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Prognostic significance of p53 and bcl-2 abnormalities in operable nonsmall cell lung cancer

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Prognostic significance of p53 and bcl-2 abnormalities in operable nonsmall cell lung cancer. J. Laudanski, W. Niklinska, T. Burzykowski, L. Chyczewski, J. Niklinski. ©ERS Journals Ltd 2001.

ABSTRACT: The association of p53 abnormalities and bcl-2 protein expression with clinical data and prognosis in 102 patients with resected nonsmall cell lung cancer (NSCLC) was investigated.

Deoxyribonucleic acid analysis of exons 5–8 of the p53 gene showed mutations (p53-M) in 47% of resected NSCLC, serum p53 antibodies (p53-Abs) were detected in 25%, p53 protein overexpression (p53-PE) in 54% and bcl-2 protein overexpression (bcl-2-PE) in 48%. A statistically significant association was found between p53-PE, serum p53-Abs and the presence of a p53 gene alteration. No significant associations were found between results of the p53-M, p53-Abs, bcl-2-PE tests and clinicopathological parameters. In the case of the p53-PE test there were significantly fewer positive results for adenocarcinoma than for squamous cell carcinoma and large cell carcinoma.

Survival analysis showed that both p53 abnormalities and negative staining for bcl-2, when analysed separately, were associated with poor overall survival. In a multivariate analysis, only the positive result of the p53-M test remained an independent, statistically significant, unfavourable prognostic factor for survival. When the p53 mutation test was removed from the model, positive results of the p53-PE test and the p53-Abs test became statistically significant, unfavourable prognostic factors.

To conclude, among p53 and bcl-2 abnormalities, only p53 gene mutations seem to have a strong and independent effect on prognosis. When deoxyribonucleic acid sequence information is not available, p53 protein expression and the presence of p53 antibodies in serum may be used to obtain important prognostic information.
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Despite major advances in cancer treatment in the past two decades, the prognosis of patients with lung cancer has improved only minimally. Although primary tumour, regional nodes, metastasis (TNM) stage is the most significant prognostic factor, the variation in survival within staging groups requires information about additional factors influencing the outcome, independent of stage [1–3].

Advances in molecular biology have provided clues to the pathogenesis of cancer and have shown the involvement of oncogene activation and tumour-suppressor gene inactivation. Recent evidence suggests that the genetic regulation of apoptosis is also of critical importance during tumourigenesis and that oncogenes and tumour suppressor genes can regulate the apoptotic rate, or the susceptibility of cells to undergo apoptosis [4, 5]. Among several genetic aberrations that have been implicated in lung cancer, mutations in the p53 gene are the most common [6–8]. Mutations in the p53 gene usually result in increased steady-state levels of p53, which may play a role in carcinogenesis through trans-dominant mechanisms, perhaps involving oligomerization between mutant and wild-type proteins. While the importance of p53 mutations (p53-M) in the pathogenesis of lung cancer is clear, it is still not clear whether the presence or absence of p53-M or overexpression of p53 protein adversely affects an individual patient's chances for survival. Recent studies have demonstrated the appearance of p53 antibodies in serum of patients with lung cancer, probably due to the accumulation of mutant p53 protein in tumour cells [9]. Although most patients with serum p53 antibodies (p53-Abs) harbour a p53-M or p53 protein overexpression, the opposite is not true, and only 30% of patients with p53 alterations developed a humoral response to p53. Insight into the importance of deregulated apoptotic cell death during carcinogenesis has been provided by studies of the bcl-2 proto-oncogene. It has also been suggested that abnormal expression of the bcl-2 protein may have relevance to the clinical behaviour of many types of tumours [10]. In lung cancer, bcl-2 protein expression (bcl-2-PE) may be a feature of less aggressiveness and may result in a favourable prognosis [11, 12].

In this study, p53 gene mutation, p53 protein expression, p53-Abs and bcl-2-PE were simultaneously evaluated and the findings correlated with the clinicopathological features and prognosis of patients with surgically treated nonsmall cell lung cancer (NSCLC).

Material and methods

Patient characteristics

The study included 102 NSCLC patients examined by the Chest Oncology Group and operated in the Thoracic Surgery Unit at the Bialystok Medical School. All the patients underwent surgical resection.

