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Cyclin A and Cyclin E Expression in Resected Non-small Cell Lung Cancer Stage I-IIIA

MONIKA KOSACKA, PAWEŁ PIESIAK, IRENA PORĘBSKA, ANNA KORZENIEWSKA, TOMASZ DYŁA and RENATA JANKOWSKA

Department of Pulmonology and Lung Cancer, Silesian Piasts University of Medicine in Wroclaw, 53-439 Wroclaw, Poland

Abstract. *Background: Lung cancer is the leading cause of cancer death in the majority of developed countries. Cyclin E regulates the the G_1-S phase transition of the cell cycle. Cyclin A increases during the S- and G_2-phases, and is a regulator of the transition to mitosis.The aim of this study was to evaluate the prognostic significance of cyclin A and cyclin E expression in primary, resected stage I-IIIA non-small cell lung cancer (NSCLC). Materials and Methods: The expression of cyclin A and E was investigated in the paraffin-embedded tumor tissue of 71 patients (53 men and 18 women; age 59.27±8.50 years), using a monoclonal antibodies to cyclin A and to cyclin E. Results: Forty-seven out of 71 (66%) tumor tissue specimens were positive for cyclin A and twenty-six (37%) were positive for cyclin E. In the majority of cases, nuclear staining was apparent. Cyclin A and cyclin E expression was significantly higher in squamous cell carcinoma than in adenocarcinoma (cyclin A: Chi^2 Yates'a 4.6; p=0.032; cyclin E: Chi^2 Yates'a 5.12: p=0.023). The prognostic value of cyclin A and E expression was examined in all patients and in patients with squamous cell lung cancer and adenocarcinoma and separately for every stage, but no correlations were found. Conclusion: No prognostic value of cyclin A and E expression was found in NSCLC, but significantly higher cyclin A and E expression was found in squamous cell carcinomas than in adenocarcinomas.*

Lung cancer is the leading cause of cancer death in the majority of developed countries and in Poland. Non-small cell lung carcinoma (NSCLC) accounts for ~80% of all lung carcinomas (1, 2). The overall 5-year survival rates of lung cancer patients remain relatively poor with rate in Europe being worse than in the USA, amounting to 9.7% in males and 9.6% in females (3). Despite improved diagnostic and therapeutic as well as supportive care options, the prognosis remains unfavourable and long-term survival has remained practically unchanged (2, 4). Only patients whose tumours are surgically resectable have better prognosis with survival ranging from 70% for stage IA to 25% for stage IIIA (1).

Although standard treatment of stage I NSCLC consists of surgical resection alone, approximately 50% of clinical stage I and 30% to 40% of pathological stage II patients have disease recurrence and die following curative resection (5). Differing survival outcomes among patients within a stage suggests the existence of other tumour factors affecting prognosis. Such factors could potentially be used to further classify patients into groups according to substages that may be treated differently. The possibility that adjuvant chemotherapy might improve the survival of patients with resected NSCLC has encouraged efforts at identification of prognostic features of these tumors (5-6). The ability to predict survival after lung cancer surgery is even more important because this information could help target therapies to those patients which would obtain the most benefit (7).

Uncontrolled cell proliferation is the hallmark of malignant tumours, which is why the evaluation of the prognostic significance of the expression of proteins involved in regulation of cell proliferation remains promising. Cellular proliferation is regulated by protein complexes composed of cyclins and cyclin-dependent kinases (cdks). Five major families of cyclins (termed A, B, C, D and E) have been isolated and characterized (8-9).

Cyclin E enters into a complex with its catalytic partner cdk2 and collaborates with the cyclin D-dependent kinases to complete Rb phosphorylation. Cyclin E-cdk2 also phosphorylates substrates other than Rb. The activity of the cyclin E-cdk2 complex peaks at the G_1-S transition, after which cyclin E is degraded and replaced by cyclin A (8-10).

