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The level of phosphorylated Akt predominantly reflects the expressive status of CerbB2 in invasive breast cancer

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Summary. Although some evidence has been documented on EGFR/PI3K mediation of Akt activation in breast cancers, ILK and DNA-PK have not been investigated so far. The aim of this study was to analyze the expression of phosphorylated Akt (pAkt) in breast cancer, with respect to its upstream regulators. The immunostaining of pAkt (Ser473) in 70 invasive breast cancers revealed that status of CerbB2 could play a major role in Akt phosphorylation, while ILK was also involved in the stimulated level of pAkt. The results would provide an important clue for the activation of Akt and potential targeted therapy in breast cancer.

Key words: pAkt, CerbB2, ILK, DNA-PK, Breast cancer

Introduction

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a central node in cell signaling downstream of growth factors, cytokines and other cellular stimuli. It has been confirmed that Akt takes part in a variety of cellular processes, including cell survival, growth, proliferation, angiogenesis, cellular metabolism, and cell migration and invasion. Several laboratories have reported Akt phosphorylation is increased in tumors of the breast, prostate, ovary, and pancreas. Phosphorylated Akt(pAkt) exerts its effects in the cell by phosphorylating a wide variety of downstream substrates, including caspase 9, MDM2, androgen receptor (AR), p21, p27, GSK (glycogen synthase kinase) and others (Paez and Sellers, 2004).

Activation of Akt1 is a multistep process initiated primarily through the binding of exogenous growth factors to receptors which have intrinsic tyrosine-kinase activity, such as the epidermal growth factor receptor(EGFR). Such binding phosphorylates (activates) the intracellular tyrosine-kinase-containing moiety of the receptor (Jiang and Liu, 2009). One of the several proteins recruited and activated by tyrosine phosphorylated growth factor receptor is PI3-kinase, which converts phosphatidylinositol-4,5- bisphosphate (PIP2) to PIP3, leading to recruitment of Akt1 to the plasma membrane. Akt1 phosphorylation is mediated by phosphatidyl inositol-3-phosphate-dependent kinase, PDK1, which phosphorylates Akt1 at Threonine (Thr) 308 (Vogt et al., 1999; Woodgett, 2005; Jiang and Liu, 2009). And another kinase, PDK2, which phosphorylates Akt1 at Serine (Ser) 473, has a big family, including DNA-PK, ILK, oxidative stresses, HSP90 (heat shock protein-90KDa) and PKC-b (protein kinase C-Beta), etc (Sarbassov et al., 2005; Matheny and Adamo, 2009).

CerbB2 amplification and overexpression have been reported in 18-25% of human breast cancers (Baselga and Swain, 2009). CerbB2 amplification is an early event in the development of breast cancers (Park et al., 2006), and the overexpression of CerbB2 is associated with a poor prognosis in breast cancers. Targeting CerbB2 with humanized monoclonal antibodies is a viable approach for inhibiting CerbB2 and achieving anti-tumouractivity (Furberg et al., 2002; Akamatsu et al., 2003; Beskow et al., 2006). Since it is a member of the EGFR family, currently, an abundance of basic and pre-clinical studies have been conducted to elucidate the implications of CerbB2/Akt pathway derangements in breast cancer using different detection systems and tissue platforms (Blanco-Aparicio et al., 2007; Al-Bazz et al.,

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2009). The use of immunohistochemistry (IHC) has been collectively considered as a useful means of identifying pAkt status in a wide range of human cancers, including breast cancer, with most studies reporting that increased pAkt expression correlates with clinical aggressiveness and progression (Shtilbans et al., 2008; Wu et al., 2008), while others found no such associations (Frogne et al., 2009). Therefore, a more comprehensive understanding of the signaling intricacies is necessary, not only to identify the diagnostic specific marker, but also to develop agents to target specific molecules.

In order to elucidate the correlations of CerbB2 and pAkt overexpression, as well as the relationship between CerbB2 status and Akt upstream molecules, including DNA-PK and ILK in invasive breast cancers, we detected several relevant antibodies in 70 invasive breast cancer cases by immunohistochemistry.

Materials and methods

Patients and tissue specimens

In this study, we selected 70 patients with invasive breast cancer and 21 patients with ductal carcinoma in situ who had been histologically confirmed by two experienced pathologists , according to WHO Classification. All of them underwent radical operations in the Pathology Department of Peking University Third Hospital. TNM clinical stage was classified according to WHO standard, and histologic score was according to Nottingham Histologic Score.

