Diverse Prognostic Roles of Akt Isoforms, PTEN and PI3K in Tumor Epithelial Cells and Stromal Compartment in Non-small Cell Lung Cancer

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Abstract. *Aim: By tissue microarray methodology, the expression of altered Akt isoforms, PTEN and PI3K and their prognostic significance in 335 non-small cell lung cancer (NSCLC) tumors were investigated. Patients and Methods: Tumor tissue was sampled and immunohistochemicaly quantified in 335 tumors from stage I to IIIA NSCLC patients both from tumor epithelial cells and surrounding stromal tissue in resected specimens. Correlations were made with clinicopathological variables. In addition, the expression of these markers was compared to 20 lung tissue cores from patients without any history of malignancy. Results: A significantly higher PTEN expression was observed in control tissue when compared with tumor (p=0.001). There was a significantly negative correlation between PI3K expression in control versus tumor tissue (p=0.001, r=–0.2). In univariate analyses, high tumor epithelial cell expression of non-phosphorylated Akt2 (p=0.014) was a positive prognostic indicator for disease-specific survival (DSS), while high tumor epithelial cell expression of p-Akt Thr^{308} (p=0.045) was a negative prognosticator. High stromal expression of total Akt3 (p=0.0008) and total PI3K (p=0.0003) correlated with a good prognosis. In the multivariate analysis, tumor epithelial cell expression of p-Akt Thr^{308} (p=0.0009) and Akt2 (p=0.004) and the stromal cell expression of Akt3 (p=0.0008) and PI3K (p=0.012) were independent prognostic factors for DSS. Conclusion: High expression of non-phosphorylated Akt2 and low expression of p-Akt Thr^{308} in tumor epithelial cells are independent predictors of improved survival in patients with primary NSCLC. In stromal cells, high expression of total Akt3 and total PI3K are both favourable independent prognostic indicators.*

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Today, lung cancer leads to more deaths than any other malignancy. An annual incidence worldwide of more than 1.3 million lung cancer cases and more than 1.1 million lung cancer deaths per year are estimated (15, 23). Due to diagnosis at late stages of the disease and the poor treatment efficacy in metastatic disease, overall survival is less than 15% and has not improved substantially for the last 30 years (27).

As a result of the research focus on molecular cancer biology, biochemical alterations such as activation of signal transduction pathways during tumorigenesis are being increasingly elucidated. Dysregulation of these pathways has been linked to alterations in cellular proliferation and survival and are proposed to confer a poor prognosis (8). One of these pivotal pathways is the phosphoinositide-3-kinase/protein kinase B (Akt) signaling pathway.

The Akt family consists of three serine/threonine protein kinase isoforms Akt1/PKB α, Akt2/PKB β and Akt3/PKB γ, which function as key regulators for cell growth, survival and proliferation. Other processes regulated by Akt isoforms include cell size, cell response to nutrient availability, intermediary metabolism, angiogenesis and tissue invasion (13).

Deregulations of these kinases have been described in human malignancies (22). In order to be activated, Akt1 is recruited to the cellular membrane by binding of its amino terminal pleckstrin homology (PH) domain to membrane-bound phosphatidylinositol 3,4,5 triphosphate (PIP_3) (31), which is followed by the phosphorylation of two key amino acids: i) threonine 308 (Thr^{308}) in the P-loop of the protein kinase domain and ii) serine 473 (Ser^{473}) in the carboxy-tail region (2). In a former study (2), these two amino acids were shown to be phosphorylated in the activated Akt3

isoform (p-Akt3). For activation of the Akt2 isoform, phosphorylation has been described to occur on the amino acids Thr^{309} and Ser^{474} (19).

Phosphatidylinositol-3-kinase (PI3K) activates Akt by catalyzing the production of its dependent kinases phosphoinositide-dependent kinase and integrin-linked kinase (3, 11, 24). The tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a phospholipid phosphatase which negatively regulates phosphatidylinositol triphosphate levels thus antagonizing PI3K (7). Frequent inactivation and loss of function mutations have been described for PTEN in different malignancies (16, 32).

Being disrupted in many human malignancies with wide-ranging biological consequences, the PI3K/Akt signaling pathway is considered a key determinant of tumor aggressiveness and an essential target for therapeutic intervention (12). A recent study by Tsurutani and colleagues (29) proposed that the evaluation of two phosphorylation sites improves the prognostic significance of Akt activation NSCLC.

