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Apoptotic Markers p53, Bcl-2 and Bax in Primary Lung Cancer

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Abstract. *Background: Apoptosis is the fundamental process necessary for eliminating damaged or unwanted cells. Alterations in the apoptotic pathway appear to be key events in cancer development and progression. The aim of the study was to determine the p53, Bcl-2 and Bax expressions in lung cancer, taking into account histological heterogeneity and the adjacent bronchial resection margin. Materials and Methods: Tissue specimens from 60 histopathologically verified lung cancer specimens and 12 bronchial stumps were evaluated. The presence of the studied markers was revealed by immunocytochemistry on paraffin-embedded tissue. Results: The percentage of p53- and Bax-positive lung cancers was comparable (51.6% for both proteins), while Bcl-2 immunoreactivity was observed in fewer (31.6%) cases. There was no p53 accumulation in bronchial stumps, while Bcl-2 and Bax staining formed a repeatable specific pattern in bronchial epithelium. The differences in apoptotic marker expression between non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) were revealed, especially regarding p53 and Bax expression (60% vs. 10%, p=0.005 and 58% vs. 20%, p=0.04, respectively). Taking into account the histological structure of NSCLC, Bax expression appeared to be more frequent in adenocarcinoma than in squamous cell lung cancers (88% vs. 42%, p=0.004). No interrelationship between the studied proteins in lung cancer tissue was revealed. Conclusion: The expression of p53, Bcl-2 and Bax was altered in lung cancer tissue compared to histologically normal bronchial epithelium. The difference between apoptotic marker expression in NSCLC and SCLC could reflect the different pathogenesis of these two lung pathologies.*

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Mutations in the *p53* tumor suppressor gene are well-known molecular events in human malignancy. Cells with a mutated *p53* gene resulting in functionally inactive p53 protein cannot control genomic integrity and tend to escape from apoptosis, leading to the development of malignancy (1). The Bcl-2 family proteins, including pro- and anti-apoptotic members, participate in the p53 apoptotic pathway and the equilibrium between those positively and negatively regulatory proteins is essential for the susceptibility to apoptosis. It is known that all Bcl-2 family-associated proteins are functionally connected with mitochodria. Bcl-2, representative of anti-apoptotic proteins, and Bax, widely described as a pro-apoptotic factor, are involved in the late signaling phase of programmed cell death presenting opposite functions. A high level of Bcl-2 expression prevents cells from apoptosis caused by cytotoxic factors or cellular stress. This phenomenon explains the oncogenic potential of Bcl-2 contributing to the accumulation of cells with DNA damage that should be eliminated under normal conditions (2). Bax-associated proteins appear to be dominant inhibitors of Bcl-2 action, they promote apoptosis *via* mitochondrial membrane damage facilitating the release of other apoptotic mediators, especially cytochrome c, resulting in caspase cascade activation followed by cell death (2).

The mutual interactions between pro- and anti-apoptotic proteins in lung cancer remain poorly understood and controversial. The heterogeneous biology and behaviour of lung cancer which can be partially explained by their significant histopathological diversity were not often considered in previous reports. Only few reports described the relationship between p53, Bcl-2 and Bax in this type of cancer (3-5). The aim of our investigation was the analysis

of p53, Bcl-2 and Bax expression in 60 lung cancers, and in 12 normal bronchial tissue specimens taking into account the histopathological structure.

Materials and Methods

Patients and tissue samples. Fifty non-small cell lung cancers (NSCLC), 10 small cell ones (SCLC) and 12 bronchial stumps tissues (resection margin) containing only histologically normal epithelium were analysed. Bronchial stump tissue and most NSCLC specimens were obtained during thoracic surgery. SCLC and 6 cases of advanced NSCLC samples were collected by fibreoptic bronchoscope biopsy. Nineteen women and 31 men with a mean age of 60.1 (range 43-75) years were included in the study.

