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Clinicopathologic and Molecular Significance of phospho-Akt expression in Early Invasive Breast Cancer

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Abstract

Akt/PKB serine/threonine kinase is a leading signalling modulator for several cellular processes including metabolism, growth, proliferation, and survival. However, complexity and diversity in the upstream/downstream arms of Akt pathway, as recent genetic studies reported, challenge considerably the evolvement of effective targeted therapies.

Aim: To study the expression of phospho-Akt1 (pAkt) in breast cancer (BC), with respect to different component proteins upstream/downstream of Akt pathway activation, clinicopathologic parameters, and patients' outcome. pAkt (ser473) was evaluated by immunohistochemistry, on tissue microarrays containing 1,202 early invasive BC with long term clinical follow-up.

Seventy six percent of the studied tumours overexpressed pAkt, where it was associated with expression of estrogen and androgen receptors, PIK3CA, cytokeratin (CK)18, CK19 and PTEN. Loss of pAkt was correlated with high grade, CK5/6, p53 and high Ki67 labelling index. Higher proportions of luminal tumours were pAkt positive relative to triple negative/basal subtypes. However, pAkt overexpression was not associated with breast cancer specific (BCSS) or metastasis free survival (MFS). Four tumour phenotypes were identified based on PIK3CA and pAkt expression, with substantial proportions being PIK3CA⁻/pAkt⁺ or PIK3CA⁺/pAkt⁻. These four combinatorial phenotypes were significantly associated with BCSS (p=0.001) and MFS (p=0.002).

Although pAKT is an oncogene correlated with poor prognostic variables, it was not a prognostic marker. Combinatorial phenotypic groups of PIK3CA/pAkt denoted functional complexity, at translational level, within the upstream and downstream arms of Akt activation with significant impact on patients' outcome. These findings may help development more adequate therapeutic regimens for specific subgroups of this key cancer pathway.

Introduction

Akt (also termed Protein Kinase B, PKB), a serine/threonine kinase, is an important signalling mediator between extracellular stimuli and the modulation of diverse cellular processes including metabolism, cell growth, proliferation, and survival [1,2]. Akt is a major downstream target of the phosphoinositide 3-kinase (PI3-K) signalling pathway [3]. Recent studies have revealed that the PI3-K/Akt signalling network harbours some of the most prevalent genetic derangements in cells and tissues derived from almost all human solid cancers [4]. Therefore, PI3K/Akt has emerged as a key target for cancer treatment strategies [5]

Activation of Akt1 is a multistep process initiated primarily through the binding of exogenous growth factors to receptors having intrinsic tyrosine-kinase activity. Such binding phosphorylates, and activates the intracellular tyrosine-kinase-containing moiety of the receptor [6]. One of the several proteins recruited to and activated by the tyrosine phosphorylated growth factor receptor is PI3-kinase, which converts phosphatidyl inositol-4, 5-bisphosphate (PIP₂) at the 3' position of the inositol ring to PIP₃, leading to recruitment of Akt1 via its pleckstrin homology (PH) domain to the plasma membrane. Akt1 phosphorylation is mediated by phosphatidyl inositol-3-phosphate dependent kinases; PDK1 which phosphorylates Akt1 at Threonine (Thr) 308, and another kinase that phosphorylates it at Serine (Ser) 473 [7-9]. In addition, other Akt1 activators have been reported including DNA-PK, oxidative stresses, HSP90 (heat shock protein-90KDa), PKC- β (protein kinase C-Beta), integrins via focal adhesion kinase (FAK), and others [10,11]

Phosphorylated Akt, pAkt, exerts its effects in the cell by phosphorylating a wide variety of downstream substrates including; caspase9, MDM2, Androgen receptor (AR), p21, p27, GSK (Glycogen Synthase Kinase), and others [12]. These alterations culminate in pleiotropic biological events, including cell growth, survival and migration, which contribute not only to oncogenesis but also to tumour progression [1,2].

Constitutive activation of Akt1 occurs in human cancer through different mechanisms. Firstly, deletion and mutation of PTEN (Phosphatase and Tensin Homolog); the phosphatase that dephosphorylates PI3-K and hence negatively regulating Akt [13]. Secondly, through amplification of the Akt gene [14], or PH domain mutation of Akt1 leading to increased its affinity for phosphoinositides and membrane localisation [15], and thirdly through amplification or mutation of the PIK3CA [16].

Currently, an abundance of basic and pre-clinical studies have been conducted to elucidate implications of pAkt pathway derangements in breast cancer using different detection systems and tissue platforms [17,18]. The use of immunohistochemistry (IHC) has been collectively considered as a useful means of identifying the Akt status in a wide range of human cancers including BC with most studies reporting that increased pAKT expression correlates with clinical aggressiveness and progression [19,20], while others find no such associations [21]. However, the complexity and diversity in the upstream and downstream arms of this pathway, reinforced by recent genetic studies [22,23], bear considerable challenges and limitations that might confront the clinical evolution of effective targeted therapies [5]. This retrospective study adheres to REMARK criteria [24].