Herein, we sought to determine the prognostic significance of all three known Akt isoforms (Akt1 phosphorylated on either or both sites, non-phosphorylated Akt2 and phosphorylated and non-phosphorylated Akt3) as well as PTEN and PI3K, upstream members of the PI3K/Akt signaling pathway.

Patients and Methods

Patients and clinical samples. Primary tumor tissues from anonymized patients diagnosed with NSCLC pathological stage I to IIIA (21) at the University Hospital of North Norway (UNN) and Nordland Central Hospital (NLSH) from 1990 through 2004 were used in this study. In total, 371 patients were registered from the hospital database. Of these, 36 patients were excluded from the study due to: (i) radiotherapy or chemotherapy prior to surgery (n=10); (ii) other malignancy within five years prior to the NSCLC diagnosis (n=13); (iii) inadequate paraffin-embedded fixed tissue blocks (n=13). Thus, 335 patients with complete medical records and adequate paraffin-embedded tissue blocks were eligible. This report includes follow-up data as of September 30, 2005. The median follow-up was 96 (range 10-179) months. Complete demographic and clinical data were collected retrospectively. Formalin-fixed and paraffin-embedded tumor specimens were obtained from the archives of the Departments of Pathology at UNN and NLSH. The tumors were staged according to the International Union Against Cancer's TNM classification and histologically subtyped and graded according to the World Health Organization (33). Staging and classification of tumors were performed by two independent pathologists at the time of diagnosis, according to in-house regulations at the UNN. An additional review of stage and classification was performed by an independent pathologist while selecting representative tumor areas for microarray construction. Regarding N-status, ipsilateral peribronchial or hilar nodes and intrapulmonary nodes are defined as N1, while N2 includes ipsilateral mediastinal or subcarinal nodes.

Tissue microarray (TMA) construction. All cases were histologically reviewed by two pathologists (S.A.S and K. A.S) and the most representative areas of viable invasive carcinoma tissue (epithelial cells) and surrounding tumor stroma from central parts within the tumor were carefully selected and marked on the hematoxylin and eosin (H/E) slides and sampled for the TMA blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA). The detailed methodology has been reported previously (5). Briefly, we used a 0.6 mm diameter stylet and the study specimens were routinely sampled in duplicate from epithelial cancer cells and from tumor-surrounding stroma intervening malignant epithelial areas. Normal lung tissue localized distant from the primary tumor and from non-cancer patients was used as negative controls. To include all core samples, eight tissue array blocks were constructed. Multiple 5-μm sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemistry (IHC) analysis.

Immunohistochemistry (IHC). The applied antibodies had been subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. The antibodies used in the study were as follows: Phospho-Akt (Ser473) (1:5; rabbit monoclonal, clone 736E11; #3787; Cell Signaling Technology, Danvers, USA), which detects Akt1 only when phosphorylated at serine 473, and Akt2 and Akt3 only when phosphorylated at equivalent sites; Phospho-Akt (Thr308) (1:50; rabbit monoclonal, clone 244F9; #4056; Cell Signaling Technology), which recognizes all three Akt isoforms when phosphorylated at threonine 308; Akt2 (1:18; rabbit monoclonal, clone 54G8; #4057; Cell Signaling Technology), which preferentially binds to non-phosphorylated endogenous levels of Akt2 and does not cross-react with recombinant Akt1 or Akt3; Akt3 (1:8; rabbit polyclonal, #4059; Cell Signaling Technology), which detects endogenous levels of total Akt3, but does not recognize the truncated form of rat Akt3 nor does it cross-react with recombinant Akt1 or Akt2; PTEN (1:10; rabbit monoclonal; #9559; Cell Signaling Technology), which detects endogenous levels of total PTEN protein; PI3K (1:25; rabbit polyclonal; #4254; Cell Signaling Technology), which detects endogenous levels of total PI3K.

Sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimen in 0.1 mol/l citrate buffer at pH 6.0 and exposing it to two repeated microwave heating of 10 minutes at 450 W. The DAKO EnVision+ System-HRP (DAB) kit (Glostrup, Denmark) was used to block endogenous peroxidase. Primary antibodies were incubated overnight at 4˚C (except PI3K, for 32 minutes at room temperature). The DAKO EnVision+ System-HRP (DAB) kit was used to visualize the antigens for all stains. This yielded a brown reaction product at the site of the target antigen. As negative staining controls, the primary antibodies were replaced with the primary antibody diluent. Finally, all slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative controls, all TMA stainings were performed in one single experiment.