The histological diagnosis of lung cancer was performed according to the WHO 1999 classification (6) after routine haematoxylin eosin staining. Among the NSCLC, 31 were recognized as squamous cell, 16 as adenocarcinoma and 3 as non-small cell without determination of the subtype. Twelve bronchial stump samples were examined and none of them showed neoplastic or dysplastic lesions, the histological structure of the bronchial mucosa was determined as normal.

The staging of lung cancer was established according to guidelines of the American Joint Committee on Cancer (7). Based on the TNM staging system, 18 patients in the NSCLC group had stage I disease, 13 stage II, 13 stage III A and 6 cases were non-operable advanced ones (IIIB and IV stages of disease). The SCLC cases were recognized as limited stage (n=9) or extend disease (n=1).

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissue using the ABC (avidin-biotin complex) method. Sections from each specimen were cut 5-μm thick, mounted on glass and dried overnight at 37˚C. All sections were then deparaffinised in xylene (3x5 min), rehydrated through a graded series of alcohol (2x10 min) and washed in distilled water (2x5 min). The deparaffined sections were boiled in a citrate buffer (pH 6.0) at 800W in a microwave oven (2x15 min). Non-specific endogenous peroxidase reactivity was blocked with periodic acid (2.28%) for 30 sec and then with sodium borohydride (0.02%) for 2 min.

Anti-p53 and anti-Bcl-2 (clone DO-7 and bcl-2/100/D5 respectively, both from Novocastra Laboratories Ltd., Newcastle-upon-Tyne, United Kingdom) and anti-Bax (clone B-9, Santa Cruz Biotechnology Inc., Santa Cruz, USA) monoclonal antibodies were applied.

The immunohistochemistry using the ABC immunoperoxydase kit was performed according to manufacturer's instructions. The slides were incubated with the primary antibodies for 30 min at room temperature for the p53 and Bcl-2 proteins and overnight at 4˚C for the Bax protein. The antibody-antigen reaction was visualised by 8-min incubation with 3,3'-diaminobenzidin-tetrachloride (DAB) as a chromogen and haematoxylin as a nuclear counterstaining. The PBS buffer was used for all subsequent washes and for dilution of the antibodies. Negative controls for each specimen were performed leaving out the primary antibody. All controls gave satisfactory negative results.

The immunohistochemical staining was interpreted by two independent observers (PI, WE) using a double-headed BHS microscope. The p53 nuclear protein accumulation was described as positive if more than 10% of cells were stained. For the Bcl-2 and Bax proteins, the cytoplasmic accumulation of stain exceeding more than 20% of cells was regarded as positive. In addition, the intensity of staining was also determined as + (low), ++ (medium) or +++ (high).

Table I. *The comparison of p53, Bcl-2 and Bax expression in lung cancer specimens and bronchial stumps.*

	Immunoreactivity positive/all cases (%)	
Marker	Cancer	Bronchial stump
p53	31/60 (51.6)	0/12 (0)
Bcl-2	19/60 (31.6)	10/12 (83.3)
Bax	31/60 (51.6)	10/12 (83.3)

Statistics. The expression of p53, Bcl-2 and Bax in NSCLC, SCLC and bronchial resection margin, as well as the associations between the expression of the studied proteins and the histological structure of NSCLC were compared using Fisher's exact test. The mutual relationships between the accumulation of the studied proteins were also statistically assessed with the Pearson correlation test. A p-value ≤ 0.05 was used to indicate statistical significance.

Results

p53, Bcl-2 and Bax expressions in lung cancer and bronchial stumps. The percentages of p53-, Bcl-2- and Bax-positive cases in lung cancer (regardless of histological structure) and in bronchial stumps are listed in Table I.

p53 presence was detected only in cancer tissue and no p53 reactivity was observed in normal bronchial tissue. The p53 protein immunoreactivity was observed typically in nuclear localisation (Figure 1a). The expression was heterogeneous according to the extent and intensity of staining and individually differentiated nuclear accumulation of p53 ranging from 10% to 90% positive cancer cells was observed.