This study was conducted to determine the pAkt status in a large series of human invasive breast cancer to study the relationship of its expression with different upstream/downstream component proteins, as well as correlations with clinicopathological parameters and disease outcome.

Material and Methods:

Patients and tumours

This study was conducted on a well-characterised series of early stage (pT1 & pT2) primary operable invasive breast carcinoma from patients enrolled into the Nottingham Tenovus Primary Breast Carcinoma Series that presented at Nottingham City Hospital between 1990 and 1998. Patients were under the age of 70 years and managed in a uniform manner. Patients' clinical history and tumour criteria, information on therapy and outcomes were available and prospectively maintained. Outcome data included survival status (whether alive, or deceased), survival time (in months), cause of death (died from breast cancer, other cause, or lost to follow-up), disease free interval (DFI), time to loco-regional recurrence, and/or distant metastasis. The Breast Cancer Specific Survival (BCSS) is defined as the time (in months) from the date of primary surgery to the date of breast cancer-related death. DFI is defined as the duration (in months) from the date of primary surgery to the appearance of loco-regional recurrence or distant metastasis. Data on a wide range of biomarkers of known clinical and biological relevance to breast cancer were available [25]. These include, hormone receptors [estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR)], epidermal growth factor receptor family members [HER1 (EGFR), HER2, HER3 and HER4], cytokeratins (CKs); basal CKs (CK5/6 and CK14), and luminal CKs (CK18 and CK19), tumour suppressor genes (p53 and PTEN), the Akt pathway activator PIK3CA, proliferation-related and cell cycle regulator markers (Ki-67, p21, p27, Bcl2 and cyclin D1), and cadherin family members (E-cadherin and P-cadherin) [25,26].

The patients had a median age at diagnosis of 54 years (range 18-70 years) with a median overall survival of 125 months (range 4-243 months) and the median

time of event-free survival of 112 months (range 2-239 months). Distant recurrence occurred in 375 cases (31%), while 313 (26%) patients died from breast cancer, while 689 (59%) patients were alive at the end of follow-up.

This study was approved by Nottingham Research Ethics Committee 2 under the title of “Development of a molecular genetic classification of breast cancer”.

Immunohistochemistry

Expression of pAkt1 in breast cancer was assessed using immunohistochemistry. Four- μ m sections were cut from paraffin processed block of previously prepared tissue microarrays (TMAs) and mounted on Superfrost slides (Surigpath). Sections were deparaffinised and heat induced retrieval of antigen epitope was performed in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 minutes. The sections were incubated at room temperature for 60 minutes with polyclonal antibody against S473pAkt1 (RB-10369-P1, Neomarkers, Fremont, CA) optimally diluted 1:150, Following wash in TBS, sections were for 60 minutes in Strept AB complex (Dako, Denmark) diluted in 1:100 in normal swine serum/TBS, followed by chromagen for visualisation [3,3'-Diaminobenzidine tetrahydrochloride (Dako liquid DAB Plus, K3468; Dako, Denmark)]. Sections were counterstained with Mayer's haematoxylin. Negative (primary antibody replaced by TBS) and positive (a known pAkt positive full-face BC tissue section) controls were included in the staining run.

Validation of anti Phospho-AKT (S473) antibody specificity:

For validation of anti Phospho-AKT (S473) antibody specificity, Western blotting was performed on whole cell lysates of MCF-7 and MDA-MB231 human breast cancer cell lines (obtained from the American Type Culture Collection;

Rockville, MD, USA) using 1:100 dilution of the primary antibody dilution, and 1:2000 of the HRP labelled secondary anti-rabbit antibody. BSA was used for blocking. ECL was used to visualize the membrane as previously described [27].

TMA IHC scoring

Stained TMAs sections were scored using the semi-quantitative H-score (Histochemical score) visual approach, taking into consideration the intensity of staining and the percentage of stained cells [28] within each tissue core. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate and strong, respectively. The proportion (percentage) of positive cells for each intensity were subjectively estimated. Final scores were obtained by the sum of multiplying each staining intensity by its proportion, producing an H-score ranging from 0–300. All cases were scored without prior knowledge of the patients' pathologic or outcome.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Cut-off values for the different biomarkers included in this study were chosen before statistical analysis. Standard cut-offs were used for established prognostic factors and were the same as for previously published patient series [25,26]. Determination of the optimal pAkt cut-offs for pAkt staining was obtained using pAkt H-score frequency distribution histogram. Analysis of categorical variables was performed using the appropriate statistical test. Survival estimates were analyzed by Kaplan-Meier method with significance determined by the Log Rank test. Multivariate analysis was performed by Cox proportional hazard analysis. A *p*-value <0.05 (two sided) was considered significant.