Correspondence to: Monika Kosacka, Department of Pulmonology and Lung Cancer, Silesian Piasts University of Medicine in Wroclaw, ul. Grabiszynska 105, 53-439 Wroclaw, Poland. Tel: +48 713349559, Fax: +48 713349596, e-mail: mokka113@hotmail.com

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Cyclin E overexpression shortens the G_1 cell cycle, alters S-phase progression and causes chromosomal instability (CIN) (11-12).

Cyclin A expression increases later in the cell cycle, during the S- and G_2-phases, and it is considered regulator of the transition to mitosis together with cyclin B (8-9). Two types of cyclin A have been described: cyclin A1 and cyclin A2 (also known as cyclin A). The protein cyclin A1 is 60% identical to cyclin A2 (13). Recent data indicate that cyclin A1 is a p53-induced gene. Cyclin A1 can induce G-2 cell cycle arrest and polyploidy. It might also mediate apoptosis and mitotic catastrophe through an unscheduled or inappropriate activation of cdk1 (14).

The aim of this study was to evaluate the prognostic significance of cyclin A and E expression in primary, resected stage I-IIIA NSCLC.

Materials and Methods

Seventy-one patients with resected NSCLC (53 men and 18 women) were evaluated. The mean age of the patients was 59.27±8.50 years. All patients had undergone surgical treatment, consisting of lobectomy, bilobectomy or pneumonectomy. The histopathological diagnosis was squamous cell carcinoma (SCC) in 43 patients, adenocarcinoma in 17 patients, large cell carcinoma in 6 patients and NSCLC of unspecified type in 5 patients. Based on the TNM staging system, 29 patients were in stage I (including 8 in IA and 21in IB), 14 in II (2 in IIA and 12 in IIB) and 28 in IIIA.

Twenty-seven patients received chemotherapy treatment: 22 received neoadjuvant chemotherapy based on cisplatin (in the majority of cases cisplatin and etoposide). Five patients received adjuvant chemotherapy.

In all patients, the 24 month survival rate was evaluated: forty-seven (66%) patients were alive and 24 (34%) had died. The average survival time was 19.24 months.

This study was approved by the appropriate Ethical Committees related to the Institution.

Immunohistochemistry. Formalin-fixed, well-preserved tumour tissue blocks from surgically resected lung cancer specimens were used for immunohistochemical study. The 4 μm-sections of formalin-fixed tissues were mounted on silanized slides, deparaffinized in xylene and rehydrated through a series of alcohol to water. The hydrated sections were treated in 3% hydrogen peroxide for 10 minutes to eliminate endogenous peroxidase activity and washed in phospate-buffered saline (PBS).

The primary antibodies used in this study were a monoclonal antibody to cyclin A (NCL-CYCLIN A clone 6E6 NOVO CASTRA United Kingdom) and a monoclonal antibody to cyclin E (NCL-L-CYCLIN E clone 13A3 NOVO CASTRA United Kingdom).

The monoclonal antibody-treated slides were rised in PBS solution and incubated with a biotinylated secondary antibody ($LSAB^R$+ Kit; DAKO Denmark). The slides were washed in PBS and then incubated with an avidin-biotin-peroxidase complex ($LSAB^R$+ Kit; DAKO K 0675) for 15 minutes. After washing with PBS, a chromogenic reaction was developed by incubating with 3,3-diaminobenzidine tetrahydrochloride (DAB+, Liquid K 3486; DAKO).

Positive staining appeared as brown cell plasma or nucleus. Cyclin A and E accumulation was described as positive if more than 10% of cells were stained. A single representative tissue section from each tumor was surveyed microscopically at ×100 for at least three areas with the highest intensity of positive tumor cells. Cell counts were performed at ×400 in at least five fields in these areas. All immunohistochemical studies were performed without knowledge of the clinical data.

Statistical methods. Statistical analysis was performed using the CSS Statistica for Windows (version 5.0 Poland). Chi-square test was used among two or multiple groups. Differences between samples were considered significant at $p<0.05$.Survival curves were constructed using the Kaplan-Meier method.

Results

Forty-seven out of 71 (66%) tumor tissue specimens were positive for cyclin A. Only nuclear staining was observed.