Bcl-2 protein was detected in the cytoplasm of the cells with granular appearance. The percentage of Bcl-2-positive cancer cells varied from 20 to 100%. In the same cancer specimen, foci of Bcl-2-positive cells with strongly intense staining as well as Bcl-2-negative cells were observed (Figure 1b). In most of cases, positive Bcl-2 lymphocytes appeared to serve as positive intrinsic control (Figure 1c). In bronchial stumps, the Bcl-2 immunoreactive cells were situated in the basal layer of the bronchial epithelium (Figure 1d).

As for Bcl-2, Bax protein expression in cancer tissue restricted to cytoplasmatic staining varied from 20 to 100% of cells, although a pattern with a high percentage of Bax-immunopositive cells was predominant (Figure 1e). In the bronchial stumps, Bax-positive cells were seen within the

entire thickness of the epithelium with strong expression in the luminal epithelial cells (Figure 1f). Cytoplasmatic staining of bronchial glands was also sometimes detected, although the intensity of staining was rather weak and varied.

Comparison of p53, Bcl-2 and Bax expression in NSCLC, SCLC and bronchial stumps. The comparative immunoreactivity of the proteins studied is shown separately for NSCLC, SCLC and bronchial stumps in Figure 2. The expression of the p53 protein was significantly higher in NSCLC than SCLC (60% and 10%, respectively $p=0.005$) and no reactivity was observed in the histologically normal epithelium of the bronchial stumps.

Considering Bcl-2 expression, no marked differences between SCLC or NSCLC were observed. As for p53, the Bax protein was detected in a markedly higher percentage of NSCLC than SCLC cases (58% and 20%, respectively, $p=0.04$). Moreover, the intensity of Bax immunoreactivity was higher and affected a higher percentage of NSCLC than SCLC tumor cells. The cellular localisation of Bax protein was comparable in these two types of lung cancer.

As shown in Figure 2, Bax expression was observed more often in the bronchial resection margin than in lung cancer tissue, however statistical significance was shown only for SCLC (83% and 20%, $p=0.008$).

Similar to Bax, Bcl-2 protein reactivity was observed more often in the bronchial margin than in the lung cancer tissue and statistical differences were detected for both types of cancer (83% and 32%, $p=0.002$ for NSCLC and 83% and 30%, $p=0.03$ for SCLC, respectively).

Comparison of p53, Bcl-2 and Bax presence in histological subtypes of NSCLC. In the NSCLC histological subtypes, a clear difference was associated only with Bax protein expression, which was detected in a significantly higher percentage of adenocarcinomas than squamous cell lung cancers (88% *vs.* 42%, $p=0.0044$). The analysis of Bcl-2 and p53 status revealed no difference in the intensity or extent of staining associated with the histology of NSCLC.

Mutual relationship between p53, Bcl-2 and Bax in lung cancer. Independently of histopathological structure of the lung cancer specimens there were no statistically confirmed interrelationships between the apoptotic markers studied.

In the bronchial resection margin, the presence of p53 was undetectable and in the majority of cases, the expression of Bcl-2 and Bax was observed.

Discussion

There is a large quantity of data concerning p53 expression in lung cancer although the reported percentage of p53-positive NSCLC cases varies from 22% to 61% (5, 8-10). In our study, p53 immunoreactivity was observed in 60% cases, confirming that p53 defects are relatively frequent event in NSCLC (4). Moreover, the significantly smaller percentage of p53-positive cases in SCLC revealed in our study indicates that the apoptosis alteration in this type of tumour might differ from that of NSCLC and p53 mutation might not be an obligatory event in SCLC tumorigenesis. This observation, however, possesses limited value since the number of SCLC cases recruited in our study was relatively small due to damage to the tissue samples obtained by transbronchial biopsy. In addition, these biopsy samples are not representative for the whole tumour, taking into account the heterogeneous and sometimes focal immunostaining (9). Based on our series of cases of unoperable lung cancer, the evaluation of the immunohistochemically studied apoptotic marker expressions should be interpreted with caution.

