

## ORIGINAL ARTICLE

## Cell cycle related proteins as prognostic parameters in radically resected non-small cell lung cancer

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**Background:** Experimental evidence suggests that lung cancer development and progression can be linked to an increased proliferation rate.**Aims/Methods:** To evaluate the immunohistochemical expression of seven components of the cell cycle machinery in a series of well characterised non-small cell lung cancer (NSCLC) specimens (n = 105).**Results:** Multivariate analysis revealed that simultaneous loss of expression of three of these factors—cyclin D1, the cyclin dependent kinase inhibitor p16, and the tumour suppressor retinoblastoma protein Rb2/p130—correlated with survival, confirming the hypothesis that the cyclin D1–p16–retinoblastoma tumour suppressor pathway is inactivated in most lung cancer samples.**Conclusions:** These results suggest that loss of control of cell cycle checkpoints is a common occurrence in lung cancer and support the idea that functional cooperation between different cell cycle regulatory proteins constitutes another level of regulation in cell growth control and tumour suppression.

In most industrialised countries, lung cancer is the main cause of cancer death and has a poor prognosis.<sup>1</sup> Non-small cell lung carcinoma (NSCLC) accounts for approximately 75% of cases and represents a heterogeneous group of cancers consisting mainly of squamous cell carcinoma, adenocarcinoma, and large cell cancer. The molecular alterations in lung cancer have been studied extensively.<sup>2</sup> They consist mainly of inactivating mutations of tumour suppressor genes, activating mutations of oncogenes, loss of heterozygosity (deletion of one of two copies of allelic DNA sequences in particular chromosomal regions), and amplification of chromosomal regions. Recent evidence suggests that not only genetic changes but also alterations in epigenetic regulation are crucially important during tumorigenesis. Studies on the functions of cellular protooncogenes and tumour suppressor genes indicate that most of these genes encode proteins involved in signal transduction pathways and cell cycle control, which play crucial roles in cell proliferation and differentiation. One of the most important checkpoints involved in oncogenesis is the restriction point in the late G1 stage. Defects in G1 regulatory proteins, particularly deregulation of the p53–p21WAF1,<sup>3–4</sup> retinoblastoma protein (pRb)–cyclin D,<sup>5</sup> and cyclin E–p16 pathways,<sup>6</sup> seem to be essential for the development of lung cancer.

“To improve prognostication, clinical markers that might predict prognosis and response to specific treatments should be established”

Despite major advances in cancer treatment in the past two decades, the prognosis of patients with lung cancer has improved only minimally, with overall survival rates between 10% and 15%.<sup>7</sup> Although TNM stage is the most important prognostic parameter to be considered, the variability of survival within staging groups requires additional parameters that influence outcome, independent of stage factors.<sup>8–10</sup> To improve prognostication, clinical markers that might predict prognosis and response to specific treatments should be established. Although many biological markers, including p53 abnormality, have been investigated, no biological