Scoring of IHC. By light microscopy, representative viable tissue sections were scored semiquantitatively for cytoplasmic staining (Figure 1). The dominant staining intensity in both tumor epithelial cells and stromal cells was scored as: 0=negative; 1=weak; 2=intermediate; 3=strong. The cell density of the stroma was scored as: 1=low density; 2=intermediate density; 3=high

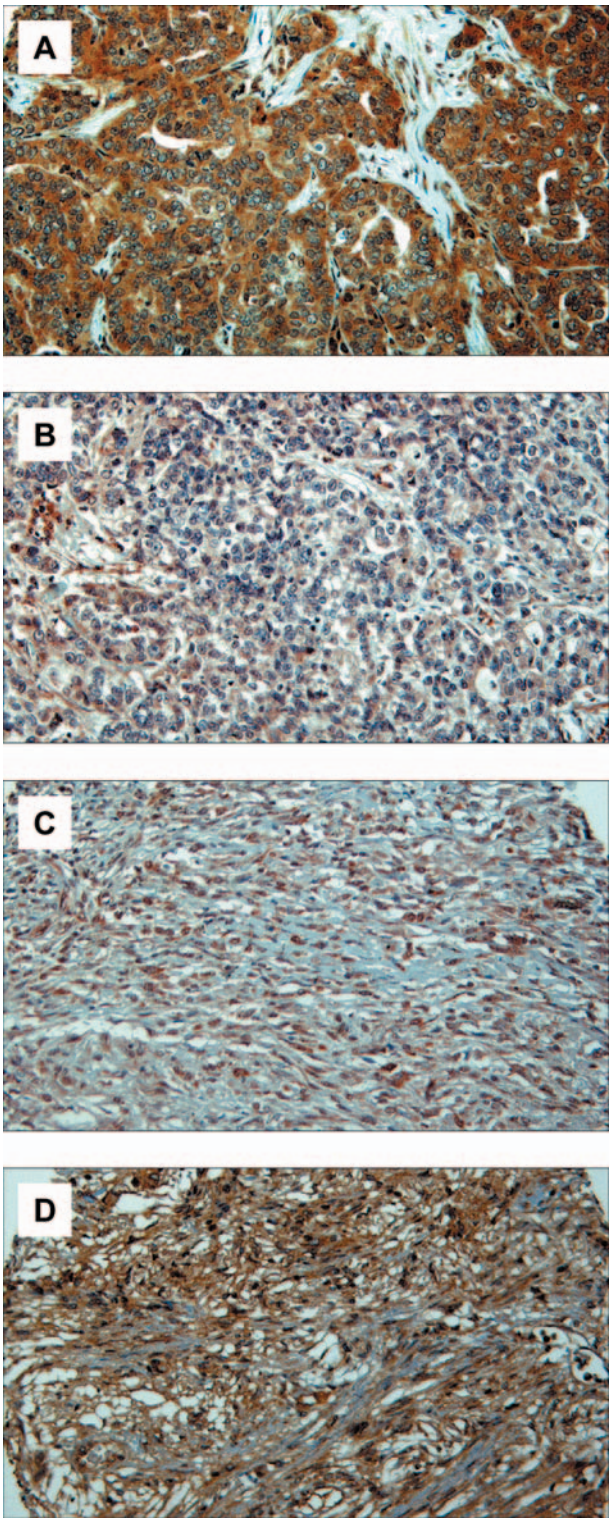

Figure 1. *Immunohistochemical analysis of PI3K/Akt signaling pathway markers in NSCLC representing high expression of p-Akt Thr^{308} in tumor epithelial cells (A), low expression of non-phosphorylated Akt2 in tumor epithelial cells (B), low expression of total Akt3 in the stromal compartment (C) and high expression of total PI3K in the stromal compartment (D). (Magnification ×200.)*

Table I. *Prognostic clinicopathological variables as predictors for disease-specific survival in 335 NSCLC patients (univariate analysis; log-rank test).*