Our results indicating the absence of p53 protein in the bronchial margin differs from the observations of Walker *et al.* (11). In their series of cases 17 out of 32 histopathologically normal bronchial resection margins originating from resected carcinomas were p53-positive although the p53 immunoreactivity was weak to moderate. In contrast to their results, in our group of bronchial resection margins, no p53 immunoreactivity was found, while the corresponding cancer tissue was occasionally positive.

The Bcl-2 protein expression in lung cancer has been evaluated by many authors but the results remain controversial. Most of the data revealed Bcl-2 presence in 19-46% of lung cancer cases (8, 10, 12-14), while in individual reports Bcl-2 reactivity was reported as high as 68% or as low as 8% (9, 15). Our data showing 31% of Bcl-2-positive cases is comparable with the results of Borner *et al.* (10) and Shibata *et al.* (13).

Bax immunoreactivity in lung cancer is also unresolved due to different percentages of positive cases described by various authors (3, 15). The percentage of Bax-positive cases in our study is close to the incidence of Bax immunoreactivity in lung cancer reported by Krug *et al.* (15) although their analysis included only NSCLC. Those discrepancies might reflect differences in Bcl-2 and Bax immunoreactivity interpretation criteria (different border percentage of cells) and different types of antibody applied or fixation techniques used. The decreased expression of Bax in lung cancer tissue compared with the bronchial resection margin observed in our study indirectly indicate the alteration in the signaling phase of apoptosis in cancer cells.

The Bcl-2 immunoreactivity detected in the normal bronchial epithelium of our study was comparable with the results described by Walker *et al.* (16). In our opinion, the high Bcl-2 expression in the basal layer of the bronchial epithelium probably protects this group of cells from apoptosis and is essential for proper tissue