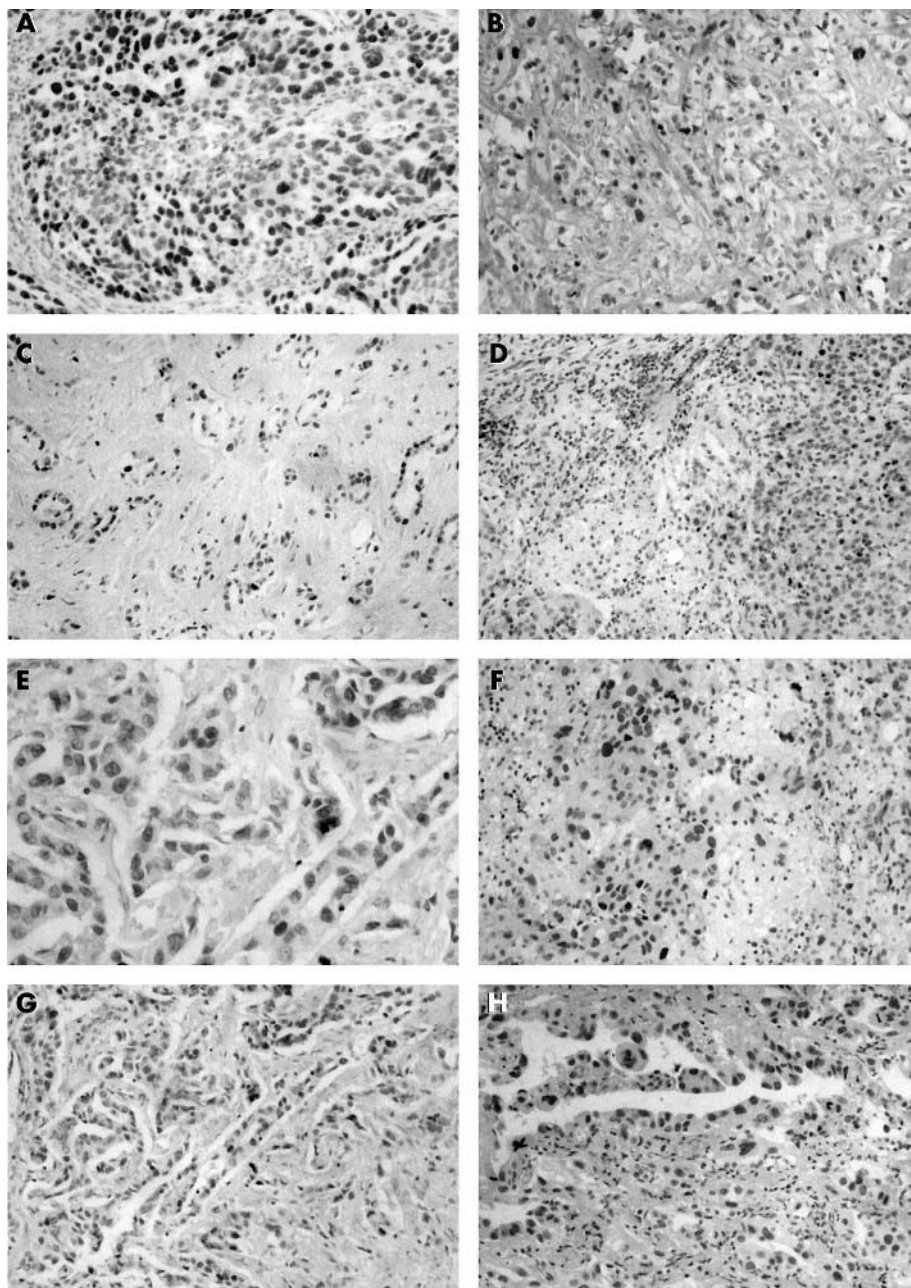
marker has been established as a clinical marker in the treatment of patients with NSCLC.<sup>11</sup>

The aims of our present study were to determine the prognostic role of proliferating cell nuclear antigen (PCNA),

**Table 1** Patients' characteristics

Characteristic	Number
Total number	105
Median age (range)	63 (45–83) years
Sex	
Male/female	78/27 (74.3%/25.7%)
Neoplasm histotype	
Squamous cell carcinoma	46 (43.8%)
Adenocarcinoma	42 (40.0%)
Others	17 (16.2%)
T stage	
T1	22 (20.9%)
T2	59 (56.2%)
T3	19 (18.1%)
T4	5 (4.8%)
N stage	
N0	43 (41.0%)
N1	40 (38.1%)
N2	22 (20.9%)
Clinical stage	
I	45 (42.9%)
II	33 (21.4%)
III (A and B)	27 (25.7%)
Grade	
1–2	45 (42.8%)
3	60 (57.2%)
Postoperative radiotherapy	
Yes	25 (23.8%)
No	80 (76.2%)
Postoperative chemotherapy	
Yes	31 (29.5%)
No	74 (70.5%)

**Abbreviations:** CI, confidence interval; NSCLC, non-small cell lung cancer; PCNA, proliferating nuclear cell antigen; pRb, retinoblastoma protein



**Figure 1** Immunohistochemical staining of cell cycle proteins in non-small cell lung cancer specimens: (A) p53, (B) p53, (C) p27, (D) p16, (E) proliferating cell nuclear antigen, (F) cyclin D1, (G) retinoblastoma protein (pRb)/p105, and (H) pRb2/p130.

p53, p27, pRb/p105, pRb2/p130, cyclin D1, and p16 expression in a well defined set of patients who had undergone radical surgical treatment for NSCLC and had longterm follow up. Moreover, we explored the association between molecular markers and the pathological and clinical characteristics of this population. The availability of the expression status of all tumour markers in the same set of patients provided a unique opportunity to determine whether alterations in p53, p27, pRb/p105, pRb2/p130, cyclin D1, and p16 expression have a cooperative or synergistic effect on lung cancer progression, metastasis, and survival.