Thin slices of tumor tissue of all cases received in our histopathology unit were fixed in 4% neutral - buffered formaldehyde solution (pH 7.0) for periods not exceeding 24 h. The tissues were processed routinely for paraffin embedding, and 4 μm-thick sections were cut for immunohistochemistry.

Immunohistochemistry

Paraffin-embedded sections were hydrated by series treatments of xylene and graded alcohols. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 60 min. The antigen retrieval was done by heating at 95°C in "A" (0.02-mol/l citrate buffer, pH 6.0) or "B" (EDTA buffer, PH 8.0) for 20 min. After blocking with horse serum (1:100), primary antibodies were diluted in PBS of different ratios (Table 1) and incubated at -4°C overnight, followed by washing in PBS. Color developing was achieved by PV-9000 (GBI,USA) and DAB (Dako liquid DAB Plus, K3468; Dako, Denmark). Sections were counterstained with Mayer's haematoxylin. Negative (primary antibody replaced by PBS) and positive (a known pAkt positive section) controls were included in the staining run.

Staining evaluation

The slides were evaluated separately by two independent observers blinded to the clinical data.

pAkt generally presented moderate cytoplasmic and nuclear staining and was classified as: -: <10% cells in weak or scattering positive; +: >10% cells stained in diffused or multiple foci. CerbB2 was scored by the widely accepted criteria that assessed the intensity and completeness of membrane staining as previously described (Jacobs et al., 1999; Seidman et al., 2001). DNA-PKcs staining was moderate brown uniformly in nuclear of normal and cancer component. While γH2AX staining focused in nuclear in scattered distribution pattern, and was classified as followed: "-", occasionally cells stained less than 5%; "+", >5% cells stained in multiple foci pattern of the nuclear. ILK staining was moderate/strong brown in the cytoplasm of cancer and stromal cells, and the cut-off was set at 10%: "-", <10% cells were stained; "+", >10% of the cells was positive. ·-pAkt-MOTIF staining was generally moderate or strong brown in the cytoplasm and nuclear : "-", <10% the cells were stained; "+", >10% the cells were stained. ER, PR and p53 was evaluated as previously described (Ellis et al., 2001; Furberg et al., 2002).

Statistical analysis

All data were analyzed with SPSS statistics software (Version 13.0, Chicago, IL, USA). Relationships between tumor markers and other parameters were studied using χ^2-test, Pearson Chi-square, Continuity

Table 1. Immunohistochemistry primary antibodies introduction.

Antibody	Source	Dilution	Antigen retrieval	Corporation
pAkt	rabbit , monoclonal	1:50	Heat, B	Cell Signaling Technology
α-pAkt-MOTIF	rabbit , monoclonal	1:800	Heat, A	Cell Signaling Technology
DNA PKcs	rabbit, polyclonal	1:500	Heat, A	Abcam
ILK	mouse, monoclonal	1:100	Heat, A	Millipore
γH2AX(Ser139)	mouse, monoclonal	1:300	Heat, A	Millipore
p53	rat, polyclonal	1:100	Heat, A	Cell Signaling Technology
ER	mouse, monoclonal	1:100	Heat, A	Dako
PR	mouse, monoclonal	1:100	Heat, A	Dako
CerbB2	mouse, monoclonal	1:100	Heat, A	Dako

Correlation and Fisher's exact test. A P-value of less than 0.05 was considered to be statistically significant.

Results

Immuno-staining of pAkt in invasive breast cancers

Phosphorylated Akt was stained in about 47.14 % (33/70) cases of invasive breast cancer, presenting both cytoplasmic and nuclear staining. The immuno-staining in cancer cells was generally in multiple foci or diffuse pattern. In contrast, the surrounding normal ducts were usually negative, except in a few cases, in which a few scattered cells were occasionally and weakly stained (Fig. 1a, cancer and normal). Meanwhile, all 21 cases of ductal carcinoma in situ were negatively stained for pAkt, except in a few cases, with occasional cells with weak staining. The difference between invasive cancer and in situ was statistically significant (P<0.05). The results suggested that Akt phosphorylation could be an indicator for invasive breast cancers.

