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ORIGINAL ARTICLE

Prognostic significance of protein kinase B/Akt pathway in patients with non-small cell lung cancer

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Summary

Purpose: *Akt, also known as protein kinase B (PKB), is an intracellular signal transduction protein activated by growth hormones. PKB/Akt is frequently activated in a variety of cancer types, but its role in the development and progression of lung cancer has not been completely elucidated yet. The aim of the present study was to determine the prognostic value of PKB/Akt in non-small cell lung cancer (NSCLC).*

Methods: *A total of 32 tumor samples from NSCLL patients were examined before treatment. The staining characteristics of the cases were evaluated in terms of age, stage (T and N), response to therapy, histological type, tumor size, and ECOG performance status (PS).*

Results: *No statistical correlation was found between PKB/Akt expression and gender, ECOG PS and stage (T and N), while significant correlation between cytoplasmic PKB/akt expression and age was detected (p<0.05). In addition, squamous cell carcinoma histology was significantly associated with both nuclear and cytoplasmic staining (p=0.033), and tumor size (<5 cm) was correlated with nuclear PKB/Akt expression (p=0.03). Both overall survival (OS) and progression-free survival (PFS) were similar in patients with and without both nuclear and cytoplasmic PKB/Akt expression.*

Conclusion: *Our results showed that although PKB/Akt was not associated with survival in NSCLC patients, it may be a potential therapeutic target for NSCLC; more studies with higher numbers of patients are needed to verify this hypothesis.*

Key words: *intracellular signal transduction, non-small cell lung cancer, phospho-Akt (p-Akt), prognosis, protein kinase B*

Introduction

Lung cancer genesis is based on extremely complex molecular events. The two mechanisms that have been blamed until today for the development of lung cancer are activation of proto-oncogenes and inactivation of tumor suppressor genes. Ras and Myc families are among the most blamed oncogenes. Of these, Ras family generally plays a role in NSCLC by means of point mutations, whereas Myc family plays a role in small cell lung cancer (SCLC) by means of amplification [1,2]. Furthermore, cERB1-2 plays a role, particularly in NSCLC, and c-met, c-src, and c-raf-1 play a role in SCLC [2,3]. Of tumor suppressor genes, variations regarding p53 and RB genes are seen in 75-100% of SCLC and 15-50% of NSCLC. Moreover, apoptosis and cell cycle related genes also play a role in the development of lung cancer [4,5]. Understanding the biology of lung cancer may lead to the identification of novel targets for the treatment of this disease.

PKB/Akt is a serin-threonine kinase that regulates a variety of cellular functions such as cell survival, cell growth, cell differentiation and progression of cell cycle, and participates in a variety of cellular events such as apoptosis and protein synthesis [6,7]. PKB/Akt is activated by

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phosphoinositide-3 kinase (PI3K) which is activated by the growth stimulators in the cells, such as epidermal growth factor and insulin-like growth factor-I [8]. This series begins with the activation of Ras, and the kinase cascade progresses in turn by Raf, MEK and Erk proteins. Ras and Raf are proto-oncogenes. Ras proteins are inactive (Ras-GDP) in resting cells [9,10]. Under normal conditions, the efficacy of Ras is minimal in the stimulation of PI-3K pathway by growth factors. On the contrary, oncogenic Ras is a strong activator of PI-3K pathway and suppresses apoptosis by activating the above mentioned pathway and forms one of the critical factors of the carcinogenesis process [11,12].

The role of PKB/Akt in the development and progression of lung cancer has not been completely defined. In the present study, the prognostic value of PKB/Akt in NSCLC and its correlation with clinicopathologic variables was evaluated.

Methods

This study was conducted at the Dr. Lutfi Kirdar Kartal Education and Research Hospital, Department of Radiation Oncology and Medical Oncology, between 2008 and 2009. Thirty-two patients, who had been diagnosed with NSCLC and had not received any anticancer therapy were included. The eligibility criteria consisted of measurable disease determined radiologically (chest X-ray, thorax CT scan or PET-CT), ECOG PS of 0-2, adequate hematological (absolute neutrophil count > 1500 mm^3, platelet count >100000 mm3), hepatic (total serum bilirubin < 1.5 times the upper limit of normal (ULN), ALT and AST < 2.5 times the ULN), and renal function (serum creatinine level < 1.25 mg/dl).