Results

pAkt expression in stained TMA sections (n=1,202) showed homogenous cytoplasmic immunoreactivity in invasive tumour cells (Figure 1A&B). The specificity of the antibody was confirmed using a Western blotting analysis of MCF-7 and MDA-MB231 human breast cancer cell lines, which showed a specific band at the correct predicted size (~ 60 KDa) of the pAkt protein confirming the specificity of the antibody used in the IHC staining (Figure 1C). The distribution of pAkt H-score did not display normal distribution (median =100). Using the pAkt H-score frequency distribution histogram, the optimal cut-off point for pAkt positivity was set at 65 (taken at the mid-point of the observed trough in the distribution histogram of pAkt), that dichotomised the cohort into two categories: pAkt negative (H-score<65), and pAkt positive expression (H-score \geq 65). Therefore, 914/1202 (76%) cases were positively stained with pAkt, with the remaining cases 288 (24%) were negative.

Association of pAkt with clinico-pathologic parameters

Table 1 summarises the associations between pAkt expression and clinico-pathological variables. pAkt overexpression showed significant positive association with patients' age ($p= 0.003$) and menopausal status ($p= 0.002$), while a significant negative association was observed with tumour grade ($p= 0.005$). Interestingly, a trend toward significant negative associations was observed between pAkt and grade components; mitosis, tubule formation and pleomorphism ($p= 0.07$). In addition, pAkt was associated with histologic tumour type ($p= 0.008$). Importantly, special types such as pure tubular and lobular carcinomas showed high proportions of pAkt overexpression compared with medullary-like carcinomas. pAkt expression was not significantly associated with tumour size, axillary nodal involvement, Nottingham Prognostic Index (NPI), or with lympho-vascular invasion (LVI); $p> 0.05$). Similar

associations were obtained when the pAkt (H-score) expression was used as a continuous variable (data not shown).

Correlation between pAkt expression and the expression of other biomarkers and molecular classes of breast cancer:

A significant positive association was found between pAkt and expression of ER, androgen receptor (AR), luminal CKs, HER4, PIK3CA, and PTEN. In addition, loss of pAKT expression was associated with p53 positive, basal CKs positive, and tumour growth fraction as assessed by the Ki-67/MIBLI. However, no associations were found between pAkt and the expression of PR, the adhesion molecules E-cadherin and p-cadherin, or with the cell cycle regulators p21, p27, and Bcl-2.

The correlation between pAkt status and expression of the HER family members was investigated. Although there was a positive association between pAkt expression and the expression of HER4, no associations were found between pAkt and the expression of EGFR, HER2 or HER3. Tumours were profiled based on their HER family members expression into three groups; a) HER2⁻/HER1,3&4⁻ (i.e. all negative), b) HER2⁺/HER1⁺ and/or HER3⁺ and/or HER4⁺, and c) HER2⁻/ HER1⁺ and/or HER3⁺ and/or HER4⁺. However, no correlations were noticed between these groups and the pAkt status ($\chi^2=3.028, p= 0.220$).

pAkt expression was significantly associated with the molecular BC subtypes as defined by their immunohistochemical profiles; luminal-like (ER⁺ and/or PR⁺), HER2⁺ (HER2 positive), triple negative basal-like BC (BLBC; ER⁻, PR⁻, HER2⁻, and positive for CK5/6, and/or CK14 and/or EGFR) and triple negative non-basal BC (TNnon-B; ER⁻, PR⁻, HER2⁻, CK5/6⁻, and/or CK14⁻ and/or EGFR⁻), ($\chi^2=28.6, p<0.001$). Interestingly, BLBC and TNnon-B subtypes displayed significantly higher proportions (38.4%, and 42%, respectively) of pAkt negativity, compared to the

luminal-like tumours and HER2 over-expressing BC (21.6% and 20.2%, respectively), Table 2.

Association of pAkt with patients' outcome

Although univariate survival analyses showed that there was no significant difference between patients with pAkt positive and negative tumours for BCSS and metastasis free-survival, MFS, (log rank, (LR) =2.643, $p=0.104$, and LR=2.265, $p=0.132$, respectively), patients with pAkt positive tumours showed a trend towards a relatively shorter BCSS and MFS when compared to those showing pAkt negativity, Figure 2 A&B.

PIK3CA/pAkt combinatorial groups

To address interactions between pAkt and PIK3CA expression and its correlation with clinicopathological parameters and patients' outcomes, tumours were re-defined based on their expression of both pAkt and PIK3CA, resulting in four phenotypic groups; PIK3CA⁻/pAkt⁻, PIK3CA⁻/pAkt⁺, PIK3CA⁺/pAkt⁻, and PIK3CA⁺/pAkt⁺. Table 3 displays the resulting numbers and percentages of this combination. The majority of tumours showed PIK3CA⁺/pAkt⁻ phenotype while PIK3CA⁻/pAkt⁺ was the least common phenotype.

Significant positive association was found between these phenotypic groups and patient age ($p=0.001$), menopausal status ($p=0.001$), tumour grade ($p<0.001$), LVI ($p<0.001$), NPI ($p<0.001$), and molecular subtype ($p<0.001$). Importantly, PIK3CA⁺ groups showed more aggressive features regardless of the expression of pAkt (PIK3CA⁺/pAkt⁻ and PIK3CA⁺/pAkt⁺). These groups showed association with higher histological grade, LVI positivity and were in the moderate and poor NPI categories ($p>0.05$).