Twenty-six of the examinated tumors (37%) were positive for cyclin E. Two distinct patterns of staining were observed: in the majority of cases nuclear staining was observed; in only 4 cases was cytoplasmatic staining revealed. Figure 1 shows representative examples of immunohistochemical staining.

We revealed that in squamous cell carcinoma, cyclin A and cyclin E expression was higher than in adenocarcinoma and the differences were significant. Cyclin A expression was positive in 40 patients out of 53 with SCC, but in only 12 out of 25 with adenocarcinoma (Chi^2 Yates'a 4.6; p=0.032). Cyclin E expression was positive in 24 cases of SCC, but in only 4 adenocarcinoma cases (Chi^2 Yates'a 5.12: p=0.023).

Moreover, we observed that in stage I tumours positive cyclin A expression was more frequent than in stage IIIA tumours. Cyclin A expression was positive in 23 cases out of 29 stage I and in 14 out of 28 stage IIIA (Chi^2 Yates'a 4.16; p=0.041).

We analyzed the prognostic value of cyclin A and E expression in all patients with NSCLC and separately in patients with SCC and adenocarcinoma, but did not find any correlations (Figures 2 and 3 and Tables I and II).

Discussion

The enormous potential of immunohistochemical methods in contributing to a better understanding of carcinogenesis and in the search for prognostic factors in lung cancer is confirmed by many studies (7, 15). In a major review of the literature 462 articles and 12 reviews relating immunochemistry to prognosis in NSCLC were found (15). The review of Singhal *et al.*, which focused on biomarkers primarily involved in one of three major pathways: cell cycle regulation, apoptosis, and angiogenesis, also confirms the utility of immunochemistry (7). In our previous study, we analysed the apoptotic markers p53, Bcl-2 and Bax in lung cancer and we demonstrated the differences between its expression in NSCLC and SCLC,