Treatment plan

A weekly chemotherapy schedule including 30 mg/m^2 docetaxel and 20 mg/m^2 cisplatin was administered simultaneously with radiotherapy. The treatment continued only with radiotherapy in patients with severe chemotherapy toxicity (grade 3/4 hematological toxicity, liver and renal function impairment). Radiotherapy was delivered with 6-15 MV photons in all patients using linear accelerator (GE Saturn 41) as 2 Gy daily fractions 5 days a week for a total of 23 fractions (46 Gy) to the primary tumor and mediastinum, followed by a boost to the primary tumor and involved lymph nodes (2 Gy daily fractions, 10 fractions, 26 Gy). The total dose of radiation was 66 Gy in 33 fractions. Response to therapy (complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD)) were determined according to the criteria defined by the World Health Organization.

Immunohistochemical staining

Thirty-two NSCLC samples were immunohistochemically stained. Three to 5 mμ sections, obtained from paraffin blocks were selected by the pathologists, put on slides and covered with poly-L-lysine. They were kept at 37°C in an incubator overnight. After being kept in xylene for 15 min, they were re-hydrated in 96% alcohol for 15 min, and washed with distilled water for 5 min. Then, they were put in citrate buffer (pH 6) and boiled for 5 min in a microwave oven 4 times (750W, 350W, 350W, 350W). Then, they were kept at room temperature within the same buffer for 20 min for cooling, and washed with distilled water. In order to eliminate endogenous peroxidase activity, 3% hydrogen peroxide solution was dripped. After 10 min the sections were washed with PBS (Phosphate Buffer Solution) for 3 times, and protein block (LabVision, Large Volume Ultra V Block, TA-125-UB) was applied for 5 min. The excess amount of block solution was poured and primary (AKT [Ser473] rabbit polyclonal antibody RB-10369-P1 (P&D System, USA) was incubated for 40 min without being washed. Afterwards it was washed in PBS 3 times. Secondary antibody (Labvision, Biotinylated Goat Anti Polyvalent TP-125-BN, Thermo Scientific) was applied for 15 min, and washed in PBS 3 times. Tertiary antibody (Labvision, Large Volume Streptavidin Peroxidase, and TS-125-HR, Thermo Scientific) was incubated with the sections for 15 min, and washed in PBS 3 times. After dripping AEC chromogene (Labvision, Large Volume AEC Substrate System, TA-125-HA, Thermo Scientific) for 10 min, the sample was washed with distilled water. The tissues were counterstained with Mayer's Hematoxylin (Bio-Optica, Mayer's hematoxylin 06002L, Milano, Italy) for 1 min. Hematoxylin turned into purple under tap water and the excess amount was washed. The sample was coated with aqueous coating material (Bio-Optica, Mount quick aqueous mounting medium, 05-1740, Milano, Italy) (Figure 1).

Immunohistochemical PKB/Akt expression was examined under light microscope. Samples were evaluated according to the intensity and extensiveness of nuclear and cytoplasmic staining of tumor cells. Nuclear and cytoplasmic staining percentage of the stained tumor cells was taken into consideration for the degree of extensiveness. The nuclear and cytoplasmic staining was analyzed as either present or absent [13,14]. The staining characteristics of the cases were evaluated in terms of age, gender, stage (T and N), response to the therapy, histological type, tumor size and ECOG PS.

Statistics

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software. Chi-square

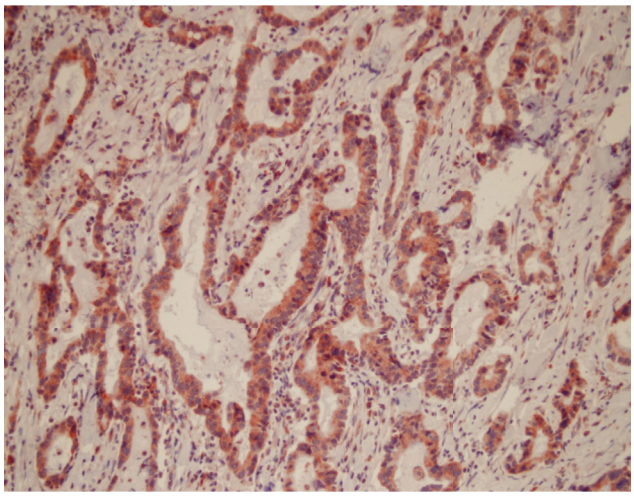

Figure 1. Immunoperoxidase staining with pAkt (Ser473) antibody (Neomarkers USA). Strong cytoplasmic staining (×200).

