Pathol. Oncol. Res. (2010) 16:239–248
DOI 10.1007/s12253-009-9216-3

Immunohistochemical Detection of Phospho-Akt, Phospho-BAD, HER2 and Oestrogen Receptors α and β in Malaysian Breast Cancer Patients

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Received: 4 May 2009 / Accepted: 7 October 2009 / Published online: 1 November 2009

Abstract Activation of Akt signaling pathway has been documented in various human malignancies, including breast carcinoma. The objective of this study is to determine the incidence of Akt phosphorylation in breast tumours and its relationship with expression of ER-α, ER-β, HER2, Ki-67 and phosphorylated Bcl-2 associated death domain (p-BAD). Immunohistochemical staining was performed to detect these molecules on 43 paraffin-embedded breast tumour tissues with commercially available antibodies. Eighteen (41.9%), 3 (7.0%), 23 (53.5%), 35 (81.4%), 21 (48.8%), 29 (67.4%), and 34 (81.0%) of breast tumours were positive for nuclear ER-α, nuclear ER-β, membranous HER2, cytonuclear p-Akt (Thr308), p-Akt (Ser473), p-BAD and Ki-67, respectively. ER-α expression was inversely correlated with HER2 and Ki-67 (P=0.041 and P=0.040, respectively). The p-Akt (Ser473) was correlated with increased level of p-BAD (Ser136) (P=0.012). No relationship of Akt phosphorylation with HER2, ER-α or ER-β was found. The p-Akt (Ser473) immunoreactivity was significantly higher in stage IV than in stage I or II (P=0.036 or P=0.009). The higher Ki-67 and lower ER-α expression showed an association with patient age of <50 years (P=0.004) and with positive nodal status (P=0.033), respectively. Our data suggest that the Akt phosphorylation and inactivation of its downstream target, BAD may play a role in survival of breast cancer cell. This study does not support the simple model of linear HER2/PI3K/Akt pathway in breast cancer.

Keywords Breast cancer · Akt · Oestrogen receptor · HER2

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Introduction

Breast cancer affects woman worldwide and in Malaysia, it is the most frequent type of cancer in females [1]. The presence of oestrogen receptor (ER)-α in breast tumours is used clinically to predict the likelihood of response to hormonal therapies. However, even though it has been reported that the response rate is >70% in ER positive cases [2] with >40% of these patients will eventually develop resistance [3]. Drug resistance problems have also been reported for trastuzumab, a monoclonal antibody which targets the human epidermal growth factor receptor 2 (HER2) [4]. A number of reasons for drug resistance have been proposed including the hyperactivation of Akt/protein kinase B (PKB). High phosphorylated Akt (p-Akt) level has been associated with tamoxifen-resistant breast cancer patients [5]. Akt has also been shown to contribute to chemoresistance and radioresistance in non-small lung cancer and breast cancer cell lines [6, 7]. This suggests that inhibition of Akt activity may be a useful strategy to overcome chemoresistance. Thus, potent and selective inhibitors targeting Akt are potentially promising drug candidates for hormone insensitive or chemoresistant tumours. An inhibitor of Akt has been shown to inhibit

the growth of tumours [8]. Perifosine, an Akt inhibitor, is currently being evaluated in a multitude of Phase I and II studies [9].

Akt1 is a serine/threonine protein kinase that was first discovered as the human homologue of the transforming gene of an oncogenic virus [10, 11]. Two additional isoforms, Akt2 and Akt3 have been identified and all three isoforms have transforming ability [12, 13]. Akt is a downstream target of signaling pathways stimulated by various receptors such as HER2 and insulin-like growth factor 1 receptor. In vitro studies show that activation of Akt by growth factors depends on the binding of phosphoinositide-3,4,5-triphosphate (PIP3) and the phosphorylation of Akt at Ser473 and Thr308. Phosphorylation of both sites is necessary for full activity of Akt in vitro. Distinct enzymes are responsible for the phosphorylation of Akt, for example, phosphoinositide-dependent kinase 1 (PDK-1) phosphorylates Akt at threonine residue 308 [14, 15] and rictor-mTOR is responsible for Ser473 phosphorylation [16, 17]. P-Akt is a powerful promoter of cell survival as it antagonises the components of the apoptotic cascade such as the Bcl-2 associated death domain (BAD) protein. Akt is also implicated in angiogenesis and metastasis which are the two important processes in cancer development [18, 19]. Thus, it is not surprising that frequent activation of Akt occurs in a wide spectrum of cancers such as prostate, breast and ovarian carcinomas [20]. In addition, HER2-overexpressing tumour with high p-Akt expression has been associated with poor disease survival [21]. However, majority of these studies used antibodies against p-Akt (Ser473) but not p-Akt (Thr308). It is unknown whether the phosphorylation of Akt at Ser473 and Thr308 occurs equally in the same tumour.

The applications of molecular-targeted therapies such as Akt inhibitors require clinical trials such as exemplified by tamoxifen, trastuzumab and gefitinib (an inhibitor of epidermal growth factor receptor). A systematic examination of ER-α, HER2 and signal transduction proteins such as Akt in primary breast tumour tissues is required as a prelude to clinical studies to assess the efficacy of Akt inhibitors in patients. Thus, the objectives of this study were, firstly, to determine the frequency of p-Akt (Ser473), p-Akt (Thr308), ER-α, ER-β, HER2, p-BAD, and Ki-67 expression and their relationships in breast tumour tissues, and secondly, to investigate the association of these biomolecules with the clinicopathological features of tumour. We also examined the ER-β expression since it has been suggested that loss of ER-β expression could be one of the events leading to breast cancer development [22]. Moreover, its frequency in breast tumours in Malaysia is still unknown. We are also aware about the significance of progesterone receptor expression but this was not elucidated in our endeavors for identifying molecular targets.