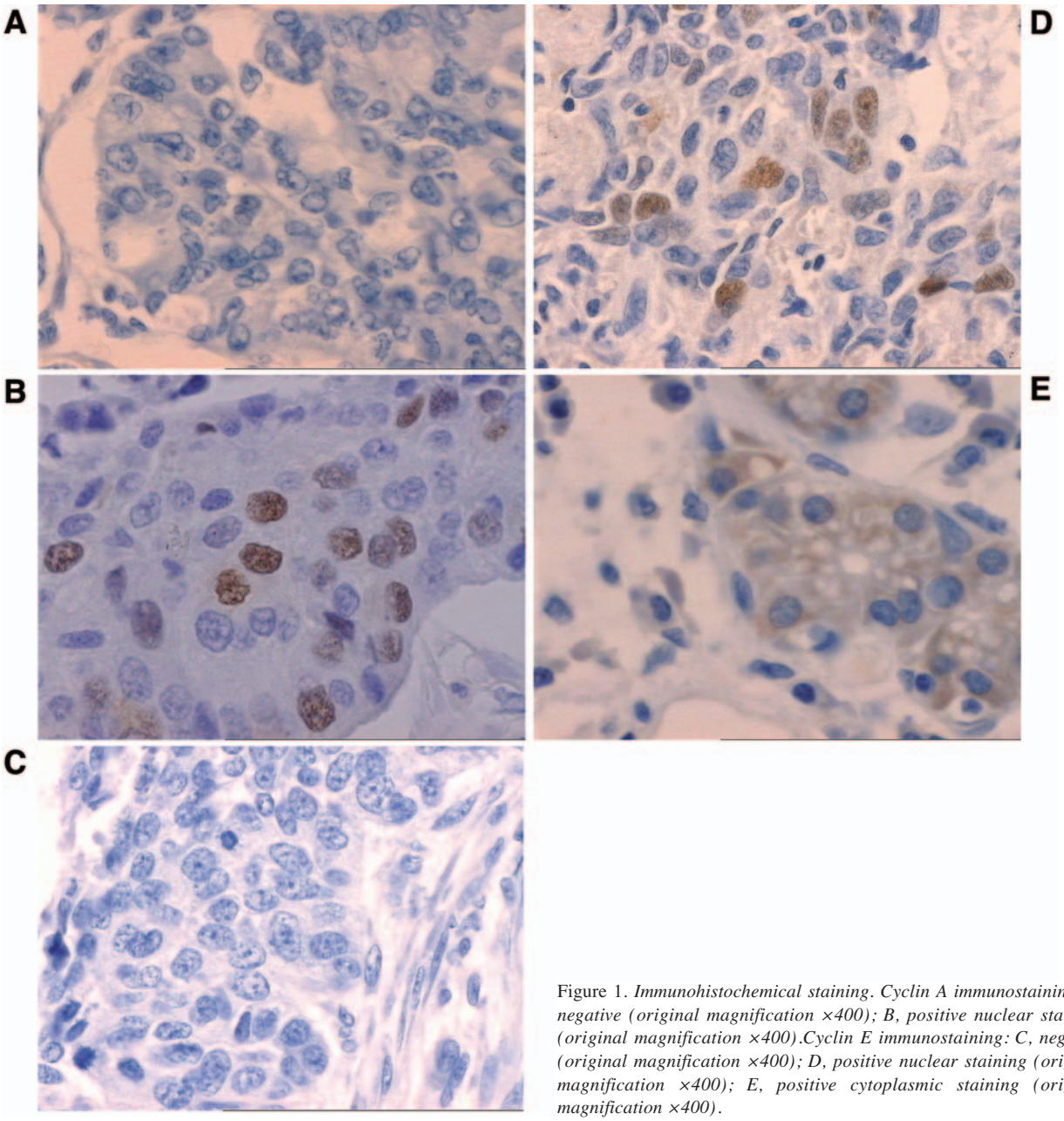

Figure 1. *Immunohistochemical staining. Cyclin A immunostaining: A, negative (original magnification ×400); B, positive nuclear staining (original magnification ×400).Cyclin E immunostaining: C, negative (original magnification ×400); D, positive nuclear staining (original magnification ×400); E, positive cytoplasmic staining (original magnification ×400).*

which could reflect the different pathogenesis of these two lung cancer histological types (16). Despite the large number of studies no single marker has yet been shown to be perfect in predicting patient outcome.

The expression of different cyclins has been often evaluated in many types of cancer and in lung cancer, but their prognostic value remains disputable. In esophageal SCC and hepatocellular carcinoma, the expression of cyclin D1 has been reported to be associated with poor outcomes (17-19). Many studies indicate that cyclin E has oncogenic potential. Transgenic cyclin E expression in the mammary gland caused hyperplasia and carcinoma (20).Expression of cyclin E occurs frequently in pulmonary SCC and is detected in lesions before the development of invasive carcinoma. Cyclin E was not detected in normal bronchial epithelium, squamous metaplasia or low-grade dysplasia, but it was found in 9% of atypias, in 33% of high-grade bronchial dysplasia and in 54% of SCCs (21). Furthermore tobacco carcinogens can transform immortalized human bronchial epithelial cells and augment cyclin E expression (22).

