Bcl-2 protein: a prognostic factor inversely correlated to p53 in non-small-cell lung cancer

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Summary Non-small-cell lung cancer (NSCLC) prognosis is strictly related to well-established clinicopathological parameters which have unfortunately become insufficient in the prognostic evaluation of this type of cancer. As p53 and bcl-2 gene deregulations are frequently involved in several types of epithelial malignancies, we investigated the Bcl-2 and p53 protein expression in 91 and 101 cases of NSCLC respectively. The expression was then compared with established indicators of prognosis and biological behaviour of the tumours. No relationship was observed between Bcl-2 and either clinicopathological or biological parameters such as histology, grading, tumour status, nodal metastasis and proliferative activity evaluated by scoring proliferating cell nuclear antigen expression and Ki-67 immunoreactivity. However, the mean Bcl-2 expression was significantly lower in patients who developed metastasis during follow-up or died of metastatic disease ($P = 0.006$ and $P = 0.01$ respectively). Moreover, survival probability was higher in patients who expressed the Bcl-2 protein ($P = 0.0002$). In contrast with this, p53 protein accumulation was observed in tumours with metastatic nodal involvement ($P = 0.02$) or in patients who developed metastasis during follow-up ($P = 0.01$), although no correlation was found between p53 expression and overall survival. An inverse relationship was also found between Bcl-2 and the anti-oncogene protein product p53 ($P = 0.01$). Thus, a high proportion of NSCLCs express p53 and Bcl-2 proteins and their expression may have prognostic importance.

Keywords: oncogenes; NSCLC; prognosis; p53; Bcl-2

Lung cancer has now become the leading cause of cancer deaths in both men and women in the USA (Minna et al., 1989). In particular, non-small-cell lung cancer (NSCLC) represents a heterogeneous subgroup in terms of both behaviour and therapeutic response.

Several studies have clearly demonstrated that multiple genetic events are associated with the development of lung cancer, including a range of chromosomal abnormalities, mutations activating the dominant cellular proto-oncogenes and genetic events inactivating tumour suppressor genes (Minna, 1993).

p53 alterations and aberrant nuclear accumulation of this protein have been recently studied with particular interest. Many experimental data indicate that the p53-suppressor gene is the most commonly altered tumour-suppressor gene (Hollstein et al., 1991). This gene codes for a nuclear phosphoprotein, normally undetectable in human cells, which is able to regulate cell growth and division (Levine et al., 1991; Lane, 1992). p53 protein may be detected by immunocytochemistry in cancer cells as a consequence either of mutational events in p53 gene or of stabilisation by other factors such as some viral proteins. p53 alterations appear early during NSCLC progression; these alterations are maintained during invasion and metastatic spread of cancer cells (Quinlan et al., 1992; Fontanini et al., 1994), inducing a proliferative advantage and conferring a particularly aggressive phenotype.

The bcl-2 gene was originally discovered owing to its involvement in the t(14;18) chromosomal translocation occurring in the majority of non-Hodgkin's B-cell lymphomas (Tsujimoto and Croce, 1986; Aisemberg et al., 1988). This translocation places the bcl-2 gene at chromosomal location 18q21 in juxtaposition with the immunoglobulin heavy-chain locus at 14q32, resulting in transcriptional deregulation of the bcl-2 gene (Cleary et al., 1984; Tsujimoto and Croce, 1986; Tsujimoto et al., 1987) and abnormally high levels of the Bcl-2 protein. Furthermore, overexpression of the Bcl-2 protein has been observed in different types of

solid tumours, including prostate (Colombel et al., 1993), lung (Pezzella et al., 1993a), thyroid (Pilotti et al., 1994) and breast (Silvestrini et al., 1994). In contrast with lymphomas, little or no evidence of gross alterations in the bcl-2 gene structure was obtained for these other types of cancer, suggesting that alternative mechanisms of deregulation of Bcl-2 expression may exist in human cancer (Leek et al., 1994).

The high incidence of p53 alterations and the aberrant expression of Bcl-2 in many human cancers together with their putative prognostic significance in lung (Pezzella et al., 1993a) and breast cancer (Silvestrini et al., 1994) induced us to study the p53 and Bcl-2 expression in a series of NSCLCs, with particular regard to their relationship, according to metastatic assessment and overall survival.