Expression of CerbB2 positively correlated with pAkt levels in invasive cancers

EGFR/PI3K is the major signaling pathway for Akt activation, and thus, the expression status of CerbB2, a member of EGFR family, was under analysis.

The expression distribution of CerbB2 in 70 cases of breast cancer samples consisted of 21.42 % (15/70) negative, 25.71% (18/70) 1+, 27.14 % (19/70) 2+, and 25. 71% (18/70) 3+ (Fig. 1b). The status of CerbB2 closely correlated with the level of Akt phosphorylation, and high phosphorylation of Akt usually reflected the over-expression of CerbB2 (P=0.001, Table 2).

Expression of ILK in invasive cancers and its relationship with pAkt level

Evidence has been documented for years that ILK plays an important role in Akt phosphorylation in cancer development or progression. In this study, ILK positive staining was generally detected in stromal components of all breast cancers with varying staining intensity. Most fibroblasts, endothelia, or macrophages in stroma were usually positive, while in 25 of 66 (37.9%) cases cancer cells expressed ILK in cytoplasmic staining (Fig. 1c). However, there were no correlations between ILK expression and pAkt (P?0.05) (Table 2), implying that ILK has no direct influence on Akt phosphorylation.

Since the staining of ILK was mainly in stromal components and some invasive cancers, 21 cases of ductal carcinoma in situ of breast were also analyzed. ILK was positive in only 3 cases, with the stromal cells in weak staining. It indicated that ILK activation in cancer cells should need an interaction with stromal cells.