Pretreatment staging procedures included physical and blood examinations, chest radiographs and tomographs, bronchoscopy, computed tomography (CT) of the thorax and ultrasound scanning of the liver. In addition, radioisotope scans of bones, examination of bone marrow aspirates, and abdominal and brain CT were performed when necessary. Selected patients underwent mediastinoscopy. During operation, radical lymph node dissection was performed uniformly. Nodes were identified and submitted separately at all levels. Pathological material has been specially reviewed for this study by the same pathologist. Postoperative, pathological staging pTNM was performed by correlating the operative and histological findings [13].

p53 status

p53 mutation

Deoxyribonucleic acid (DNA) was extracted from formalin-fixed paraffin-embedded tissue, as described previously [14]. Exons 5, 6, 7, and 8 of the p53 genes were amplified by polymerase chain reaction (PCR). The reaction mixtures contained one-tenth of the DNA extract as a template, 10 mM Tris (pH 8.3), 50 mM KCl, 0.125 mM deoxyribonucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.15–0.2 μM of each upstream and downstream primer, and 1.25 U of Taq polymerase in a volume of 50 μL.

For sequencing, 20 μL of PCR product was purified by size filtration with Microcon 50 (Amicon Inc., Beverly MA, USA). DNA sequencing was performed by the dideoxy termination method with (α-^{35}S) deoxyadenosine triphosphate (dATP) incorporation using the CircumVent DNA sequencing kit (New England Biolab, Beverly, MA, USA). Sequences were then resolved with a 6% polyacrylamide/8M urea gel and autoradiography was carried out for 3–5 days of radiograph film exposure.

p53 protein expression

Five-micron-thick sections were tested for p53 protein by immunohistochemistry. For staining enhancement, slides were pre-treated in a microwave oven (900W, two cycles, 1×1 min and 1×7 min) and then stained with a monoclonal mouse antihuman p53 antibody (DO-7; Dako, Denmark) at a 1:100 dilution. The antigen-antibody complex was visualized using biotin-streptavidin-peroxidase staining technique (Dako LSAB + Kit, Peroxidase) and 3-amino-9-ethylcarbazole; (AEC; Sigma, St Louis, MO, USA) as chromogen. The sections were then counterstained with Meyer's haematoxylin. A lung carcinoma with high p53 protein expression was used as positive control; for a positive external control, an oesophageal carcinoma known to express p53 was used. A negative control was obtained by omission of the primary antibody. All slides were reviewed independently by two investigators. p53 expression (nuclear staining) was evaluated by counting 1,000 cells·section^{-1} in five randomly chosen high-power fields (400 ×) of tumour, and percentages of positive cells were determined by light microscopy. The threshold values for p53 immunostaining were chosen at 20%. Tumours with $>20\%$ of tumour cells positive, were included as positive, and p53 staining was scored in two categories: negative (0) and positive (1) where $0 = \leqslant 20\%$ and $1 = > 20\%$.

Anti-p53 antibody in sera

Serum samples were obtained from patients at the time of diagnosis and stored at -80°C until use. An anti-p53 autoantibody sandwich enzyme-linked immunosorbent assay (ELISA) (Dianova GmbH, Hamburg, Germany) with solid-phase recombinant p53 protein was purchased from Oncogene Research Products (Cambridge, USA) and the analyses of sera performed according to the manufacturer's recommendations. Mean absorbance in the wells coated with p53 and in those coated with control protein was calculated for each serum sample. The anti-p53 index was calculated using the formula according to the manufacturer's recommendation: (E450 (serum sample)-E450 (anti-p53 low control))/(E450 (anti-p53 high control)-E450 (anti-p53 low control)). A ratio of >1.2 from two independent assays was considered as a critical cut-off [15].

bcl-2 protein expression

Sections for immunohistochemical examinations were fixed in acetone for 24 h and embedded in paraffin blocks. Five-micron-thick sections were tested for bcl-2 protein by immunohistochemistry. For staining enhancement, slides were pretreated in a microwave oven and then stained with a monoclonal mouse antihuman bcl-2 antibody (Clone 124; Dako) at a 1:100 dilution.

