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Activated Akt expression in breast cancer: Correlation with p53, Hdm2 and patient outcome

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Abstract

Activation of protein kinase-B/Akt (pAkt) is mediated by oestrogen and involves HER-2 *in vitro*, to phosphorylate Hdm2 and influence p53 cytoplasmic localisation and degradation. Expression of all active Akt isoforms (pAkt) were examined, together with p53/Hdm2 subcellular expression in invasive ductal breast cancers (IDCs), to evaluate whether *in vitro* findings were related to clinical data and determine the effect on outcome. Immunohistochemical expression of serine 473 specific phosphorylated Akt (pAkt) isoforms (Akt-1,2,3) was evaluated in 97 patients, together with subcellular expression of p53/Hdm2. The results show that pAkt was evaluable in 95 patients with cytoplasmic expression in 81% and more likely to be associated with larger tumours ($P = 0.007$), with no correlation with HER-2 expression. pAkt correlated with increasing levels of cytoplasmic p53 ($P = 0.025$) and was associated with a reduced disease-free survival ($P = 0.04$; univariate). In conclusion, pAkt has implications in breast cancer growth through mechanisms inactivating p53 with an association with immunohistochemical p53 expression, which is preferentially cytoplasmic. Despite *in vitro* associations, pAkt appears to be a variable marker of HER-2 expression.

Keywords: HER-2; Hdm2; Immunohistochemistry; p53; Phospho-Akt

1. Introduction

Growth of breast cancers result from pathways driving cellular proliferation and inhibition of apoptosis, mediated by the phosphatidylinositol 3-kinase (PI3-K) induced activation of protein kinase B (PKB)/Akt [1]. Oestrogen, insulin-like growth factors as well as the epidermal growth factor (EGF) family members including HER-2, play a key role in activating PI3-K/Akt signalling [2]. Activation of Akt influences cell cycle progression through dysregulation of the cell cycle checkpoint protein p53 and its cell cycle inhibitor, $p21^{WAF1/CIP1}$ [3,4].

Human Akt exists as three closely related isoforms: Akt-1, -2 and -3 that are catalytically inactive in the cytosol and are recruited to the plasma membrane through phosphorylation activation at threonine 308 and serine 473 by PI3-K [5], with a rapid nuclear localisation of Akt [6]. All Akt-isoforms may be amplified and overexpressed in breast tumours, with Akt-1 universally predominant in most tissues, compared with Akt-2 in insulin-responsive tissues and Akt-3 in association with oestrogen receptor (ER) negative breast tumours [7–9]. *In vitro*, breast carcinoma cell lines have increased levels of Akt-1 and Akt-2 expression, compared with normal breast epithelial cells with activated Akt (pAkt)

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representative of equivalent phosphorylation sites in all Akt isoforms [7–9]. *In vivo* studies, in large part affirm the correlation between pAkt and levels of Akt-1 and Akt-2 in breast tumours [7–9].

HER-2 signalling via Akt is suggested by a variable correlation between HER-2 expression and pAkt in breast cancers, with a less consistent relationship between HER-2 and respective Akt-1 and -2 isoforms [7–9]. Potential clinical implications relate to evidence of activated Akt in tamoxifen-resistant MCF-7 breast cancer cells, with breast cancers demonstrating diminished efficacy to radiotherapy [7,11]. *In vitro* HER-2/neu activates Akt to block apoptosis through inactivation of p53 and $p21^{WAF1/CIP1}$ [3,4,12]. p53 is a nuclear transcription factor that maintains genomic integrity, in part through the universal cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ [13]. Akt targets $p21^{WAF1/CIP1}$ through phosphorylation to produce cytoplasmic $p21^{WAF1/CIP1}$ that blocks apoptosis and correlates with HER-2 expression in breast cancers [14]. Inactivation of p53 occurs through other Akt pathways that target Hdm2 to enhance p53 degradation [12]. p53 transactivates Hdm2 (Human Mdm2; Murine double minute 2) that negatively regulates p53 through ubiquitination and Hdm2-mediated nuclear export of p53 for its cytoplasmic degradation [15]. Nuclear import and export is a feature of both p53 and Hdm2 with implications for their functional regulation, such that cytoplasmic p53 is associated with poor prognostic tumours [16–18]. Both wild-type and mutant p53 are evident in cytoplasmic p53-expressing cell lines and breast cancers [17,19]. Akt phosphorylates Hdm2 to produce its nuclear localisation and enhanced degradation of p53 with some evidence of preferential nuclear Hdm2 expression in HER-2 expressing breast cancers [12,20,21].