Characteristic	Patients		Median survival (months)	5-Year survival (%)	*p*-value
	n	(%)			
Age					
≤65 years	156	47	104	57	0.62
>65 years	179	53	NR	58	
Gender					
Female	82	25	127	65	0.19
Male	253	75	84	55	
Smoking					
Never	15	5	19	43	0.13
Present	215	64	NR	60	
Previous	105	31	84	54	
Performance status					
Normal	197	59	NR	62	0.04
Slightly reduced	120	36	61	52	
In bed >50%	18	5	36	40	
Weight loss					
<10%	303	90	127	57	0.92
>10%	32	10	NR	57	
Histology					
SCC	191	57	NR	65	0.30
Adenocarcinoma	95	28	52	44	
BAC	18	5	NR	67	
LCC	31	9	84	54	
Differentiation					
Poor	138	41	48	48	0.001
Moderate	144	43	NR	64	
Well	53	16	NR	65	
Surgical procedure					
Lobectomy + wedge*	243	73	NR	61	0.0009
Pneumonectomy	92	27	35	46	
Stage					
I	212	63	NR	68	<0.0001
II	91	27	41	46	
IIIa	32	10	18	22	
Tumor status					
1	90	27	NR	75	0.002
2	218	65	84	52	
3	27	8	42	43	
Nodal status					
0	232	69	NR	66	<0.0001
1	76	23	37	43	
2	27	8	18	20	
Surgical margins					
Free	307	92	127	58	0.34
Not free	28	8	64	51	
Vascular infiltration					
No	284	85	NR	61	0.0005
Yes	51	15	25	35	
Postoperative radiotherapy					
No	276	82	NR	61	0.002
Yes	59	18	41	42	

NR, Not reached; *wedge, n=10; SCC; squamous cell carcinoma; BAC, bronchioalveolar carcinoma; LCC, large-cell carcinoma.

density. All samples were anonymized and independently scored by two pathologists (S.A.S and K.A.S). In case of disagreement, the slides were re-examined and a consensus was reached by the observers. When assessing one variable for a given core, the observers were blinded to the scores of the other variables and to outcome. To evaluate the interobserver agreement with respect to IHC scoring, 100 consecutive tumor epithelial cell cores and tumor stroma cores stained for two rabbit polyclonal markers, vascular endothelial growth factor (VEGF)-C and VEGF receptor-3, were examined (10). The mean correlation coefficient (r) between them was 0.95 (range 0.93-0.98) and was assessed for both antibodies in tumor epithelial and stromal areas. The mean score for duplicate cores from each individual was calculated separately in tumor epithelial cells and stroma, and high expression in tumor epithelial cells was defined as a score ≥2. Stromal expression was calculated by summing density score (1-3) and intensity score (0-3) and the mean score was used prior to categorizing into low and high expression. High expression in the stroma was defined as score ≥2. Evaluation of both phosphorylation sites of activated Akt (p-Akt Ser^{473} and p-Akt Thr^{308}) was performed by summing their intensity scores (0-3) and mean score was used prior to categorizing into low and high expression. High expression was defined as score ≥2. The same procedure was performed for stromal expression, taking the mean score for both intensity and density of stromal cells.

Statistical methods. Statistical analyses were carried out using the SPSS statistical package (Chicago, IL, USA), version 14. The Chi-square test and Fisher's exact test were used to examine the association between molecular marker expression and various clinicopathological parameters. Univariate analyses were made by using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log-rank test. Disease-specific survival (DSS) was determined from the date of surgery to the time of death from lung cancer. To assess the independent value of different pretreatment variables on survival in the presence of other variables, multivariate analysis was carried out using the Cox proportional hazards model. Only variables of statistical significance from the univariate analyses were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.1, respectively. Prevalence assessment of markers in tumor tissue and normal lung tissue and their correlation was carried out using Spearman's test.

Ethics clearance. The National Data Inspection Board and The Regional Committee for Research Ethics approved the study.

Results

Patient data. Demographic, clinical and histopathological variables are shown in Table I: the median age was 67 years (range, 28-85 years) and 75% of the patients were males. The patient population of 335 cases represented the four major subtypes of NSCLC with 191 squamous cell carcinomas (SCCs), 95 adenocarcinomas (ACs), 31 large-cell carcinomas (LCCs) and 18 bronchioalveolar carcinomas (BACs). Due to nodal metastases or non-radical surgical margins, 18% (59) of patients received postoperative radiotherapy.

Expression pattern and correlations with clinicopathological variables. Our IHC analyses included the three isoforms of Akt including both phosphorylation sites of Akt1, serine 473 (p-Akt Ser^{473}) and threonine (p-Akt Thr^{308}), non-phosphorylated Akt2 and total (phosphorylated and non-phosphorylated) Akt3. Additionally we analyzed total PI3K and total PTEN.

All the investigated Akt markers were expressed in the cytoplasm of the tumor epithelial cells. A weak positive nuclear staining was observed in some cores. Nuclear staining was seen to be related to cytoplasmic staining, with more pronounced nuclear staining in cores with high cytoplasmic expression of the investigated markers. Based on morphological criteria, pneumocytes in control cores from normal lung tissue, distant from the primary tumor, generally showed weak positive immunostaining. In tumor stroma and in control cores, inflammatory cells (macrophages, lymphocytes, granulocytes and plasma cells) and endothelial cells frequently showed positive staining, while fibroblast-like cells only occasionally presented positive staining.