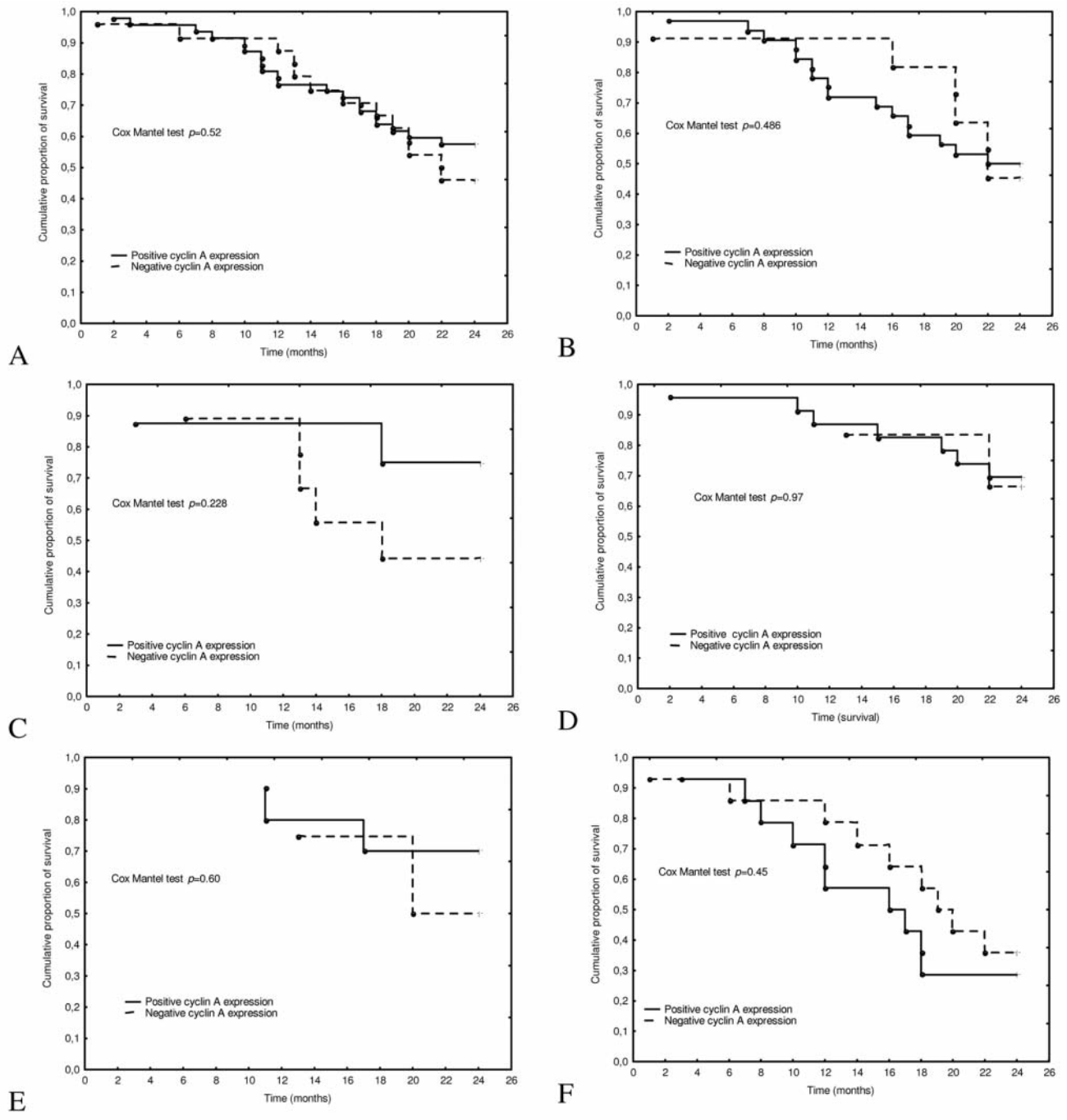

Figure 2. *Cumulative proportion of survival by Kaplan-Meier analysis according to cyclin A expression. A, All patients with non-small cell lung cancer; B, patients with squamous cell lung cancer; C, patients with adenocarcinoma; D, patients with stage I tumour; E, patients with stage II tumour; F, patients with stage IIIA tumour.*

The expression of cyclin E in SCC of the larynx does not seem to have a prognostic significance but could be involved in the development of laryngeal lesions, implicated in cell proliferation, with other cell cycle- related proteins. It has been shown that a high level of cyclin E expression correlated with increased Ki-67 score (23). The same correlation has been confirmed in lung cancer: tumors having high-level cyclin E expression showed a significantly higher Ki-67 expression than tumors having low-level cyclin E expression ($p<0.001$) (24).