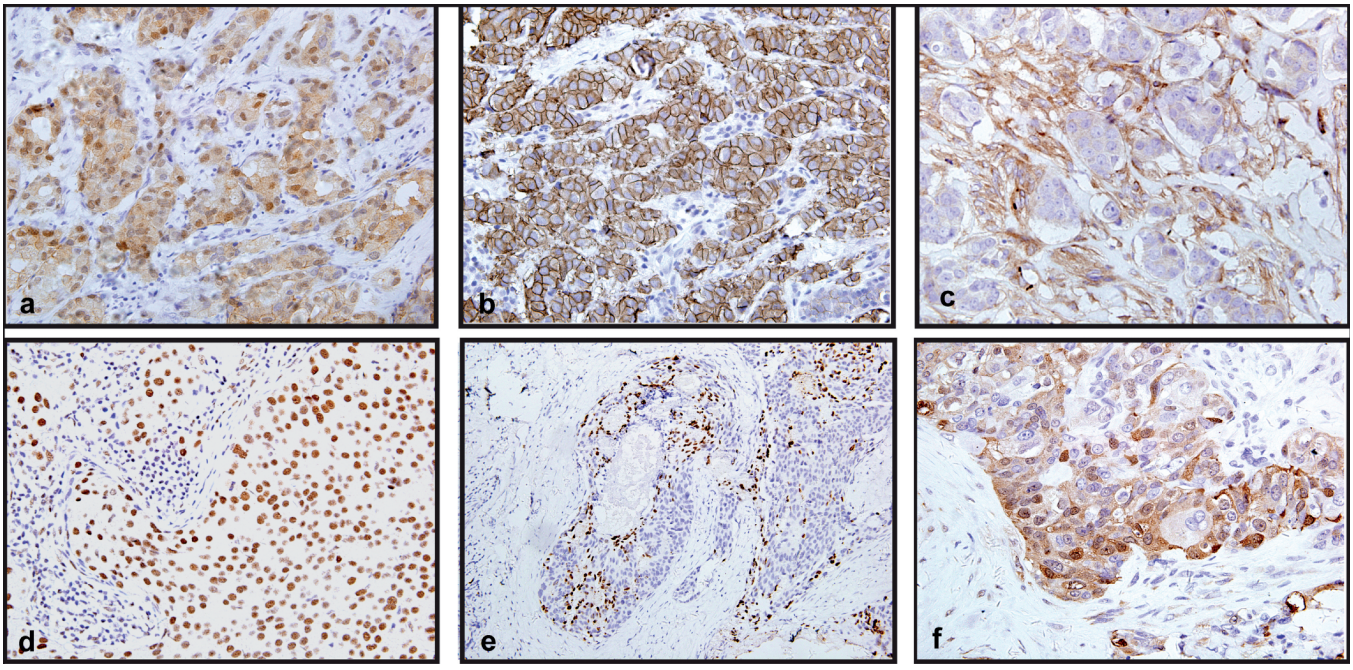

Fig. 1. Representative images of immuno-staining in breast cancers. **a.** Staining of pAkt both in cytoplasmic and nuclear. **b.** CerbB2 expression, 2+. **c.** The ILK showed positive cytoplasmic staining in both cancer cells and stromal cells. **d.** DNA-PK expressed in the nuclei of both normal and malignant breast tissues. **e.** The positive staining of γH2AX in the nuclei of cancer cells. **f.** Expression of pAkt-MOTIF in cytoplasm and nuclei of cancer cells. a-d, f, x 400; e, x 200

Expression of DNA-PKcs/γH2AX in invasive cancers and its relationship with pAkt level

DNA-PK is a member of the phosphatidylinositol-3-kinase (PI-3-K) superfamily and has been identified as a factor required for activation of Akt (PKB) via phosphorylation in response to DNA damage (Bozulic et al., 2008). The detection of DNA-PKcs, the core-subunit of DNA-PK, was carried out and the results showed that DNA-PKcs generally presented a nuclear staining in both cancer cells and the surrounding non-neoplastic breast tissue (Fig. 1d). A similar immunostaining of DNA-PKcs has been described in other reports (Akamatsu et al., 2003; Beskow et al., 2006).

Stucki and Jackson (2006) has found that cells express DNA-PKcs at a constant level, and DNA-PKcs is activated through a complex formation to phosphorylate the downstream effector, such as H_2AX. Therefore, immunostaining of phosphorylated H_2AX (γH_2AX) was performed and showed that positive staining was detected only in cancer cells without any staining in normal breast tissue, reflecting a cancer-specific status (Fig. 1e). About 35.8% (24/67) cases were positively stained with γH_2AX. However, there were no correlations between pAkt and γH_2AX expression (P>0.05) (Table 2). This implied that the DNA-PK pathway is not involved in the activation of

Table 4. Correlation between pAkt expression and CerbB2/γH2AX or ILK co-expression.

Markers of coexpression	pAkt expression		Significance	
	-	+	χ^2	p value
CerbB2/ILK			9.078	0.003
-	31	19		
+	3	13		
CerbB2/γH2AX			2.345	0.126
-	29	23		
+	5	10		

Table 2. Correlation between pAkt staining and the expression of CerbB2, γH2AX, ILK, pAkt-MOTIF.

Marker	pAkt expression		Sum	Significance	
	-	+		χ^2	P value
CerbB2			70	16.924	0.001
-	14	1			
1+	10	8			
2+	9	10			
3+	4	14			
ILK					
-	23	18	66	0.901	0.340
+	11	14			
γH2AX			67	0.175	0.676
-	22	21			
+	11	13			
pAkt-MOTIF			69	10.054	0.002
-	9	0			
+	26	34			

Table 3. Correlation between pAkt staining and the expression of CerbB2, γH2AX, ILK.

Tissue marker	pAkt negative expression			pAkt positive expression		
	CerbB2 (-/1+)	CerbB2 (2+/3+)	p value	CerbB2 (-/1+)	CerbB2 (2+/3+)	p value
ILK			0.465			0.044
-	13	10		8	10	
+	8	3		1	13	
γH2AX			0.709			0.108
-	15	8		7	13	
+	6	5		1	12	

Table 5. Correlation between pAkt staining and the expression of ER, PR, P53.

Tissue marker	pAkt expression		Significance	
	negative	positive	χ^2	p value
ER			1.377	0.241
-	13	17		
+	23	17		
PR			0.051	0.822
-	6	5		
+	30	29		
P53			0.229	0.632
-	19	16		
+	17	18		

Table 6. Correlation between pAkt expression and clinicopathological features.

	pAkt expression		Significance	
	Negative	positive	χ^2	p value
Menopausal status			0.000	1.000
Premenopausal	19	19		
Postmenopausal	16	16		
TNM Clinical Stage			1.200*	0.273*
T1N0	14	14	3.590	0.058
T1N1	4	8		
T2N0	9	2		
T2N1	3	6		
Histologic grades			2.116	0.347
Grade 1(scores of 3-5)	11	6		
Grade 2(scores of 6/7)	10	14		
Grade 3(scores of 8/9)	15	14		

Akt in invasive breast cancer.