## MATERIALS AND METHODS

### Population study

Archival, formalin fixed, paraffin wax embedded lung cancer specimens (n = 105) were obtained from patients who

underwent surgical resection for lung cancer (lobectomy or pneumonectomy) in the department of cardio-thoracic surgery of the University Hospital of Vienna (Vienna, Austria) between 1998 and 2002. Patients received no chemotherapy or radiotherapy before surgical resection. Outcome data were collected from the “Central Institute of Statistics of Austria”, hospital charts, and periodic interviews with patients and their families. Approval to use these cases for scientific purposes was obtained from the medical ethics committee. The histological diagnosis and tumour classification were based on the World Health Organisation criteria. The postoperative pathological TNM stage was determined according to the guidelines of the American Joint Committee on Cancer.<sup>12</sup> Median follow up time was 25 months (range, 1–56). Eighty five of 105 patients considered in this evaluation died (80.1%). Table 1 summarises the main characteristics of the patients.

**Table 2** Immunohistochemical parameters in patients with non-small cell lung cancer

Marker	Number (%)
p53	
Negative	73 (69.5%)
Positive	32 (30.5%)
p16	
Negative	44 (41.9%)
Positive	61 (58.1%)
PCNA	
Negative	60 (57.1%)
Positive	45 (42.9%)
p27	
Negative	61 (58.1%)
Positive	44 (41.9%)
Cyclin D1	
Negative	42 (40.0%)
Positive	63 (60.0%)
Rb/p105	
Negative	59 (56.2%)
Positive	46 (43.8%)
Rb2/p130	
Negative	50 (47.6%)
Positive	55 (52.4%)

PCNA, proliferating cell nuclear antigen; pRb, retinoblastoma protein.

### 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 and rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) specific for p16 (sc-1661), p27 (sc-1641), PCNA (sc-56), pRb/p105 (sc-102), and pRb2/p130 (sc-317) were used at a 1/100 dilution, whereas the 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. Moreover, the expression of some of the markers analysed in the normal lung tissue close to the tumour tissue, when available, was used as an internal control. All of the samples were processed under the same conditions. Three pathologists (BV, FB, and AB) independently evaluated the staining patterns, and each scored the specimens for the percentage of positive nuclei by scanning the entire section. The level of concordance, expressed as the percentage of agreement between the observers, was 90.5% (95 of 105 specimens). In the remaining specimens, the score was obtained after collegial revision and agreement. All immunoreactive nuclei were regarded as positive, irrespective of the intensity of staining. The pathologists were blinded to clinical outcome at the time of evaluation.

### Statistical analysis

The prognostic variables considered for statistical analysis of overall survival included: sex, age, histological type, pathological T stage, pathological N stage, clinical tumour stage, and p53, p27, p16, PCNA, cyclin D1, pRb/p105, and pRb2/p130 expression. For purposes of survival analysis, tumour

**Table 3** Survival and pathological and immunohistochemical parameters in patients with non-small cell lung cancer (univariate analysis)

