

## ORIGINAL ARTICLE

## Analysis of cell cycle regulator proteins in non-small cell lung cancer

V Esposito, A Baldi, G Tonini, B Vincenzi, M Santini, V Ambrogi, T C Mineo, P Persichetti, G Liuzzi, V Montesarchio, E Wolner, F Baldi, A M Groeger

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**Background/Aims:** Abnormalities of the proteins involved in cell cycle checkpoints are extremely common among almost all neoplasms. This study aimed to investigate the expression of four components of the cell cycle machinery—p21, p16, p53, and proliferating cell nuclear antigen (PCNA)—in non-small cell lung cancer (NSCLC).

**Methods:** The expression of p21, p16, p53, and PCNA was examined in 68 well characterised NSCLC specimens using immunohistochemistry. The coregulation of these proteins and their influence on survival were analysed using both univariate and multivariate analyses.

**Results:** By univariate analysis, the expression of all the proteins examined, except for PCNA, was significantly correlated with survival. In multivariate analysis, the only immunohistochemical parameter able to influence overall survival was p16, confirming the hypothesis that the RB–p16 tumour suppressor pathway is inactivated in most lung cancer samples. Finally, the group of patients with NSCLC who were negative for both p21 and p16 had a significantly shorter overall survival.

**Conclusions:** These results suggest that loss of control of cell cycle checkpoints is a common occurrence in lung cancers, and support the idea that functional cooperation between different cell cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumour suppression.

See end of article for authors' affiliations

Correspondence to:  
Dr A Baldi, Via G. Orsi 25,  
80128 Naples, Italy;  
[alfonsobaldi@tiscali.it](mailto:alfonsobaldi@tiscali.it)

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Lung cancer is one of the most prevalent and lethal tumours in western countries. Despite recent advances in oncological treatment, the prognosis for this neoplasm continues to be poor.<sup>1,2</sup> This situation exists because of difficulty in reaching an early diagnosis and because several aspects of lung cancer pathogenesis have not been clarified yet. Nevertheless, great progress has been made in understanding the molecular and cellular pathogenesis of lung cancer.<sup>3</sup> One area that has been the focus of much research is cell cycle control. The precise regulation of the cell cycle is a fundamental requirement for the homeostasis of the eukaryotic cell. During the past decade, scientists have successfully delved into the molecular machinery controlling the fine regulation of the cell cycle, identifying and characterising several genes and gene products involved.<sup>4</sup> A key role is played by cell cycle kinases (CDKs), relatively small proteins with an apparent molecular mass between 33 kDa and 43 kDa. The activity of these molecules is regulated by their arrangement in a multimeric complex with larger proteins, called cyclins because of their cyclical expression and degradation during the cell cycle. Different CDK–cyclin complexes, formed with precise timing throughout the cell cycle, together with their phosphorylation/dephosphorylation, efficiently regulate the activity of the multimeric holoenzyme. Conversely, CDK–cyclin complexes are negatively modulated by the binding of a family of small proteins called CDK inhibitors; namely the CIP (p21 and p27) and the INK (p16) families.<sup>5,6</sup> The p53 tumour suppressor gene is also involved in cell cycle checkpoints because it encodes a protein that acts as a transcription factor for several cell cycle regulatory proteins, including the p21 gene.<sup>7</sup> In contrast, proliferating cell nuclear antigen (PCNA) is involved in activation of DNA polymerase  $\delta$ , which is required for DNA replication and repair.<sup>8,9</sup> Finally, the p53–p21 pathway also inhibits DNA replication by merit of the interaction between p21 and PCNA, without affecting the DNA repair function of PCNA.<sup>10,11</sup>

“Cyclin dependent kinase (CDK)–cyclin complexes are negatively modulated by the binding of a family of small proteins called CDK inhibitors; namely the CIP (p21 and p27) and the INK (p16) families”

Although several of the factors involved in regulating cell cycle control have been investigated in lung cancer, few studies have examined multiple factors in the same tumour series. Therefore, the aim of our study was to evaluate the expression of the p53, p21, p16, and PCNA proteins in a large series of non-small cell lung cancers (NSCLCs) to assess the integrity of cell cycle checkpoints in these tumours, to evaluate the coexpression of these proteins, and to examine the relation between these cell cycle regulators and the clinicopathological features of NSCLCs, including their ability to predict survival in patients with NSCLC.