Materials and Methods

Tissue Specimens

A total of 43 surgically resected breast cancer specimens were retrieved from archived clinical material collected between 1999 and 2002. Ethics approval was obtained from the institutional ethics review committee. Demographics of the 43 samples used for this study are summarised in Table 1. According to the TNM staging system adopted by the American Joint Committee on Cancer, there were 11 (25.6%) stage 0, 8 (18.6%) stage I , 15 (34.9%) stage II, and 9 (20.9%) stage IV tumours. All the invasive breast cancers are infiltrating ductal carcinoma. Tumours were graded as well (grade I), moderately (grade II) or poorly (grade III) differentiated according to the predominant pattern of the tumour.

Immunohistochemical Staining

Immunohistochemistry was performed by the standard biotin–streptavidin-peroxidase method on 4 μm-thick formalin-fixed, paraffin-embedded tissue sections. After deparaffinization in xylene and rehydration in descending concentrations of alcohol, an antigen retrieval step was performed by microwaving in 10 mM citrate buffer (pH 6) for 20 min at boiling temperature. Endogenous peroxidase

Table 1 Patient characteristics

Characteristics	Number of cases (%)
Age (years)	
<50	22 (51)
≥50	21 (49)
Race	
Malay	5 (11.6)
Chinese	29 (67.4)
Indian	6 (14.0)
Others	3 (7.0)
Pathological stage	
0	11 (25.6)
I	8 (18.6)
II	15 (34.9)
III	0 (0.0)
IV	9 (20.9)
Histological grade	
I	13 (30.2)
II	22 (51.2)
III	8 (18.6)
Lymph node status	
Positive	14 (32.6)
Negative	29 (67.4)

was blocked by 3% hydrogen peroxide. After blocking with 3% bovine serum albumin for 1 h, the sections were incubated with the diluted primary antibody for appropriate time, as listed in Table 2. The immunoreactivity was detected by using LSAB+ Kit (Dako, Carpinteria, CA) in room temperature according to the manufacture's instructions. The 3,3′-diaminobenzidine (DAB) (Liquid DAB+; Dako, Carpinteria, CA) solution was used as a chromogen. Sections were lightly counterstained with hematoxylin. The section without primary antibody served as negative control.

Evaluation of Immunohistochemical Staining

Immunostained tissue sections were evaluated under ×200 magnification by light microscopy. A total of 5–10 fields were viewed and scored. A modified semi-quantitative scoring system adopted from previous studies [23, 24] was used to evaluate the immunoreactivity of p-Akt (Thr308), p-Akt (Ser473), p-BAD (Ser136), and Ki-67. The score for percentage of positive cells was assigned using the following scales: 0, no staining of cells in any field; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%. The intensity of staining was scored using the following scales: 1+, mild staining; 2+, moderate staining; 3+, strong staining. The final total score was obtained by the sum of the score for percentage of positive cells and the score of staining intensity. Hence, the possible total scores were 0 and 2–7. For assessment of the positivity, total scores of ≥3 were considered positive.

The Allred scoring system [25] was used to determine the positivity of ER-α and ER-β. Briefly, a proportion score was assigned representing the estimated proportion of positive staining tumor cells (0, none; 1, >0 to 1/100; 2, >1/100 to 1/10; 3, >1/10 to 1/3; 4, >1/3 to 2/3; 5, >2/3 to 1). An intensity score was also assigned, which represented the estimated average staining intensity of positive tumour cells (0, none; 1, weak; 2, intermediate; 3, strong). The proportion score and intensity score were then added to obtain a total score (0 or 2–8). A positive result for both ER-α and ER-β was defined as total score ≥3.

HER2 immunoreactivity was scored according to the criteria specified by Dako for the interpretation of the HercepTest [26]. Immunoreaction was determined as positive if more than 10% of the tumour cells showed weak to moderate complete membrane staining (2+) or strong complete membrane staining (3+). All other staining patterns were interpreted as negative (0, no staining; 1+, a faint and incomplete membrane staining was detected in more than 10% of the tumour cells).

Statistical Analysis

The total scores of immunostaining were used to perform the statistical analysis. The association of biomolecules with clinicopathological variables was performed by the Mann–Whitney *U*-test or Kruskal–Wallis one-way analysis of variance by ranks. Spearman rank correlation test was used to assess the correlation among the expression of biomolecules. A 2-sided p value of less than 0.05 was considered statistically significant. Statistical analysis was performed by using SPSS 11.5 statistical software.

Results

Analysis of ER-α, ER-β, HER2, p-Akt (Ser473), p-Akt (Thr308), p-BAD (Ser136), and Ki-67 Immunoreactivity

The case distribution of the various biomolecules according to their immunohistochemical score is shown in Fig. 1. Positive nuclear ER-α, ER-β and membranous HER2 immunoreactivity (Fig. 2a–c) was observed in 18/43 (41.9%), 3/43 (7.0%), and 23/43 (53.5%) breast tumour tissues, respectively (Fig. 1a–c). Comparison of positive ER-α and ER-β scores showed that ER-β scores were lower (score 3–4), compared to that of ER-α (score 3–8). ER-α and ER-β were expressed by 3/13 (23.1%) and 7/13 (53.8%) apparently normal adjacent breast tissues, respectively (data not shown). All of the apparently normal adjacent breast tissues (13/13; 100%) did not express HER2 (data not shown). There was definitely a larger proportion

Table 2 Primary antibodies used for immunohistochemical analysis

Protein	Clone	Company	Catalog no.	Antibody dilution/incubation time
ER-α	F-10	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-8002	1:100/1 h
ER-β	14C8	Abcam (Cambridge, MA)	ab288	1:200/Overnight[a]
HER2 (c-erbB-2)	Polyclonal	Dako (Carpinteria, CA)	A0485	1:300/1 h
p-Akt (Ser473)	Polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-7985-R	1:50/1 h
p-Akt (Thr308)	Polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-16646-R	1:100/1 h
p-BAD (Ser136)	Polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-7999	1:100/1 h
Ki-67	MIB-1	Dako (Carpinteria, CA)	M7240	1:100/1 h