	Median survival (months)	95% CI	p Value
Tumour stage			
I–II	39.83	24.30 to 50.37	0.003
III (A and B)	21.00	19.77 to 30.23	
T stage			
T1–2	27.00	22.76 to 31.24	0.6480
T3–4	26.00	18.93 to 33.07	0.6480
N stage			
N0	26.00	20.40 to 31.60	0.0122
N1–3	32.00	28.86 to 42.44	
Grade			
G1–2	25.00	16.99 to 33.01	0.8177
G3	27.00	23.09 to 30.91	
p53			
Negative	34.00	25.00 to 43.00	0.040
Positive	21.00	15.37 to 26.63	
p27			
Negative	25.00	20.98 to 29.02	0.0279
Positive	40.00	29.14 to 50.86	
Cyclin D1			
Negative	46.00	39.79 to 52.21	<0.0001
Positive	19.00	16.44 to 21.56	
p16			
Negative	14.00	10.03 to 17.97	<0.0001
Positive	39.00	30.29 to 47.71	
PCNA			
Negative	26.00	18.41 to 33.59	0.1164
Positive	30.00	19.99 to 40.01	
pRb/p105			
Negative	29.00	21.60 to 36.40	0.2318
Positive	25.00	16.35 to 33.65	
pRb2/p130			
Negative	17.00	12.42 to 21.58	<0.0001
Positive	38.00	28.47 to 47.53	

CI, confidence interval; PCNA, proliferating cell nuclear antigen; pRb, retinoblastoma protein.

pathological stage (pT1–2 v pT3–4), grade (grades 1 and 2 v grade 3), lymph node status (N0 v N1 and N2), and tumour stage (stage I–II v stage IIIA/B) were evaluated as dichotomised variables. Moreover, to carry out analysis, a dichotomised scoring system was used for the molecular markers, as follows: p53, cyclin D1, p27, pRb/p105, pRb2/p130, and p16 expression in more than 5% of tumour cells was defined as positive expression,<sup>4 13–15</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>16 17</sup>

A univariate survival analysis for each prognostic variable on overall survival was estimated according to the Kaplan–Meier method.<sup>18</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>19</sup> The Cox proportional hazards model was applied to the multivariate survival analysis.<sup>20</sup> Relations between ordinal data and continuous variables (before dichotomisation) were assessed by means of Fischer's exact test or Spearman's test. A p value < 0.05 was regarded as significant in two tailed tests. SPSS software (version 11.00; SPSS, Chicago, Illinois, USA) was used for statistical analysis.

### RESULTS

Immunohistochemical analysis of p53, p27, p16, PCNA, cyclin D1, pRb/p105, and pRb2/p130 was carried out on 105 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. Figure 1

**Table 4** Multivariate Cox regression analysis of overall survival in patients with non-small cell lung cancer

	RR	95% CI	p Value
Clinical stage			
III (A and B)	1	–	0.130
I-II	0.567	0.272 to 1.182	
N stage			
N0	1	–	0.066
N1-3	2.165	0.951 to 4.928	
p53			
Negative	1	–	0.645
Positive	1.062	0.822 to 1.371	
p27			
Negative	1	–	0.427
Positive	0.901	0.684 to 1.1.65	
pRb2/p130			
Negative	1	–	0.0001
Positive	0.525	0.395 to 0.697	
p16			
Positive	1	–	0.005
Negative	0.635	0.462 to 0.872	
Cyclin D1			
Negative	1	–	0.001
Positive	1.749	1.251 to 2.447	