Using the Kaplan-Meier survival plots and log rank statistics, significant differences were noticed between these phenotypes for patients' BCSS and MFS, (LR=15.5, $p=0.001$, HR=1.319, 95% CI=1.140-1.526, and LR=14.4, $p=0.002$, HR=1.271, 95% CI=1.115-1.449, respectively, Figure 2A&B) independently of patients' age, menopausal status, LVI, molecular subtypes, and adjuvant therapy ($p=0.034$, HR=1.184, 95% CI=1.013-1.483 for BCSS, and $p=0.024$, HR=1.173, 95% CI=1.021-1.348 for MFS). However, inclusion of the grade into Cox proportional hazard models for BCSS and MFS left the PIK3CA/pAkt combinations as a grade-dependant prognostic variable ($p>0.05$). Importantly, tumours positive for PIK3CA showed worse outcome regardless of the expression of pAkt and no significant survival difference was observed between patients having PIK3CA⁺/pAkt⁻ and PIK3CA⁺/pAkt⁺ tumours.

Discussion

The expression of pAkt in this study, as determined by IHC, demonstrated that 914/1202 (76%) of investigated tumours overexpressed pAkt. This relatively high prevalence of may be a reflection of pAkt oncogenetic role that was reported to occur early during the cascade of carcinogenic events, seen in the *in-situ* stage carcinomas prior to their progression to invasive carcinomas [29].

A significant inverse association was found between pAkt and tumour grade, with a trend toward significance with individual grade components especially mitosis, which is reinforced by the significant negative association with proliferative fraction as assessed by MIB1-LI and are in accordance with previous studies [30]. However, other investigators reported direct association of pAkt with tumour grade [31,32]. In line with others [33], a direct association was revealed between pAkt expression and patients' age and menopausal status. Moreover, histologic tumour types of favourable outcome (invasive tubular, lobular, and invasive papillary carcinomas) displayed high proportions of pAkt overexpression, relative to medullary carcinomas; a type with relatively less favourable prognosis; which is inverse to the expression of PIK3CA in the same cohort [34].

Importantly, pAkt expression was positively associated with ER status, a further demonstration of previous reports of Akt activation downstream of ER [35]. However, of the HER family; (EGFR, HER2, HER3 and HER4), which are well-known Akt upstream activators, only HER4 showed positive association with pAkt, while no associations were found with EGFR, HER2 status, or HER3. The cooperation between the HER receptors with HER2 to produce a more aggressive tumour phenotype is well-evidenced [36]. Therefore, we profiled our tumour series based on their HER2 status with respect to the other HER family members.

Interestingly, no association was found between the resulting subgroups and the pAkt status. These findings may point to other mechanisms of constitutive Akt activation, other than growth factor stimulation via receptor tyrosine kinase [14-16]. In line with this hypothesis is the significant positive association between pAkt and PTEN expression, which was available in only a subset of tumours of this series, despite the expected and reported inverse relationship between the two [37].

In addition, pAkt overexpression was directly correlated to the expression of AR, a known downstream pAkt target. Lin *et al* demonstrated that AR phosphorylation via pAkt abrogates AR-induced apoptosis resulting in increased cell survival [38]. On the other hand, pAkt was inversely associated with the IHC expression of p53 in our series, where p53 negative/wild cases showed higher proportions of pAkt positivity than p53 positive cases. However, this could not solely be explained through the action of pAkt on the p53 degradation mediator Mdm2, which showed no correlation with pAkt expression. In this respect, growing evidence challenge the conventional simplistic view that proposes Mdm2 as the principal mediator for p53 turnover, where many other factors are integral members of the multi-faceted p53 pathway [39].

Of utmost interest is the correlation seen between pAkt expression and the BC molecular subtypes, where the luminal-like subtype displayed a higher frequency of pAkt positivity than the basal-like and TN subtypes. This association might mirror the positive association between pAkt with ER, luminal CK18, 19 and the negative association with basal CK14 found in our series; and on the other hand supports other reports attributing the resistance to adjuvant hormonal therapy to the activation of Akt in hormone receptor positive cases [40].

Despite the strong correlation with histologic grade, hormonal receptor status and BC molecular subtypes, the association of pAkt expression with patients' BCSS or MFS during the period of follow-up did not reach statistical significance. These findings might not be surprising, firstly because of the dependency of pAkt status in our series on ER expression whose prognostic significance is reported to be dependant on age, lymph node status and length of follow up time [41]. Secondly, it may be due to the high proportion of tumours showing pAkt expression implying an early activation of Akt pathway in these cases, rather than a feature of tumour progression [29].