[a] Incubation was at 4°C. For other antibodies, incubation was at room temperature

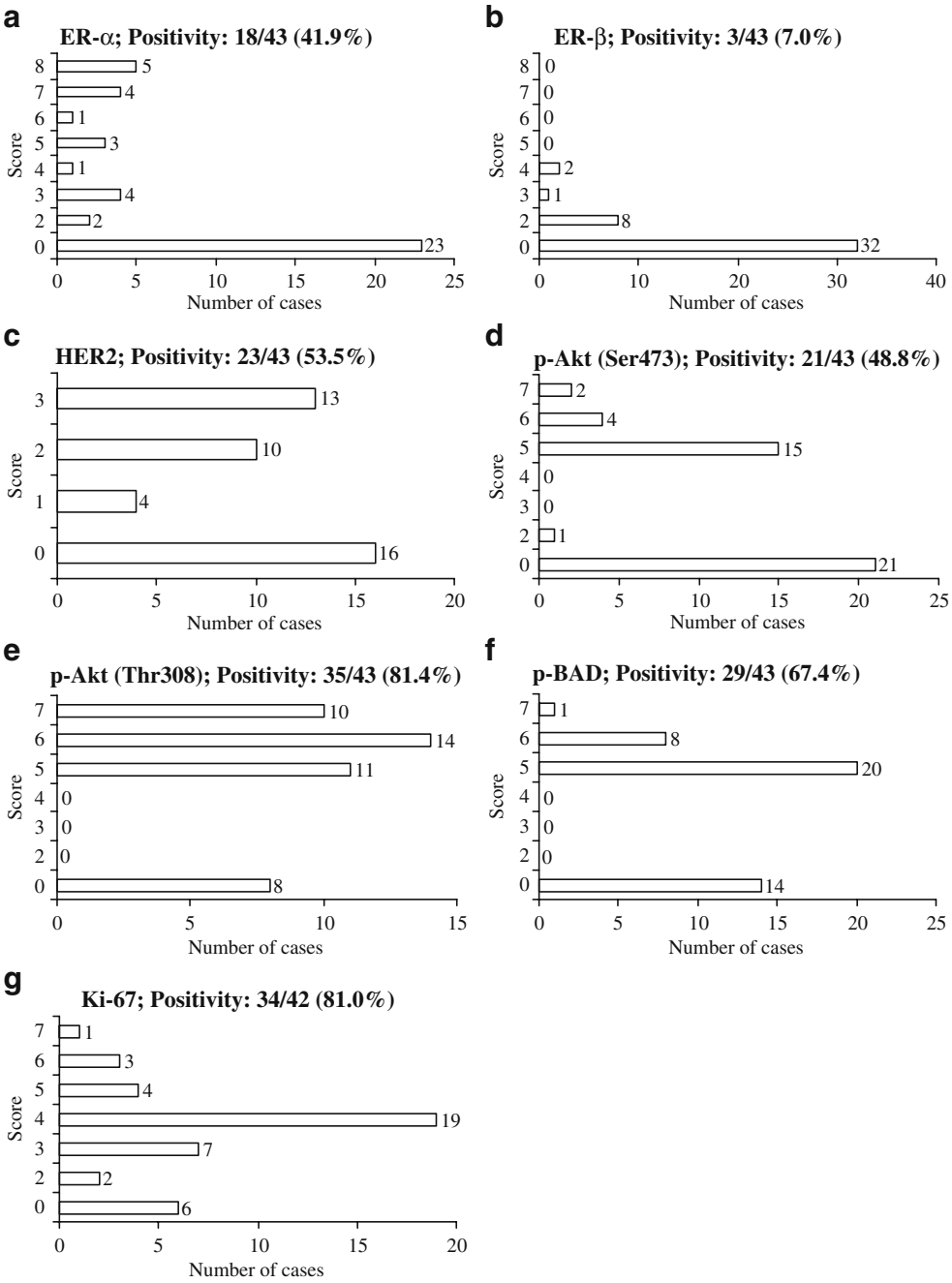
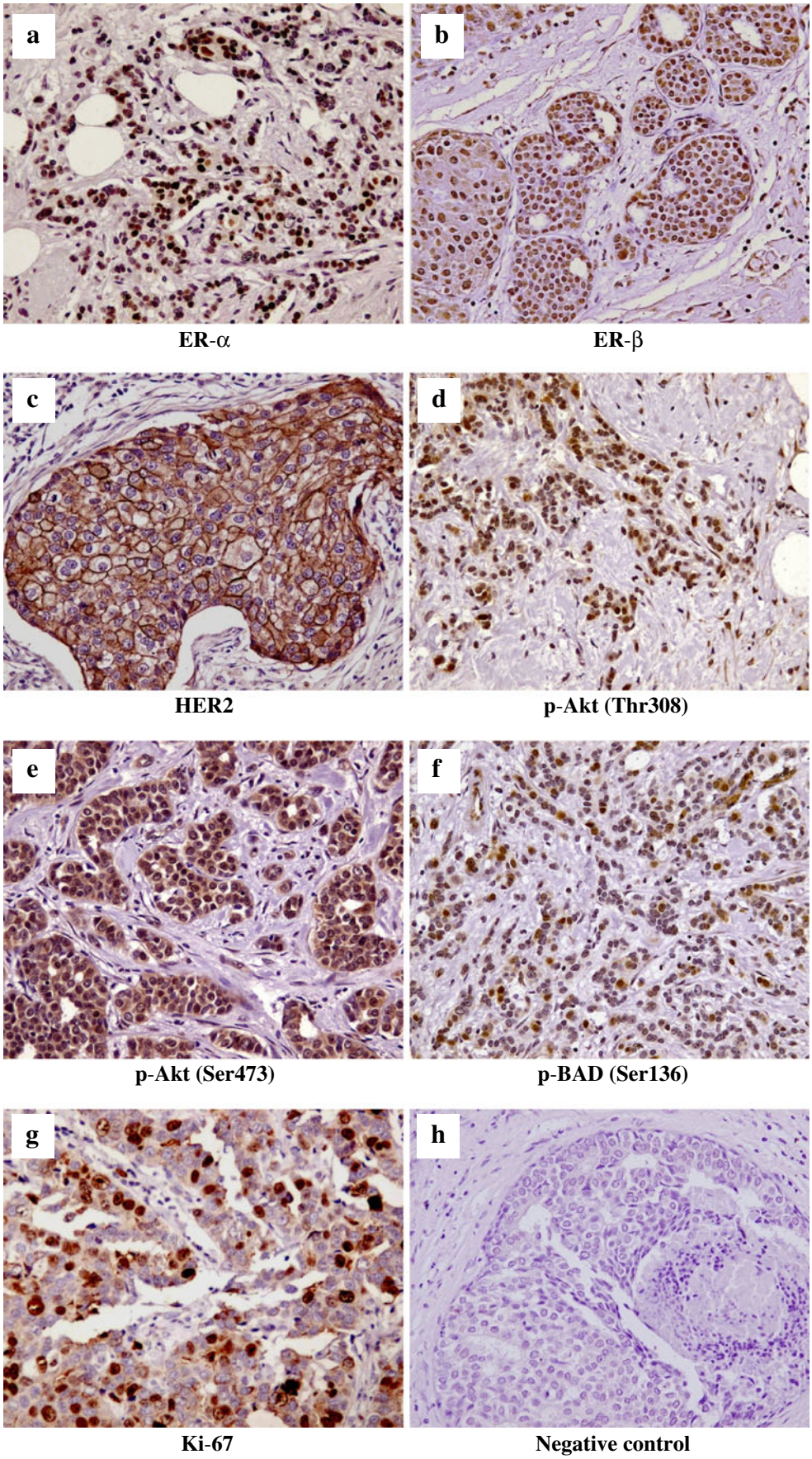


