed paraffin wax sections (Table 1). In contrast to the other studies,[45,47] a strong inverse association between phospho-Akt expression and tumour grade has been observed, indicating that Akt activation may be associated with tumour initiation rather than progression. However, it is at variance with the result of Perez-Tenorio and Stal,[13] who reported that phospho-Akt expression in breast carcinomas was not associated with tumour grade, and was not related to ER or nodal status.

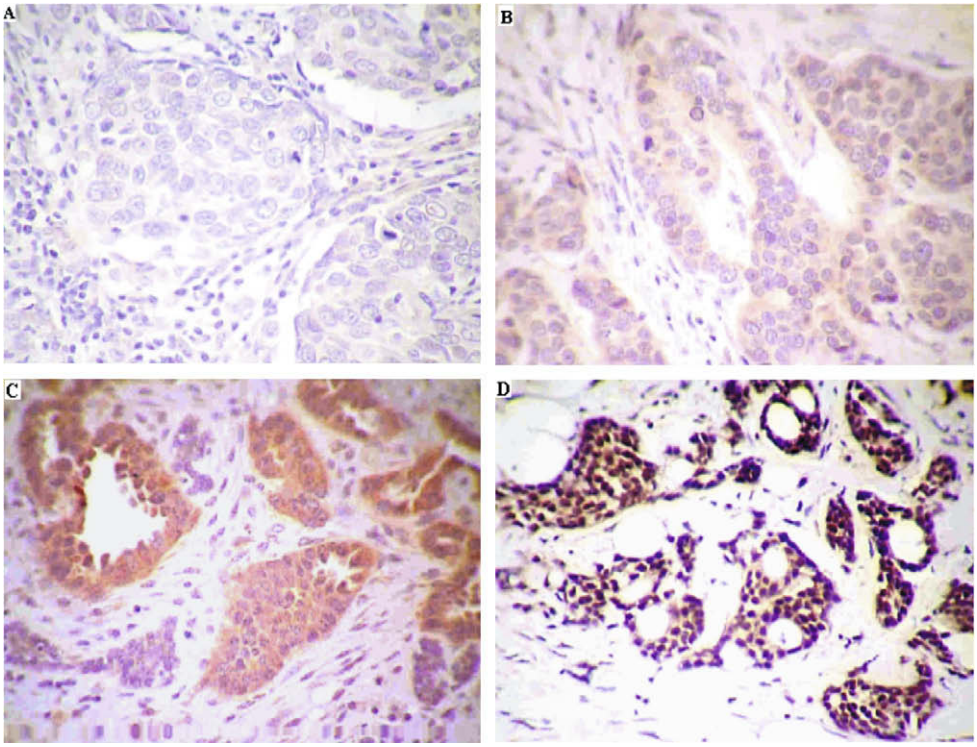

Fig. 4 – Immunohistochemical staining of phosphorylated Akt in paraffin-embedded human breast tumours using phospho-Akt (Ser473) antibody (IHC specific). Representative immunohistochemical staining of phospho-Akt paraffin fixed specimens. Image A: negative control (lack of phospho-Akt staining in invasive ductal carcinoma of breast); image B: invasive breast tissue that was graded as phospho-Akt negative because it showed weak cytoplasmic staining; image C: invasive breast carcinoma staining positive (two plus) for phospho-Akt and image D: invasive cribriform carcinoma showing three plus staining for phospho-Akt.

Table 3 – Univariate Cox analysis for survival in breast cancer constructed on 51 patients[a].

Factors	Disease-free survival			Overall survival		
	P-Value (univariate)	RR	95% CI for RR	*P*-Value (univariate)	RR	95% CI for RR
Akt/PKB expression	0.63	0.87	0.48–1.56	0.54	0.8	0.46–1.5
BAD expression	0.0361	0.17	0.11–0.27	0.33	1.33	0.75–2.34

Note: Only BAD positivity was associated with disease-free survival.
Abbreviations: RR, relative risk and CI, confidence interval.
a About 51 frozen tumours were evaluated.

Table 4 – Univariate Cox analyses of clinicopathological factors for survival in breast cancer constructed on 106 patients[a].