Correlation between pAkt staining and the co-expression of CerbB2, DNA-PKcs/γH2AX, or ILK in invasive cancers

When pAkt expression was positive, the correlations between the co-expression of CerbB2/γH2AX and pAkt had no significance (P>0.05, Table 3). However, when pAkt was positive, 13 of 32 (44.8%) cases had positive co-expression of CerbB2 and ILK, and there were close correlations between CerbB2 and ILK (P<0.05, Table 3). Furthermore, the correlations between co-expression of CerbB2/ILK and pAkt had close significance (P<0.05, Table 4). Thus, it seemed that DNA-PK had little influence on Akt phosphorylation, but the ILK pathway had a minor effect on Akt phosphorylation.

Correlation between pAkt staining and the expression of pAkt-MOTIF in invasive cancers

Activated Akt induces its downstream targets by phosphorylation, in which pAkt usually phophorylates a conserved sequence (pAkt-MOTIF) in its substrates: -RXXRS/T-. To confirm the effect of activated Akt on its down-stream effectors, we analyzed the level of global pAkt-MOTIF in breast cancers. The staining of pAkt-MOTIF (n=70) showed both cytoplasmic and nuclear immunoreactivity, and most cases 60/69 of invasive breast cancers were positive (Fig. 1f). There were close positive correlations between the expression of pAkt-MOTIF and pAkt (P>0.05, Table 2), indicating that pAkt activates its downstream effectors in breast cancers.

Correlation between pAkt staining and the expression of ER, PR or P53 in invasive cancers

There was no significant correlation of ER, PR expression and P53 nuclear accumulation with pAkt expression (P>0.05, Table 5).

Correlation between pAkt expression and clinicopathological features in invasive cancers

There was no correlation between pAkt expression and menopausal status in these cases, or pAkt and TNM Clinical Stage or histologic grade (P>0.05, Table 6). However, the statistical difference between pAkt expression and lymph node metastasis was near to being significant (P=0.058>0.05, Table 6) (see Discussion).

Discussion

In view of the known biological importance of the PI3K/Akt pathway in breast cancer, we used immunohistochemistry to assess the abundance and localization of the phosphorylated form of Akt, pAkt, in a series of 70 invasive breast cancers. Expression of pAkt in this study demonstrated that 33/70 (47%) of breast cancer cells overexpressed pAkt. This frequency of pAkt positivity was lower than that reported by Mohammed A (Aleskandarany et al., 2011), who reported 76% in their cohort. The reasons for the differences between these results could come from several points, including the primary antibodies used, the standard of evaluation. In this analysis, the positive staining of pAkt was generally more than 50% of cancer cells, representing its high phosphorylated status. Then this prevalence of pAkt in breast cancers is believed to be sufficient for its oncogenetic role that was reported to occur early during the cascade of carcinogenic events (Bose et al., 2005). In our study, there was a close correlation between pAkt staining and CerbB2 expression (P<0.05). Thus, a direct association was revealed between pAkt expression and CerbB2 status.

CerbB2(HER2) is one member of the HER family. The HER family includes EGFR, HER2, HER3 and HER4, which are well-known Akt upstream activators. The cooperation between the HER receptors with HER2 to produce a more aggressive tumor phenotype is well-evidenced (Zaczek et al., 2005). As is well-known, breast cancer is a heterogeneous disease, including several molecular subtypes, such as luminal, ER positive, CerbB2-rich, basal-like or triple-negative. On the basis of our results, CerbB2 could be a major regulator of Akt in breast cancer, and pAkt expression could be a useful predictor of CerbB2 status. In addition, the Akt pathway induces resistance to the apoptotic response, and the inhibition of this pathway is now considered to be a promising strategy to improve the effect of therapies for various kinds of cancers [reviewed in (Thompson and Thompson, 2004)], so in breast cancer inhibiting Akt phosphorylation should be the target of clinical therapy. Another member of the HER receptors, CerbB3(HER3), usually forms a heterodimer with HER2 and also plays an important role in activation of Akt. HER2 could phosphorylate HER3 and make the latter bind directly to p85, the regulator of PI3 kinase, through its YXXM motifs (Way and Lin, 2005). Knockdown or blocking binding of Neuregulins by monoclonal antibody of HER3, or disruption of interaction between HER2 and HER3 has been demonstrated to suppress the phenotypes that HER2 mediates (Blackburn et al., 2012). It is reasonable that some cases with low levels of HER2 also displayed high levels of pAkt. Therefore, pAkt level could also reflect the combined effects of HER receptors.