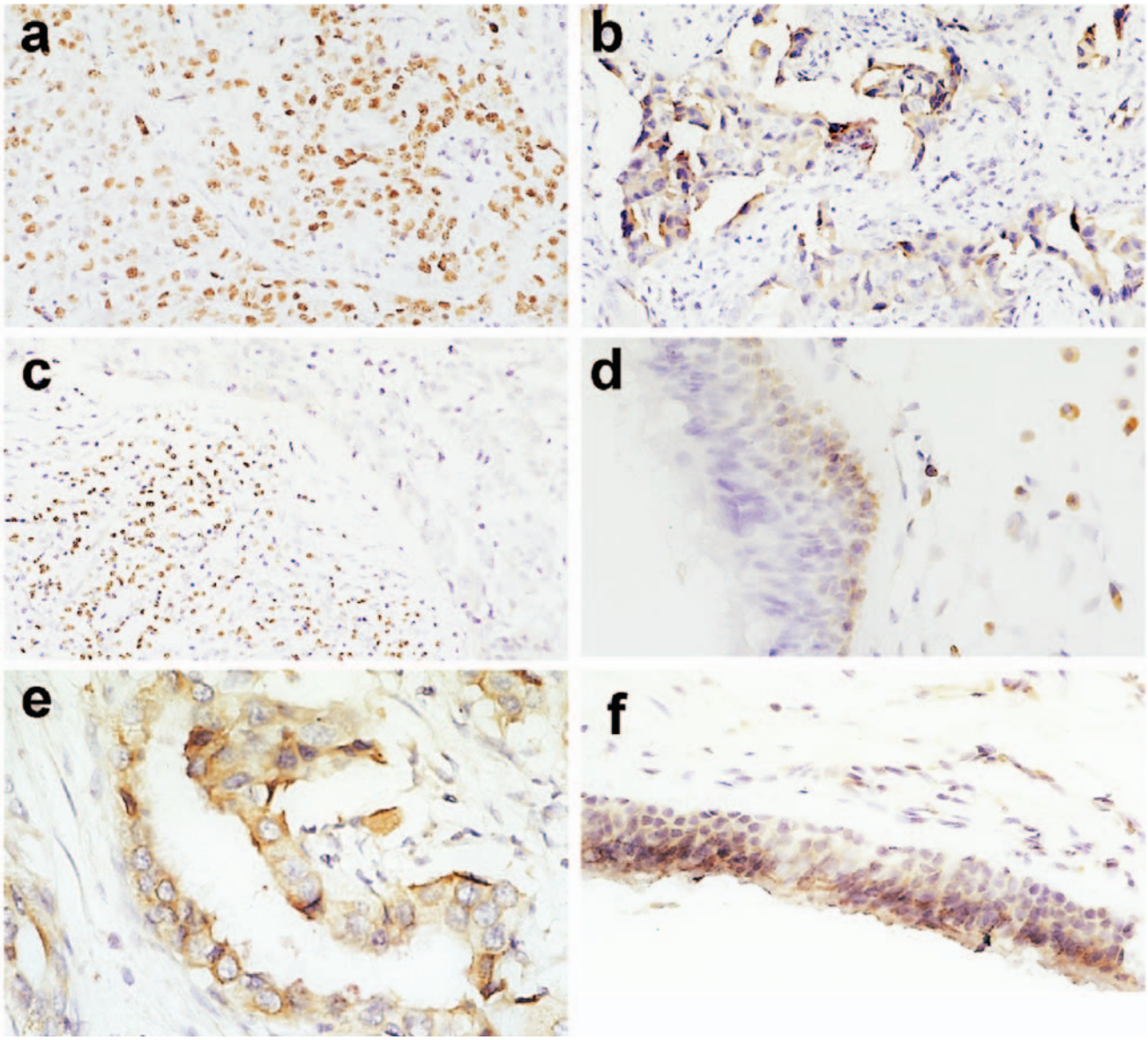

Figure 1. *Representative immunostaining of p53, Bcl-2 and Bax. a. Positive p53 nuclear expression in lung cancer (original magnification x200). b. Heterogenous immunoreactivity of Bcl-2 in lung cancer tissue (original magnification x200). c. Bcl-2 positive lymphocytes with negative cancer cells in lung cancer tissue (original magnification x200). d. Bcl-2 presence in basal layer of bronchial epithelium(original magnification x400). e. Bax cytoplasmatic immunoreactivity in lung cancer tissue (original magnification x400). f. Bax expression in bronchial stump (original magnification x400).*

regeneration. Similar to our observations the intense Bax expression in all epithelial cells was reported by Jeanmart *et al.* and the higher Bax than Bcl-2 expression (Bax/Bcl-2 ratio >1) is thought to be typical for normal bronchial epithelial tissue (17).

The differences in molecular structure of NSCLC subtypes remain unclear. Data indicating the elevated expression of p53 and Bcl-2 in squamous cell lung cancer compared with other histological types were reported (9, 18). Our results did not confirm this observation and are in agreement with the previously published data of Greatens *et al.* (19) in which there were no significant differences between p53 and Bcl-2 in relation to the histological structure of NSCLC. There is only scarce information about Bax expression which takes into account the different types of NSCLC. A higher Bax expression in adenocarcinoma than in squamous cell lung carcinomas was reported by Mori *et al.* (4) and is in agreement with our results.

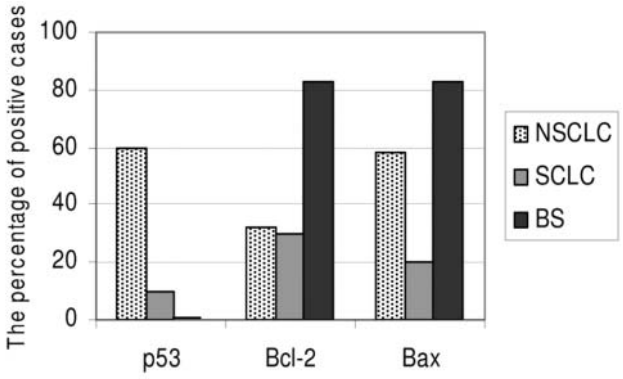

Figure 2. *Comparison of apoptotic marker immunoreactivity in NSCLC, SCLC and bronchial stump (BS).*

However, further analysis seems to be necessary to establish the expression of apoptotic markers in adenocarcinoma and squamous cell lung cancer.

