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An Immunohistochemical Evaluation of Phosphorylated Akt at Threonine 308 [pAkt(Thr308)] in Invasive Breast Cancer

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Abstract. *Background: Akt is a serine/threonine kinase which is fully activated when phosphorylated (pAkt). The aim of this study was to investigate the expression pattern of phosphorylated Akt at Threonine 308 [pAkt(Thr308)] in association with clinicopathological parameters and various biological markers. Materials and Methods: Immunohistochemistry was performed on paraffin-embedded tissue specimens from 152 invasive breast carcinomas to detect the expression of the proteins pAkt(Thr308), estrogen (ER) and progesterone (PR) receptors, p53, Ki-67 and c-erbB-2. Results: pAkt(Thr308) protein was immunodetected in the cytoplasm and the nuclei of the malignant cells. pAkt was found to be positively associated with the lobular histological type, while it was found to exert no impact on patients' survival. pAkt(Thr308) immunopositivity was inversely related to Ki-67 and p53 (p=0.013 and p=0.020, respectively), while being positively associated with cerbB2 expression (p=0.005). Conclusion: This is the first study to show a frequent detection of pAkt(Thr308) in lobular breast carcinomas and an association of its expression with indices of proliferation (c-erbB2, Ki-67) and apoptosis (p53).*

The serine/threonine protein kinase, protein kinase B or Akt (PKB/Akt) has emerged as a crucial regulator of widely divergent cellular processes including cell proliferation, apoptosis and motility (1, 2). Akt has been shown to be a downstream target for PI3K (phosphoinositide 3-kinase), being activated by tyrosine kinase receptors, including erbB family receptors (3). Akt activation is a multistep process involving both membrane translocation and phosphorylation (1). In unstimulated cells, Akt is basally phosphorylated at Ser-124 and Thr-450. There is data which support that PI3K activation results in the translocation of Akt to the plasma membrane, where it is phosphorylated at Thr308 and Ser473 by two distinct kinases (1, 2, 4), PDK1 (3-phospho inositide-dependent kinase) and PDK2, respectively. Thr-308 phosphorylation is necessary for Akt activation and additional phosphorylation at Ser-473 is required for maximal activity (1, 4).

Akt has been shown to block apoptosis through phosphorylation and inactivation of proapoptotic proteins such as Bad, forkhead transcription factor and caspase-9 (2). Moreover, Akt is invoved in cells proliferation by regulation of the cyclin-dependent kinase inhibitors (1).

Previous studies regarding the PI3K-Akt pathway in breast cancer were mainly focused on the role of the phosphorylated form of Akt at Ser-473 [pAkt(Ser-473)] (5-8). Our aim was to investigate the expression pattern of the phosphorylated form of Akt at Thr-308 [pAkt(Thr308)] in invasive breast carcinomas, in order to determine its clinicopathological and prognostic value and its relationship with proliferation and apoptosis.

Materials and Methods

Patients and samples studied. One hundred and fifty two paraffin blocks with tumor samples were available from patients with resectable breast cancer who had undergone surgery. We only selected women with histologically proven, clearly invasive breast carcinomas, regardless of their initial stage, in whom axillary lymph node dissection had been performed and who had all their resected materials studied histologically. The patients, aged from 25 to 86 years (mean age 56.74 years), had not received radiation or chemotherapy preoperatively. Lastly, the material acquired was used in research after informed consent had been obtained.

Routine histological examination was performed with heamatoxylin-eosin staining. All carcinomas were classified according to the criteria of the World Health Organization (9) and were recorded as invasive ductal or invasive lobular. The combined

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Key Words: pAkt(Thr308), breast cancer, p53, lobular, cerbB-2, Ki-67.

Table I. *Association of pAkt(Thr308) protein expression in the cytoplasm and nuclei of cancer cells with clinicopathological parameters and various biological markers (Fisher's exact test).*