For further scrutiny of the results of pAkt expression in our series relevant to the upstream Akt activator PI3K, tumours were redefined according to their combined pAkt and PIK3CA IHC status, resulting in four phenotypic groups; PIK3CA⁻/pAkt⁻ (double negative), PIK3CA⁻/pAkt⁺, PIK3CA⁺/pAkt⁻, and PIK3CA⁺/pAkt⁺ (double positive). Significant differences were noticed between these combinatorial phenotypic groups for patients' BCSS and MFS, independently of other prognostic parameters except grade; a similar pattern to that previously reported for PIK3CA expression in this cohort [34]. Interestingly, a substantial proportion (~16%) of tumours was PIK3CA⁻/pAkt⁺, meaning that the Akt was activated whereas the main upstream PI3K activator was inactive. The activation of Akt in this subset of tumours may be an indication of other mechanisms of Akt activation other than PI3K [13-15]. In addition, a comparable subset of tumours was PIK3CA⁺/pAkt⁻, indicating diminished pAkt levels in the presence of PI3K over-activity. A growing body of evidence has recently reported a diminished Akt activation in breast tumours and cell lines harbouring the activating PIK3CA mutations, denoting an Akt-independent execution of downstream oncogenic signals of PI3K [22,23]. Furthermore, the

largest subset of tumours was the double positive tumours (~60%), in which both PI3K/Akt was active. In this subset, the activation of Akt is most probably a downstream effect of PI3K activation, although other Akt activators might also have contributed. This phenotype displayed a 10 year BCSS and MFS of 72% and 68% respectively, nearly equal to these displayed by the PIK3CA⁺/pAkt⁻ phenotype. In other terms, the outcome of patients relative to pAkt status has been mostly driven by PIK3CA status, thus when the later was positive, the prognosis was more or less the same to that of PIK3CA⁺/pAkt⁻ phenotype, but was dramatically poorer than PIK3CA⁻/pAkt⁺ tumours.

It is noteworthy that the PI3K/Akt pathway inhibitors, as specific cancer therapies, are currently in their early phase of clinical trials [5]. In addition, current research strategies to limit PI3K/Akt signalling focus primarily on inhibitors of the PI3K catalytic subunit, which would not be as effective in cases of constitutively active Akt [22,42]. With the findings of the current study in mind, specific inhibitors that target Akt should be considered within the specific context of Akt activation whether it is a result of an upstream stimulation, genetic mutation, or gene amplification [13-15]. For instance, the prevalence and importance of Akt pathway activation with or without PI3K activation, represented in our series by the double positive and PIK3CA⁻/pAkt⁺ phenotypes respectively, might warrant the clinical effectiveness of specific Akt inhibitors. However, the pervasiveness of Akt-independent effectors of PI3K signalling, represented in our series by the PIK3CA⁺/pAkt⁻ phenotype, might therefore substantially limit the clinical effectiveness of Akt inhibitors in this subset of tumours.

Therefore, to develop effective new cancer therapeutics that could limit constitutively active Akt signals, it will be important to gain a more complete insight of the precise

upstream and downstream members of this complex kinase pathway, and the specific PI3K/Akt axis in particular, in more specific rather than general signalling terms.

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Figure legends

Figure 1: pAkt (Ser473) immunohistochemical expression in invasive breast carcinoma TMA cores and Western blotting of MCF-7 and MDA-MB231 cell lysates. (A) Negative IHC expression, (B) Positive IHC expression; (200x magnification). C: Western blotting analysis using anti Phospho-Akt (S473) rabbit monoclonal antibody showing band at the expected size (56 KD), lane (1)- MCF-7 lysates and lane (2)- MDA-MB231 lysates.

Figure 2: Kaplan-Meier survival plot for patients' BCSS showing the association between pAkt expression and: Breast cancer specific survival (A) and the probability of distant recurrence (B).

Figure 3: Kaplan-Meier survival plot for patients' BCSS showing the association between the patients' subsets resulted from PIK3CA and pAkt expression: Breast cancer specific survival (A); the probability of distant recurrence (B).

Table Legends:

Table 1: Correlation between pAkt expression and the clinicopathologic parameters in the studied cohort

Table 2: Table 2: Correlations between pAkt expression and the expression of other biomarker

Table 2 (continued): Correlations between pAkt expression and the expression of other biomarkers

Table 3: Numbers and percentages within each subset of PIK3CA/pAkt combinations:

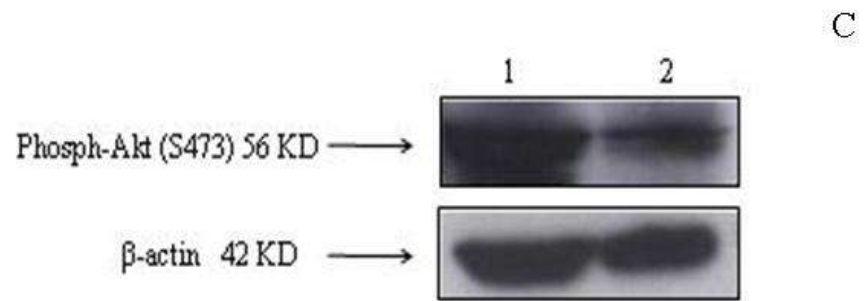
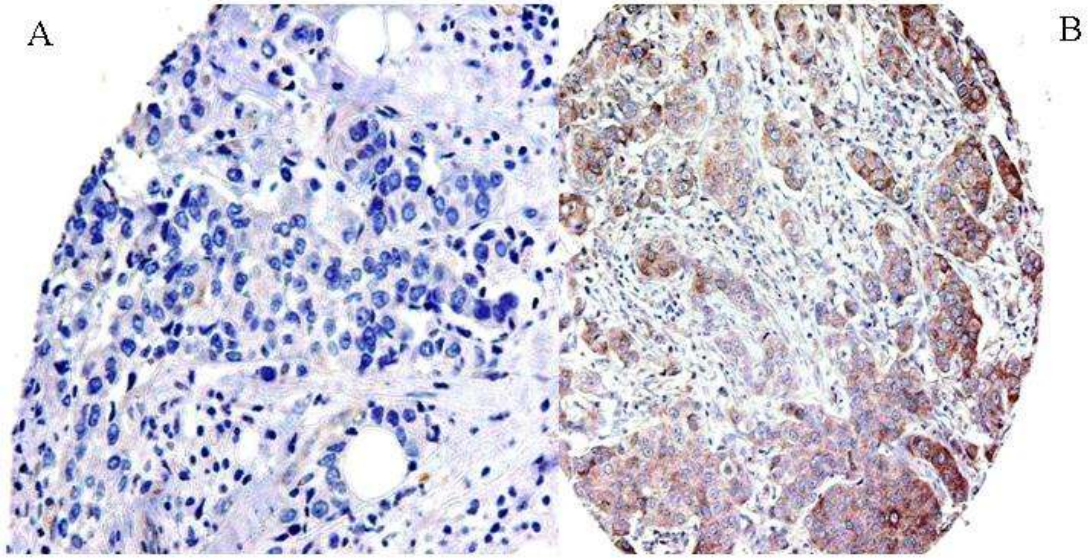


Figure 2: Kaplan-Meier survival plot for patients' outcome showing association between pAkt expression and (A): Breast cancer specific survival and, (B) metastasis free survival probability (MFS).

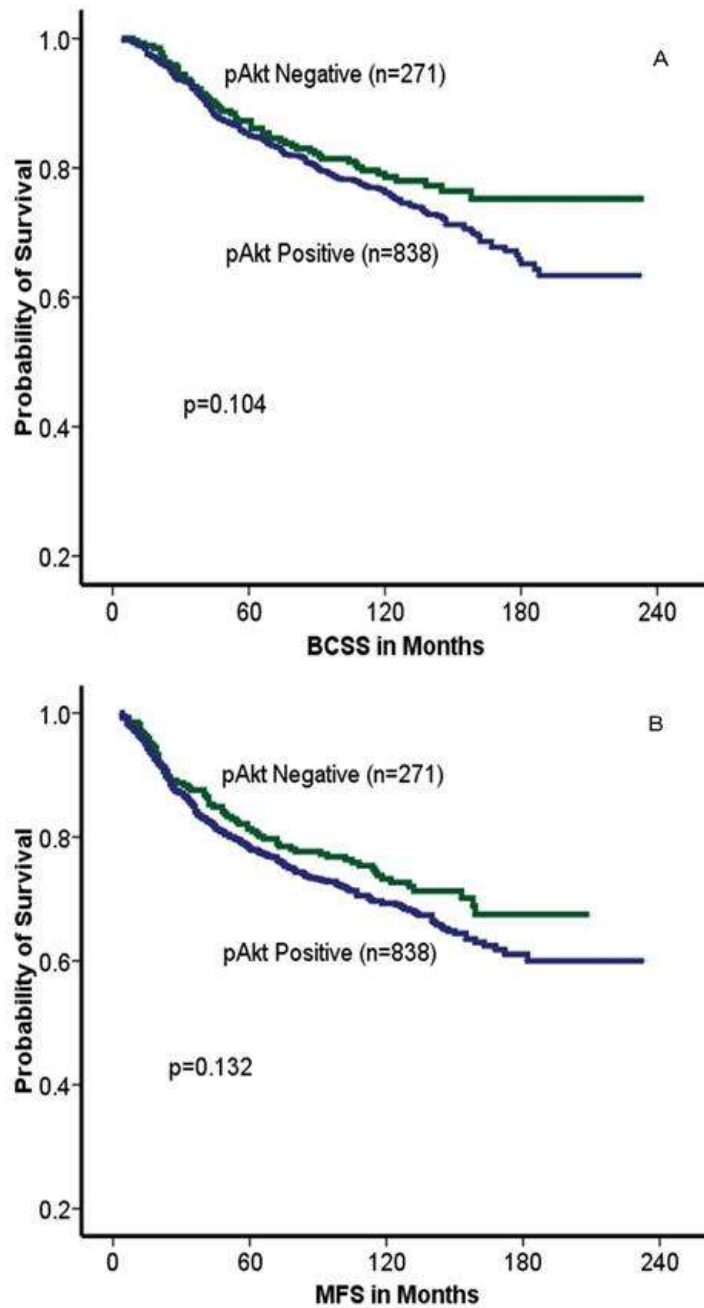


Figure 3: Kaplan-Meier survival plot for patients' outcome showing the association between the patients' subgroups of combinatorial PIK3CA and pAkt expression (A): Breast cancer specific survival; (B): probability of distant recurrence.