Variables	Disease-free survival			Overall survival		
	P-Value (univariate)	RR	95% CI for RR	*P*-Value (univariate)	RR	95% CI for RR
ER [b]status	0.001	0.33	0.15–0.73	<0.001	0.37	0.17–0.79
Histological grade	0.49	0.8	0.5–1.5	0.53	0.9	0.6–1.4
Lymph node status	0.03	0.16	0.1–0.25	0.04	0.15	0.10–0.24
Phospho-Akt	0.9	1.005	0.63–1.6	0.68	0.89	0.55–1.5
BAD	0.02	0.63	0.42–0.93	0.033	0.64	0.43–0.97

Variables examined for possible association with disease-free and overall survival included lymph node status (LN), histological grade and immunohistochemical parameters.
Abbreviations: RR, relative risk associated with dying or progressing while being in a greater risk category compared with that of being in lower risk category.
a About 106 paraffin samples were evaluated.
b Oestrogen receptor status defined using an immunohistochemical method; cut-off 10% of reactive cells.

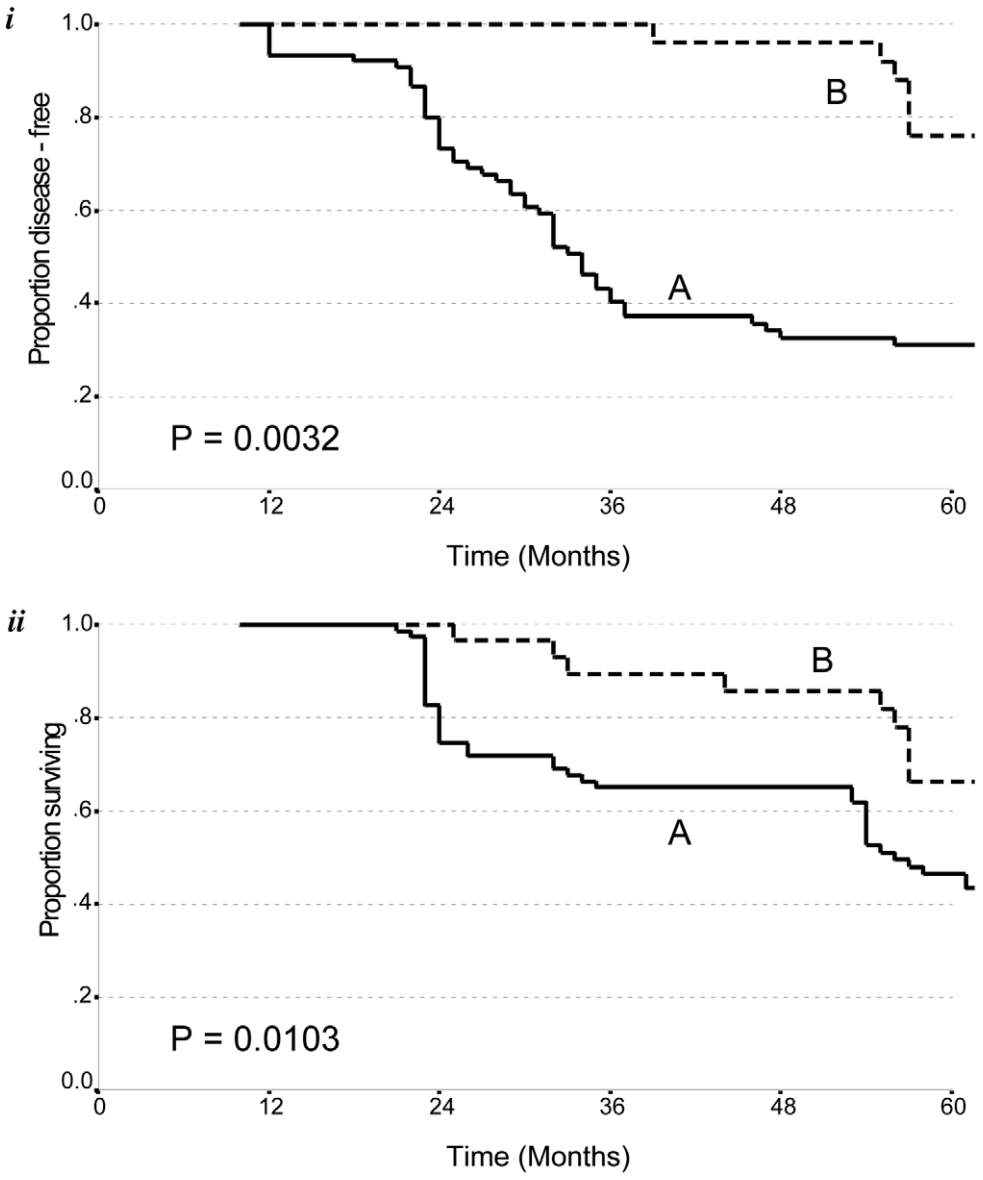

Fig. 5 – Kaplan–Meier plots for survival for BAD positive cases compared with BAD negative cases in patients with primary breast cancer. Survival curves for 106 patients stratified by BAD immunohistochemical reactivity. Group A represents 69 patients having invasive breast tumours with weak or no BAD expression. Group B represents 37 patients with BAD overexpression tumours: (i) shows disease-free survival and (ii) shows overall survival.