		Cytoplasmic pAkt(Thr308)			Nuclear pAkt(Thr308)		
	Total	N	%	*P*-value	N	%	*P*-value
Menopausal status							
before	44	23	52.3	NS	5	11.4	NS
after	93	54	58.1		14	15.1	
Histological type							
ductal	107	55	51.4	0.021	18	16.8	NS
lobular	29	22	75.9		1	3.4	
Histological grade							
1	16	6	37.5	0.028	2	12.5	NS
2	82	53	64.6		11	13.4	
2	35	15	42.9		6	17.1	
Nuclear grade							
1	46	28	60.9	NS	3	6.5	NS
2	46	28	60.9		7	15.2	
3	44	21	47.7		9	20.5	
Tumor size							
<2 cm	28	16	57.1	NS	3	10.7	NS
2 to 5 cm	86	50	58.1		12	14.0	
>5 cm	22	11	50.0		4	18.2	
LN							
non-infiltrated	52	29	55.8	NS	7	13.5	NS
infiltrated	84	48	57.1		12	14.3	
Stage							
1	21	13	61.9	NS	2	9.5	NS
2	91	52	57.1		13	14.3	
3	24	12	50.0		4	16.7	
ER							
negative	62	33	53.2	NS	11	17.7	NS
positive	75	44	58.7		8	10.7	
PR							
negative	65	33	50.8	NS	12	18.5	NS
positive	72	44	61.1		7	9.7	
Ki-67							
negative (< or =10)	66	47	71.2	0.013	11	16.7	NS
positive (>10)	51	24	47.1		6	11.8	
p53							
negative (< or =10)	98	62	63.3	0.020	12	12.2	NS
positive (>10)	38	15	39.5		7	18.4	
c-erbB-2							
negative (<10)	54	22	40.7	0.005	5	9.3	NS
positive (> or =10)	83	55	66.3		14	16.9	

histological grade (1, 2 and 3) of infiltrating ductal carcinoma was obtained according to a modified Scarff-Bloom-Richardson histological grading system with guidelines as suggested by Nottingham City Hospital pathologists (10). Nuclear grading was based on nuclear polymorphism and mitotic activity. Staging at the time of diagnosis was based on the TNM system (11). Tumor size (<2 cm, 2-5 cm, >5 cm) and lymph node status were evaluated separately. The clinicopathological characteristics of the series are shown in Table I. During the immunohistochemical procedure some specimens were destroyed, while others were considered to have too little tissue to be evaluated. Therefore, the samples which were finally included in the statistical evaluation were 137.

Follow up data were available for 135 patients, of whom 32 died of breast cancer and 103 had a recurrence. Mean survival time was 95.57 months (range 5 to 135 months). Patient outcome was defined as disease-free and overall survival. All patients received conventional post-operative treatment depending on the extent of the disease, including radiation therapy and medical antiestrogen therapy, when indicated. Premenopausal patients with axillary involvement were treated with six courses of adjuvant chemotherapy.

Immunohistochemistry. Immunohistochemical staining for p-Akt(Thr308) was performed on 4 μm thick formalin-fixed paraffin sections, after overnight heating at 37˚C subsequent

