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


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.

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.1,2 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.1 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.3 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.4,5 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.6 In contrast, proliferating cell nuclear antigen (PCNA) is involved in activation of DNA polymerase δ, which is required for DNA replication and repair.7,8 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.9,10

“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.11 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...
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, 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. Fischer’s exact test was used to assess relations between ordinal data (correlation matrix between immunostaining parameters). A univariate survival
analysis for each prognostic variable on overall survival was estimated according to the Kaplan–Meier method. 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. The Cox proportional hazards model was applied to the multivariate survival 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 (p = 0.022), 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
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; 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 clinico-pathological data and the expression of cell cycle proteins, we found a negative correlation between lymph node status and p21 and p16 expression, suggesting a possible role for these two proteins in the progression of the disease. Interestingly,
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. 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. 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. 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. Moreover, it has been shown that members of the p21 family of proteins promote the association of D-type cyclins with CDKs by counteracting the effects of p16 molecules. Therefore, it has been proposed that functional cooperation between different cell cyclin inhibitor proteins constitutes another level of regulation in cell growth control and tumour suppression.

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.

ACKNOWLEDGEMENTS

This work was funded by grants from: International Society for the Study of Comparative Oncology, Inc (ISSCO, President HE Kaiser) Silver Spring, MD, USA; FUTURA-Onlus; Ministero della Salute; MIUR; and Second University of Naples.

Authors’ affiliations
V Esposito, V Montesarchio, Third Division of Infective Diseases, D. Catogno Hospital, Naples 80100, Italy
A Baldi, F Baldi, Department of Biochemistry and Biophysical 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 Ambrogi, T C Mineo, Department of Thoracic Surgery, Tor Vergata University, Rome 00100, Italy
O Livazzi, A.O. ‘‘L. Spallanzani” Rome 00100, Italy
E Wolner, A M Groegeer, Department of Cardio-Thoracic Surgery, University of Vienna, Vienna 1008, Austria

REFERENCES
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 and A M Groeger

doi: 10.1136/jcp.57.1.58

Updated information and services can be found at:
http://jcp.bmj.com/content/57/1/58

These include:

References
This article cites 28 articles, 8 of which you can access for free at:
http://jcp.bmj.com/content/57/1/58#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
- Lung cancer (oncology) (120)
- Lung cancer (respiratory medicine) (120)
- Breast cancer (506)
- Immunology (including allergy) (1664)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/