Fig. 1 Frequency and case distribution of **a** ER-α, **b** ER-β, **c** HER2, **d** p-Akt (Ser473), **e** p-Akt (Thr308), **f** p-BAD (Ser136), and **g** Ki-67 expression by immunohistochemical score in breast tumour tissues. The scoring cutoff points used for establishing positivity are stated in the 'Materials and Methods'

of positive cases for p-Akt (Thr308) (35/43; 81.4%) compared to p-Akt (Ser473) (21/43; 48.8%) (Fig. 1d,e). The positive immunohistochemical scores of the cytonuclear staining (Fig. 2d,e) were similar for both phosphorylation sites (score 5–7). Positive cytoplasmic p-BAD (Ser136) and nuclear Ki-67 immunoreactivities (Fig. 2f,g) were observed in 29/43 (67.4%) and 34/42 (81.0%) breast tumour tissues, respectively (Fig. 1f,g). Majority of p-BAD (Ser136)-positive cases had a score 5 (20/43). Eight samples had a score 6 and one sample had the highest score. As for the Ki-67 staining, majority of the immunopositive tumour tissues had a score 4 (19/42).

Fig. 2 Representative sections showing immunohistochemical staining of ER-α (**a**), ER-β (**b**), HER2 (**c**), p-Akt (Thr308) (**d**), p-Akt (Ser473) (**e**), p-BAD (Ser136) (**f**), and Ki-67 (**g**) in breast tumour tissues. The representative negative control is also shown (**h**). Strong and moderate nuclear immunoreactivity for ER-α and ER-β, respectively, were observed in virtually all tumour cells. Strong membranous HER2 immunoreactivity was detected in one of the breast tumour tissues. All tissue sections shown above are infiltrating ductal carcinoma except in **b** and **h**, which are ductal carcinoma in situ. Original magnification, ×200


a
b
ER-α
ER-β
c
d
HER2
p-Akt (Thr308)
e
f
p-Akt (Ser473)
p-BAD (Ser136)
g
h
Ki-67
Negative control

Further analysis of the coexistence of ER-α and HER2 on the same tumour specimen showed that 20.9% (9/43) of breast tumour tissues were ER^+HER2^+ as similar to ER^+HER2^-, 32.6% (14/43) expressed ER^-HER2^+, and 25.6% (11/43) were ER^-HER2^- (data not shown). If ER-α and HER2 are used as predictive markers for drug response, our data implies that 18/43 (41.9%) and 23/43 (53.5%) could be selected for anti-oestrogen (e.g. tamoxifen) and anti-HER2 (e.g. trastuzumab) monotherapy, respectively. However, it should be noted that ER-negative tumours can also respond to anti-oestrogen treatment.

Correlation Among the ER-α, ER-β, HER2, p-Akt (Ser473), p-Akt (Thr308), p-BAD (Ser136), and Ki-67 Immunoreactivities in Breast Tumour Tissues

Using the Spearman rank correlation test, HER2 and Ki-67 both showed a negative correlation with ER-α immunoreactivity (P=0.041 and P=0.040, respectively; Table 3). The p-Akt (Ser473) was correlated with increased level of p-BAD (Ser136) (P=0.012). No statistically significant correlation was seen among other biomolecules. Since phosphorylation of Akt on both Ser473 and Thr308 has been shown to be required for full activation of the kinase [16, 27], we categorized the breast tumour cases into 4 groups as follows: positive for both Ser473 and Thr308, positive for Ser473 only, positive for Thr308 only, and negative for both phosphorylations. Phosphorylation of Thr308 only was detected in 19/43 (44.2%) or co-occurred with Ser473 phosphorylation in 16/43 (37.2%) breast tumours (Table 4). A lower number of immunoreactivity of Ser473 phosphorylation only was detected in 5/43 (11.6%) tumour tissues. Statistical analysis showed that the Akt phosphorylation groups were significantly associated with p-BAD (Ser136) (P=0.032) (Table 4). By using the Mann–Whitney's U-test, the group with phosphorylated Akt on both sites or Ser473 only had significantly higher level of p-BAD (Ser136) immunoreactivity (P=0.033 or P=0.018, respectively), compared to the group with non-phosphorylated Akt (Table 4). This relationship was not observed with ER-α, HER2 or Ki-67.