A recent study in breast cancer shows increased activation of Akt-isoforms (pAkt) and their correlation with disease-free survival, with implications that pAkt may be important in breast cancer growth and outcome [7,8,10]. The aim of this study was to evaluate the expression of one phosphorylated site (serine 473) of all the Akt isoforms (Akt-1, -2 and -3; pAkt) in invasive ductal carcinoma (IDC) of the breast using an immunohistochemical (IHC)-specific antibody and to examine pAkt in relation to other factors involved in the pathway, such as HER-2 expression as well as p53 and Hdm2 levels and subcellular localisation on IHC. Furthermore, it was important to evaluate pAkt in relation to clinicopathological parameters and prognosis in breast cancer. We have shown pAkt to be expressed ubiquitously in the cytoplasm of 81% of IDCs, with no activation of Akt in 19% of tumours. pAkt is more likely associated with larger tumours and increasing levels of cytoplasmic p53 with implications for patient outcome.

2. Patients and methods

2.1. Patients

The study included 97 patients aged 26–88 years (median 59 years) with diagnoses of IDC of the breast between 1996 and 2000 at the Bristol Royal Infirmary, Bristol, UK (Table 2). Regional Ethics Committee approval was granted prior to commencement of the study. Forty patients underwent a wide local excision (WLE) and a mastectomy was performed in the remaining women ($n = 55$) in whom pAkt was evaluable. Axillary lymphadenopathy was evaluable in 84 patients with 34 lymph node negative, and 50 node positive (N1: mobile ipsilateral, or N2: fixed ipsilateral) patients. No axillary surgery was undertaken in the remaining 11 patients due to age-related co-morbidity. Clinicopathological subgroups were analysed according to the Nottingham prognostic index (NPI) [22]. Evaluation of the NPI was precluded in 13 patients due to non-evaluable regional lymphadenopathy and tumour size. Adjuvant treatment groups comprised: tamoxifen alone in 54 patients, CMF- and anthracycline-containing regimes in 15 and 21 patients, respectively. Five patients received no adjuvant treatment. The median follow-up duration was 51 months (range 6–120 months). All were primary tumours.

Tumour samples were collected and freshly fixed in buffered formalin according to a standardised protocol at a single institution. Tumours were classified according to the NHS breast screening programme (NHSBSP) Guidelines and were graded by the modified Bloom's grading system [23]. Oestrogen receptor (ER) immunostaining was performed according to standard methods and assessed using the quick-score (0–8) [24]. Tumour proliferation was assessed using nuclear Ki67 immunostaining as described, with tonsillar tissue used as a positive control and primary antibody substitution with Tris-buffered saline (TBS) as a negative control. The percentage of Ki67 staining was scored and analysed as a continuum on univariate and multivariate analyses, with a median cut-off of 10% ($<10\%$ *vs.* $\geqslant 10\%$), (Table 2) [25]. HER-2 immunostaining was performed and was scored according to described methods [25]. Lymphovascular invasion was assessed as present or not, and together with ER, HER-2 and Ki67 was analysed in the Department of Pathology (by CS and CC).

2.2. Immunohistochemistry

pAkt immunoreactivity, specifically phosphorylation of serine 473, was evaluated using the rabbit polyclonal antibody (Cell Signaling Technology # 9277, New England Biolabs, Beverly, MA, USA) at a dilution of 1:800 [26]. The anti-pAkt antibody is recommended specifically for immunohistochemistry and has been validated by

ELISA and flow cytometry [26]. pAkt immunostaining of IDCs was compared with formalin-fixed paraffin-embedded cell buttons, using LNCAP prostate carcinoma cells as a positive control for pAkt, and LNCAP cells treated with the specific PI3-K inhibitor (LY291002) as a negative control.

Hdm2 expression was assessed using the mouse monoclonal antibody OP46 (Oncogene Research, CN Biosciences, Nottingham, UK) that detects C-terminal Hdm2 with specificity for the 90 kDa isoform [25]. OP46 was selected following testing with other antibodies including 2A10 and 3G5 (A.J. Levine, Rockefeller University, New York, USA), both of which had technical or other limitations. OP46 was used at a 1:80 dilution of 100 μg/ml and evaluated in the context of Hdm2-overexpressing A375 malignant melanoma cells, and negatively controlled using IgG2b that was substituted for the primary antibody. Biotinylated rabbit anti-mouse IgG secondary antibody (DAKO E0354, 1:300 dilution of 1.26 mg/ml, Ely, Cambridgeshire, UK) was used for Hdm2. p53 immunostaining was performed as described elsewhere [25] using the DO7 monoclonal antibody (Novocastra Laboratories, Newcastle-upon-Tyne, UK). Breast carcinomas known to overexpress p53 with known TP53 gene mutations and protein accumulation were used as positive controls. Negative controls were obtained by omitting the primary antibodies.