## MATERIALS AND METHODS

## Patients and tissue samples

We retrospectively evaluated surgical specimens from 68 patients with NSCLC who had undergone surgical resection or biopsy in the departments of thoracic surgery, University of Vienna, Austria and University of Rome, Italy. The case series under investigation was representative of unselected series of NSCLC. Tumour staging was performed according to the international system for staging lung cancer.<sup>12</sup> The patients consisted of 50 men and 18 women (median age, 58 years). All patients underwent surgery (50 patients) or biopsy (18 patients) without preoperative treatment. According to the international system for staging lung cancer, there were 23 patients with clinical or pathological

**Abbreviations:** CDK, cyclin dependent kinase; CI, confidence interval; NSCLC, non-small cell lung cancer; PCNA, proliferating cell nuclear antigen

**Table 1** Patient characteristics

Characteristic	N
Total number	68
Median age (range)	58 (43–81)
Male/female	50/18
Tumour histotype	
Squamous cell carcinoma	35
Adenocarcinoma	29
Other	4
Clinical stage	
I	23
II	21
IIIA	18
IIIB	6
Grading	
1–2	38
3	30
Surgery	
Yes	55
No	13
Postoperative radiotherapy	
Yes	18
No	50
Postoperative chemotherapy	
Yes	11
No	57

**Table 2** Dichotomised expression levels of cell cycle control proteins in the 68 tumours

Protein	Expression		
	Cutoff point (%)	Negative	Positive
p53	5	24	44
p21	5	33	35
p16	5	31	37
PCNA	42.35	23	45

PCNA, proliferating cell nuclear antigen.

stage I, 21 patients with pathological stage II, and 24 patients with clinical or pathological stage III (18 IIIA and six IIIB). The morphological classification of the carcinomas was conducted according to the World Health Organisation specifications: 35 were squamous carcinomas, 29 were adenocarcinomas, and four were less frequent histotypes.

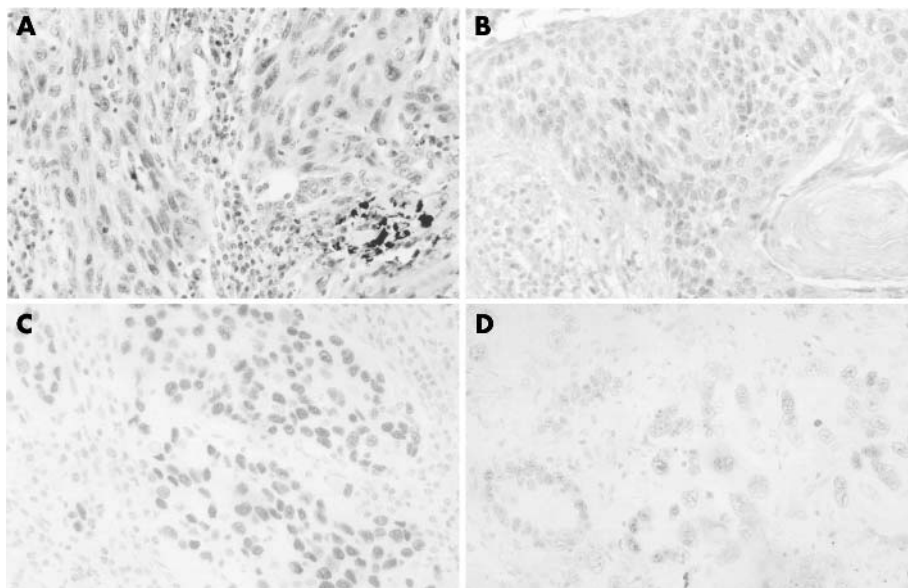
Postoperative radiotherapy was administered to 18 patients with stage II and III disease, whereas 11 patients with stage III disease received postoperative chemotherapy. During follow up, all the 68 patients died of lung cancer. Table 1 summarises the main characteristics of the patients.