Association of the Expression of Biomolecules with Clinicopathological Features

Statistical analysis of the association of biomolecules with the clinicopathological features (listed in Table 1) showed that the p-Akt (Ser473) immunoreactivity was significantly higher in stage IV than in stage I or II (P=0.036 or P=0.009, respectively; Fig. 3a). Furthermore, we observed that the Ki-67 expression was significantly associated with patient age of <50 years (P=0.004; Fig. 3b). In addition, patients with lymph node metastases tended to have a lower ER-α expression (P=0.033; Fig. 3c). No significant association of other biomolecules with the clinicopathological features was found (data not shown).

Discussion

Immunohistochemical analysis of ER-α and HER2 has become a standard of care for breast cancer patients. The presence of nuclear ER-α and membrane HER2 correlates with increased likelihood of response to antioestrogen and anti-HER2 therapy, respectively. The difficulties encountered in immunohistochemical studies include subjectivity of scoring, choice of source of primary antibody, length and type of fixatives, antigen retrieval methods and cut-off values to determine positivity. Our study showed a lower percentage of ER-α positivity compared to 50–80% in previous studies [28]. However, our data with anti-ER-α (clone F-10 from Santa Cruz) was similar to an ER-α immunopositivity of 21% from a previous study from

Table 3 Correlations among the total score of ER-α, ER-β, HER2, p-Akt (Ser473), p-Akt (Thr308), p-BAD, and Ki-67 in breast tumour tissues

		ER-α	ER-β	p-Akt (Thr308)	p-Akt (Ser473)	p-BAD	Ki-67
HER2	C.C	−0.314	0.035	0.198	0.060	−0.024	0.053
	P	0.041[a]	0.825	0.203	0.702	0.877	0.738
ER-α	C.C		−0.008	0.157	0.109	0.185	−0.318
	P		0.958	0.315	0.486	0.235	0.040[a]
ER-β	C.C			0.259	0.050	0.128	0.173
	P			0.094	0.749	0.415	0.273
p-Akt (Thr308)	C.C				−0.062	−0.030	−0.274
	P				0.694	0.848	0.079
p-Akt (Ser473)	C.C					0.379	0.202
	P					0.012[a]	0.200
p-BAD	C.C						−0.186
	P						0.239

Spearman rank correlation test

C.C Correlation coefficient

[a] A 2-sided $p<0.05$ indicates statistical significance

Table 4 Association between Akt phosphorylation and the score of ER-α, HER2, p-BAD (Ser136), and Ki-67 in breast tumour tissues

		Akt phosphorylation				
	n/43 (%)	S473&T308 16 (37.2)	S473 only 5 (11.6)	T308 only 19 (44.2)	None 3 (7.0)	*p*
ER-α	M.R	23.00	23.40	20.84	21.67	0.943
HER2	M.R	25.22	17.30	22.66	8.50	0.121
p-BAD (Ser136)	M.R	24.91[a]	30.10[a]	19.71	7.50[a]	0.032*
Ki-67	M.R	23.13	26.00	19.16	20.67	0.608

Statistical significance of the differences was analyzed using Kruskal–Wallis test

MR mean rank of score

[a] Using Mann–Whitney's *U*-test, S473 only versus none, $P=0.018$ and S473&T308 versus none, $P=0.033$

*$p<0.05$ indicates statistical significance

Malaysia [29]. Monoclonal antibody F-10 binds to the C-terminus of ER-α. Looi et al. [29] used a monoclonal antibody, ER1D5 from Dako which detects the N-terminus of ER-α. All splice variants will also be positive with these two antibodies [30, 31]. It is unclear whether the lower rates of ER-α could be due to age as highest proportion of ER-α positive cases are in the age group of >65 [32]. In our study, about half of the samples (21/43) were ≥50 years of age and only 6/43 (14%) was aged >65.

Overexpression of HER2 has been reported in 25–30% of invasive breast cancer [33]. Our data shows that HER2 positivity rate was high (51.2%) consistent with positivity rate of 44% from a previous Malaysian study [29]. These two Malaysian studies show that a relatively high percentage of breast tumour samples are HER2 positive. We noted that a lower proportion of specimens used in this study were ER-α^{+}HER2^{+} [9/43 (20.9%)] in contrast to 33.3% in the previous study [29]. However, our data show an inverse correlation between HER2 and ER-α expression, which supports the findings reported by others [34, 35]. Moreover, as previously reported [36, 37], we found a negative correlation of ER-α with the proliferation marker, Ki-67, suggestive of more aggressive behaviour of breast cancer with less or no ER-α expression. Indeed, a *ras* superfamily gene has been identified to be up-regulated by ER and able to suppress growth of breast cancer cells [38]. A previous study [39] showed that the ER-α status is determined by the activity of Forkhead box protein FOXO3a, which plays a critical role in cell death and cell cycle arrest and therefore may also explain the finding of inverse correlation between proliferation and ER-α expression.