Formalin-fixed paraffin sections of breast cancer tissue and cell pellets (controls) were subjected to immunohistochemical staining with heat antigen retrieval in citrate buffer (pH6). Primary antibodies for pAkt and Hdm2 were incubated at 4 °C overnight (18 h), except p53 that was incubated for 1 h at room temperature. Biotinylated goat anti-rabbit (DAKO E0432, 1:300 dilution of 1.09 mg/ml, Ely, Cambridgeshire, UK), was used for pAkt and biotinylated rabbit anti-mouse (DAKO E0354) for Hdm2 and p53 for 30 min at room temperature. Signal enhancement was achieved with streptavidin and biotinylated horseradish peroxidase (Strept ABComplex/HRP, DAKO 0377, Glostrup, Denmark), with visualisation in chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) for 5–10 min prior to counterstaining with haematoxylin.

The degree of staining intensity and the proportion of cells with pAkt, Hdm2 and p53 immunoreactivity in the nucleus and cytoplasm were graded semi-quantitatively to produce an intensity distribution score (IDS) for each localisation in IDCs. Initial scoring was of 10 HPFs, however, in view of the homogeneous staining this was reduced to 5 HPFs. pAkt scoring was analysed as reported previously for activated Akt-1 and was evaluated as negative (0; +); weak/moderate (++) and strongly positive (+++) [8]. A score of 0 or 1+ was considered negative and positivity was evaluated as 2+ or 3+. Scoring of pAkt was analysed as a continuum in statistical calculations of correlations with other variables. Nuclear and cytoplasmic Hdm2 and p53 immunoreactivity were scored using the principles of the IDS or *H*-score, but adapting this system to the quick-score previously used to describe nuclear immunoreactivity [24]. A modified quick-score (MQS) giving an overall score of 0–8 was standardised for nuclear as well as cytoplasmic expression [24,25]. Sections were independently scored by two pathologists (CS and CC) in the Department of Pathology. Scores were assessed as a continuum for the purposes of statistical correlation, unless otherwise stated.

2.3. Statistical analysis

Data was analysed with the SPSS 10.0 for Windows statistics software and summarised with descriptive statistics. The association between pAkt and patient characteristics was assessed using the Spearman non-parametric test for continuous variables and the χ^2 test for categorical factors. The relationship between pAkt and the subcellular localisation of p53 and Hdm2 was analysed using the Spearman correlation for non-parametric data, with the pAkt and p53/Hdm2 used as continuous variables. Analyses of survival data were performed using the log-rank test and the Cox regression model, with survival curves computed using the Kaplan–Meier method. For pAkt, univariate and multivariate analyses were performed, the latter adjusting for NPI score and treatment received (tamoxifen/chemotherapy/none). As the NPI is based on nodal involvement, on tumour size and on grade, patients ($n = 13$) with non-evaluable lymphadenopathy and tumour size were excluded from the multivariate regression analyses (Table 3).

3. Results

3.1. pAkt expression and association with clinicopathological factors

pAkt immunoreactivity was evaluable in 95 cases, and scored positively in 77 (81%) with a pattern of staining that was homogeneous and cytoplasmic. Activation of Akt was analysed (Fig. 1 and Table 1) as described (Methodology). Strong expression was seen in 22% ($n = 21$) of IDCs. The majority of tumours expressed moderate (2+) levels of pAkt, with few showing no activation of Akt (0/1+). pAkt overexpression was tumour specific in the absence of stromal staining with a similar pattern of pAkt expression in synchronous and adjacent ductal carcinoma *in situ* (DCIS) (data not shown). Activated Akt was analysed in relation to clinicopathological criteria (Table 2), with no association to HER-2 (either where scores were analysed categorically or as a continuum) that may relate in part to the small number