Expression of the investigated markers in tumor epithelial cells and stroma did not correlate with age, gender, smoking, clinical performance status, vascular infiltration, tumor differentiation or histological type.

To compare the prevalence of our markers in tumor tissue against normal lung tissue (control cores), we immuno-histochemically examined epithelial cell expression in 20 cores of lung tissue from 20 patients without any history of malignancy. Data are presented in Figure 2. While there was no significant difference in the expressions of Akt isoforms, there was a significantly higher PTEN expression in control cores (p=0.0001). All control cores showed weak to strong PTEN staining, while 42% of tumor cores did not express PTEN, resulting in an almost inverse expression between tumor epithelial and control cores. Moreover, there was a significant negative correlation between PI3K expression in control tissue and tumor tissue (p=0.001).

Univariate analysis. Marker expression in tumor epithelial cells and stroma are presented in Table II. In addition to the statistically significant clinical variables (Table I), tumor epithelial cell expression of p-Akt Thr^{308} (p=0.045) and non-phosphorylated Akt2 (p=0.014, Figure 3), as well as stromal cell expression of total Akt3 (p=0.0008) and total PI3K (p=0.0003, Figure 4), were prognostic indicators for DSS in univariate analyses. It has to be noted that while high tumor expression of p-Akt Thr308 indicated a worse prognosis, high tumor expression of Akt2 appeared to be favourable. High stromal expression of both Akt3 and PI3K indicated better survival. Stromal cell expression of PTEN tended towards statistical significance (p=0.051). Tumor epithelial cell expression of p-Akt Thr^{308} had an even stronger prognostic significance among patients with