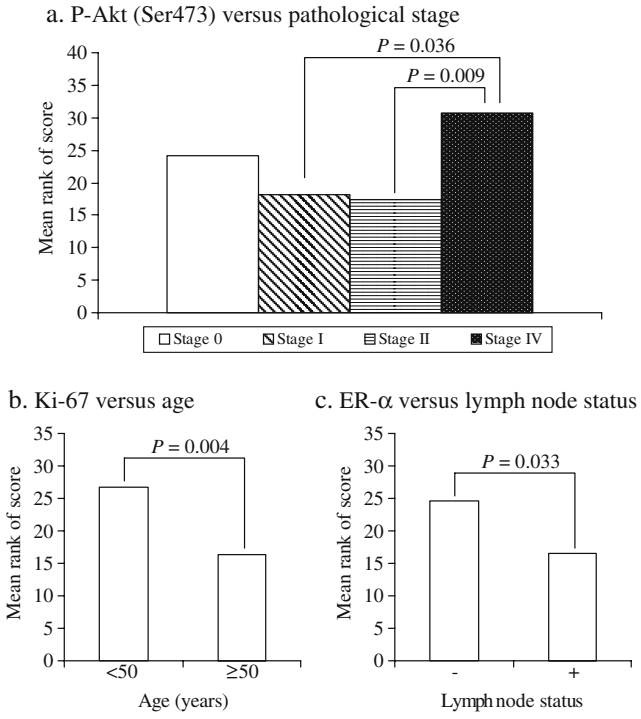


Fig. 3 Biomolecules that were found to be significantly associated with the clinicopathological features include **a** p-Akt (Ser473), **b** Ki-67, and **c** ER-α. Statistical significance ($P<0.05$) of the differences was analyzed using Mann–Whitney's *U*-test

We observed that there was a lower positivity of ER-β (3/43, 7.0%) in the present study compared to that of Fuqua et al. [40] (76%) and Skliris et al. [41] (79%), which used the same antibody clone (14C8) and scoring system (Allred) with ours. However, a difference between these studies was the cut-off point for determination of ER-β positivity. Fuqua et al. [40] and our studies used a value greater than 2, whereas Skliris et al. [41] used a value greater than 4. The low frequency of ER-β in our study may be explained by differences in the size, ethic and clinicopathological background of the breast cancer cohort. We noted that the ER-β level tended to be lower in breast cancers (7.0%) than apparently normal adjacent breast tissues (7/13, 53.8%; data not shown). It has been hypothesized that the progressive loss of ER-β may lead to abnormal cellular proliferation and/or transformation, thus impacting the process of carcinogenesis in breast cancer [42].

In contrast to a study by Al-Bazz et al. [43], our analysis of the relationship between Akt phosphorylation and oestrogen receptor status showed no correlation of p-Akt (Ser473) or p-Akt (Thr308) expression with ER-α or ER-β. Our data suggest that Akt phosphorylation in breast cancer could occur independently from the activity of ER-α. This is consistent with a previous tissue microarray study on 285 cases [44] and also with the in vitro studies, whereby, oestrogen can increase Akt activation in ER-α positive [45] and negative [46] breast cancer cell lines. Conversely, Akt activation was reported to be linked to activation of ER-α, via phosphorylation, in tamoxifen-treated ER-α-positive breast cancers [47].

HER2, also known as c-erbB-2 or neu, is a member of the Type 1 tyrosine kinase growth factor receptor family. It forms heterodimers with other members of its receptor family, such as epidermal growth factor receptor (EGFR/c-erbB-1) and responds to circulating ligands (e.g. epidermal growth factor) to generate the intracellular signal transduction involving multiple proto-oncogene products such as protein kinase C, phosphatidylinositol 3-kinase (PI3K), and Akt kinase [48]. Their constitutive activation occurs as a consequence of overexpression of HER2 [49, 50] and may be sufficient to cause transformation [51]. In the present study, no relationship between p-Akt (Thr308) or (Ser473) and HER2 was found and this is consistent with the previous reports [44, 52]. In contrast, a positive correlation of phosphorylated Akt with HER2 overexpression [53–55] and activation via phosphorylation [47] was reported suggesting that HER2 is an upstream regulator of the Akt signaling in breast cancer.

The inhibition of apoptosis and promotion of cell proliferation, angiogenesis and metastasis are key processes in the development of various malignancies. Akt, a serine/threonine kinase has emerged as an essential mediator of these biological processes [56]. For example, activated Akt phosphorylates BAD at Ser136 which results in its association with 14-3-3 proteins, and thus preventing p-BAD from interacting with Bcl-2 or Bcl-xL and subsequent apoptosis [57]. In a recent study, there was no association between p-Akt level and BAD expression but a significant correlation was found between expression of Akt and BAD [43]. However, the phosphorylation of BAD, indicating of inactivation, was not reported in that study. In the present study, we found a significant correlation of p-Akt (Ser473) with increased level of p-BAD (Ser136). Although the phosphorylation of Akt on both Ser473 and Thr308 has been shown to be required for full activation of the kinase [16, 27], we found no difference between co-phosphorylated Akt and only one of these residues in phosphorylation of the downstream target, BAD. However, we observed that the level of p-BAD (Ser136) is significantly higher in tumours with Akt phosphorylation at both sites or Ser473 only than those that express none of them (Table 4). These findings suggest a possible role of Akt activation in phosphorylation and inactivation of BAD, thus promoting cell survival in breast cancer. In concordance with an in vitro study, inactivation of BAD through ERK (extracellular-regulated kinase) and Akt signaling pathways can abrogate apoptosis of a breast cancer cell line, MCF-7 [58]. We found no correlation between p-Akt and Ki-67 expression although the activation of PI3K/Akt pathway through HER2 is known to increase cell growth and survival [59].

Analysis of the association of biomolecules with clinicopathological features showed that the p-Akt (Ser473) level was significantly elevated in stage IV breast cancer compared to stage I and II (Fig. 3a), which is consistent with a previous report [60]. In a study of trastuzumab-containing neo-adjuvant chemotherapy, however, p-Akt was not associated with the clinical outcome [61]. Moreover, our data showed that the Ki-67 expression was higher in younger (<50 years) breast cancer patients, which is in agreement with the published reports [62, 63]. Unlike reported data that show lack of association between ER-α expression and lymph node status [34, 35], however, Hussein et al. [64] and we found a significantly lower ER-α level in positive nodal status than negative status in our breast cancer cases. Taken together, these findings suggest increased p-Akt (Ser473) level, lower age and reduced/negative ER-α expression are markers for aggressive clinical behavior in breast cancer patients.