Table 5 – Agreement of Akt/PKB protein positivity and negativity determined by immunohistochemistry for paraffin and frozen specimens.

IHC (paraffin sections)	IHC (frozen sections)	
	Negative (%)	Positive (%)
Negative	12 (38.7)	6 (19.4)
Positive	2 (6.5)	11 (35.5)

IHC: immunohistochemistry.
Kappa = 0.5. Kappa measures the agreement between two rates when both are rating the same object. The difference between the observed proportion of cases in which the rates agree and the proportion expected by chance is divided by the maximum difference possible between the observed and expected proportions, given the marginal totals. A value of 1 indicates perfect agreement. A value of 0 indicates that agreement is no better than chance.
Negative IHC, tumour cells showed no or weak staining (– or +).
Positive IHC, tumour cells showed moderate or strong immunostaining (++ or +++).

Table 6 – Agreement of BAD protein positivity and negativity by immunohistochemistry for paraffin and frozen specimens.

IHC[a] (paraffin sections) (%)	IHC[b] (frozen sections)	
	Negative (%)	Positive (%)
Negative	10 (32.3)	5 (6.5)
Positive	2 (16.1)	14 (42.2)

Kappa =0.55.
a Negative IHC, tumour cells showed no or weak staining (– or +).
b Positive IHC, tumour cells showed moderate or strong immunostaining (++ or +++).

Other studies have shown discrepant results for the expression of phospho-Akt in breast tumours[13] using formalin-fixed paraffin sections. This conflicting level of positivity compared with our results indicates that using routine formalin-fixed paraffin wax-embedded material may underestimate the percentage of positivity in tumours studied. In the current study, there were 26 (24.5%) patients, who were BAD positive and phospho-Akt negative. Biologically, activation of Akt results in the inhibition of pro-apoptotic signals from BAD proteins, thus enhancing cell survival,[5,48] and since it was found that phospho-Akt expression is an up-regulator for BAD,[6,49,50–52] it could be predicted that cases with high phospho-Akt expression should have low BAD levels. However, no relation was observed between phospho-Akt expression and BAD levels. Additionally, phospho-Akt expression did not provide any new information regarding the survival of the patients, despite a strong correlation with histological grade and ER status.

This study has shown a reasonable concordance in Akt and BAD immunoreactivity between the paraffin and frozen sections (Tables 5 and 6). However, a decrease in the number of positive cases from frozen to paraffin sections with Akt and BAD has been noted. This difference may be due to fragile antigenic sites being destroyed or diminished, when exposed to fixation and heat during paraffin wax processing. Alternatively, any delay in tissue handling may lead to partial proteolysis, resulting in negative immunohistochemistry (paraffin) results. These results emphasise the importance of the evaluation of multiple fixation and processing protocols in any study or inter-laboratory comparison.

In summary, we show that Akt, phospho-Akt and BAD are expressed in frozen tissue and paraffin-embedded tissue from primary breast cancers. Akt expression had no prognostic relevance in any group of patients in this study, even when alterations of their expressions were evaluated concurrently with the alterations of BAD protein. At present, and based on our analysis, it is not possible to conclude that phospho-Akt expression provides an independent prognostic value greater than that of other, more established, tumour parameters (T stage, nodal and ER status). On the other hand, the observed inverse association with biologically more aggressive breast tumours may be both important and revealing. BAD protein expression seems to provide valuable prognostic information on patients with breast carcinoma, and may be useful as an indicator of disease-free survival. In any event, the choice of fixative is critical in the demonstration and evaluation of apoptotic proteins by immunohistochemistry in breast carcinoma, and this must be taken into consideration when collecting and comparing data. These results could provide the basis for understanding how the Akt, phospho-Akt and BAD pathway contribute to human oncogenesis.

Conflict of interest statement

None declared.

Acknowledgement

We are grateful to Catherine Gelsthorpe and Orla Gallagher for their technical help with this study.

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