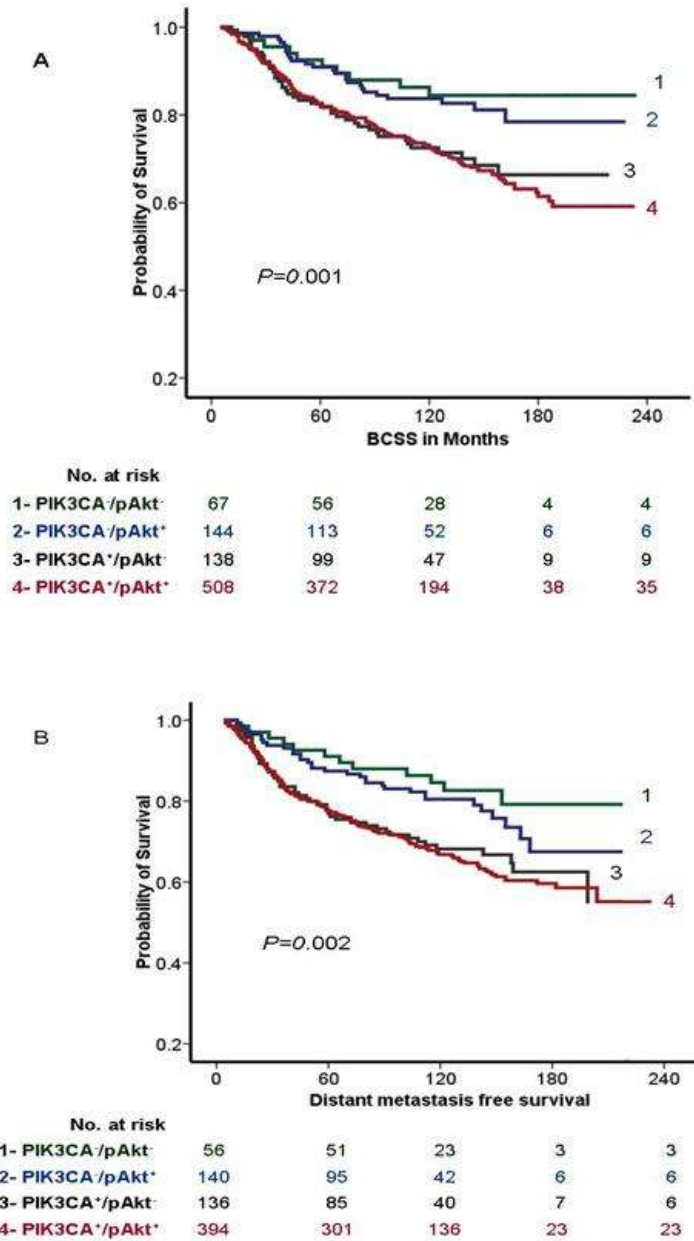


Table 3: Correlation between pAkt expression and the clinicopathologic parameters in the studied cohort

	pAkt Expression		Significance	
	Negative No. (%)	Positive No. (%)	χ^2	<i>p</i> value
Patients' Age				
≤ 50 yrs	131 (28.9)	323 (71.1)	9.358	0.003
> 50 yrs	157 (21.1)	588 (78.9)		

	288	911		
Total				
Menopausal Status				
Premenopausal	135 (47.2)	476 (52.2)	9.797	0.002
Postmenopausal	153 (52.8)	435 (47.8)		
Tissue marker			χ^2	<i>p</i> value
Total	No (n%)	Yes (%)		
Grade				
1 Negative	147 (51.2)	160 (17.5)	10.685	0.001
2 Positive	158 (54.9)	314 (34.1)		
3	167 (58.5)	434 (47.6)	10.509	0.005
Total	287	908		
LN Stage				
1 (Negative)	176 (61.3)	566 (62.2)	1.620	0.445
2 (1-3 LN involved)	91 (31.7)	262 (28.9)		
3 (>3)	20 (7.0)	81 (8.9)		
Total	287	909		
Tumour size				
≤2cm	170 (59.2)	559 (61.5)	0.527	< 0.488
>2cm	117 (40.8)	348 (38.5)		
Total	287	907	907	
Nottingham Prognostic Index				
Good NPI (<3.4)	76 (26.5)	301 (33.2)	4.717	0.09
Moderate NPI (3.41-5.4)	157 (54.4)	463 (50.9)		
Poor NPI (5.41-5)	54 (18.9)	145 (15.9)		
Total	287	909		
Vascular Invasion				
Negative	154 (53.7)	512 (56.3)	1.861	0.181
Definite	104 (36.3)	283 (31.1)		
Total	258	795		
Local recurrence				
Negative	255 (88.8)	755 (83.1)	3.366	0.073
Positive	25 (8.8)	116 (12.8)		
Total	280	891		
Regional recurrence				
Negative	253 (90.0)	804 (89.1)	0.004	0.995
Positive	27 (9.6)	87 (9.6)		
Total	280	891		
Distant metastases				
No	208 (73.3)	614 (67.8)	2.719	0.099
Yes	78 (27.7)	295 (32.2)		