In conclusion, our study shows that a higher number of breast tumours were HER2 positive (53.5%) compared to ER-α positive (41.9%). Only 7.0% of the tumours expressed ER-β. In this study, we distinguished the phosphorylation status of Akt at amino acid residue Ser473 and Thr308. No correlation of p-Akt (Ser473) or p-Akt (Thr308) with HER2 or ER-α was found. Our data shows clearly that there was a positive correlation between p-Akt (Ser473) level and the phosphorylation of downstream target, BAD suggesting a role of Akt pathway in inhibition of apoptosis and ultimately, leads to survival of the tumour. The increase of p-Akt (Ser473) level, as well as lower ER-α expression, was associated with more aggressive tumours. This study provides information on alterations of these molecules which may involve in the mechanisms that underlie breast carcinogenesis. We are aware that the sample size was relatively small and therefore, data need to be further investigated with larger sample size. Taken collectively, we suggest that Akt is a relevant target in a reasonable proportion of our Malaysian patients. However, further elucidation of the significance of Akt activation in clinical outcome is needed to provide more useful information about treatment strategy for breast cancer patients.

Acknowledgement This project is supported by a grant from The Ministry of Science, Innovation and Technology, Malaysia (06-02-04-0373-PR002/07-07).

References

1. Lim GCC, Halimah Y (2004) Second report of the national cancer registry: cancer incidence in Malaysia 2003. National Cancer Registry, Malaysia
2. Clarke R, Skaar T, Leonessa F et al (1996) Acquisition of an antiestrogen-resistant phenotype in breast cancer: role of cellular and molecular mechanisms. Cancer Treat Res 87:263–283
3. Ring A, Dowsett M (2004) Mechanisms of tamoxifen resistance. Endocr Relat Cancer 11:643–658
4. Pandolfi PP (2004) Breast cancer—loss of PTEN predicts resistance to treatment. N Engl J Med 351:2337–2338
5. Tokunaga E, Kataoka A, Kimura Y et al (2006) The association between Akt activation and resistance to hormone therapy in metastatic breast cancer. Eur J Cancer 42:629–635
6. Brognard J, Clark AS, Ni Y et al (2001) Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 61:3986–3997
7. Clark AS, West K, Streicher S et al (2002) Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther 1:707–717
8. Luo Y, Shoemaker AR, Liu X et al (2005) Potent and selective inhibitors of Akt kinases slow the progress of tumors in vivo. Mol Cancer Ther 4:977–986
9. Kondapaka SB, Singh SS, Dasmahapatra GP et al (2003) Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther 2:1093–1103
10. Jones PF, Jakubowicz T, Hemmings BA (1991) Molecular cloning of a second form of rac protein kinase. Cell Regul 2:1001–1009
11. Bellacosa A, Kumar CC, Di Cristofano A et al (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res 94:29–86
12. Masure S, Haefner B, Wesselink JJ et al (1999) Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. Eur J Biochem 265:353–360
13. Cheng JQ, Godwin AK, Bellacosa A et al (1992) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci U S A 89:9267–9271
14. Alessi DR, James SR, Downes CP et al (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7:261–269
15. Stephens L, Anderson K, Stokoe D et al (1998) Protein kinase B kinases that mediate phosphatidylinositol 3, 4, 5-trisphosphate-dependent activation of protein kinase B. Science 279:710–714
16. Sarbassov DD, Guertin DA, Ali SM et al (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098–1101
17. Hresko RC, Mueckler M (2005) mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J Biol Chem 280:40406–40416
18. Dimmeler S, Fleming I, Fisslthaler B et al (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399:601–605
19. Nakanishi K, Sakamoto M, Yasuda J et al (2002) Critical involvement of the phosphatidylinositol 3-kinase/Akt pathway in anchorage-independent growth and hematogeneous intrahepatic metastasis of liver cancer. Cancer Res 62:2971–2975
20. Altomare DA, Testa JR (2005) Perturbations of the AKT signaling pathway in human cancer. Oncogene 24:7455–7464
21. Wu Y, Mohamed H, Chillar R et al (2008) Clinical significance of Akt and HER2/neu overexpression in African–American and Latina women with breast cancer. Breast Cancer Res 10:R3
22. Lazennec G, Bresson D, Lucas A et al (2001) ER beta inhibits proliferation and invasion of breast cancer cells. Endocrinology 142:4120–4130
23. Rajnakova A, Goh PM, Chan ST et al (1997) Expression of differential nitric oxide synthase isoforms in human normal gastric mucosa and gastric cancer tissue. Carcinogenesis 18:1841–1845
24. Endo K, Terada T (2000) Protein expression of CD44 (standard and variant isoforms) in hepatocellular carcinoma: relationships with tumor grade, clinicopathologic parameters, p53 expression, and patient survival. J Hepatol 32:78–84
25. Allred DC, Harvey JM, Berardo M et al (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 11:155–168
26. Cobleigh MA, Vogel CL, Tripathy D et al (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639–2648
27. Scheid MP, Marignani PA, Woodgett JR (2002) Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Mol Cell Biol 22:6247–6260
28. Nadji M, Gomez-Fernandez C, Ganjei-Azar P et al (2005) Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. Am J Clin Pathol 123:21–27
29. Looi LM, Cheah PL (1998) C-erbB-2 oncoprotein amplification in infiltrating ductal carcinoma of breast relates to high histological grade and loss of oestrogen receptor protein. Malays J Pathol 20:19–23
30. Pavao M, Traish AM (2001) Estrogen receptor antibodies: specificity and utility in detection, localization and analyses of estrogen receptor alpha and beta. Steroids 66:1–16
31. Speirs V, Green CA, Shaaban AM (2008) Oestrogen receptor beta immunohistochemistry: time to get it right? J Clin Pathol 61:1150–1151 author reply 1151-2
32. Rhodes A, Jasani B, Balaton AJ et al (2000) Frequency of oestrogen and progesterone receptor positivity by immunohistochemical analysis in 7016 breast carcinomas: correlation with patient age, assay sensitivity, threshold value, and mammographic screening. J Clin Pathol 53:688–696
33. Slamon DJ, Godolphin W, Jones LA et al (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707–712
34. Chariyalertsak S, Chariyalertsak S, Cheirsilpa A et al (1996) Immunohistochemical detection of c-erbB-2 oncoprotein in patients with breast cancer. J Med Assoc Thail 79:715–721
35. Almasri NM, Al Hamad M (2005) Immunohistochemical evaluation of human epidermal growth factor receptor 2 and estrogen and progesterone receptors in breast carcinoma in Jordan. Breast Cancer Res 7:R598–R604
36. Rosa FE, Caldeira JR, Felipes J et al (2008) Evaluation of estrogen receptor alpha and beta and progesterone receptor expression and correlation with clinicopathologic factors and proliferative marker Ki-67 in breast cancers. Hum Pathol 39:720–730
37. Jarzabek K, Koda M, Kozlowski L et al (2005) Distinct mRNA, protein expression patterns and distribution of oestrogen receptors alpha and beta in human primary breast cancer: correlation with proliferation marker Ki-67 and clinicopathological factors. Eur J Cancer 41:2924–2934