test and Fisher's exact test were used to analyze the relationships between the groups and categorical variables. Survival probabilities and curves were obtained according to the Kaplan-Meier method and compared by the log-rank test. Univariate analysis was carried out to evaluate the significance of PKB/Akt expression and other clinicopathological features as prognostic factors, then multivariate analysis with the Cox proportional hazards model was performed in order to further analyze the PKB/Akt expression and all of the significant prognostic factors which were found in the univariate analysis. Multivariate p values were used to characterize the independence of these factors. The 95% confidence interval (95% CI) was used to quantify the relationship between survival time and each independent factor. All p values were two-sided in the tests and p values < 0.05 were considered as statistically significant.

Results

Thirty-one (96.9%) patients were male and one female, with a median age of 63 years (range 18-70). Twenty-four (75%) patients were older than 50 years. The majority of patients (71.9%) had T4 tumors. N stage was as follows: 7 (21.9%) patients had N_0, 1 (3.1%) N_1, 20 (62.5%) N_2 and 4 (12.5%) N_3. One patient (3.1%) was classified as stage IIB, 6 (18.8%) as stage IIIA, and 25 (78.1%) as stage IIIB. ECOG PS was 2 in the majority (65.6%) of the patients. Twenty-nine (90.6%) patients had a history of smoking. Histopathological subtype was squamous cell carcinoma in 21 (65.6%) patients and adenocarcinoma in 3 (9.4%) patients, while 8 (25%) patients were classified as 'not otherwise specified' NSCLC. Demographic, clinical and histopathological characteristics of patients are summarized in Table 1. All patients completed 66 Gy. Weekly chemotherapy concurrent with radiotherapy could be applied for a median 5 courses (range 3-7).

Table 1. Patient and disease characteristics

Characteristics	*N (%)*
Gender	
Female	1 (3.1)
Male	31 (96.9)
Age (years)	
≤50	8 (25.0)
>50	24 (75.0)
ECOG PS	
0	9 (28.1)
1	23 (71.9)
T stage	
T1	1 (3.1)
T2	3 (9.4)
T3	5 (15.6)
T4	23 (71.9)
N stage	
N0	7 (21.9)
N1	1 (3.1)
N2	20 (62.5)
N3	4 (12.5)
TNM stage	
IIB	1 (3.1)
IIIA	6 (18.8)
IIIB	25 (78.1)
Histopathology	
Squamous cell carcinoma	21 (65.6)
Adenocarcinoma	3 (9.4)
Non-small cell lung cancer, NOS	8 (25.0)

NOS: not otherwise specified, ECOG PS: Eastern Cooperative Oncology Group performance status

Both nuclear and cytoplasmic staining were observed in 11 of 32 (34.4%) patients. Nuclear staining was positive in 20 (62.5%) of the patients, while cytoplasmic staining was positive in 21 of 32 (65.6%) patients. In 2 (6.3%) patients staining was negative. There was significant correlation between cytoplasmic staining and age, when patients with cytoplasmic staining were >50 years compared with patients <50 years ($p<0.05$). Both nuclear and cytoplasmic staining in patients with T3-T4 stages (17 and 19, respectively) was more frequent compared with T1-T2 patients (3 and 2, respectively), but these differences were not significant (p=0.5 and 0.4, respectively). Lymph node involvement was not associated with either nuclear or cytoplasmic staining ($p>0.05$). In addition, no significant correlation was detected between stage and staining (p=0.67 and p=0.60, respectively).