**Immunohistochemistry**

Briefly, sections from each specimen were cut at 3–5 µm, mounted on glass slides, and dried overnight at 37°C. All sections then were dewaxed in xylene, rehydrated through a graded alcohol series, and washed in phosphate buffered saline. This buffer was used for all subsequent washes and for the dilution of the antibodies. Tissue sections were heated twice in a microwave oven for five minutes each at 700 W in citrate buffer (pH 6), and then processed with the standard streptavidin–biotin–immunoperoxidase method (Dako Universal kit; Dako Corporation, Carpinteria, California, USA). Mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) specific for p16 (sc-1661), p21 (sc-6246), and PCNA (sc-56) were used at a 1/100 dilution, whereas a monoclonal antibody specific for p53 (D01; Dako Corporation) was used at a 1/500 dilution. All the primary antibodies were incubated for one hour at room temperature. Diaminobenzidine was used as the final chromogen, and haematoxylin as the nuclear counterstain. Negative controls for each tissue section were performed leaving out the primary antibody. Positive controls included in each experiment consisted of tissue previously shown to express the antigen of interest. Two pathologists (FB and AB) evaluated the staining pattern of the four proteins separately and scored the protein expression in each specimen by scanning the entire section and estimating the percentage of tumour cell nuclei staining. All immunoreactive nuclei were regarded as positive, irrespective of staining intensity.

**Statistical analysis**

To carry out statistical analysis, a dichotomised scoring system was used, as follows: p53, p21, and p16 expression in more than 5% of tumour cells was defined as positive expression,<sup>13 14</sup> whereas the median value for the PCNA labelling index in this tumour series was used as a cutoff point, and tumours were classified as either less than or greater than the median value.<sup>15</sup> Fischer’s exact test was used to assess relations between ordinal data (correlation matrix between immunostaining parameters). A univariate survival



**Figure 1** Immunohistochemical staining of cell cycle proteins in tumour specimens: (A) p53, (B) p21, (C) p16, and (D) proliferating cell nuclear antigen.

**Table 3** Correlation matrix (and significance) between molecular markers and pathological parameters in patients with non-small cell lung cancer

	T	N	p53	p21	p16	PCNA
N						
R value	0.427		-0.056	-0.231	-0.301	0.256
p Value	<0.0001		NS	0.015	0.008	0.022
p53						
R value	-0.083	-0.056		-0.141	-0.101	0.153
p Value	NS	NS		NS	NS	NS
p21						
R value	0.151	-0.231	-0.141		-0.090	-0.243
p Value	NS	0.015	NS		NS	NS
p16						
R value	-0.082	-0.301	-0.101	-0.090		-0.031
p Value	NS	0.008	NS	NS		NS
PCNA						
R value	0.216	0.256	0.153	0.243	0.031	
p Value	NS	0.022	NS	0.019	NS	

PCNA, proliferating cell nuclear antigen.

analysis for each prognostic variable on overall survival was estimated according to the Kaplan–Meier method.<sup>16</sup> The terminal event was death attributable to cancer or non-cancer causes. The significance of the differences in survival distribution among the prognostic groups was evaluated by the log rank test.<sup>17</sup> The Cox proportional hazards model was applied to the multivariate survival analysis.<sup>18</sup> The prognostic variables on overall survival included sex, age, histological types, pathological T factor, pathological N factor, clinical tumour stage, p53, p21, p16, and PCNA. A p value < 0.05 was regarded as significant in two tailed tests. SPSS software (version 10.00, SPSS, Chicago, Illinois, USA) was used for statistical analysis.

## RESULTS

Immunohistochemical analysis of p53, p21, p16, and PCNA protein expression was carried out on 68 primary NSCLC specimens. All of the cell cycle associated proteins examined were present in the nuclei of tumour cells, although a small proportion of cells displayed cytoplasmic immunoreactivity in addition to nuclear staining. Table 2 details the expression of

each protein, and fig 1 shows examples of positive immunostaining.

### Clinicopathological data and cell cycle proteins

The cell cycle checkpoint proteins were analysed with respect to detailed clinicopathological information available for all patients in this cohort. A negative correlation was found between lymph nodes status and p21 (p = 0.015) and p16 expression (p = 0.008), whereas a positive correlation was found between lymph nodes status and PCNA (p = 0.022). No correlations were detected with the other clinical features, such as age, sex, clinical tumour stage, tumour grading, and tumour histology. Finally, as expected, a positive correlation was found between T and lymph nodes status (p < 0.0001). Remarkably, no correlation was found between p16, p21, and p53 differential expression. Table 3 summarises these results.