The antigen-antibody complex was visualized using biotin-streptavidin-peroxidase staining technique (Dako LSAB + Kit, Peroxidase) and AEC (Sigma) as chromogen. The sections were then counterstained with Meyer's haematoxylin. A lung carcinoma with high bcl-2-PE was used as positive control; for a positive external control, oesophageal, laryngeal or endometrial carcinomas known to express bcl-2 were used. Additionally, infiltrating lymphocytes were used as an internal positive control in every section of lung tumour. A negative control was obtained by omission of the primary antibody. All slides were reviewed independently by two investigators. bc1-2 expression (cytoplasmic or perinuclear staining) was evaluated by counting 1,000 cells·section^{-1} in five randomly chosen high-power fields (400 ×) of tumour, and percentages of positive cells were determined by light microscopy. Tumours with >20% of tumour cells positive were

included as positive, and bcl-2 staining was scored in two categories: negative (0) and positive (1), where $0 = \leqslant 20\%$ and $1 = > 20\%$.

Statistical analysis

Comparisons based on contingency tables were performed using Pearson's Chi-squared test or Cochran-Armitage's test for trend [16]. Multivariate analysis of the probability of obtaining a positive result of the p53-M test was performed using logistic regression [16]. Significance of covariates included into the model was checked using the likelihood ratio test. The fit of the model to the data was assessed using the likelihood ratio statistic and standardized Pearson's residuals [16].

For purposes of the analysis of survival time, August 31, 1997, was taken as the end of follow-up. The survival status of all but six patients was confirmed on that day. Survival time was calculated from the date of surgery to the date of death or the end of follow-up. Patients alive at the end of the follow-up were regarded censored observations. Six patients lost to follow-up before August 31, 1997, were treated as censored observations at the date of their last observation.

Survival curves were estimated using the Kaplan-Meier method. In the univariate analysis of survival time, the logrank test (unstratified and stratified) and the logrank test for trend [17] were used. In the multivariate analysis, Cox's proportional hazard model was used. Comparisons based on the model were performed using the score test. The fit of the model was checked graphically using Martingale residuals [18]. Proportionality of hazard functions was checked using the test for time-dependent covariates and plots of cumulative hazard function [19].

All the applied tests were two-sided. The analysis was performed using BMDP PC90 (programs 4F, 1L and 2L) and STATA (v.6) software.

Results

Table 1 presents the distribution of p53 abnormalities and bcl-2-PE under consideration for sex and histological type and TNM stage of the tumour. For each of the three clinical factors, proportions of positive results of each of the molecular tests were compared using Chi-squared test at 0.01 level of significance (adjusted for multiple comparisons). Only in the case of the p53-PE test was a statistically significant difference between the proportions for different histological types found (p=0.002). There were significantly less positive results for adenocarcinoma (AdC; 28.1%) than for squamous cell carcinoma (SqCC) or large cell carcinoma (LCC; 66.7% and 62.5%, respectively).

To investigate the association between the p53-M test and the other three tests (p53-PE, p53-Abs, bcl-2-PE), a logistic regression model was used to analyse the probability of obtaining a positive result of the p53-M test. The model initially included the following covariates: sex, histological type of tumour, TNM stage of tumour, and results of the p53-PE, p53-Abs and bcl-2-PE tests. Subsequently, the covariates with coefficients nonsignificant at 0.05 level were removed from the model. The final model included only the positive results of the p53-PE and p53-Abs tests as covariates. It indicated that, independently of each other, positive results of the p53-PE and p53-Abs tests significantly (p<0.001 and p=0.001, respectively) increased the odds of obtaining a positive result of the p53-M test. For the p53-PE test, the increase (odds ratio) was equal to 18.50 (95% confidence interval (CI) (5.71, 59.94)), while for the p53-Abs test it was equal to 11.87 (95% CI (2.90, 48.55)).

The above analysis indicates a strong, positive association between results of the p53-M test and results of the p53-PE and p53-Abs tests. Sequencing gel and immunohistochemistry staining of a tumour with concordant findings are shown in figure 1.

Analysis of survival

All 102 patients were included in the analysis of overall survival. Two of them died within one month of the operation. It was checked that the results did not depend on whether the two patients were excluded or included in the analysis. Among the remaining 100 patients, the length of follow-up ranged 6.9–42.7