In patients with squamous cell carcinoma histology both nuclear and cytoplamic staining were significantly more frequent than in patients with non-squamous cell histology (47.6 vs 9.1%, p=0.033). However, there were no sig-

Table 2. Relationship between patient characteristics and nuclear and cytoplasmic staining

Characteristics	*NS (+) N (%)*	*NS (-) N (%)*	*p-value*	*CS (+) N (%)*	*CS (-) N (%)*	*p-value*
Gender			0.37			0.65
Female	20 (100)	11 (91.7)		20 (95)	11 (100)	
Male	0	1 (8.3)		1 (5)	-	
Age (years)			0.66			< 0.05
≤50	5 (25)	3 (25)		3 (14.3)	5 (45.5)	
>50	15 (75)	9 (75)		18 (85.7)	6 (54.5)	
ECOG PS			0.06			0.12
0	8 (40)	1 (8.3)		4 (19)	5 (45.5)	
1-2	12 (60)	11 (91.7)		17 (81)	6 (54.5)	
Tumor size (cm)			0.03			0.08
≤5	3 (15)	6 (50)		8 (38)	1 (9)	
>5	17 (85)	6 (50)		13 (62)	10 (91)	
T stage			0.51			0.42
T1-2	3 (15)	1 (8.3)		2 (10)	2 (8)	
T3-4	17 (85)	11 (91.7)		19 (90)	9 (82)	
N stage			0.53			0.45
N0	4 (20)	3 (25)		4 (19)	3 (28)	
N (+)	16 (80)	9 (75)		17 (81)	8 (72)	
Histopathology			0.14			0.60
Squamous cell	15 (75)	6 (50)		16 (76.2)	5 (45.5)	
Adenocarcinoma	1 (5)	3 (25)		2 (9.6)	2 (18.2)	
NSCLC,NOS	4 (20)	3 (25)		3 (14.2)	4 (36.3)	
Tumor response			0.24			0.005
CR	5 (25)	1 (8.3)		1 (4.8)	5 (45.5)	
Non-CR	15 (75)	11 (91.7)		20 (95.2)	6 (54.5)	

NS: nuclear staining, CS: cytoplasmic staining, CR: complete response, NOS: not otherwise specified, ECOG PS: Eastern Cooperative Oncology Group performance status

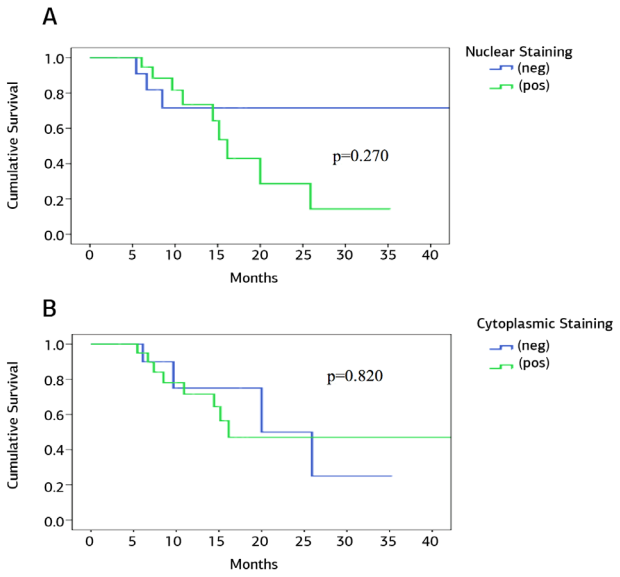

Figure 2. Kaplan-Meier estimates for overall survival in the **(A)** nuclear staining group and **(B)** the cytoplasmic staining group.

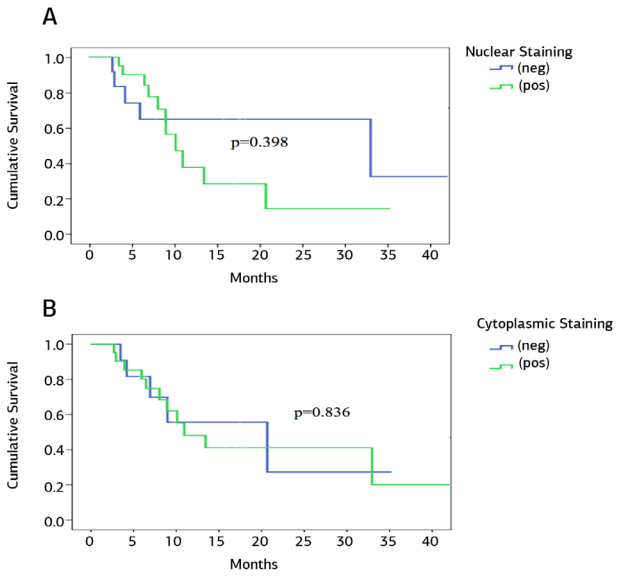

Figure 3. Kaplan-Meier estimates for progression-free survival in the **(A)** nuclear staining group and **(B)** the cytoplasmic staining group.

nificant correlations between histopathological subtypes and nuclear or cytoplasmic staining (p>0.05). Nuclear staining in patients with tumor size > 5 cm was significantly more frequent (17 of 23 patients, 85%) compared with patients with tumors ≤ 5 cm (15%, p=0.03). The relationships between these parameters and nuclear or cytoplasmic staining are shown in Table 2.

