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# Bcl-2 Protein Expression in Lung Cancer and Close Correlation with Neuroendocrine Differentiation

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***For determination of the cellular distribution of bcl-2 expression in lung cancer and clarification of its correlation with cell neuroendocrine differentiation, Bcl-2 immunostaining was carried out on a large series of formalin-fixed, paraffin-embedded lung cancer samples, and four general neuroendocrine marker and seven peptide hormone stainings were carried out on all Bcl-2-positive squamous cell carcinomas and adenocarcinomas of the lung as well as on 8 pulmonary neuroendocrine carcinomas histologically diagnosed. In addition, 3 small cell lung cancer cell lines were studied by Western blotting. Neuroendocrine differentiation in Bcl-2-negative squamous cell carcinomas and adenocarcinomas was examined with chromogranin A and α-subunit of Go protein stainings. Bcl-2 protein was detected in 104/111 small cell carcinomas, 8/8 neuroendocrine carcinomas, 0/6 typical (well differentiated) carcinoids, 23/64 squamous cell carcinomas, 4/65 adenocarcinomas, and all 3 small cell lung cancer cell lines. All 8 neuroendocrine carcinomas, 11 of the Bcl-2-positive squamous cell carcinomas, and all 4 Bcl-2-positive adenocarcinomas expressed multiple neuroendocrine markers. The distributions of Bcl-2 and neuroendocrine marker immunoreactivity closely paralleled each other on consecutive sections. In squamous cell carcinomas, Bcl-2-positive cells could be roughly subdivided into those with neuroendocrine differentiation features, usually demonstrating intense Bcl-2 staining, with basaloid tumor cells usually expressing weak to moderate Bcl-2 staining. The present study clearly shows Bcl-2 protein expression to be remarkably differentially regulated according to histological types of lung cancers and to appear to quite likely be closely associated with neuroendocrine differentiation of tumor cells, indicating that bcl-2 is importantly involved in cell development and differentiation, in addition to protecting cells from apoptosis. Bcl-2 might be usable as a neuroendocrine marker in lung cancers and possibly also in neural-crest-derived tumors. (Am J Pathol 1996, 148:837–846)***

Among oncogenes, *bcl*-2 is unique because of its unambiguous role in prolonging cell survival by blocking programmed cell death (apoptosis) without affecting cell proliferation.[1] It was first detected in the majority of follicular non-Hodgkin's B cell lymphomas with t(14;18) translocation, which consequently brought about the dysregulation of the *bcl*-2 gene and overexpression of its $M_r$ 26,000 protein.[2,3] The Bcl-2 protein has been detected in many normal hematopoietic and nonhematopoietic tissues.[4,5]

By transferring *bcl*-2 expression vectors to pre-B cells, *bcl*-2 was found to prolong cell survival in the absence of interleukin-3, a cytokine critical for maintaining hematopoietic cell survival *in vitro*.[6] Bcl-2 protein present at high levels prevents cells from apoptosis in many instances,[7–9] indicating that this protein blocks the final common pathway by which cells enter apoptosis. A limited number of solid tumors, such as lung cancer, Epstein-Barr virus-associated nasopharyngeal carcinoma, gastric adenocarcinoma, and neuroblastoma, have been shown to express *bcl*-2, implying possible oncogenic functions.[10–14] Such functions may be closely related to

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apoptosis-suppressing ability. *bcl*-2 transfection results in the neoplastic transformation of mouse NIH3T3 cells and, in *bcl*-2 transgenic mice, follicular lymphoproliferation may develop followed by high grade malignant lymphomas.[15–17]

The *bcl*-2 gene is important for suppressing apoptosis. Its protein may have other functions such as direct involvement in terminal cell differentiation in addition to merely protecting cells from apoptosis. The high frequency of *bcl*-2 expression has been clearly demonstrated in the central nervous system, neural-crest-derived cells, and their corresponding tumors.[4,5,18–22] In these studies, *bcl*-2 was extensively expressed in neurons of developing brain, but in adult brain confined to much fewer neurons and endocrine cells of the anterior pituitary gland. In developing brain, with the progress of differentiation in cortical regions, postmitotic cells contain a much greater content of Bcl-2 protein than stem cells, indicating the involvement of *bcl*-2 in differentiation.[18] In neuroblastoma cell lines, Bcl-2 protein has been detected at high levels in cells with more neuronal differentiation although only slightly or not at all in cells with more epithelial differentiation.[18] *bcl*-2 expression is thus shown possibly associated with a certain pathway of neuroectodermal cell differentiation. Bronchial carcinoids and small cell carcinomas of the lung (SCLCs) are two extremes of the extent of malignancy, each possessing distinct neuroendocrine properties.[23–25] Some non-SCLCs have also been shown to have neuroendocrine properties,[26,27] and 12 of 14 SCLC cell lines and 15 of 23 SCLCs have been reported to express *bcl*-2,[11,28] which is consistent with our recent observation that 54 of 60 SCLCs were positive for Bcl-2, as shown by immunohistochemical staining.[29] By using frozen sections, some non-SCLCs have been noted to express Bcl-2 protein.[10] It follows from these findings that *bcl*-2 expression may possibly be related to cellular neuroendocrine differentiation in lung cancer. In this study, examination was first made of the distribution of *bcl*-2 expression in major histological types of lung cancer, using a large series of formalin-fixed, paraffin-embedded samples. Secondly, *bcl*-2 expression was shown to be closely related to cellular neuroendocrine differentiation in lung cancer.