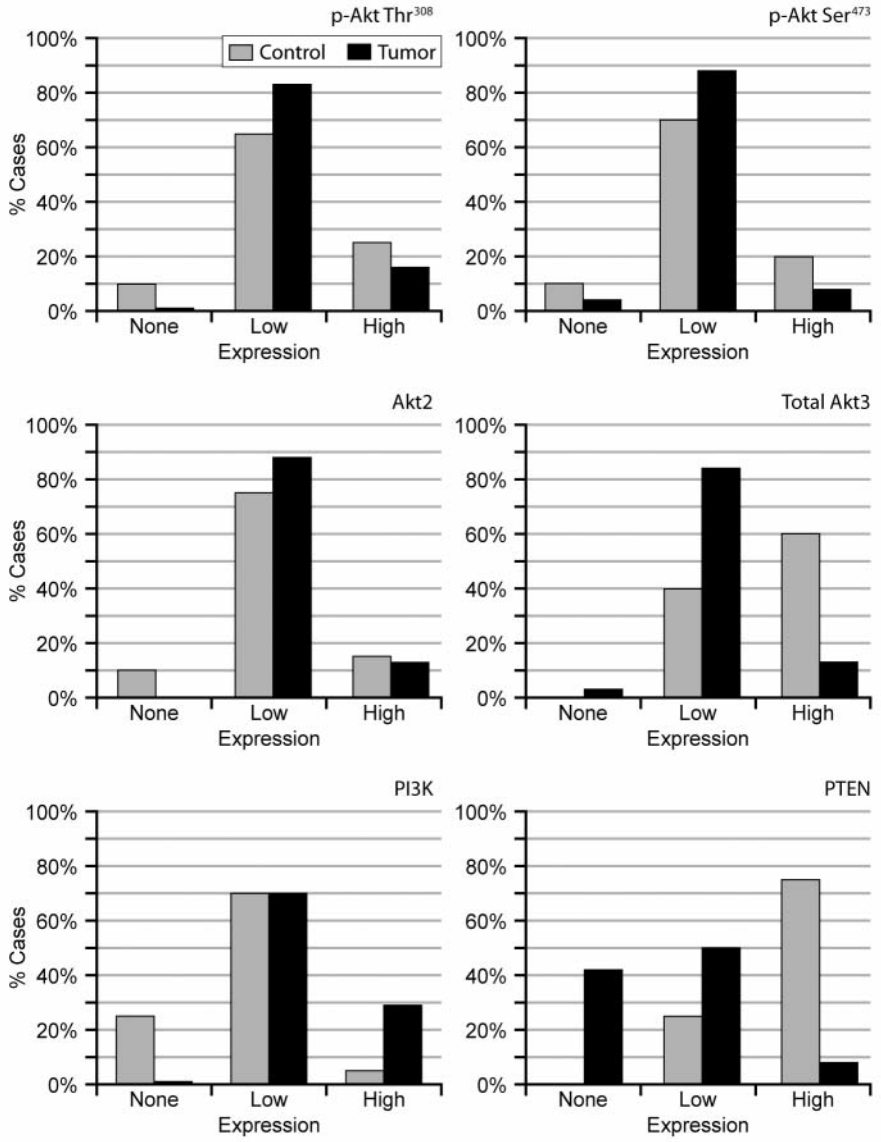

Figure 2. *PI3K/Akt signaling pathway marker expression with comparison of prevalence between tumor epithelial tissue and epithelial tissue from control patients without any history of malignancy.*

moderately differentiated tumors (p=0.008), patients in N1 nodal status (p=0.026) and patients with squamous cell carcinoma (p=0.038). There was no significant correlation between DSS and tumor epithelial cell expression of p-Akt Ser^{473} (p=0.4), total Akt3 (p=0.9), total PI3K (p=0.4) and total PTEN (p=0.08) or stromal cell expression of p-Akt Ser^{473} (p=0.8), p-Akt Thr^{308} (p=0.3) and non-phosphorylated Akt2 (p=0.3). Prognostic relevance of the concomitant phosphorylation of both sites of activated Akt1 (p-Akt Ser^{437} and p-Akt Thr^{308}) was evaluated. Ninty-eight percent of the tumors were phosphorylated at both sites. There was no significant association between DSS and the concomitant expression of phosphorylated Akt (Ser^{473} and Akt Thr^{308}) in tumor epithelial (p=0.2) or stromal cells (p=0.09).

Multivariate Cox proportional hazards analysis. All significant clinicopathological and molecular variables from the univariate analyses were entered into the multivariate analysis. Data are presented in Table III. Tumor epithelial cell expression of p-Akt Thr308 (p=0.0009) and non-phosphorylated Akt2 (p=0.004) were independent unfavourable and favourable prognostic indicators, respectively. Stromal cell expression of total Akt3 (p=0.0008) and PI3K (p=0.01) were both independent favourable prognostic factors.

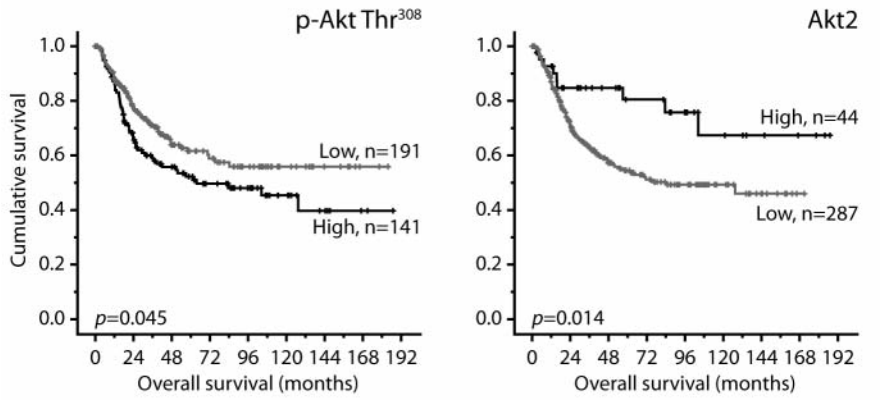

Figure 3. *Disease-specific survival curves for patients according to tumor epithelial cell p-Akt Thr308 and non-phosphorylated Akt2 expression.*

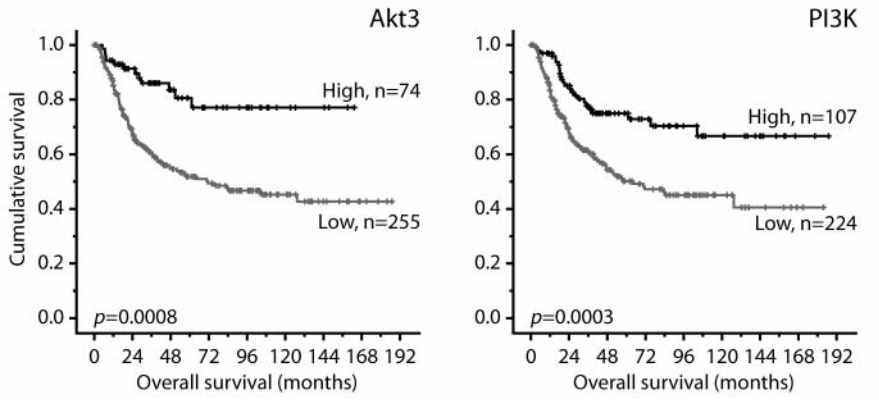

Figure 4. *Disease-specific survival curves for patients according to stromal total Akt3 and total PI3K expression.*