### Overall survival and immunohistochemical and clinical parameters

We evaluated the prognostic value of the different clinicopathological features of the patients and the

**Table 4** Survival and pathological and immunohistochemical parameters in patients with non-small cell lung cancer in univariate analysis

	Number of patients	Median survival (months)	95% CI	p Value
Clinical staging				
Stage I–II	44	50.83	34.30 to 67.37	< 0.0001
Stage III (A/B)	24	18.00	9.77 to 26.23	
Pathological T factor				
T1–2	55	22.00	14.88 to 29.12	0.5969 (NS)
T3–4	13	24.00	7.56 to 40.44	
Pathological N factor				
N0	26	36.00	22.80 to 49.20	0.0012
N1–3	42	15.00	10.16 to 19.84	
Grading				
G1–2	38	36.00	30.85 to 41.15	0.1874 (NS)
G3	30	13.00	1.51 to 29.49	
p53				
p53 negative	24	33.00	25.29 to 40.71	0.0071
p53 positive	44	18.00	9.57 to 26.43	
p21				
p21 negative	33	17.00	8.00 to 26.00	0.0146
p21 positive	35	36.00	13.57 to 58.43	
p16				
p16 negative	31	9.00	4.73 to 13.27	< 0.0001
p16 positive	37	33.00	20.77 to 45.23	
PCNA				
PCNA (2)	23	21.00	15.70 to 26.30	0.2325 (NS)
PCNA (3)	45	24.00	9.73 to 38.27	

CI, confidence interval; NS, not significant; PCNA, proliferating cell nuclear antigen.

immunohistochemical parameters both by univariate and multivariate analysis.

By univariate analysis, survival seemed to be influenced by p53, p21, and p16. Patients expressing p53 had a worse overall survival than did those negative for p53. In contrast, p21 positive patients showed a better survival than did p21 negative ones. Moreover, patients with positive staining for p16 had a better survival than did p16 negative patients. No correlations were found between overall survival and cell kinetics, as evaluated by PCNA. Among the clinical and pathological parameters, the only two that influenced survival in patients with NSCLC were lymph node status and clinical tumour stage. There was a significant difference in overall survival between patients with lymph node involvement (N1–3) and those without lymph node metastasis (N0). Furthermore, there was also a significant difference in overall survival between patients with stage I–II NSCLC and those with stage III NSCLC. Finally, no differences were found between patients with stage I and stage II NSCLC and between those with stage IIIA and stage IIIB NSCLC. Chemotherapy and radiotherapy showed no clinical impact on overall survival in our patients with NSCLC. However, surgery influenced survival in univariate analysis (median survival of the surgery group, 33 months *v* median survival of the non-surgery group, 17 months;  $p = 0.005$ ). Table 4 and fig 2A–D show the results of the univariate analysis relating to the prognostic value of the various parameters on overall survival in patients with NSCLC.