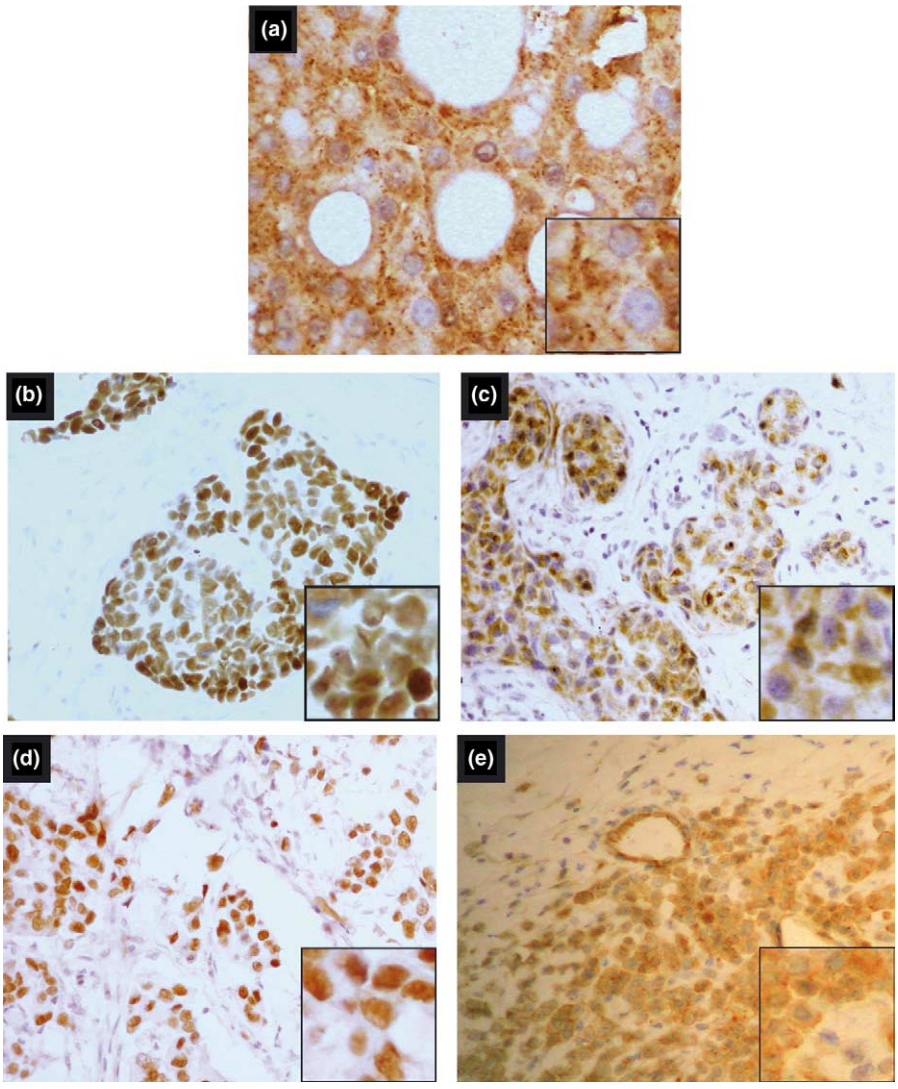

Fig. 1. Examples of pAkt and subcellular p53 and Hdm2 immunoreactivity in infiltrating ductal carcinoma of the breast. Immunostaining was performed as described in 'Materials and methods', and nuclei were counterstained with haematoxylin. (a) A tumour showing pAkt cytoplasmic expression and representative examples of (b) Predominant nuclear p53 immunoreactivity compared with (c) cytoplasmic p53 expression. Exclusive and predominant (d) nuclear Hdm2 expression was less evident compared with an association with (e) predominant cytoplasmic Hdm2 immunoreactivity. Insets in panels (a)–(e) show higher power views of the same fields. High power magnification of main photomicrographs (400×).

(16%) of HER-2 overexpressing breast cancers. pAkt expression was positive in 13 (87%; $n = 15$) cases of HER-2 – overexpressing (2+/3+) breast cancers, although this was not statistically significant (Table 2). pAkt positive (2+/3+) tumours were significantly larger ($P = 0.007$; Table 2), than IDCs in which Akt was inactive (0/1+), with a similar trend in lymph node positive patients ($P = 0.085$) and the NPI ($P = 0.069$).

3.2. Relationships between pAkt expression and subcellular expression of p53 and Hdm2

pAkt expression was measured as a continuum and was analysed in relation to p53 and Hdm2 positivity (*H*-score $\geqslant 3$ *vs.* <3) in the nucleus and cytoplasm of breast cancers (Fig. 1 and Table 1). p53 immunoreactivity and subcellular localisation was evaluable in 88 (91%) cases of IDCs, with technical exclusions based on fatty breast specimens failing to adhere to glass slides. Immunohistochemically, p53 was detectable in 25 (28%) IDCs (*H*-score $\geqslant 3$), with similar levels of nuclear (25 cases) and cytoplasmic (22 cases) expression. Weak to moderate (*H*-score 3–5) nuclear p53 expression was observed with similar cytoplasmic staining (Table 1b). There was no nuclear or cytoplasmic p53 expression (*H*-score 0–2) in the majority of invasive breast cancers. Strong nuclear p53 expression (*H*-score 6–8) was evident in 16% of IDCs, with 6% of these expressing intense cytoplasmic staining. Activation of Akt was significantly associated with cytoplasmic p53 expression (Spearman