Discussion

To further unveil the role of Akt isoforms in lung tumorigenesis, we immunohistochemically analyzed the expression of phosphorylated Akt1 isoforms on either or both phosphorylation sites (Ser^{473} and Thr^{308}), unphosphorylated Akt2, total Akt3, total PTEN and total PI3K in 335 NSCLCs. Our investigation considered alterations of expression in both tumor epithelial cells and in the stromal tumor compartment. High tumor epithelial cell expression of p-Akt Thr^{308} showed an independent negative correlation with DSS. In contrast, high tumor epithelial cell expression of non-phosphorylated Akt2 as well as high stromal expression of total Akt3 and total PI3K were independent positive prognosticators for DSS. In accordance with results published by other groups (26, 28, 29), we found high prevalence of Akt1 activation in all NSCLC subtypes. Regarding Akt1 and Akt3 activation, there was no significant difference between expression in tumor cores and control cores, whereas for PTEN and PI3K a significant difference in expression was observed. All of the control cores showed positive staining for PTEN, while 42% of all tumor cores did not. In addition, high expression of PI3K was observed in 29% of all tumor cores, while this was observed in only 4% of control cores. Several studies have reported increased expression of p-Akt Ser^{473} in NSCLC (6, 18, 20) but there are inconsistent results concerning the prognostic impact of p-Akt in NSCLC tumors. Several recent studies (17, 28, 30) have reported a lack of association between activated Akt and survival or clinicopathological variables such as histology, stage of disease and metastasis. At the same time, other research groups (9, 25, 26) indicated an association between expression of p-Akt and NSCLC prognosis. David *et al.* (9), in an investigation of various NSCLC tumors from 61 patients followed up for 10 years, concluded that overexpression of p-Akt is an independent poor prognostic factor. In 102 NSCLC cases, Tang *et al.* (26) found that patients with concomitant p-Akt Ser^{473} expression and loss

Table II. *Tumor epithelial cell and stromal cell expression of PI3K/Akt signaling pathway markers as predictor for disease-specific survival in 335 NSCLC patients (univariate analysis; log-rank test).*

Marker expression	Patients		Median survival (months)	5-Year survival (%)	*p*-value
	n	(%)			
p-Akt Thr308					
Tumor					0.045
Low	191	57	NR	62	
High	141	42	64	52	
Missing	3	1			
Stroma					0.3
Low	255	76	84	54	
High	78	23	NR	69	
Missing	2	1			
p-Akt Ser473					
Tumor					0.4
Low	270	81	127	57	
High	63	18	NR	59	
Missing	2	1			
Stroma					0.8
Low	312	93	127	58	
High	22	6	NR	60	
Missing	1	1			
Akt2					0.014
Tumor					
Low	287	13	84	55	
High	44	86	NR	80	
Missing	4	1			
Stroma					0.31
Low	138	41	127	57	
High	195	58	NR	59	
Missing	2	1			
Akt3					
Tumor					0.9
Low	233	70	104	56	
High	94	28	NR	66	
Missing	8	2			
Stroma					0.0008
Low	255	76	71	52	
High	74	22	NR	81	
Missing	6	2			
PI3K					
Tumor					0.4
Low	233	70	NR	57	
High	95	28	127	62	
Missing	7	2			
Stroma					0.0003
Low	224	67	63	50	
High	107	32	NR	75	
Missing	4	1			
PTEN					
Tumor					0.08
Low	231	69	84	54	
High	98	29	NR	68	
Missing	6	2			
Stroma					0.051
Low	222	66	83	54	
High	108	32	NR	68	
Missing	5	2			

NR, Not reached; missing, refers to both lack of staining of samples or lack of the sample itself.