Table 2: Correlations between pAkt expression and the expression of other tissue markers

PgR				
Negative	129 (26.1)	366 (73.9)	0.902	0.364
Positive	147 (23.6)	476 (76.4)		
AR				
Negative	119 (30.1)	271 (69.5)	9.312	0.003
Positive	151 (22.1)	532 (77.9)		
Tissue marker				
	Negative	Positive	χ^2	p value
HER2	No. (%)	No. (%)		
Negative	294 (24.8)	754 (75.2)	1.705	0.226
Positive	31 (20)	124 (80)	0.624	0.432
E-cadherin				
Negative	96 (23.4)	314 (76.6)	0.320	0.585
Positive	185 (25.6)	539 (74.4)	2.622	0.109
EGFR				
Negative	206 (24.7)	628 (25.3)	1.564	0.216
Positive	53 (26.6)	146 (74.4)	8.072	0.006
P-cadherin				
Negative	108 (22.8)	365 (77.2)		
Positive	143 (27.3)	381 (72.7)		
HER3				
Negative	29 (29.9)	68 (70.1)		
Positive	215 (24.1)	676 (75.9)		
PIK3CA				
Negative	71 (30.7)	160 (69.3)		
Positive	59 (30.9)	132 (69.1)	4.836	0.032
Negative	186 (23.2)	614 (76.8)		
HER2/HER family status:				
- HER2 ⁻ /HER 1,3&4 ⁻	12 (32.5)	39 (76.5)		
- HER2 ⁺ /HER1 ⁺ &/or HER3 ⁺ &/or HER4 ⁺	24 (18.8)	104 (81.3)	3.028	0.220
- HER2 ⁻ /HER1 ⁺ &/or HER3 ⁺ &/or HER4 ⁺	204 (25.9)	585 (74.1)		
p53				
Negative	184 (22.9)	618 (77.1)	4.364	0.039
Positive	93 (28.9)	229 (71.1)		
CK5/6				
Negative	221 (23.2)	731 (76.8)	6.412	0.014
Positive	62 (31.8)	133 (68.2)		
CK14				
Negative	241 (24.1)	758 (75.9)	1.485	0.236
Positive	38 (29)	93 (71)		
CK18				
Negative	43 (37.4)	72 (62.6)	41.939	< 0.001
Positive	223 (23.2)	740 (76.8)		
CK19				
Negative	33 (41.8)	46 (58.2)	36.919	< 0.001
Positive	252 (25.6)	733 (74.4)		

Positive	152 (21.6)	553 (78.4)		
MIB1				
Low proliferative	67 (21.2)	249 (78.8)		
Moderately proliferative	83 (21.1)	310 (78.9)	9.104	0.011
Highly proliferative	77 (30.6)	175 (69.4)		
PTEN				
Negative	21 (35.6)	38 (64.4)		
Positive	99 (22.1)	349 (77.9)	5.255	0.033
p21				
Negative	70 (21.7)	252 (78.3)		
Positive	147 (23.5)	476 (76.5)	0.111	0.765
p27				
Negative	19 (25)	62 (75)		
Positive	77 (17.7)	313 (80.3)	3.152	0.089
Bcl-2				
Negative	69 (25.3)	204 (47.4)		
Positive	124 (20.7)	474 (79.3)	2.239	0.136
Mdm2				
Negative	75 (21.7)	271 (78.3)		
Positive	12 (28.6)	30 (71.4)	1.024	0.328
Molecular subtype				
Luminal-like	161 (21.6)	586 (78.4)		
HER2 over-expressing	34 (20.2)	134 (79.8)		
Tripe negative	21 (42)	29 (58)	28.603	< 0.001
Triple negative basal	56 (38.4)	90 (61.6)		

Table 2 (continued): Correlations between pAkt expression and the expression of other biomarkers

Table 3: Numbers and percentages within each subset of PIK3CA/pAkt combinations:

Group	PIK3CA/pAkt status	Number	Percentage
1	PIK3CA ⁻ /pAkt ⁻	75	7.9
2	PIK3CA ⁻ /pAkt ⁺	158	16.6
3	PK3CA ⁺ /pAkt ⁻	149	15.7
4	PIK3CA ⁺ /pAkt ⁺	567	59.7
	Total	949	100%

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