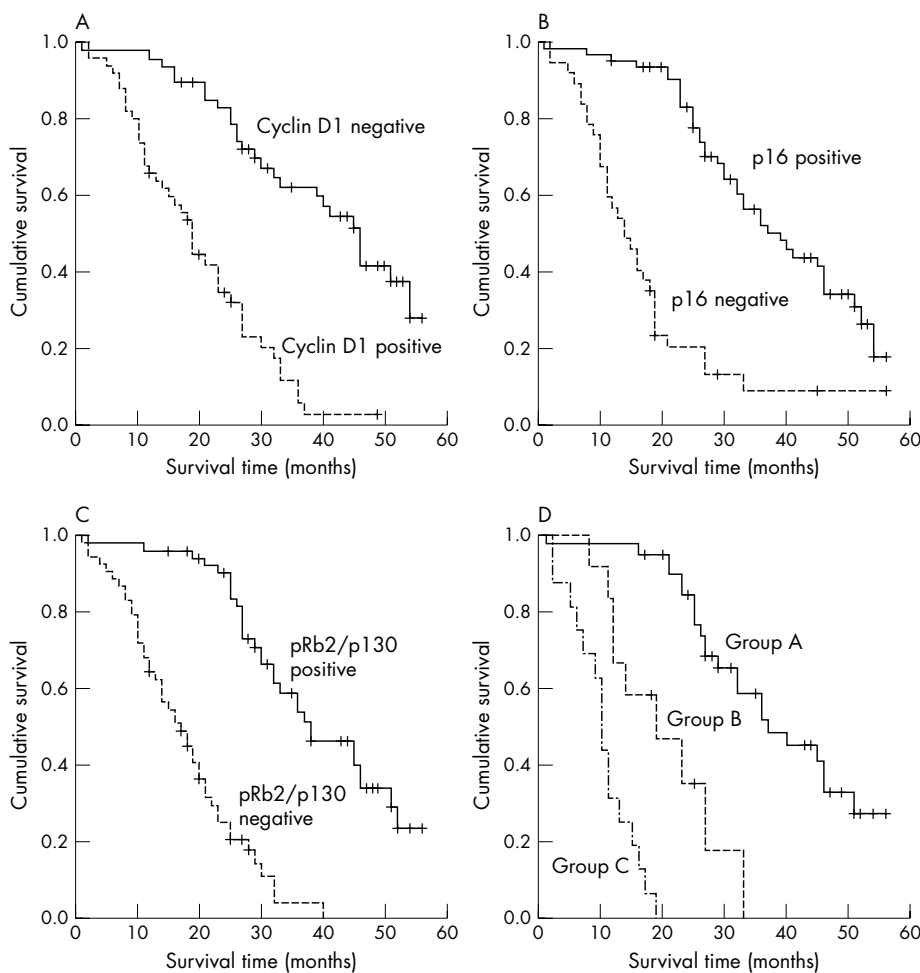
CI, confidence interval; pRb, retinoblastoma protein; RR, relative risk of death.

shows examples of immunostaining for all the markers analysed. Table 2 summarises the immunohistochemical scores.

**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. In a univariate analysis, as expected, tumour stage ( $p = 0.003$ ) and lymph node status ( $p = 0.0122$ ) correlated with survival. Among all the cell cycle proteins, only positive expression of p53 ( $p = 0.040$ ) and negative expression of p27 ( $p = 0.0279$ ), cyclin D1 ( $p = < 0.0001$ ), p16 ( $p = < 0.0001$ ), and pRb2/p130 ( $p = < 0.0001$ ) significantly correlated with survival in this cohort of patients. Finally, no correlations were found between overall survival and the patients' clinical features (age, sex, radiotherapy, or chemotherapy; data not shown). Table 3 and fig 2A–C summarise these results.

By multivariate analysis, the only immunohistochemical parameters that influenced overall survival were p16 (95% confidence interval (CI), 0.462 to 0.872;  $p = 0.005$ ), cyclin D1 (95% CI, 1.251 to 2.447;  $p = 0.001$ ), and pRb2/p130 (95% CI, 0.395 to 0.697;  $p = 0.0001$ ). Interestingly, none of the clinical parameters correlated with survival. Table 4 provides



**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) Negative expression of cyclin D1 was associated with shorter patient survival; (B) positive expression of p16 correlated with improved outcome; (C) positive expression of retinoblastoma protein 2 (pRb2)/p130 was associated with improved outcome; (D) patients with negative expression of pRb2/p130, cyclin D1, and p16 (group C) had a significant shorter overall survival (see table 5 for the definition of the patient groups).

**Table 5** Immunohistochemical staining patterns of p21, p16, and p53 in patients with non-small cell lung cancer

Cyclin D1	pRb2/p130	p16	No. of patients	Group
–	+	+	24	A (59)
–	–	+	22	
–	+	–	4	
+	+	–	9	B (25)
+	+	–	18	
+	–	+	6	
–	–	–	1	C (21)
+	–	–	21	

Group A, no or only one adverse prognostic factor; group B, two adverse prognostic factors; group C, three adverse prognostic factors.

details of the multivariate analysis of the prognostic value of the various parameters on overall survival in patients with NSCLC.

Finally, when we grouped the NSCLC cases based on the cyclin D1, pRb2/p130, and p16 scores (group A, no or only one adverse prognostic factor; group B, two adverse prognostic factors; group C, three adverse prognostic factors), we found that the group of patients with NSCLC who had all three adverse prognostic factors had a significantly shorter overall survival. Tables 5 and 6 and fig 2D depict these results.