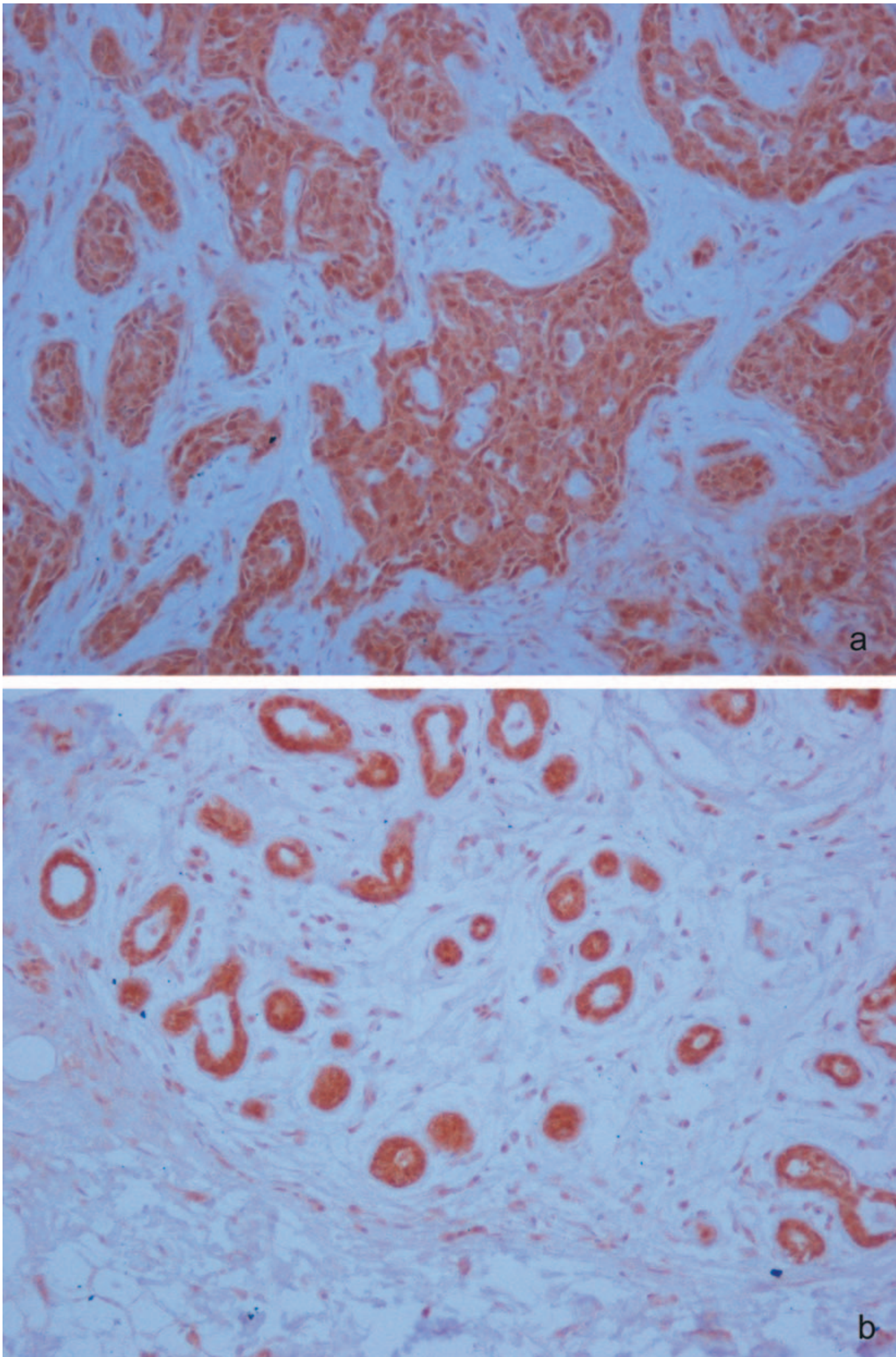

Figure 1. *Immunohistochemical staining of pAkt(Thr308) protein (a) in the cytoplasm and the nuclei of the malignant cells of breast carcinoma and (b) in the normal breast epithelium (ABC/HRP x200).*

deparaffinization, rehydration and antigen retrieval performed in one step procedure with the reagent Trilogy (Cell Marque, Hotsprings, AR, USA) in a microwave oven by heating the slides for 15 minutes. After rinsing with Tris Buffer Saline (TBS), normal horse serum was applied for 30 min to block non-specific antibody binding. Subsequently, sections were incubated overnight at 4˚C with the primary antibody. A three-step technique (Elite ABC Vector Laboratories, Burlingame, CA, USA) was used for visualization, with diaminobenzidine as a chromogen. Finally, sections were counterstained with hematoxylin and mounted.

A rabbit monoclonal antibody against the p-Akt(Thr308) (244F9) (#4056, Cell Signaling Technology, Beverly, MA, USA) was used at a dilution of 1:40. The immunomarkers assessed in the present study, in combination with pAkt(Thr308), had been previously detected with the following antibodies: a) anti-ER, clone 1D5 and anti-PR, clone IA6 (Dako, Glostrup, Denmark) at a dilution of 1:450 and 1:150, respectively; b) anti-p53, clone BP53.12.1 (BioGenex, San Ramon, CA, USA) at a dilution of 1:50; c) anti-Ki-67, rabbit polyclonal antibody (Dako, Glostrup, Denmark) at a dilution of 1:50; d) anti-c-erbB-2, clone CB11 (BioGenex) at a dilution of 1:150. The results of the aforementioned immunomarkers were obtained from our archival database.

Evaluation of immunohistochemistry. The evaluation of the immunohistochemical staining was performed by two pathologists, independently, through light microscopic observation and without knowledge of the clinical data of each patient. Cases of disagreement were reviewed jointly to reach a consensus score. The score resulted as the average of 10 distinct high-power fields observed under x400 magnification. With regards to positive control, a breast cancer tissue section previously known to be pAkt immunoreactive (external control) as well as normal ductal epithelial cells and an *in situ* component adjacent to cancer tissues (internal staining control) were used. These in particular stained with high intensity, comparable to that of the external staining control. Negative controls had the primary antibody omitted and replaced by nonimmune, normal serum from the same species as the primary antibody, or by TBS.

p-Akt(Thr308) was detected in the cytoplasm and the nuclei of the malignant cells. Staining intensity and the number of stained cells were taken into consideration throughout the evaluation process and the cytoplasmic and nuclear staining in tumor cells was scored as positive when moderate to strong staining was detected in more than 15% and 10% of the cells, respectively. In internal positive controls, pAkt(Thr308) was strongly expressed.