Table 1. – Proportions of positive results for the p53 mutation (p53-M), p53 protein expression (p53-PE), anti-p53 antibody (p53-Abs) and bcl-2 protein expression (bcl-2-PE) tests by sex, histological type and primary tumour, regional nodes, metastasis (TNM) stage

	p53-M test	p-value	p53-PE test	p-value	p53-Abs test	p-value	bcl-2-PE test	p-value
Sex		0.87		0.92		0.87		0.82
F	4/9 (44.4)		5/9 (55.6)		2/9 (22.2)		4/9 (44.4)	
M	44/93 (47.3)		50/93 (53.8)		23/93 (24.7)		45/93 (48.4)	
Histological type		0.21		0.002		0.26		0.72
SqCC	29/54 (53.7)		36/54 (66.7)		10/54 (18.5)		28/54 (51.8)	
AdC	11/32 (34.4)		9/32 (28.1)		9/32 (28.1)		14/32 (43.7)	
LCC	8/16 (50.0)		10/16 (62.5)		6/16 (37.5)		7/16 (43.7)	
TNM stage		0.03*		0.29*		0.98*		0.34*
I (Ia+Ib)	7/21 (33.3)		9/21 (42.9)		5/21 (23.8)		11/21 (52.4)	
II (IIa+IIb)	10/27 (37.0)		15/27 (55.6)		7/27 (25.9)		15/27 (55.6)	
IIIa	31/54 (57.4)		31/54 (57.4)		13/54 (24.1)		23/54 (42.6)	

Data presented as fraction (%). *: Chi-squared test for trend. F: female; M: male; SqCC: squamous cell carcinoma; AdC: adenocarcinoma; LCC: large cell carcinoma.

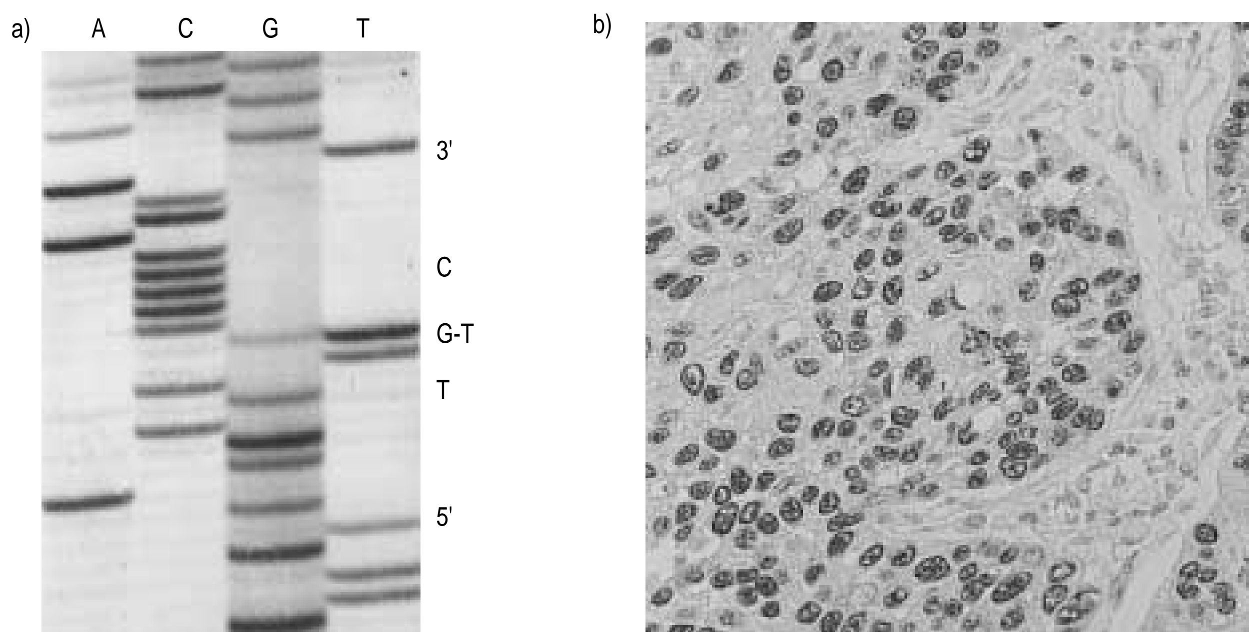

Fig. 1. – a) Nucleotide sequence of mutated p53 (Exon 5, codon 176, Cys-Phe) gene determined by direct sequencing (A: adenine; C: cytosine; G: guanine; T: thymine). and b) overexpression of p53 protein determined by immunohistochemical analysis in stage IIIa of squamous cell carcinoma.

months (median: 28 months). In this group 47 patients died during the observation period until August 31, 1997, with follow-up ranging 6.9–42.5 months (median: 22.6 months). In the group of 53 surviving patients the median follow-up time was equal to 33.4 months.