## DISCUSSION

Regulation of cell viability is an important mechanism for controlling tissue differentiation and the development of the organism. This mechanism is also thought to be involved in the development of human cancer. Cancer is now viewed not only as being the consequence of uncontrolled proliferation, but also the result of an altered balance between cell proliferation and cell death. We evaluated the immunohistochemical expression of several cell cycle regulators in a group of 105 patients with lung cancer who had undergone surgical resection to clarify the crucial events in lung cancer pathogenesis, and to evaluate the impact of these events on patient outcomes. We chose immunohistochemistry for our investigation because it avoids the complication of contamination by non-neoplastic cells that constantly affects both western blot analyses and nucleic acid based approaches. In addition, immunohistochemistry on formalin fixed, paraffin wax embedded sections is a relatively easy method that is suitable for routinely processed samples.

Using univariate analysis, we found that all the cell cycle markers analysed, except for PCNA and pRb/p105, significantly correlated with survival, in agreement with several previous reports.<sup>21–29</sup> As expected, lymph node status and clinical tumour stage significantly correlated with survival also.

Surprisingly, when we performed multivariate analysis, the only immunohistochemical parameters that influenced overall survival were p16, cyclin D1, and pRb2/p130. This result

**Table 6** Survival according to staining patterns of p21, p16, and p53 in patients with non-small cell lung cancer

Group	Median survival (months)	95% CI	p Value
A	41.00	27.09 to 49.91	<0.0001
B	19.00	7.32 to 30.68	
C	10.00	8.70 to 11.30	

Group A, no or only one adverse prognostic factor; group B, two adverse prognostic factors; group C, three adverse prognostic factors. CI, confidence interval.

## Take home messages

- In non-small cell lung carcinoma, simultaneous loss of expression of cyclin D1, the cyclin dependent kinase inhibitor p16, and the tumour suppressor retinoblastoma protein 2/p130 correlated with survival, confirming the hypothesis that the cyclin D1–p16–retinoblastoma tumour suppressor pathway is inactivated in most lung cancer samples
- Thus, loss of control of cell cycle checkpoints is probably a common occurrence in lung cancer and functional cooperation between different cell cycle regulatory proteins probably constitutes another level of regulation in cell growth control and tumour suppression

agrees with the proposed hypothesis that the p16–cyclin D1–pRb tumour suppressor pathway is inactivated in most lung cancer samples.<sup>30</sup> We demonstrated aberrant p53 expression in a small number of specimens, which did not correlate with patient survival in multivariate analysis. This finding disagrees with a previous study from our group reporting on 61 NSCLCs, and does not help clarify the still debated prognostic role of p53 in patients with lung cancer.<sup>31</sup> A recent study that aimed to review the association between p53 alterations and patient outcome by a meta-analysis of data from published papers showed that p53 mutation is a significant marker of poor prognosis in patients with lung adenocarcinoma.<sup>32</sup> Nevertheless, more studies with larger numbers of patients will be needed to settle this issue definitively, and to assess the real impact on cancer treatment of this information.

“Our results confirm a role for cell cycle regulatory proteins in the pathogenesis of lung cancer, and highlight the possible use of some of these proteins as prognostic factors in this disease”

Finally, we grouped the lung cancer specimens based on cyclin D1, pRb2/p130, and p16 status. Interestingly, we found that the group of patients with three adverse prognostic factors had a significantly shorter overall survival. Numerous data from the literature suggest the existence of a functional collaboration between distinct cyclin dependent kinase inhibitor genes.<sup>33</sup> Therefore, it has been proposed that functional cooperation between different cell cycle regulator proteins constitutes another level of regulation in cell growth control and tumour suppression.<sup>34</sup>

In conclusion, our results confirm a role for cell cycle regulatory proteins in the pathogenesis of lung cancer, and highlight the possible use of some of these proteins as prognostic factors in this disease. However, additional studies are required to define the prognostic impact of cell cycle regulators in lung cancer. Nevertheless, our data support the hypothesis that targeting multiple checkpoint proteins may represent a good therapeutic strategy for the development of new molecular treatments for lung cancer.

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