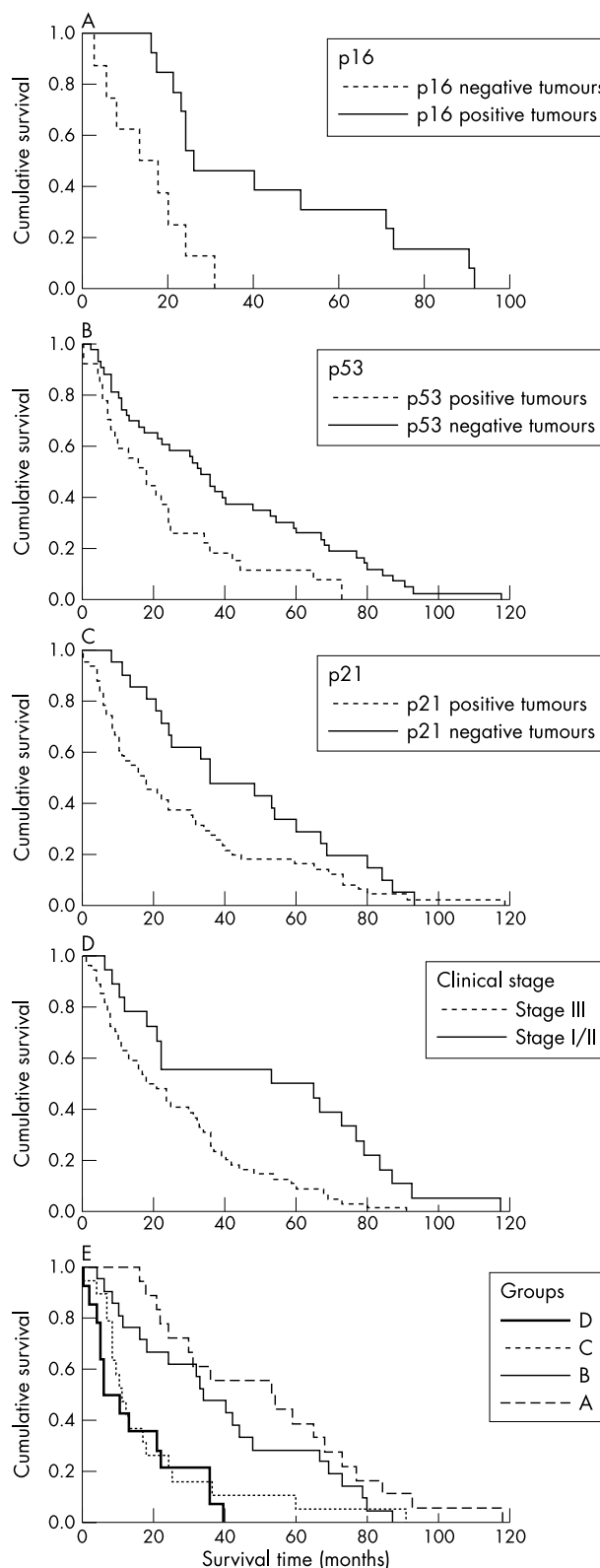
By multivariate analysis, the only clinical parameter that influenced overall survival was tumour staging. When comparing patients with stage I–II NSCLC with patients with stage III NSCLC, the relative risk of death in those with stage III disease was 3.45 (95% confidence interval (CI), 1.43 to 6.78;  $p = 0.001$ ). The only immunohistochemical parameter that influenced overall survival was p16. The calculated relative risk of death in p16 negative patients with NSCLC was 3.149 (95% CI, 1.384 to 7.164;  $p = 0.006$ ). Borderline significance was recorded for p21 and p53. The relative risk of death for patients overexpressing p53 was 1.771 (95% CI, 0.796 to 2.007;  $p = 0.053$ ), whereas for p21 negative patients it was 1.818 (95% CI, 0.912 to 3.407;  $p = 0.060$ ). Table 6 shows the results of the multivariate analysis relating to the prognostic value of the various parameters on overall survival in patients with NSCLC.

Finally, when we grouped the NSCLC cases based on the p21 and p16 scores (group A, both positive; group B, p21 negative and p16 positive; group C, p21 positive and p16 negative; group D, both negative), we found that the group of patients who were both p21 and p16 negative had significantly shorter overall survival. Table 5 and fig 2E show these data.

## DISCUSSION

The ability of a cell to control its own replication is very important for the maintenance of the structure and functions of the organ it belongs to and of the organism as a whole. Several pathologies have been linked to altered control of cellular replication, and cancer is one of the most studied of these. To date, many checkpoint proteins have been examined in lung cancer, but few studies have investigated multiple factors in the same tumours. We have analysed the expression of four key proteins involved in cell cycle checkpoints in a large series of well characterised NSCLCs.

When we looked at the correlation between the clinicopathological data and the expression of cell cycle proteins, we found a negative correlation between lymph nodes status and p21 and p16 expression, suggesting a possible role for these two proteins in the progression of the disease. Interestingly,



**Figure 2** Kaplan-Meier survival curves showing the effects of cell cycle proteins and clinical stages on overall survival of patients with non-small cell lung cancer. (A) Positive expression of p53 was associated with shorter patient survival, (B) positive expression of p21 was correlated with improved outcome, (C) positive expression of p16 was associated with improved outcome, (D) clinical stage III was correlated with shorter patient survival, (E) patients lacking both p21 and p16 expression (group D) had a significantly shorter overall survival (see table 5 for the definition of the patient groups).

**Table 5** Survival according to staining patterns of p21, and p16, in patients with non-small cell lung cancer

Group	Median survival (months)	95% CI	p Value
Group A (16 patients) p21+ and p16+	53.00	15.58 to 90.42	
Group B (21 patients) p21- and p16+	34.00	22.04 to 45.96	
Group C (19 patients) p21+ and p16-	11.00	6.73 to 15.27	
Group D (12 patients) p21- and p16-	6.00	1.58-12.11	<0.0001

CI, confidence interval.

no correlation was found between p16, p21, and p53 expression.