Figure 2 shows survival curves in relation to the four molecular tests under consideration. The curves suggest that, for the p53-M, p53-PE and p53-Abs tests, positive results had an unfavourable effect on the likelihood of surviving. In the case of the bcl-2-PE test, an unfavourable effect was observed for a negative result of the test.

The univariate analyses of the survival time were performed using the log rank test. Sex, histological type and TNM stage of the tumour were also included in the analyses. Taking into account the fact that multiple comparisons were made, a level of significance of $p<0.01$ was adopted. The results of the test for TNM stage ($p<0.001$ for trend), p53-M ($p<0.001$), p53-Abs ($p<0.001$), and bcl-2-PE ($p<0.001$) were statistically significant. The result for p53-PE was marginally significant ($p=0.008$), while the tests for sex and histological type gave nonsignificant results ($p=0.16$ and 0.24, respectively).

Since the log rank test for TNM stage of the tumour was statistically significant, survival curves for the p53-M, p53-PE, p53-Abs and bcl-2-PE tests were compared using a stratified log rank test at 0.01 significance level, with strata defined by TNM stages (I, II and IIIA). The results for the p53-M test ($p<0.001$) and the p53-Abs test ($p<0.001$) were statistically significant, in accordance with the outcome of the univariate analysis. However, the results for the p53-PE and bcl-2-PE tests became marginally nonsignificant ($p=0.014$ and $p=0.02$, respectively). Thus, for those two tests the adjustment for the effect of TNM stage qualitatively changed the conclusion obtained from the unstratified analysis.

To evaluate the prognostic value of results of all the molecular tests under consideration, with simultaneous adjustment for effects of sex, histological type and TNM stage of the tumour, a multivariate analysis using Cox's proportional hazard model was performed. The model fitted to the data is presented in table 2. Based on the results obtained previously [15], separate effects of the positive result of the p53-Abs test for different histological types of the tumour were used.

The score test for the hypothesis of a nonzero effect for at least one of the covariates included in the proportional hazard model (table 2) was statistically significant at 0.05 level of significance ($p<0.001$). This level was also adopted for the assessment of significance of coefficients of the covariates. Results shown in table 2 indicate that after adjustment for effects of sex, histological type of the tumour and TNM stage, the effects of results of the p53-PE, p53-Abs and bcl-2-PE tests were not statistically significant at 0.05 level of significance ($p=0.47$, 0.25 and 0.19, respectively). Only the positive result of the p53-M test remained as an independent, significant ($p=0.03$) unfavourable prognostic factor for survival. The relative risk associated with a positive result of the test was estimated to be equal to 2.74, with 95% CI of (1.08, 6.97).

The fact that the effects of the p53-PE and p53-Abs tests were nonsignificant in the model presented in table 2 can be explained by the strong association between the two tests and the p53-M test mentioned earlier. It was checked that when the p53-M test was removed from the model presented in table 2, positive results of the p53-PE test and the p53-Abs test became significant ($p=0.004$ and $p=0.03$, respectively), unfavourable prognostic factors.