Staining for ER and PR was evaluated semiquantitatively using the H score system and a score >50 was considered as positive for both antigens (12). The evaluation of the immunostaining of p53, Ki-67 and c-erbB2 and caspase-3 proteins was performed as previously described (13). The cut-off values of these immunomarkers are shown in Table I.

Statistics. The significance of the relationship between the expression of pAkt(Thr308) and clinicopathological parameters was evaluated with univariate analysis using chi-square test and Fisher's exact probability test. The effect of pAkt(Thr308) differential expression on postoperative survival rates was assessed using both univariate (log-rank test) and multivariate (stepwise forward Cox's proportional hazard regression model) analysis. A *p*-value of <0.05 was considered statistically significant.

Results

pAkt(Thr308) protein was immunodetected in the cytoplasm and the nuclei of the malignant cells in 56.2% and 13.9% of the cases, respectively (Figure 1a). In the cases where *in situ* carcinoma or normal epithelium (Figure 1b) were present, they demonstrated an intense immunopositivity for pAkt(Thr308).

Increased cytoplasmic expression of pAkt(Thr308) was associated with lobular carcinomas whereas increased nuclear expression tended to be associated with ductal carcinomas (p=0.021 and p=0.075, respectively). No correlation was found between pAkt(Thr308) expression (cytoplasmic or nuclear) and menopausal status, nuclear grade, tumor size, LN status, tumor stage, ER or PR receptor status. pAkt(Thr308) immunopositivity was inversely associated with Ki-67 and p53 (p=0.013 and p=0.020, respectively) (Figures 2 and 3), while a positive association was found between its expression and c-erbB2 expression (p=0.005) (Figure 4) (Table I).

With regards to its prognostic significance, pAkt(Thr308) failed to have any impact on overall (OS) or disease-free survival (DFS) (univariate analysis), a finding that was also confirmed in the multivariate analysis.

Discussion

This study examined the expression of pAkt(Thr308) in invasive breast carcinoma and found it to be mainly immunodetected in the cytoplasm and to a lesser degree, in the nuclei of tumor cells, in line with a previous study (14).

As far as we know, this is the third immunohistochemical study applying an antibody which detects endogenous levels of Akt only when phosphorylated at threonine [pAkt(Thr308)] (14, 15), in contrast to previous studies that investigated the expression of pAkt(Ser473), thus the two-sided phosphorylated form of Akt (5-8, 16-18).

In the present study, pAkt(Thr308) was immunodetected more frequently in lobular breast carcinomas, a histological subtype known to be characterized by the loss of E-cadherin, a transmembrane protein involved in homotypic cell cell contacts (19). Recent research findings suggest that down-regulation of E-cadherin is induced by Snail transcription factor, whose levels are further regulated by glycogen synthase kinase-3β (GSK3β), a downstream target of Akt (20). Thus, Zhou *et al.* (20) showed that GSK3β inactivation *via* its phosphorylation by Akt results in the up-regulation of Snail and down-regulation of E-cadherin, suggesting a possible explanation for the more frequent immunodetection of the pAkt protein in lobular breast carcinomas. To our knowledge, this is the first study to show a more frequent PI3K-Akt pathway activation in lobular breast carcinomas. There are two more immunohistochemical studies in the

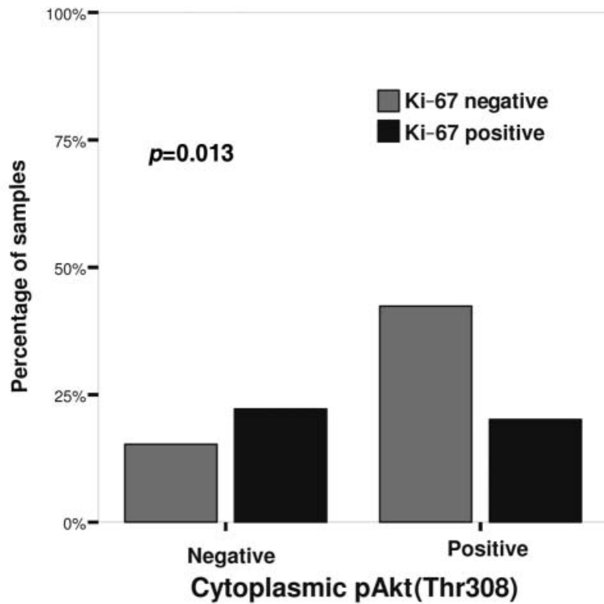

Figure 2. *Schematic representation of the relation between pAkt(Thr308) and Ki-67 (p-value refers to the difference in the percentage of pAkt positive cases between the groups of Ki-67 positive and Ki-67 negative cases).*