“We found that the group of patients whose lung cancer specimens were negative for both p21 and p16 had significantly shorter overall survival”

When we looked at the correlation between the expression of the different proteins and survival using univariate analysis, we found that all the cell cycle markers analysed, except for PCNA, were significantly correlated with survival. This result is in agreement with numerous studies published about the cell cycle checkpoint proteins investigated here and lung cancer.<sup>3 19-24</sup> As expected, lymph node status and clinical tumour stage were also significantly correlated with survival.

Surprisingly, when we performed multivariate analysis, the only immunohistochemical parameter that influenced overall survival was p16. This result is in agreement with the proposed hypothesis that the RB-p16 tumour suppressor pathway is inactivated in most lung cancer samples.<sup>23</sup> Among the clinical parameters, tumour staging was the only factor to influence survival in multivariate analysis.

Finally, we grouped the lung cancer specimens based on p21 and p16 status. Interestingly, we found that the group of patients whose lung cancer specimens were negative for both p21 and p16 had significantly shorter overall survival. Numerous data from the literature suggest the existence of a functional collaboration between distinct CDK inhibitor genes.<sup>25</sup> Indeed it has recently been demonstrated that cell cycle inhibition by p16 is associated with the post-transcriptional induction of p21 and strong inhibition of cyclin E-CDK2 kinase activity.<sup>26</sup> Moreover, it has been shown that members of the p21 family of proteins promote the

**Table 6** Multivariate Cox regression analysis of overall survival in patients with non-small cell lung cancer

	RR of death	95% CI	p Value
Clinical staging			
Stage I-II	1	-	
Stage III (A/B)	3.450	1.43 to 6.78	0.001
Pathological N factor			
N0	1	-	0.089 (NS)
N1-3	2.015	0.659 to 6.156	
p53			
p53 negative	1	-	0.053 (NS)
p53 positive	1.771	0.796 to 2.007	
p21			
p21 positive	1	-	0.060 (NS)
p21 negative	1.818	0.912 to 3.407	
p16			
p16 positive	1	-	0.006 (NS)
p16 negative	3.149	1.384 to 7.164	

CI, confidence interval; NS, not significant; RR, relative risk.

## Take home messages

- In univariate analysis, the expression of p53, p21, and p16 in patients with non-small cell lung cancer (NSCLC) was significantly correlated with survival
- In multivariate analysis, only p16 influenced overall survival and those patients who were negative for both p21 and p16 had a significantly shorter overall survival.
- Thus, loss of control of cell cycle checkpoints is common in lung cancer, and functional cooperation between different cell cycle inhibitor proteins may be another level of regulation in cell growth control and tumour suppression

association of D-type cyclins with CDKs by counteracting the effects of p16 molecules.<sup>27</sup> Therefore, it has been proposed that functional cooperation between different cell cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumour suppression.<sup>25 28</sup>

Taking into account the complicated functional network constituted by the cell cycle regulator proteins, it is evident that knowledge of the level of expression of these factors, and their coregulators, may be important in predicting patient clinical response to treatment. Targeting multiple checkpoint proteins may represent a good therapeutic strategy for the development of new molecular treatments for lung cancer. Our data support this hypothesis and the need for further work aimed at investigating the simultaneous expression of numerous cell cycle regulators in NSCLC.

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## Authors' affiliations

**V Esposito, V Montesarchio**, Third Division of Infective Diseases, D. Cotugno Hospital, Naples 80100, Italy

**A Baldi, F Baldi**, Department of Biochemistry and Biophysics "F. Cedrangolo", Section of Anatomic Pathology, Second University of Naples, Naples 80100, Italy

**G Tonini, B Vincenzi, P Persichetti**, Section of Oncology, Campus BioMedico University, Rome 00100, Italy

**M Santini**, Department of Thoracic Surgery, Second University of Naples

**V Ambrogio, T C Mineo**, Department of Thoracic Surgery, Tor Vergata University, Rome 00100, Italy

**G Liuzzi**, A.O. "L. Spallanzani", Rome 00100, Italy

**E Wolner, A M Groeger**, Department of Cardio-Thoracic Surgery, University of Vienna, Vienna 1008, Austria

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