Taking into account the functional association of p53 with the downstream regulated proteins Bax and Bcl-2, the interrelationship between the studied markers was evaluated. Our study revealed a lack of correlation between p53 and Bax or Bcl-2 in lung cancer tissue. No correlation between p53 and Bcl-2 status was also previously reported (20). Shabnam *et al.* (5) described a positive association of p53 and Bax expression, however only locally advanced squamous cell lung carcinoma was included in their study. In contrast to our findings, Gregorc *et al.* (9) revealed a negative correlation between Bcl-2 and Bax immunoreactivity. Ours is the first study to report abnormalities in the simultaneously analysed p53, Bcl-2 and Bax immunoreactivity in normal bronchial resection margin, found independently of co-existing lung cancer.

Similarly to data reported previously (21), the differences in apoptotic marker immunoreactivity reported in this study, in relation to the NSCLC and SCLC types of lung cancer indirectly confirm the different pathogenesis of these two lung pathologies. All our patients were under strict clinical control and possible associations between studied apoptotic markers and conventional disease parameters, as well as disease-free survival and overall survival will be published separately. The existing published data evaluating the interrelationships between p53, Bcl-2 and Bax expression in lung cancer indicate that further studies are recommended, especially on representative histopathologically verified subgroups of lung cancer. The evidence for the sequence of apoptotic pathways regarding the prognostic significance of the key regulators in lung cancer seems to be very important, especially for establishing the adequate therapeutic strategies for this disease.

References

1 Soussi T: The p53 tumor suppressor gene: from molecular biology to clinical investigation. Ann NY Acad Sci *910*: 121-137, 2000.

2 Cory S, Huang CSD and Adams MJ: The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene *22*: 8590-8607, 2003.

3 Groeger AM, Esposito V, De Luca A, Cassandro R, Tonini G, Ambrogi V, Baldi F, Goldfarb R, Mineo TC, Baldi A and Wolner E: Prognostic value of immunohistochemical expression of p53, bax, Bcl-2 and Bcl-x in resected non-small cell lung cancers. Histopathology *44*: 54-63, 2004.

4 Mori S, Ito G, Usami N, Yoshioka H, Ueda Y, Kodama Y, Takahashi M, Fong KM, Shimokata K and Sekido Y: p53 apoptotic pathway molecules are frequently and simultaneously altered in non-small cell lung carcinoma. Cancer *15*: 1673-1682, 2004.

5 Shabnam MS, Srinivasan R, Wali A, Majumdar S, Joshi K and Behera D: Expression of p53 protein and the apoptotic regulatory molecules Bcl-2, Bcl-XL and Bax in locally advanced squamous cell carcinoma of the lung Lung Cancer *45*: 181-188, 2004.

6 Brambilla E, Travis WD, Colby TV, Corrin B and Shimosato Y: The new World Health Organization classificantion of lung tumors. Eur Respir J *18*: 1059-1068 2001.

7 Beahrs OH, Henson DE, Hutter RVP and Kennedy BJ: Lung Manual for Staging of Cancer, 4th edition. Chicago American Joint Committee on Cancer, pp. 115-122, 1992.

8 Hwang JH, Lim SC, Kim YC, Park KO, Ahn SJ and Chung WK: Apoptosis and bcl-2 expression as predictors of survival in radiation-treated non-small cell lung cancer. Int J Radiat Oncol Biol Phys *50*: 13-18, 2001.