ILK is an essential factor in mediation of signal transduction between the extracellular matrix and intracellular processes, and regulates proliferation, migration or metastasis of cancer cells through regulation interaction with cytoskeletal actin or tubules, and its role in Akt phosphorylation has been well documented for years, although the exact mechanism remains controversial. ILK-modified mice genetically displayed accelerated development and growth of breast cancer (Oloumi et al., 2010). In this study, it was impressive that the expression of ILK was mainly detected in stromal cells surrounding the neoplastic cells,

while only in some cases the cancer cells was positive. Especially, ILK was rarely detected in non-invasive cancers. This suggests that the activation of ILK in cancer cells need interaction between the cancer cell and its microenvironment. Therefore, it is understandable that ILK was detected in some cases of pAkt positive, in which ILK could be involved in the activation of Akt in the presence of CerbB2 expression. Thus, in some invasive breast cancers Akt activation could be a cooperative result induced by both CerbB2 expression and ILK activation, of which the latter is usually triggered by tumor microenvironment. It is also assumed that the involvement of ILK in pAkt activation might play a role in invasive breast cancer progression. Actually, Inhibition of ILK by small inhibitors profoundly blocks invasiveness of breast cancers that express high level sof HER2 (Pontier et al., 2010).

Although Akt activation in human cancer tissues has been extensively investigated, the status of its downstream effectors has rarely been evaluated up to now. The generation of a specific antibody against Akt phosphorylated conservative sequence makes it possible to monitor the global phosphorylation of pAkt substrates. Our study confirmed the concordance between the staining of α-pAkt MOTIF and pAkt staining ($P<0.05$), and also indicated that the detection of global phosphorylation by pAkt could be another predictor of pAkt status in invasive breast cancer.

In recent years several reports have confirmed that DNA-PK directly phosphorylates both threonine 308 and serine 473 of Akt(PKB) in response to either irradiation or DNA-damaging agent treatment, acting as a kinase of PDK2 to activate Akt. However, the correlation of DNA-PK and Akt has not been investigated in human tumor tissues. In our analysis, DNA-PKcs was generally detected in both cancer and normal tissue, similar to a report described by Moll et al. (1999), suggesting that the expression of DNA-PKcs maintains a constant level in either tumor or normal situations. In addition, some research found that the activation of DNA-PK is usually achieved via complex formation rather than elevation of expressed quantity. Therefore, we performed a detection of γH2AX, a substrate of DNA-PK in our investigation. In contrast to DNA-PKcs, γH2AX was stained in limited cases (35.8%, 24/67 cases) and the positive staining was only in cancer cells. The data might suggest that genomic instability in breast cancers is not be a major event, and thus DNA-PK was not an essential factor in activation of Akt.

As is well-known, breast cancer is a heterogeneous disease and several molecular variants have been proposed, including luminal, ER-positive, CerbB2-rich, basal-like or triple-negative. Despite over-lapping molecular alterations, each subtype of breast cancer holds distinct genetic changes. Thus, the close correlation between pAkt and CerbB2 makes it reasonable that there was no significant correlation between pAkt expression and other routine factors, such as hormonal receptors and P53 nuclear accumulation ($P>0.05$).

TNM Clinical Stage has been successfully used in evaluation of breast cancer progression. Despite lacking a significant close correlation between TNM and pAkt expression, in statistics the correlations of pAkt and lymph node status was $p=0.058$, near the cut-off value ($p=0.05$). Considering that most cases belonged to early invasive breast cancer (T1N0), we supposed that pAkt expression might be related with clinical stage if more advanced stage cases were included.

In summary, Akt activation in invasive breast cancer may be a reflection of its oncogenetic role during the cascade of carcinogenic events. CerbB2 was the main upstream factor to activate Akt, and ILK also acts as a co-factor in breast cancer progression. More importantly, the described investigation reveals a close correlation between the level of phosphorylated Akt and CerbB2 expression, and a potential role in evaluation of EGFR/Akt signaling status.

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