Materials and methods

Patients and tissue samples

The study involved 101 patients with primary resectable non-small-cell lung cancer. The patients (91 men and ten women, mean age 63 ± 6.4 years) presented no clinical or radiological evidence of distant metastases at diagnosis and underwent lobectomy or pneumonectomy between March 1991 and December 1992 at Santa Chiara Hospital of Pisa University. The median follow-up was 25 months (range 2–41). The histopathological features of the surgical specimens were classified and staged according to the World Health Organization (1982) criteria and the TNM staging system (Mountain, 1987). Immediately after surgery a part of the tumour sample was processed by conventional histological procedures for the determination of the Bcl-2 and proliferation cell nuclear antigen (PCNA) expression. The rest of the tumour material was frozen in liquid nitrogen and stored at $-80°C$ for p53 and Ki-67 determination.

Immunohistochemistry

A total of 101 and 91 samples were examined for p53 and Bcl-2 expression respectively. In 90 cases both p53 and Bcl-2 immunoreactions were performed.

Bcl-2 immunostaining The 5 μm tumour sections were immunostained using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method (Cordell *et al.*, 1984) with the anti-Bcl-2 monoclonal antibody (clone 124) (Pezzella *et al.*, 1992) raised to a synthetic peptide. Briefly, paraffin sections were dewaxed in xylene and dehydrated through graded alcohols. The monoclonal antibody Bcl-2 124 was applied overnight at 1:20 dilution. A rabbit anti-mouse secondary antibody prediluted in 0.05 M Tris buffer containing normal swine serum was applied for half an hour. The alkaline phosphatase–mouse anti-alkaline phosphatase immune complex was applied for half an hour first and then for 10 min; during the interval the anti-mouse serum was used. The reaction was developed with alkaline phosphatase substrate containing naphthol AS-MX fast red Tr and levamisole (APAAP Kits, Dako). As a positive control for Bcl-2, we used a paraffin-embedded section from a normal peribronchial lymph node removed during post-surgical sampling of a lung tumour. At the same time positive staining of small lymphocytes provided an internal control for Bcl-2 staining. Staining without anti-Bcl-2 monoclonal antibody was performed as a negative control procedure. The Bcl-2 immunoreactivity was assessed by scoring a minimum of five high-power fields (HPFs) (40 × objective lens).

p53 immunostaining

For p53 detection the anti-p53 monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, NY, USA) was used overnight at 1:200 dilution on 5 μm frozen sections, as previously reported (Fontanini *et al.*, 1993*a*). This antibody reacts specifically with human wild-type and mutant p53 recognising an N-terminal epitope of the protein. The avidin–biotin–peroxidase method was used, developing the immunoreaction with diaminobenzidine. Simultaneous staining of a known p53[+] case was employed as a positive control for p53 expression. Incubation of parallel slides omitting the first antibody was performed as the negative control. As for Bcl-2, the number of p53-immunoreactive cells was counted by scoring a minimum of five HPFs (40 × objective lens).

PCNA and Ki-67 score

Proliferative activity in each sample was evaluated using PC10 and Ki-67 MAbs on paraffin-embedded and frozen sections respectively, as previously reported (Fontanini *et al.*, 1993*b*). Absolute counts of PC10 and Ki-67 immunoreactivity were made by scoring a minimum of five HPFs (40 × objective lens); 1% PC10- and Ki-67-positive tumour cells out of the total number of tumour cells counted provided the PCNA and Ki-67 index for each tumour.

Statistical analysis

All statistical analyses were carried out by the STATISTICA (Stat-Soft) software system. The differences between p53 and Bcl-2 expression and clinicopathological parameters were assessed by the unpaired *t*-test. The relationship between the p53 and Bcl-2 expression was evaluated by a chi-square test and by a linear regression coefficient test. Survival analysis was calculated by the Kaplan–Meier method.