CR was achieved in 6 (18.8%) patients. We observed that there were 5 patients with nuclear and 1 patient with cytoplasmic staining in the CR group. In addition no patient was found with

Table 3. Results of univariate analysis with respect to the PKB/Akt status

Staining	*Median OS (months)*	*p-value*	*Median PFS (months)*	*p-value*
Nuclear staining		0.270		0.398
Absent	21		15	
Present	16		11.5	
Cytoplasmic staining		0.820		0.836
Absent	20		14	
Present	16		11	

OS: overall survival, PFS: sprogression-free survival

both nuclear and cytoplasmic staining. Significant differences were detected in patients with non-CR with respect to cytoplasmic and both nuclear and cytoplasmic staining compared with patients with CR (p=0.005 and 0.049, respectively; Table 2).

At a median follow-up of 14.5 months (range 3-51) the median PFS was 13 months (SE: 5; 95% CI: 5-25) and the median OS was 20 months (SE: 5; 95% CI: 11-29) for the entire cohort. Median OS for patients with nuclear staining was worse than that of patients without nuclear staining, but this difference was not statistically significant (21 vs 16 months, respectively, p=0.270). Furthermore, median OS was also similar among patients with or without cytoplasmic staining (20 vs 16 months, respectively, p=0.82). Figure 2 shows OS according to the nuclear or cytoplasmic staining. Median PFS was similar for patients with and without nuclear and cytoplasmic staining (p=0.398 and p=0.836, respectively, Figure 3).

Results of univariate survival analyses with respect to the PBK/Akt staining are summarized in Table 3. Prognostic significance could not be proved in multivariate analysis.

Discussion

Akt, or protein kinase B, is a serine/threonine kinase that regulates growth factors, such as epidermal growth factor and insulin-like growth factor-I, and through them, cell survival [15]. Akt is cellular homolog product of v-akt and has 3 isoforms: Akt-1, Akt-2 and Akt-3. All 3 isoforms are expressed in normal tissues, but the level of expression may vary depending on the tissue. Akt-1 and Akt-2 are expressed in brain, thymus and lungs, whereas Akt-3 is expressed in brain and testicles. Akt is activated downstream by various growth factors including insulin, insulin like growth hormone-I and epidermal growth factor as well as by phosphotidilinositol 3-kinase (PI3K). Complete activation is provided by means of phosphorylation at Thr308/309 location in kinase activation handle and at Ser473/474 location in COOH-terminal tail [16].

The role of Akt in carcinogenesis has been well-documented and Akt is overexpressed in various types of human cancer [16,17]. Akt is associated with initiation of tumorigenesis in pancreatic carcinoma [18] and glioma [19], and seems to correlate with stage and tumor grade in prostate carcinoma [20]. Moreover, although PTEN frequently disappears or is inactivated via mutation [21], PI3K, which is the positive regulator of Akt, is frequently upregulated [22]. Finally, it has been shown that activated Akt induces cell transformation [23].

In their study including 61 patients, David et al. put forward the hypothesis that overexpression of Akt might be predictive for short survival [13]. There was a strong staining with pAkt antibody in 14 of 61 NSCLC cases and overexpression of PKB/Akt was found to be an independent prognostic factor for survival. In addition, the authors showed that age at the time of diagnosis was not significant but stage was significant. Overall survival was significantly different with respect to Akt status even after stage has been taken into account and mortality was higher in patients with strong staining as compared with those without staining. There was a tendency for the patients with strong staining to be diagnosed at lower stages as compared to those without staining. Also in their study, patients with strong staining significantly tended to be older on average than those without staining, even if they were at the same stages [13]. In the present study, cytoplasmic staining alone was seen more frequently in patients aged ≥50 years; no relationship with age and stage could be demonstrated in other groups.