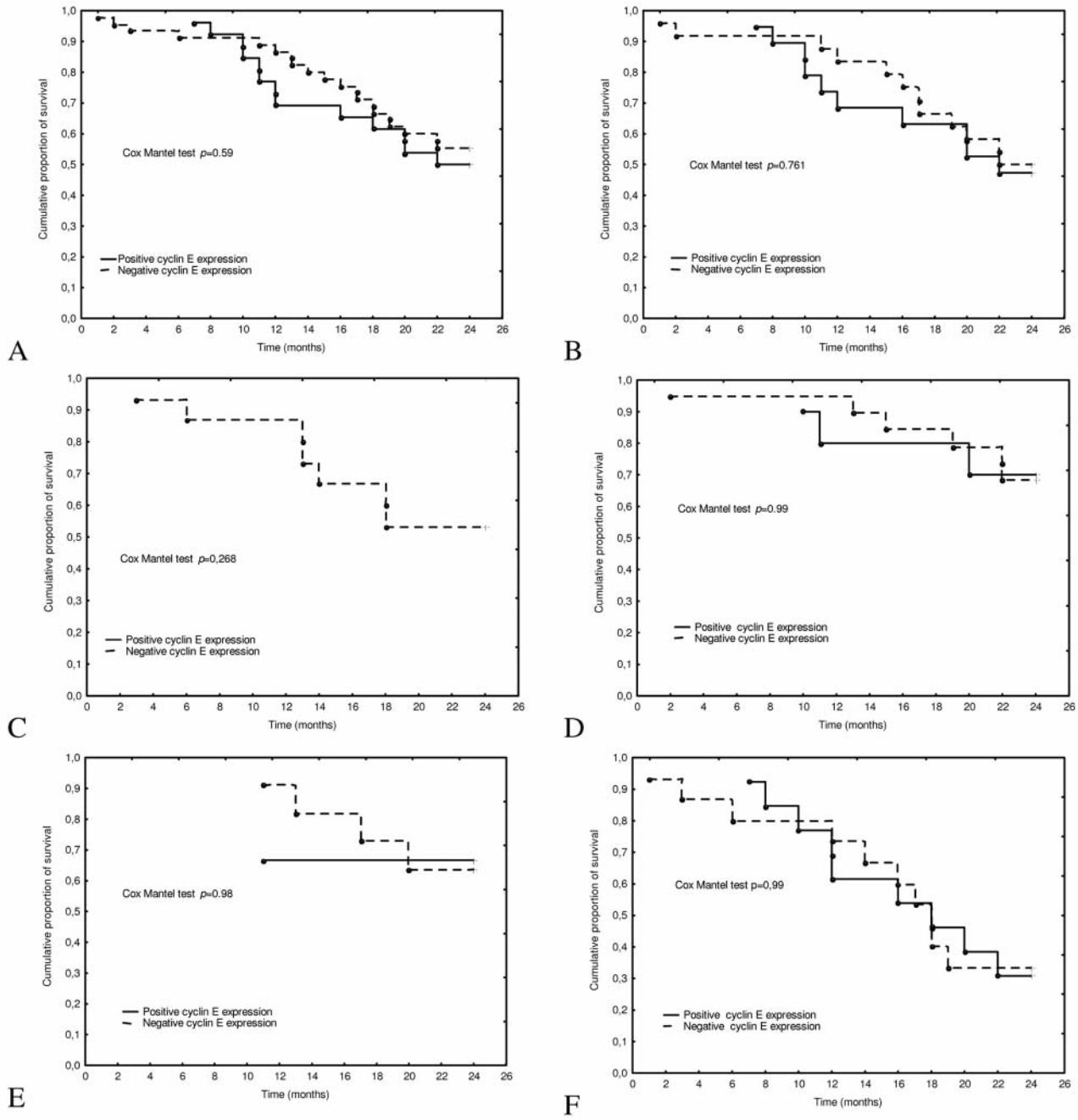

Figure 3. *Cumulative proportion of survival by Kaplan-Meier analysis according to cyclin E expression. A, All patients with non-small cell lung cancer; B, patients with squamous cell lung cancer; C, patients with adenocarcinoma; D, patients with stage I tumour; E, patients with stage II tumour; F, patients with stage IIIA tumour.*

Mishina *et al*. observed, as we did, that cyclin E expression was higher in SCC (p=0.0002). In a group of 217 resected NSCLC these authors demonstrated also high-level cyclin E expression in tumors from smokers (p=0.001) and in pT_{2-4} tumors than in pT_1 tumors. They suggest that cyclin E may play a pivotal role in the biological behavior of NSCLC and that a high level of cyclin E expression may be a new prognostic marker for NSCLC. They also showed that 5-year survival is significantly shorter in patients with high-level cyclin E expression (48% *vs*. 63%) (25). Our study did not confirm the prognostic value of cyclin E expression.