38. Finlin BS, Gau CL, Murphy GA et al (2001) RERG is a novel ras-related, estrogen-regulated and growth-inhibitory gene in breast cancer. J Biol Chem 276:42259–42267
39. Guo S, Sonenshein GE (2004) Forkhead box transcription factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway. Mol Cell Biol 24:8681–8690
40. Fuqua SA, Schiff R, Parra I et al (2003) Estrogen receptor beta protein in human breast cancer: correlation with clinical tumor parameters. Cancer Res 63:2434–2439
41. Skliris GP, Munot K, Bell SM et al (2003) Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. J Pathol 201:213–220
42. Fox EM, Davis RJ, Shupnik MA (2008) ERbeta in breast cancer—onlooker, passive player, or active protector? Steroids 73:1039–1051
43. Al-Bazz YO, Underwood JC, Brown BL et al (2009) Prognostic significance of Akt, phospho-Akt and BAD expression in primary breast cancer. Eur J Cancer 45:694–704
44. Bose S, Chandran S, Mirocha JM et al (2006) The Akt pathway in human breast cancer: a tissue-array-based analysis. Mod Pathol 19:238–245
45. Ahmad S, Singh N, Glazer RI (1999) Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 58:425–430
46. Tsai EM, Wang SC, Lee JN et al (2001) Akt activation by estrogen in estrogen receptor-negative breast cancer cells. Cancer Res 61:8390–8392
47. Kirkegaard T, Witton CJ, McGlynn LM et al (2005) AKT activation predicts outcome in breast cancer patients treated with tamoxifen. J Pathol 207:139–146
48. Lenferink AE, Busse D, Flanagan WM et al (2001) ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. Cancer Res 61:6583–6591
49. Hermanto U, Zong CS, Wang LH (2001) ErbB2-overexpressing human mammary carcinoma cells display an increased requirement for the phosphatidylinositol 3-kinase signaling pathway in anchorage-independent growth. Oncogene 20:7551–7562
50. Pianetti S, Arsura M, Romieu-Mourez R et al (2001) Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. Oncogene 20:1287–1299
51. Zhang G, He B, Weber GF (2003) Growth factor signaling induces metastasis genes in transformed cells: molecular connection between Akt kinase and osteopontin in breast cancer. Mol Cell Biol 23:6507–6519
52. Panigrahi AR, Pinder SE, Chan SY et al (2004) The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. J Pathol 204:93–100
53. Schmitz KJ, Otterbach F, Callies R et al (2004) Prognostic relevance of activated Akt kinase in node-negative breast cancer: a clinicopathological study of 99 cases. Mod Pathol 17:15–21
54. Stal O, Perez-Tenorio G, Akerberg L et al (2003) Akt kinases in breast cancer and the results of adjuvant therapy. Breast Cancer Res 5:R37–R44
55. Zhou X, Tan M, Stone Hawthorne V et al (2004) Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. Clin Cancer Res 10:6779–6788
56. Hill MM, Hemmings BA (2002) Inhibition of protein kinase B/Akt. implications for cancer therapy. Pharmacol Ther 93:243–251
57. Zha J, Harada H, Yang E et al (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87:619–628
58. Fernando RI, Wimalasena J (2004) Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. Mol Biol Cell 15:3266–3284
59. Krasilnikov MA (2000) Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. Biochemistry (Mosc) 65:59–67
60. Liao Y, Hung MC (2003) Regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenoviral protein E1A-mediated sensitization to apoptosis. Mol Cell Biol 23:6836–6848
61. Yonemori K, Tsuta K, Shimizu C, et al (2009) Immunohistochemical expression of PTEN and phosphorylated Akt are not correlated with clinical outcome in breast cancer patients treated with trastuzumab-containing neo-adjuvant chemotherapy. Med Oncol 26:344-349
62. Klauber-DeMore N (2005) Tumor biology of breast cancer in young women. Breast Dis 23:9–15
63. Hartley MC, McKinley BP, Rogers EA et al (2006) Differential expression of prognostic factors and effect on survival in young (< or =40) breast cancer patients: a case-control study. Am Surg 72:1189–1194 discussion 1194-5
64. Hussein MR, Abd-Elwahed SR, Abdulwahed AR (2008) Alterations of estrogen receptors, progesterone receptors and c-erbB2 oncogene protein expression in ductal carcinomas of the breast. Cell Biol Int 32:698–707