Results

Bcl-2 protein immunostaining

Bcl-2 immunoreactivity was localised in the cytoplasm of neoplastic cells (Figure 1a); no nuclear Bcl-2 positivity was found in this series of cancers. We evaluated as positive tumours with only 1% of stained cells, provided this positivity was very defined and localised in the cytoplasm of neoplastic cells. Heterogeneous staining was sometimes detected in the basal layer cells of the normal bronchial epithelium adjacent to tumour areas. The frequency of cells
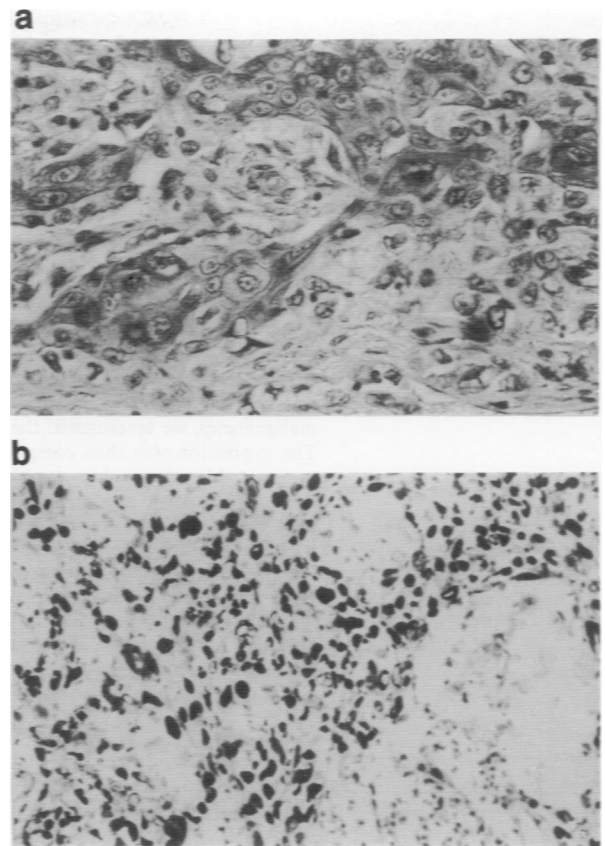
Figure 1 (**a**) Bcl-2 expression in the cytoplasm of tumour cells (anti-Bcl-2 MAb, APAAP method, 25 ×). (**b**) p53 nuclear immunoreactivity detected in neoplastic cells using PAb 1801 (ABC method, 25 ×).

expressing the Bcl-2 protein varied widely from one tumour to the other. Bcl-2 protein immunopositivity was detected in 61 out of the 91 (67%) tumour samples examined, ranging from 1.5% to 90% positive cells (mean 27.1 ± 25.1; median 15).

Bcl-2 expression and clinicopathological parameters As reported in Table I, no statistical differences in Bcl-2 expression were found between: (1) tumour histotype, (2) tumour grade, (3) tumour status and (4) nodal metastasis.

Bcl-2 expression and proliferative activity Highly proliferating tumours (cut-off 30% for PCNA and 13% for Ki-67) express a percentage of positive cells similar to that of tumours with low proliferative activity (Table II). These results suggest that the Bcl-2 protein expression is independent of the proliferative status of the tumours.

Bcl-2 expression in relation to distant metastasis and survival In 89 out of 91 patients the data regarding development of metastases and overall survival were available (two patients died during the post-operative period). In order to determine whether the alterations in the Bcl-2 protein may be a prognostic indicator in NSCLC, we analysed Bcl-2 expression both in tumours from patients with and without metastases and in patients still alive or dead from neoplastic disease. Bcl-2 expression was significantly higher ($P < 0.006$) in tumours from metastasis-free patients than in tumours from patients with distant metastases. We also found that tumours from living patients presented a higher number of Bcl-2-immunoreactive cells than tumours from dead patients ($P < 0.01$) (Table III). The same results obtained from survival analysis are reported in Figure 2a. NSCLC patients with Bcl-2-positive tumours had a higher probability of survival than patients with Bcl-2-negative cancers (Kaplan–Meier analysis, $P = 0.0002$). We obtained the same results

Table I Clinicopathological data and immunocytochemical reactivity for Bcl-2 protein in 91 cases of NSCLC

Variables	No. of cases	Bcl-2 immunoreactivity Mean (± s.d.)	P[a]
Sex			
Male	81	17.6 ± 23.7	NS
Female	10	22.9 ± 28.8	
Histology			
Squamous	55	19.9 ± 23.2	NS
Non-squamous	36	15.4 ± 26.6	
Grading			
G1	16	17.7 ± 24.4	
G2	38	18.1 ± 23.5	NS
G3	37	17.1 ± 251	
T-status			
T1	16	22.8 ± 23.8	
T2	64	17.4 ± 24.2	NS
T3	11	15.7 ± 25.7	
N-status			
N0	64	17.5 ± 21.8	NS
N1-2	27	19.6 ± 29.4	

[a]Unpaired t-test.