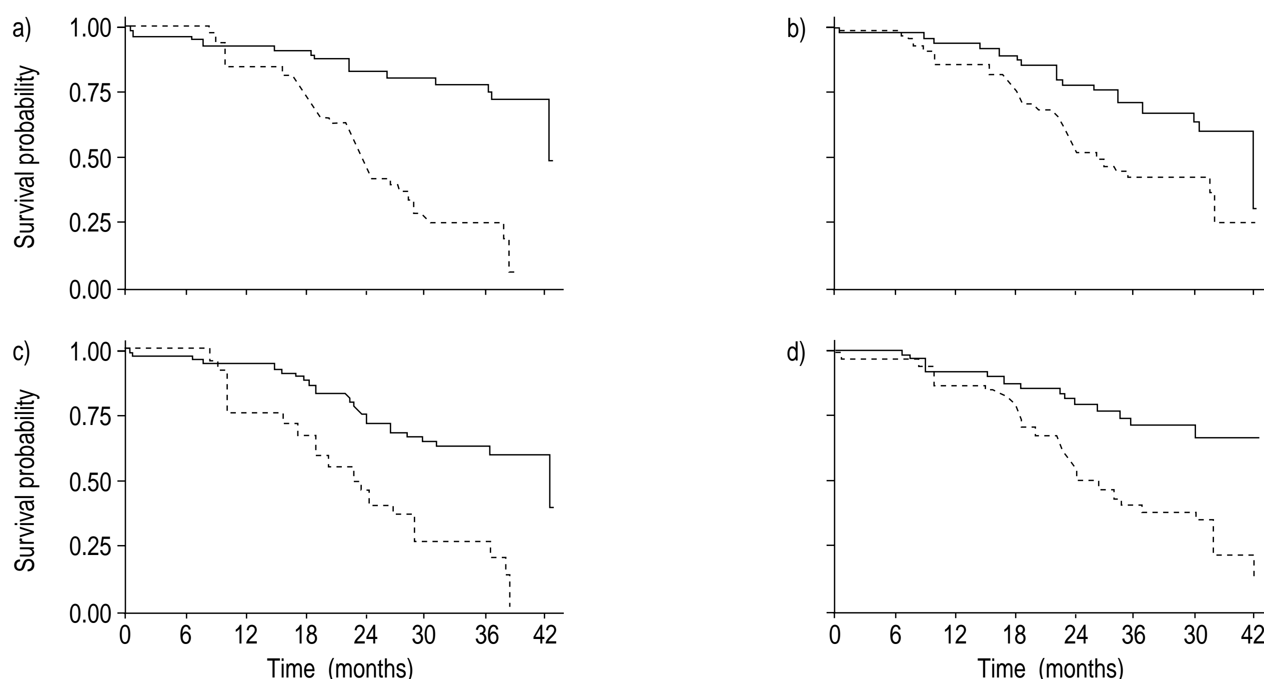

Fig. 2. – Survival curves for: a) p53 mutation (-ve, n=54; +ve, n=48); b) p53 protein expression (-ve, n=47; +ve, n=55); c) p53 antibody (-ve, n=77; +ve, n=25); and d) bcl-2 protein expression (-ve, n=53; +ve, n=49) tests. —: negative; - - - : positive. Log rank test: p<0.001 for a, c and d and p=0.008 for b.

Table 2. – A proportional hazard model for survival

	Relative risk (95% CI)	p-value
Sex		
Female	1	-
Male	1.50 (0.34, 6.57)	0.59
Histological type		0.05*
SqCC	1	-
AdC	2.58 (1.11, 5.99)	0.02
LCC	2.57 (0.76, 8.74)	0.12
TNM		<0.001*
I (Ia+Ib)	1	-
II (IIa+IIb)	1.34 (0.38, 4.66)	0.64
IIIA	5.16 (1.78, 14.95)	0.001
bcl-2-PE		
negative	1	-
positive	0.64 (0.32, 1.26)	0.19
p53-PE		
negative	1	-
positive	1.37 (0.58, 3.25)	0.47
p53-Abs		0.25*
negative	1	-
positive, SqCC	2.41 (0.92, 6.30)	0.07
positive, AdC	1.22 (0.41, 3.62)	0.72
positive, LCC	2.03 (0.48, 8.59)	0.33
p53-M		
negative	1	-
positive	2.74 (1.08, 6.97)	0.03

*: the score test for nonzero effect of at least one of the effects associated with the factor. SqCC: squamous cell carcinoma; AdC: adenocarcinoma; LCC: large cell carcinoma; TNM: primary tumour, regional nodes, metastasis staging; bcl-2-PE: bcl-2 protein expression; p53-PE: p53 protein expression; p53-Abs: p53 antibody serum; p53-M: p53-mutation.

Discussion

In this study, p53 abnormalities in the DNA, protein and immune response against p53 levels and bcl-2-PE in 102 NSCLCs were analysed.

p53-M was found in 47% of the analysed cases, with the majority of base substitutions in exon 5 (42%) and G residues (48%), which is not much different from the data reported by other investigators [20, 21]. p53 overexpression and p53-Abs were detected in 54% and 25% of cases, respectively. A strong, statistically significant association was found between p53 protein overexpression, p53-Abs and the presence of a p53 gene alteration in exons 5–8. For p53 overexpression this is in agreement with previous reports [22, 23]. This finding provides further evidence that the presence of an aberrant p53 gene leads to the accumulation of a highly stabilized protein. On the other hand, p53 nonsense mutations were negative in immunohistochemical staining. However, there were also NSCLC tumours that demonstrated p53 positive staining but in which no mutations were found. These differences between molecular and immunohistochemical results might be due to mutations in the p53 gene outside exons 5–8, within the p53 promoter region, or to the overexpression of p53 caused not only by p53-Ms but also by some other factors which bind to the p53 protein and thus increase its half-life [24]. As previously discussed, a statistically significant, positive association between p53 gene mutation and the presence of antibodies against p53 in serum was found. Previous studies have also suggested that positivity of p53 auto-antibodies is related to p53-M or

abnormal accumulation of p53 in the primary tumour [25, 26].