Table 1
Immunohistochemical expression of pAkt and the subcellular localisation of p53 and Hdm2 in invasive ductal breast cancers

(a) pAkt IHC score				
	0	1+	2+	3+
Invasive $n = 95$ (%)	3 (3)	15 (16)	56 (59)	21 (22)
(b) p53 Modified quick-score				
	Total no. tumours (%)	0–2 (0)	3–5 (+)	6–8 (++)
Invasive				
Nucleus	88	62 (70)	11 (13)	14 (16)
Cytoplasm	88	66 (75)	17 (19)	5 (6)
(c) Hdm2 modified quick-score				
	Total no. tumours (%)	0–2 (0)	3–5 (+)	6–8 (++)
Invasive				
Nucleus	85	45 (53)	31 (36)	9 (11)
Cytoplasm	85	40 (47)	30 (35)	15 (18)

Tumour numbers (n) scored (0, 1+, 2+, 3+) on immunohistochemistry (IHC) for (a) pAkt, and using the MQS (0–8) for (b) p53 and (c) Hdm2 expression in the nucleus and cytoplasm of invasive cancers. pAkt expression was cytoplasmic and evaluated as negative (0/1+) or positive (2+/3+). IHC expression was individually assessed in the nucleus and cytoplasm for p53 and Hdm2, and defined as negative (0) (MQS-score 0–2), weak/moderate (1+) (MQS-score 3–5) and strong (2+) (MQS-score 6–8). Positivity for p53 and Hdm2 expression was defined as an MQS of $\geqslant 3$ (1+/2+).

correlation coefficient (cc) 0.241, $P = 0.025$), with no association with nuclear p53 (cc -0.141, $P = 0.196$) when all variables were analysed as a continuum (data not shown). Hdm2 expression and subcellular distribution was evaluable in 85 (88%) cases of IDCs (Fig. 1, Table 1(c)). Nuclear Hdm2 was detectable (H-score $\geqslant 3$) in 40 (47%) cases, with a similar proportion of cytoplasmic staining. A minority of IDCs strongly expressed nuclear and cytoplasmic Hdm2. Approximately half of invasive cancers were negative for nuclear and cytoplasmic Hdm2. There were no associations between pAkt and a specific nuclear or cytoplasmic localisation of Hdm2.

3.3. Relationship of clinicopathological factors to prognosis and the predictive potential of pAkt expression

Overall survival (OS) and disease-free survival (DFS) were determined in 87 and 95 patients, respectively, (primary tumours), with a median follow-up of 51 months (range 6–120 months). Disease relapse (local or distant recurrences) occurred in 29 women, in whom 25 were post-mastectomy. The majority of recurrences were distant, with a single cutaneous recurrence following breast conservation surgery and a further four local recurrences associated with synchronous metastatic disease. Of those who relapsed, death was confirmed in 22 patients, with 8 suspected deaths in the absence of a recorded mortality date that were consequently not included in the regression analyses (Table 3 and Fig. 2). Locoregional recurrence occurred at a median duration of 26 months (range 3–65 months) from diagnosis. Breast cancer related mortality occurred at a median of 26 months (range 8–98 months) from presentation. The mean duration of OS and DFS were 94 and 88 months, respectively. Four-year DFS and OS were 70% and 77%, respectively. The relationship of established clinicopathological features with OS and DFS were analysed using Cox regression analysis (Table 3). Generally poor prognostic factors, such as large tumour size, high tumour grade and lymph node metastases, were significantly associated with decreased OS and DFS, so that NPI was highly significantly associated with outcome. ER-negative and HER-2 overexpressing breast cancers were associated with reduced OS and DFS on univariate analysis, but were not independent prognosticators (Table 3). High tumour proliferation (Ki67 on IHC), although associated with a lower percentage of patients remaining disease-free (DFS) and alive (OS) at four years, did not reach statistical significance. Although mastectomy was associated with a reduced DFS and OS (univariate, Table 3) the type of surgical excision was not an independent predictor of outcome (multivariate), and may reflect stage at presentation.

Univariate and multivariate analysis using the continuous score for pAkt expression were used to investigate possible relationships to patient outcome. In univariate analysis activation of Akt was associated with a reduced DFS ($P = 0.04$) (Table 3), with a similar trend, although not significant, in pAkt positive (2+/3+) patients on Kaplan–Meier survival curves (Figs. 2(a) and (b)). Activation of Akt was not an independent prognosticator for DFS or OS (Table 3) in this study.