In a study carried out by Balsara et al. in 110 patients, high Akt activity was determined in 23 (21%) patients. In that study, it was demonstrated that there was no relationship between PKB/Akt expression and histological subtypes or survival. Median OS time was 26 months for the negative group and 23 months for the positive group. The prevalence of PKB/Akt positivity was similar among patients in lower stages (stages I-II) and those with higher stage. Furthermore, PKB/Akt positivity gave similar outcomes both in well-differentiated and poorly-differentiated

tumors. Consequently, this outcome possibly implies that Akt activation occurs in the early period of tumor progression [24]. Our results were compatible with their study with respect to stage, but not with the histological subtypes.

Ninety-six percent of 78 cases that had been evaluated by Shah et al. [14]. were immunoreactive for PBB/Akt. PBB/Akt expression is limited only to tumor cells. The percents of tumor cells that were positively stained in each individual section ranged between 1-90% (median 15); however, cytoplasmic PBB/Akt staining (CP-Akt) was observed in all positively stained tumor cells. In addition, nuclear staining was observed in 42% of the cases. Nuclear staining was classified as "positive" or "negative". Membranous staining was observed in only 5% of the cases, and 1-15% of tumor cells stained positively. CP-Akt and NP-Akt had strong correlation with well-differentiated tumors. NP-Akt was correlated with the presence of nodal involvement as well as with squamous histology as compared with non-squamous types [14].

In the study performed by Lee et al. [25] only 43 patients were included and no significant correlation was observed between CP-Akt and NP-Akt and other pathological factors. PBB/Akt/alpha-actin and the N stage were the only independent prognostic factors. Gender, histological subtype, tumor stage and nodal status were not prognostic factors in univariate analysis [25]. In our study both nuclear and cytoplasmic staining in patients with squamous cell carcinoma histology were significantly more frequent than in patients with non-squamous cell histology (p=0.033).

The relation between PKB/Akt and distant metastasis has been previously reported in other types of tumors and evidence raises the hypothesis that PKB/Akt may have a role in the progression of the disease rather than in its development [26]. Analyzing the percentage of PKB/Akt positively stained tumors ignoring subcellular localization, Shah et al. obtained similar outcomes [14]; however, it had been surprising to find out a correlation between NK-Akt and lymph node metastasis.

Tsurutani et al. evaluated 252 patients to study for any association between Akt phosphorylation with clinical outcomes [27]. Two phosphorylation events are needed for complete activation of Akt. However, a single phosphorylation site (S473) has been determined up to now in clinical samples of NSCLC, and this led to conflicting results concerning the prognostic significance of Akt activation in NSCLC. In the above mentioned study, the authors tried to determine whether Akt phosphorylation enhances the prognostic accuracy at T308 or not. Phosphorylation of S473 or T308 was positive in most of NSCLC samples, but was rarely determined in surrounding tissues. Defining Akt activation using both phosphorylation sites, Akt activation was specific for NSCLC as compared with surrounding tissues (73.4 vs 0%; $p<0.05$). It was significantly higher in adenocarcinoma as compared to squamous cell carcinoma (78.5 vs 68.5%; p=0.040), and associated with shorter survival in all disease stages (p=0.041). In multivariate analysis, increased phosphorylation of T308 only was a poor prognostic factor in stage I patients or for tumors <5 cm (log-rank p=0.011 and p=0.015, respectively). These results raised the thought that observing Akt phosphorylation at T308 facilitates the evaluation of Akt activation and show that Akt activation is a poor prognostic factor for all stages of NSCLC [27]. In the present study we proved that nuclear staining in patients with tumor size > 5 cm was significantly more frequently observed compared with patients with tumors ≤5 cm (85 vs 15%).

In conclusion, no statistical correlation was demonstrated between PKB/Akt expression and gender, stage, ECOG PS, lymph node involvement, and T stage. In contrast, significant correlation was detected between cytoplasmic PKB/Akt expression and age. In addition, squamous cell histology was significantly associated with both nuclear and cytoplasmic staining and tumor size (<5 cm) was related with nuclear PKB/Akt expression. Although PKB/Akt activation may be associated with poor prognosis and chemotherapy and radiotherapy resistance, no significant difference could be demonstrated between nuclear or cytoplasmic staining and survival. We maintain that our results need to be confirmed by prospective studies including larger numbers of NSCLC patients.

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