9 Gregorc V, Ludovini V, Pistola L Darwish S, Floriani I, Bellezza G, Sidoni A, Cavaliere A, Scheibel M, De Angelis V, Bucciarelli E and Tonato M: Relevance of p53, bcl-2 and Rb expression on resistance to cisplatin-based chemotherapy in advanced non-small cell lung cancer. Lung Cancer *39*: 41-48, 2003.

10 Borner MM, Brousset P, Pfanner-Meyer B, Bacchi M, Vonlanthen S, Hotz MA, Altermatt HJ, Schlaifer D, Reed JC and Betticher DC: Expression of apoptosis regulatory proteins of Bcl-2 family and p53 in primary resected lung cancer. Br J Cancer *79*: 952-958, 1999.

11 Walker C, Robertson LJ, Myskow MW, Pendleton N and Dixon GR: p53 expression in normal and dysplastic bronchial epithelium and in lung carcinomas. Br J Cancer *70*: 297-303, 1994.

12 Ohsaki Y, Toyoshima E, Fujiuchi S, Matsui H, Hirata S, Miyokawa N, Kubo Y and Kikuchi K: Bcl-2 and p53 protein expression in non-small cell lung cancers: correlation with survival time. Clin Cancer Res *2*: 915-920, 1996.

13 Shibata Y, Hidaka S, Tagawa Y and Nagayasu T: Bcl-2 protein expression correlates with better prognosis in patients with advanced non-small cell lung cancer. Anticancer Res *24*: 1925-1928, 2004.

14 Laudanski J, Chyczewski L, Niklinska WE Kretowska M, Furman M, Sawicki B and Niklinski J: Expression of bcl-2 protein in non-small cell lung cancer: correlation with clinicopathology and patient survival. Neoplasma *46*: 25-30, 1999.

15 Krug LM, Miller VA, Filippa DA Venkatraman E, Ng KK and Kris MG: Bcl-2 and bax expression in advanced non-small cell lung cancer: lack of correlation with chemotherapy response or survival in patients treated with docetaxel plus vinorelbine. Lung Cancer *39*: 139-143, 2003.

16 Walker C, Robertson L, Myskow MW and Dixon GR: Expression of the BCL-2 protein in normal and dysplastic bronchial epithelium and in lung carcinomas. Br J Cancer *72*: 164-169, 1995.

17 Jeanmart M, Lantuejoul S, Fievet F, Moro D, Sturm N, Brambilla Ch and Brambilla E: Value of immunohistochemical markers in preinvasive bronchial lesions in risk assessment of lung cancer. Clin Cancer Res *9*: 2195-2203, 2003.

18 Moldvay J, Scheid P, Wild P Nabil K, Siat J, Borrelly J, Marie B, Farre G, Labib T, Pottier G, Sesboue R, Bronner C, Vignaud JM, Martinet Y and Martinet N: Predictive survival markers in patients with surgically resected non-small cell lung carcinoma. Clin Cancer Res *6*: 1125-1134, 2000.

19 Greatens TM, Niehans GA, Rubins JB Jessurun J, Kratzke RA, Maddaus MA and Niewoehner DE: Do molecular markers predict survival in non-small cell lung cancer? Am J Respir Crit Care Med *157*: 1093-1097, 1998.

20 Appolinairo RM, van der Valk P, de Jong JS, Deville W, van Ark-Otte J, Dingemans AM, van Mourik JC, Postmus PE, Pinedo HM and Giaccone G: Prognostic value of the expression of p53, bcl-2 and bax oncoproteins and neovascularisation in patients with radically resected non-small-cell lung cancer. J Clin Oncol *15*: 2456-2466, 1997.

21 Dursun BA, Memis L, Dursun A, Bayiz H and Ozkul M: Clinical importance of correlations between p53 immunoreactivity and clinicopathological parameters in lung carcinoma. Pathol Oncol Res *5*: 285-290, 1999.

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