4. Discussion

Increased Akt levels are a feature of tumourigenesis with *in vitro* evidence in human breast carcinoma cell lines and increased expression in breast cancers [7–9]. Few studies have clarified the functional implications of any particular Akt isoform in breast cancers. This study evaluates all serine 473-phosphorylated Akt

Table 2
Relationship between pAkt expression and clinicopathological variables

Characteristics	pAKT score			
	Negative (%)	Positive (%)	*P*-value (χ^2)	Patients *n*(%)
Invasive tumours	18	77		95[a]
Age (years)				
⩽50	5(20)	20(80)	0.876	25(26)
>50	13(19)	57(81)		70(74)
Lymph node stage				
Stage I (node –ve)	9(26)	25(74)	0.085	34(36)
Stage II (1–3 +ve nodes)	6(23)	20(77)		26(27)
Stage III (>3 +ve nodes)	1(4)	23(96)		24(25)
				11(12)
Invasive tumour grade				
Grade I	3(23)	10(77)	0.668	13(14)
Grade II	10(21)	37(79)		47(49)
Grade III	5(14)	30(86)		35(37)
Invasive tumour size (cm)				
⩽2	14(30)	32(70)	**0.007**	46(48)
>2	4(9)	43(91)		47(49)
Multifocal				2(2)
Lymphovascular invasion				
Present	7(15)	39(85)	0.318	46(48)
Absent	11(23)	36(77)		47(49)
NA				2(2)
NPI				
GPG < 3.4	4(22)	14(78)	0.069	18(19)
MPG 3.4–5.4	10(29)	24(71)		34(36)
PPG > 5.4	2(7)	28(93)		30(32)
Not possible to calculate				13(14)
ER (quick-score)				
Positive (4–8)	11(19)	47(81)	0.927	58(61)
Negative (0–3)	6(18)	27(82)		33(35)
(NA)				4(4)
HER-2 IHC				
Negative (0/1+)	16(20)	64(80)	0.545	80(84)
Positive (2+/3+)	2(13)	13(87)		15(16)
Ki67 IHC				
Low proliferation <10%	6(13)	41(87)	0.128	47(49)
High proliferation ⩾10%	12(25)	36(75)		48(51)

NPI, Nottingham prognostic group; GPG, good prognostic group; MPG, moderate prognostic group; PPG, poor prognostic group; ER, oestrogen receptor; IHC, immunohistochemistry. Significant *P*-values ($P < 0.05$) indicated in **bold.**

[a] Patient numbers reflect evaluable pAkt.

isoforms and shows positive pAkt expression in 81%, compared with similar studies showing 54% and 27%, respectively, [7,8]. This ubiquitous pAkt expression is likely to relate to multiple pathways involving tyrosine kinase-related growth factors and oncogenes. Immunohistochemical pAkt expression did not reveal particular information regarding plasma membrane and nuclear staining, in large part as these phenomena relate to the rapidity of Akt activation.

Oestrogen-related breast cancer growth involves the PI3-K/Akt pathway with inhibition of Akt activation by ICI182780 (Faslodex) [10]. No clear association has been shown between pAkt and ER status in this study or previous reports [7–9]. *In vitro*, HER-2 is strongly associated with activation of Akt in breast cancer cell lines and in tamoxifen-resistant MCF-7 cells [10,11]. In large part there is further evidence for this association *in vivo*, although some studies are inconsistent, more particularly with respect to correlations between HER-2 and particular Akt-isoforms [7–9]. The limited number of HER-2 overexpressing breast cancers ($n = 15$) in this study may explain the lack of an association between HER-2 and pAkt. HER-2 mediated breast cancer growth is also likely to activate cytoplasmic signalling through MAP kinase pathways with no specificity for PI3-K/Akt signalling [27]. The association between pAkt and larger breast cancers emphasises the importance of the PI3-K/Akt signalling pathway in promoting cell survival and cell growth (Table 2), as well as confirming resistance to apoptosis.