Table II Bcl-2 protein expression in NSCLC according to PCNA and Ki-67 immunoreactivity

Proliferative activity	No. of cases	Bcl-2 immunoreactivity Mean (± s.d)	P[a]
PCNA			
≤ 30[a]	33	15.3 ± 25.9	0.6
> 30	26	18.1 ± 26.5	
Ki-67			
< 13[a]	35	17.6 ± 27.8	0.6
> 13	24	15.6 ± 24.7	

[a]Unpaired t-test.

Table III Relationship between Bcl-2 protein expression, survival and metastasis in 89 patients with NSCLC

	No. of cases	Bcl-2 expression Mean (± s.d)	P[a]
Alive	51	22.8 ± 24.2	0.01
Dead	38	10.8 ± 22.3	
No metastasis	48	24.2 ± 24.4	0.006
Metastasis	41	10.2 ± 21.5	

[a]Unpaired t-test.

when considering Bcl-2 expression as a dichotomous variable (negative vs positive tumours) (Table IV). Taken together, these results suggest that Bcl-2 expression may be considered as a favourable prognostic marker in this type of cancer.

p53 protein immunostaining

p53 immunostaining was performed in 101 cases. Sixty-nine cases were found to be positive and 32 negative (mean 39.9 ± 28.5; range 1-85). Immunoreactivity was confined to the nuclei of neoplastic cells (Figure 1b) with the following staining pattern: (a) tumours with only a few scattered positive cells (< 1%) or none at all were considered negative; tumours with more than 1% positive cells with either (b) heterogeneous or (c) homogeneous distribution were evaluated as positive.

p53 expression and clinicopathological parameters The mean p53 immunoreactivity according to clinicopathological parameters is summarised in Table V. As is shown, no correlation was found between p53 expression and clinicopathological parameters such as T status, histiotype and tumour grade. By contrast, mean p53 immunoreactivity
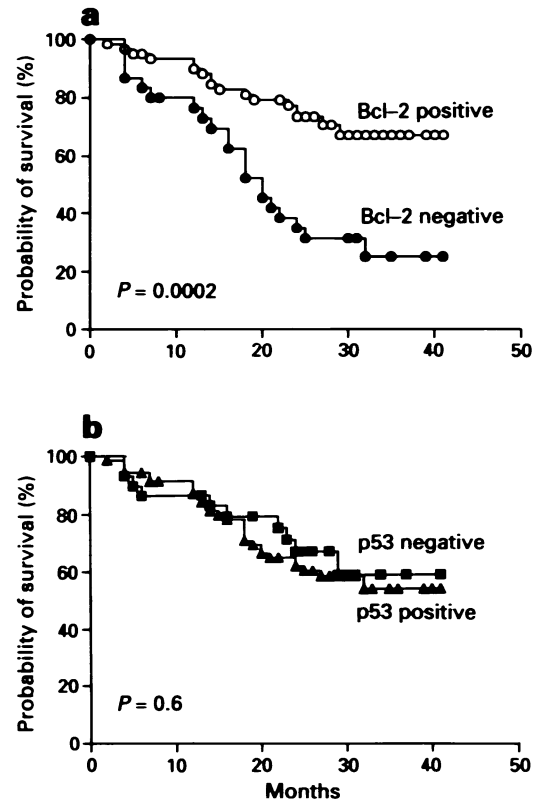

Figure 2 Survival of patients with non-small-cell lung cancer according to status of Bcl-2. (a) and p53 (b) proteins (Kaplan-Meier method).

Table IV Survival and metastasis in 89 patients with NSCLC according to Bcl-2 expression

	Bcl-2 expression Positive cases/ total	Negative cases/ total	P[a]
Alive	42	9	0.0005
Dead	17	21	
No metastasis	40	8	0.0006
Metastasis	19	22	

[a]Contingency tables.