Table III. *Results of Cox regression analysis summarizing significant independent prognostic factors.*

Factor	Hazard ratio	95% CI	*p*-value
Tumor status			0.0002*
1	1.000		
2	2.344	1.410-3.898	0.001
3	4.040	1.995-8.181	0.0001
Nodal status			0.0004*
0	1.000		
1	2.002	1.293-3.100	0.002
2	2.438	1.395-4.260	0.002
Performance status			0.007*
Normal	1.000		
Slightly reduced	1.948	1.287-2.949	0.002
In bed >50%	1.454	0.611-3.458	0.398
Differentiation			0.005*
Well	1.000		
Moderate	1.951	1.045-3.644	0.036
Poor	1.001	0.525-1.909	0.998
Vascular infiltration			0.003*
No	1.000		
Yes	2.126	1.294-3.495	
p-Akt Thr^{308} Tumor			0.0009*
High	1.000		
Low	0.412	0.279- 0.610	
Akt2 Tumor			0.004*
High	1.000		
Low	2.912	1.395-6.078	
Akt3 Stroma			0.0008*
High	1.000		
Low	3.823	1.963-7.446	
PI3K Stroma			0.012*
High	1.000		
Low	1.841	1.146-2.956	

*Overall significance as a prognostic factor. CI, Confidence interval.

of PTEN had a significantly reduced 5-year survival rate. Whereas Shah *et al*. (25) made observations in 82 NSCLC stage I-IIIA patients suggesting p-Akt Ser^{473} expression to be a favourable prognostic factor in NSCLC. Inconsistent results between previous studies may be associated with tissue specificity, technical differences, and/or the use of immunohistochemical antibodies obtained from different providers. In addition, different scoring methods, the study size and the number of statistical variables entered in the multivariate analysis may also contribute to the discordance. We present the first large-scale study to investigate the prevalence of the three known Akt isoforms in NSCLC. We found a high prevalence of all Akt isoforms in the NSCLC tissues. High epithelial tumor p-Akt Thr^{308} expression correlated with a worse prognosis in accordance with the accumulating evidence indicating Akt as a driver of tumorigenesis and an inhibitor of apoptosis (4, 14). But whereas tumor cell expression of activated p-Akt Thr^{308}

indicated an adverse prognosis, no prognostic correlation was observed for tumor epithelial cell expression of p-Akt Ser^{473}. Moreover, phosphorylation at both p-Akt sites (Ser^{473}+Thr^{308}) did not improve the prognostic significance of Akt in NSCLC as previously reported (29). Several research groups have studied the prevalence of phosphorylated Akt and its relevance to prognosis in NSCLC (25, 26, 29). Through PubMed searches, we could not identify any studies investigating the prognostic significance of the non-phosphorylated Akt-isoform expression in resected primary NSCLC tissues. In the present study, we found high non-phosphorylated Akt2 expression in tumor epithelial cells to be a positive prognosticator. Whether the observed overexpression of non-phosphorylated Akt2 indicates a lower presence of phosphorylated (active) Akt2 or whether these findings represent opposing prognostic roles of phosphorylated (active) and non-phosphorylated Akt isoforms deserves further investigation. An apparent limitation for such an investigation is to find a sufficiently specific antibody for phosphorylated Akt2 on paraffin-embedded material. Immunohistochemistry as a method for detecting protein expression in paraffin-embedded tissue is both highly sensitive and specific (34), but the specificity of an immunohistochemical test will never exceed the specificity of the antibody provided.

Stromal epithelial interactions are considered critical for regulating tissue development and for the maintenance of tissue homeostasis. Consequently, it seems essential to study the tumor stroma and its expression of different molecular markers for a better understanding of tumorigenesis. In the present study, the term 'stroma' comprises all groups of non-epithelial cells and structures intervening between islands of tumor epithelial cells, *i.e.* mesenchymal cells (fibroblasts and fibroblast-like cells), leukocytes, macrophages, endothelial cells and extracellular matrix (ECM) including collagen. Investigating the stromal compartment can, however, be complicated as the stroma is not static. Cellular and ECM compositions evolve over time, adapting to changes related to the surrounding epithelial cells.

Studying the expression of our markers in stroma, we found high expression of total PI3K and total Akt3 to correlate with a favourable prognosis. In an attempt to explain this finding, we propose the hypothesis that overexpression of a protein kinase in different compartments can have different effects on tumorigenesis. This implies that stromal overexpression of the antiapoptotic proteins Akt3 and PI3K enhances stromal cell function by preventing tumor cell proliferation in concert with the assumed function of the immune system. Similar stromal findings for other antiapoptotic markers have been reported in previous papers from our group (1, 10).

These unexpected, and apparently opposing, prognostic roles of Akt isoforms in epithelial tumor cells *versus* stroma could have important implications in the therapeutic development of anticancer agents targeting Akt. This is in keeping with the observed, almost inverse prevalence of PTEN and PI3K expression in lung tumor *versus* normal cells. Further studies within this field should contribute to a wider understanding of this extremely complex pathway, hence establishing the foundation for more effective therapeutic strategies.

Conflict of interest

There are no financial or ethical conflicts of interest.

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