Cellular commitment to apoptosis is likely to relate to the integration of two key pathways including p53-induced apoptosis and the aversion of cell death through Akt/PKB. A reciprocal cross-talk between p53 and Akt involves Akt inactivation of p53, as well as a p53-dependent downregulation of Akt [28]. This suggests an anticipated inverse association between pAkt and p53 levels, whereby mutant p53 or immunohistochemical overexpression of p53 is associated with increased levels of Akt and its subsequent activation (pAkt). Similarly, the implication is that non-functional p53 is unable to destroy Akt. Proportionately, more mutant or wild-type p53 may be detectable in the cytoplasm and has been implicated in poor prognostic breast cancers [17,18]. In this study, the association of cytoplasmic p53 with increasing levels of pAkt ($P = 0.025$) suggests the loss of a p53-dependent destruction of Akt and an association between two key tumourigenic pathways [29]. Stenmark-Askmalm *et al.* [18] suggest that cytoplasmic p53 expression, compared with exclusive nuclear staining, in breast cancers is more likely to be associated with adverse pathobiological variables, such that our preliminary findings may reflect an association between two potential prognostic factors and merits future investigation [18]. The lack of a similar association between pAkt and nuclear p53 on IHC may support Stenmark-Askmalm *et al.* [18] and imply other pathways involving p53/Akt that include regulation through changes in subcellular localisation [12,16,18]. Activated Akt (serine 473) increases Hdm2 activity to degrade p53 through a process that involves p53 nuclear export to the cytoplasm, and essentially depends on p53 transcriptional activation of Hdm2 [12,20]. A possible association between wild-type cytoplasmic p53 as a

Table 3
Relationships between clinicopathological criteria, pAkt and patient outcome

Variable	Univariate analysis		Multivariate analysis	
	95% CI	*P*	95% CI	*P*
Overall survival				
Age	0.99–1.04	0.4	0.98–1.05	0.56
NPI	1.48–3.36	**<0.001**	1.55–3.79	**<0.001**
ER quick score	0.66–0.92	**0.002**	0.73–1.10	0.29
HER-2 IHC score	0.94–2.02	**0.04**	0.62–1.69	0.94
Ki67 proliferative index	0.99–1.00	0.4	0.10–1.00	0.87
Lymphovascular invasion (+/−)	1.46–9.38	**0.006**	0.59–7.15	0.26
Treatment (tamoxifen/chemotherapy/none)	0.96–2.42	0.075	0.44–1.43	0.43
Surgery (WLE *vs.* mastectomy)	1.80–20.6	**0.004**	0.55–12.1	0.23
pAkt IHC score	0.87–2.82	0.133	0.70–3.08	0.30
Disease-free survival				
Age	0.97–1.02	0.9	0.97–1.04	0.88
NPI	1.46–3.17	**<0.001**	1.71–3.90	**<0.001**
ER quick score	0.66–0.89	**0.001**	0.73–1.05	0.16
HER-2 IHC score	1.04–1.92	**0.02**	0.87–1.95	0.21
Ki67 proliferative index	0.99–1.00	0.2	0.99–1.00	0.29
Lymphovascular invasion (+/−)	1.63–6.95	**0.002**	0.77–7.93	0.13
Treatment (tamoxifen/chemotherapy/none)	0.98–2.14	0.067	0.50–1.37	0.47
Surgery (WLE *vs.* mastectomy)	2.05–17.0	**0.001**	0.80–15.8	0.10
pAkt IHC score	1.02–3.07	**0.044**	0.84–3.43	0.14

Ninety-five percent confidence intervals (95% CI) and *P*-values are given for the results of both the univariate and multivariate analyses. Data for univariate analysis was evaluable in 95 patients and a multivariate analysis on 84 patients that excluded non-evaluable NPI in 11 patients. The multivariate analysis is adjusted for NPI (nodes, grade and size), surgical (WLE/mastectomy) and adjuvant treatment (tamoxifen/chemotherapy/none). All clinicopathological variables were analysed as a continuum. Univariate and multivariate analyses for pAkt (OS) were evaluated on 87 and 77 patients, respectively, (pAkt (DFS) univariate (95 patients) and multivariate (84 patients)). Relationships that reached significance ($P < 0.05$) are highlighted in **bold**.
NPI, Nottingham prognostic group; ER, oestrogen receptor; IHC, immunohistochemistry.

prerequisite for Akt-mediated degradation of p53 may also exist [19,29]. p53 immunostaining is not necessarily tightly correlated with *TP53* gene function, with detection of *TP53* point mutations in 89%, as well as wild-type p53 protein [17]. A study of inflammatory breast cancers showed the presence of wild-type cytoplasmic p53 in 37% of cases, with further evidence that cytoplasmic p53 expression occurs in normal lactating breast tissue and is associated with intact p53 function in neuroblastoma cell lines [17,19].