Table V p53 immunoreactivity in 101 cases of NSCLC according to clinicopathological parameters

Variables	No. of cases	p53 immunoreactivity Mean (± s.d.)	P[a]
Sex			
Male	91	27.9 ± 27.6	NS
Female	10	21.1 ± 33.3	
Histology			
Squamous	64	24.7 ± 27.4	NS
Non-squamous	37	31.6 ± 29.2	
Grading			
G1	17	24.7 ± 28.9	
G2	43	26.1 ± 27.7	NS
G3	41	31.1 ± 28.7	
T-status			
T1	17	25.4 ± 30.6	
T2	73	27.7 ± 27.4	NS
T3	11	22.04 ± 31.8	
N-status			
N0	62	22.9 ± 26.6	0.02
N1-2	39	36.6 ± 29.4	

[a]Unpaired t-test.

in tumours from patients with hilar and/or mediastinal nodal involvement was significantly higher than in patients without nodal metastasis ($P = 0.02$).

Table VI p53 expression in NSCLC according to the development of metastasis and survival

	No. of cases	p53 immunoreactivity Mean (± s.d)	P[a]
Metastasis	44	35.4 ± 28.3	0.01
No metastasis	55	21.7 ± 26.7	

[a]Unpaired t-test.

Table VII Bcl-2 and p53 expression in 59 cases of non-small cell lung cancer

p53 expression	No. of positive cases/Total	%	Bcl-2 expression No. of negative cases/Total	%	P[a]
p53 positive	36	60	26	86.7	0.01
p53 negative	24	40	4	13.3	

[a]Contingency tables.

Figure 3 Relation between Bcl-2 and p53 expression in 90 cases of non-small-cell lung cancer (linear regression coefficient test).

p53 expression in relation to distant metastases and survival

In 99 out of 101 cases we obtained data concerning either the development of metastases during follow-up or overall survival (two patients died during the post-operative period). Forty-four out of 99 (44.4%) patients who had developed distant metastasis showed a higher mean p53 positivity (35.4%) than metastasis-free patients (21.7%) (P = 0.01; Table VI). On the other hand, overall survival was not affected by p53 overexpression (Figure 2b; Kaplan–Meier method).

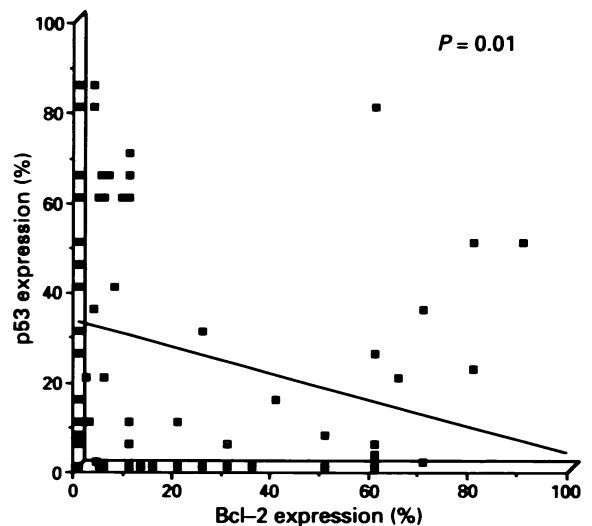## Bcl-2 and p53 protein expression

Staining for both Bcl-2 and p53 was available in 90 cases. The results are summarised in Table VII. Of 60 Bcl-2-positive tumours, 60% showed p53 overexpression, whereas of 30 Bcl-2-negative tumours 87% showed p53 immunoreactivity (P = 0.01). Regression analysis is reported in Figure 3; a clear inverse correlation was found between the p53 and Bcl-2 protein expression (P = 0.01).

Discussion

Bcl-2 and p53 proteins are both related to programmed cell death or apoptosis and thus their relationship is of interest. In this series of NSCLCs the results showed that Bcl-2 and p53: (a) are detectable by the immunohistochemical technique in about 60% and 70% of cases respectively; (b) are inversely associated; and (c) provide information regarding metastasis onset and overall survival probability.

Bcl-2 represents the product of the proto-oncogene involved in the 14;18 translocation; its distribution in reactive lymph nodes and lymphomas has already been described (Villeundas et al., 1992; Pezzella et al., 1993b; Piris et al., 1994). Bcl-2 expression has been observed not only in the B-lymphoma but also in different types of solid tumours such as breast (Leek et al., 1994; Silvestrini et al., 1994), prostate (Colombel et al., 1993) thyroid (Pilotti et al., 1994) and lung cancer (Pezzella et al., 1993a).