Studies on the relations between p53 abnormalities and clinicopathological parameters of NSCLC are few. In this study, from all possible investigations of p53 and bcl-2 abnormalities, only in the case of the p53 protein overexpression was a statistically significant difference in proportion of positive results for different histological types found. Significantly fewer positive results were found for AdC than for SqCC or LCC.

Recent studies indicate that, in addition to conventional oncogenes, other genes that encode proteins preventing apoptotic cell death may also play a critical role in carcinogenesis [27]. In this study, the expression of bcl-2 and its relation to the altered p53 in NSCLC was taken into account. Although the mechanism by which bcl-2 is overexpressed in solid tumours is currently poorly defined, some studies have indicated the clinical value of determination of bcl-2-PE in lung cancer. In this study it was found that immunohistochemical bcl-2 demonstration was not associated with any clinicopathological parameters. Discrepancies have already been reported between the relationship of bcl-2 expression and p53 accumulation in solid tumours [28, 29]. Kitagawa *et al.* [30] and Fontanini *et al.* [12] showed an inverse relationship between overexpression of bcl-2 and accumulation of p53 protein in NSCLC. However, the recent data of Fleming *et al.* [31] showed a lack of correlation between bcl-2 and either p53 immunostaining or p53-M. In the present study, the authors also found no statistically significant relationship between bcl-2 and p53.

There is still considerable controversy as to exactly how p53 and bcl-2 alterations affect the outcome of patients with NSCLC. Most of the studies have focused on the potential prognostic value of p53-PE. Some of these papers suggest that p53 immunohistochemistry is indicative of a poor prognosis [32, 33], while others demonstrate the contrary [34, 35]. Similarly, the potential role of p53 gene mutations is also debatable. Carbone *et al.* [33] did not associate p53-Ms with major aggressiveness in NSCLC patients, while other investigators observed the opposite effect [36, 37]. Fukuyama *et al.* [38] suggested that p53-Ms were an independent unfavourable prognostic marker especially in the early stage of NSCLC. Tomizawa *et al.* [39] reported that mutations rather than expression of the p53 would be important as a prognostic marker in the management of early stage NSCLC. Most of these investigations, however, analysed relatively insensitive, indirect techniques of screening for p53-Ms, such as the single-strand conformational polymorphism (SSCP).

There have been some studies suggesting that overexpression of bcl-2 may indicate a more favourable prognosis in NSCLC. Pezzella *et al.* [11] found that bcl-2 protein was negatively associated with adenocarcinoma and positively associated with improved 5-yr survival in patients overall, in patients of all ages with SqCC, and in patients $\geqslant$60 yrs of age with any types of NSCLC. Ritter *et al.* [40] found a trend towards an increased disease-free survival in cases with bcl-2 staining, which did not achieve statistical significance. A subsequent study by Fontanini *et al.* [12] reported increased survival probability in patients with bcl-2 staining of NSCLC. On the other hand, Anton *et al.* [41] reported that in series of 427 NSCLC cases, bcl-2 immunopositivity was not an independent prognostic factor.

In this present study it was found that both p53 abnormalities and negative staining for bcl-2, when analysed separately, were associated with poor overall survival. However, in the multivariate analysis only the positive result of the p53-M test remained an independent, statistically significant, unfavourable prognostic factor for survival. When the p53-M test was removed from the model, positive results of the p53-PE test and the p53-Abs test became statistically significant, unfavourable prognostic factors.

The presented results prompt the conclusion that from p53 abnormalities analysed at the deoxyribonucleic acid, protein and immune response levels and bcl-2 abnormalities analysed at the protein level, only p53 gene mutation seems to have a strong and independent effect on survival prognosis. However, when deoxyribonucleic acid sequence information is not available, p53 protein expression and the presence of p53 antibodies in serum may provide important prognostic information.

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