Akt associates with Hdm2 with *in vitro* evidence of binding to, and phosphorylation of Hdm2 (serine 166 and 186) to increase levels of Hdm2 expression and its enhanced nuclear localisation [12,20,21,30]. As discussed, this mechanism underlines Akt-induced inactivation of p53. In this study, there were no clear associations between pAkt and Hdm2 levels of expression. Hdm2 expression is a feature of tumourigenesis, with enhanced transcription, translation and an extended protein half-life. The subcellular localisation of Hdm2 involves evidence of cytoplasmic Hdm2 *in vitro,* and in a series of breast cancers *in vivo* [12,15,21]. Akt phosphorylation of Hdm2 in proximity to the nuclear localisation and export signals is necessary for the translocation of Hdm2 from the cytoplasm into the nucleus [12,21]. In a preliminary series of breast cancers Akt-positive tumours were associated with predominant nuclear Hdm2 expression, compared with both nuclear and cytoplasmic Hdm2 in Akt-negative breast cancers [12]. By contrast, this study shows no specific association between Akt expression and a preferential subcellular localisation of Hdm2. Possible confirmation of our findings and a suggestion as to the complexity of these pathways is highlighted by another *in vitro* study in which Akt-induced phosphorylation of Hdm2 had little effect on its subcellular localisation [30].

It is likely that activated Akt is more discriminatory as a prognosticator than Akt-1 and Akt-2 levels, respectively [7–9]. Its clinical significance is suggested with a single study in 93 breast cancers demonstrating its independent prediction of a shorter DFS, and another study implying that pAkt predicts radioresistance [7,8]. Our study examining serine 473 phosphorylation of all Akt isoforms suggests an association between their activation and a shorter interval to disease progression ($P = 0.04$; univariate) (Table 3 and Fig. 2). Lack of independent prognostic significance may reflect small patient numbers, although similar to the study of Pérez-Tenorio and colleagues

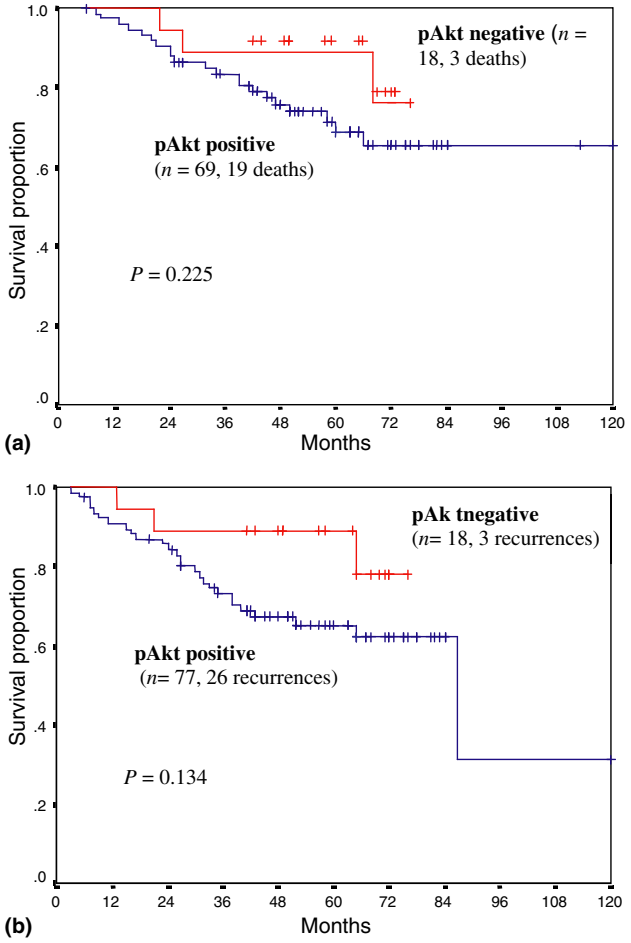

Fig. 2. The relationship of pAkt to (a) overall survival (OS) and (b) disease-free survival (DFS) in breast cancer. Curves according to pAkt positive (2+/3+) and negative (0/1+) breast cancers. The *P*-values are given for the log-ranks.

[7] and Stål and colleagues [8]. PI3-K/Akt signalling has also been implicated in both tamoxifen and taxol resistance to suggest a possible link between this pathway and treatment failure, which may have implications for disease recurrence [11].

5. Conclusion

Activation of Akt/PKB by PI3-K has important implications for breast cancer growth through mechanisms inactivating p53 that involve Hdm2. We have demonstrated that universal activation of Akt isoforms is associated with immunohistochemical p53 expression, which is preferentially cytoplasmic. Alternative pathways, whereby Akt regulates p53, could involve its cytoplasmic localisation. Although HER-2 is likely to activate Akt, variable associations *in vivo* suggest a greater complexity of these pathways in human cancers.

Conflict of interest statement

None declared.

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