In particular, in NSCLCs, which are believed to originate from the respiratory epithelium, Bcl-2 overexpression was related with better overall survival (Pezzella et al., 1993a). Our results obtained from a group of 91 NSCLC patients with median follow-up of 24 months agree with those reported by Pezzella et al. However, both studies found that Bcl-2 overexpression seems to be able to induce a less aggressive tumour phenotype. The reason for this remains to be clarified. Interestingly, in other types of cancers such as breast (Leek et al., 1994; Silvestrini et al., 1994), thyroid (Pilotti et al., 1994) and prostate (Colombel et al., 1994) a strong association has been found between Bcl-2 expression and tumour differentiation, suggesting that this gene may somehow act to switch off proliferation during tumour pro-

gression. However, this hypothesis cannot be confirmed in lung cancer since we failed to find any differences in the Bcl-2 expression of tumours of different grade. It is known that Bcl-2 promotes cell survival even when the cell proliferation rate is not elevated. This could provide a growth advantage eventually leading to neoplastic transformation (Vaux et al., 1988). McDonnel et al. (1989) suggested that for cellular clones in which a low proliferative rate is offset by Bcl-2 expression the acquisition rate of complementary defects is slower than in clones with a higher proliferative rate.

The association of a growth advantage owing to cellular survival with low proliferative rate and slower acquisition of further genetic defects could explain the slow evolution of follicular lymphoma in which Bcl-2 expression is a frequent primary aberration (Vaux et al., 1988; McDonnel et al., 1989). Different authors have suggested recently that alterations in Bcl-2 could be present as a frequent aberration not only in follicular lymphoma (Korsmeyer, 1992), but also in other types of cancer such as breast (Silvestrini et al., 1994; Leek et al., 1994) and lung (Pezzella et al., 1993a) carcinomas. This relation could be responsible for the increasing likelihood of mutational aberrations in other oncogenes, such as those interfering with growth and proliferation of tumour cells.

Our observations of a subgroup of patients with slowly progressing Bcl-2-positive tumours suggest that in these tumours Bcl-2 expression is likely to occur as an initial alteration leading to a less aggressive behaviour of tumours. This is in agreement with the inverse relationship between Bcl-2 and p53 which we found in our series of cancers, supporting the hypothesis that either one or the other is sufficient to modify the apoptotic pathway in NSCLC.

In our study Bcl-2 expression is not associated with cell proliferation evaluated as a percentage of PC10- or Ki-67-immunoreactive cells. In addition, we found that the bronchial epithelium expresses Bcl-2 in a proportion of basal cells, and that there is a total lack of Bcl-2-positive cells in the upper differentiated layers of the epithelium although hyperplasia is sometimes present in these areas. These findings have been confirmed by Lu et al. (1993), who have recently reported the lack of Bcl-2 expression in non-proliferating syncytiotrophoblast and in psoriasis despite evident mitotic activity. This suggests that Bcl-2 is mainly associated with activated and undifferentiated cells undergoing terminal differentiation which need protection from apoptosis.

p53 is now well characterised as a tumour-suppressor gene, with loss of normal p53 function recorded as the most common genetic event associated with human malignancies (Holl-stein, 1991). p53 alterations with consequent aberrant nuclear accumulation have been correlated with progression and

poor prognosis in some solid tumours such as breast (Thor et al., 1992; Allred et al., 1993; Silvestrini et al., 1993), gastric (Martin et al., 1992), bladder (Sarkis et al., 1993) and lung (Quinlan et al., 1992) carcinomas. In a series of 103 NSCLCs (Fontanini et al., 1993a) we found that p53 protein overexpression correlated with metastatic involvement of hilar and/or mediastinal lymph nodes, supporting other findings by Quinlan et al. about the negative prognostic role of p53 alterations in NSCLC. In this group of patients it was confirmed that p53 accumulation may predict the metastatic behaviour of NSCLC, and that it is overexpressed not only in cancer with nodal metastatic involvement at diagnosis but also in tumours which develop distant metastases during follow-up.

Our present results indicate on the one hand an inverse relationship between Bcl-2 and p53 expression and, on the other hand, an inverse prognostic significance of these variables in NSCLC behaviour. The loss of Bcl-2 expression is in fact associated with shorter overall survival, metastatic development during follow-up and other poor prognostic markers such as p53 positivity. For this reason, the role of Bcl-2 in lung cancer progression may differ from that seen in lymphomas in which the translation 14;18 occurs.

Further efforts are needed to assess the prognostic significance of Bcl-2 and its relation with other gene products involved in the regulation of apoptosis and proliferation.

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