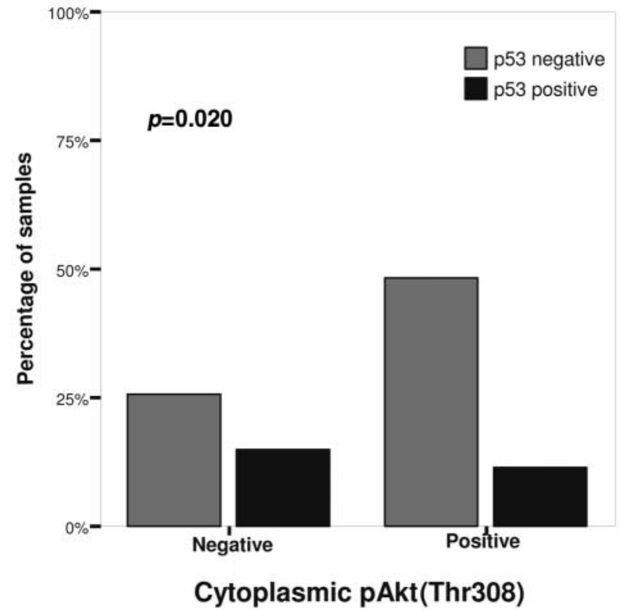

Figure 3. *Schematic representation of the relation between pAkt(Thr308) and p53 (p-value refers to the difference in the percentage of pAkt positive cases between the groups of p53 positive and p53 negative cases).*

literature which investigate the clinicopathologic and prognostic value of pAkt(Thr308) in breast cancer. Neither of them reported any clinicopathological value for pAkt, while its prognostic significance remains controversial. Xia *et al.* found an adverse prognostic value of pAkt(Thr308) in univariate analysis (15), while Kirtegaard *et al.* did not confirm such a value (14), in accordance with our findings.

Furthermore, pAkt expression was found to be positively associated with cerbB2, while negatively associated with p53 which are a well-established activator and downstream target, respectively, of the PI3K/Akt signaling pathway (1, 2). These associations are described for the first time for pAkt(Thr308) and seem to reflect the well-known implication of the c-erbB2-activated PI3K/Akt signaling pathway in the Mdm-mediated reduction of p53 levels and the inhibition of p53-dependent cell cycle arrest and apoptosis (21).

Moreover, this is the first study to mention an inverse relationship between pAkt(Thr308) expression and Ki-67, a proliferation index. At first sight, it is an interesting finding, since the PI3K/Akt pathway is thought to promote cell proliferation *via* the induction of cytoplasmic localization of $p21^{WAF1}$ and $p27^{Kip1}$ (1). However, tumorigenesis is the net result of both cell proliferation promotion and apoptosis inhibition. Given the well-known antiapoptotic role of pAkt, one could suggest that Akt overexpression may contribute to oncogenesis by inhibiting apoptosis rather than promoting proliferation (7).

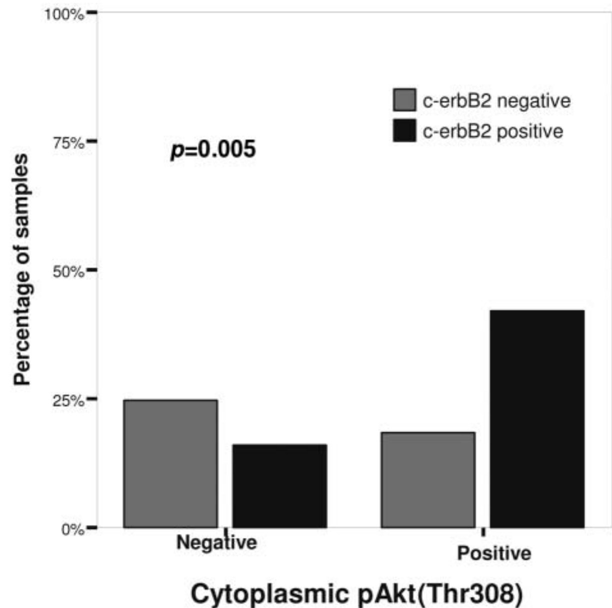

Figure 4. *Schematic representation of the relation between pAkt(Thr308) and c-erbB-2 (p-value refers to the difference in the percentage of pAkt positive cases between the groups of c-erbB2 positive and c-erbB2 negative cases).*

In conclusion, this is the first study to mention a more frequent detection of pAkt(Thr308) in lobular breast carcinomas and an association of its expression with indices of proliferation (c-erbB2, Ki-67) and apoptosis (p53).

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