Table I. *Comparison of 24-month survival and cyclin A expression in selected groups of patients.*

Survival	Cyclin A expression		Chi2 Yates'a	*P*-value	Cox Mantel
	Positive n (%)	Negative n (%)			
NSCLC					
<24 months	20 (42.55%)	13 (54.17%)	0.46	0.498	0.52
>24 months	27 (57.45%)	11 (45.83%)			
SCC					
<24 months	16 (50.00%)	6 (54.55%)	0.01	0.928	0.48
>24 months	16 (50.00%)	5 (45.45%)			
Adenocarcinoma					
<24 months	2 (25.00%)	5 (55.56%)	0.61	0.43	0.22
>24 months	6 (75.00%)	4 (44.44%)			
Stage I					
<24 months	7 (30.43%)	2 (33.33%)	0.13	0.719	0.97
>24 months	16 (69.57%)	4 (66.67%)			
Stage II					
<24 months	3 (30.00%)	2 (50.00%)	0.01	0.929	0.60
>24 months	7 (70.00%)	2 (50.00%)			
Stage IIIA					
<24 months	10 (71.43%)	9 (64.29%)	0.00	1.00	0.45
>24 months	4 (28.57%)	5 (35.71%)			

Table II. *Comparison of 24 month survival and cyclin E expression in selected groups of patients.*

Survival	Cyclin E expression		Chi2 Yates'a	*P*-value	Cox Mantel
	Positive n (%)	Negative n (%)			
NSCLC					
<24 months	13 (50.00%)	20 (44.44%)	0.04	0.837	0.59
>24 months	13 (50.00%)	25 (55.56%)			
SCC					
<24 months	10 (52.63%)	12 (50.00%)	0.02	0.892	0.76
>24 months	9 (47.37%)	12 (50.00%)			
Adenocarcinoma					
<24 months	0 (0.0%)	7 (46.67%)	0.24	0.620	0.26
>24 months	2 (100%)	8 (53.33%)			
Stage I					
<24 months	3 (30.00%)	6 (31.58%)	0.11	0.737	0.99
>24 months	7 (70,00%)	13 (68.42%)	Stage II		
<24 months	1 (33.33%)	4 (36.36%)	0.34	0.56	0.98
>24 months	2 (66.67%)	7 (63.64%)			
Stage IIIA					
<24 months	9 (69.23%)	10 (66.67%)	0.07	0.79	0.99
>24 months	4 (30.77%)	5 (33.33%)			

However, we analysed 5-year survival, not 2-year survival and in a smaller group of patients.

Yoo *et al*. investigated 219 patients and found cyclin E expression in 38.4% of cases. These authors observed no correlation between cyclin E expression and survival, but they revealed that in adenocarcinoma, cyclin E staining was associated with differentation (cyclin expression in 50% cases in poorly differentiated tumours *vs*. 10% in well-differentiated) (26).

The prognostic significance of cyclin A remains disputable. In 144 NSCLC cases examined immunohistochemically Dobashi and co-workers revealed, that the labelling index of cyclin A had an inverse correlation with histological differentiation and high cyclin A indicated a poor prognosis in all histological types (27).

Although Volm *et al*. confirmed in Kaplan-Meier analysis this correlation, cyclin A expression was correlated with poorer survival only in stage III, not in I-II. These authors found, that the clinical parameters (age, stage, histology, extent of tumour size, lymph node involvement) had no influence on expression of cyclin A, but they detected a significant correlation between the expression of cyclin A and the response to doxorubicin *in vitro* (28).

The studies in renal cell cancer indicate, that the expression of cyclin A could be related to venous invasion, high nuclear grade, high mitotic rate and high Ki-67 labelling (17).

Conclusion

We did not find any prognostic value of cyclin A and E expression in NSCLC, but cyclin A and E expression was significantly higher in SCC than in adenocarcinoma.

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