## Materials and Methods

### Tissue Samples

Formalin-fixed, paraffin-embedded surgical, biopsy, and autopsy tissue samples of 111 cases of SCLCs obtained from the pathology files of Kitasato University Hospital from 1986 to 1994 and of the East National Cancer Center Hospital from 1986 to 1991 were used. According to the World Health Organization histological typing criteria of lung tumors,[30] SCLCs were diagnosed and subtyped into 52 oat cell and 59 intermediate cell type SCLCs. From the pathology files of Kitasato University Hospital dating from 1980 to 1994, surgically resected tissue samples of 64 squamous cell carcinomas and 65 adenocarcinomas of the lung were randomly picked up, and 6 typical (well differentiated) pulmonary carcinoids and 8 pulmonary neuroendocrine carcinomas were independently chosen for this study. The latter were defined morphologically as malignant tumors that were different from SCLC and typical carcinoid but consisted of cells with abundant cytoplasm, coarse chromatin, frequent nucleoli, and mitotic figures. The cells were arranged in organoid, trabecular, palisading, rosette-like patterns and showed frequent necrosis[31,32] (Figure 1). For comparison, six

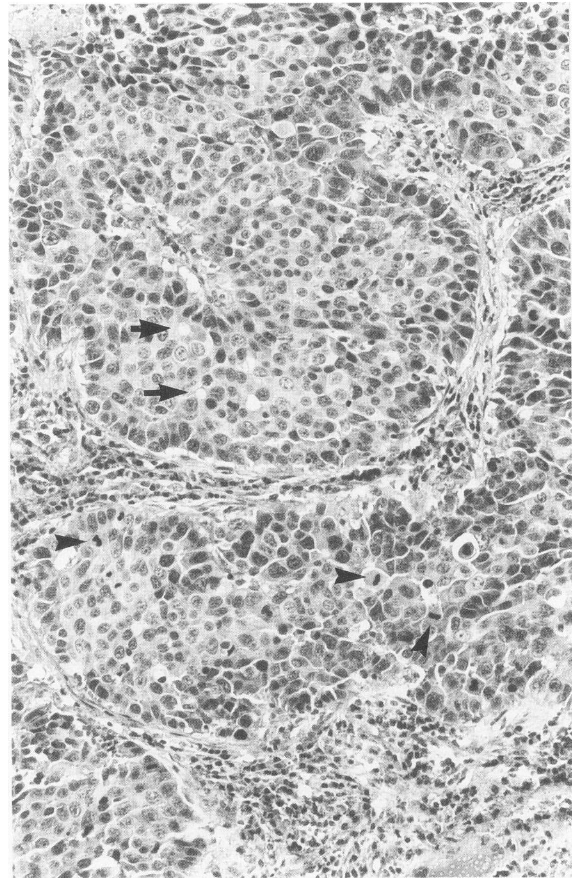

**Figure 1.** *Neuroendocrine carcinoma (case 5 in Table 3) is histologically different from SCLC and typical carcinoid and shows cell pleomorphism, increased number of mitotic figures* (**arrowhead**)*, and hyperchromatic nuclei. Nucleoli in some cells are prominent. A hint of palisading at the edge of tumor cell nests and rosette-like structures* (**arrow**) *can be observed. Hematoxylin and eosin; magnification,* ×*200.*

extra-pulmonary small cell carcinomas of the pancreas, skin, cervix, urinary bladder, esophagus, and larynx were used. According to the extent of tumor differentiation, squamous cell carcinomas of the lung were divided into 27 poorly differentiated and 37 moderately to well differentiated cases, whereas the 65 adenocarcinomas consisted of 17 poorly differentiated and 48 moderately to well differentiated ones.

### Antibodies and Immunohistochemical Stainings

By the labeled streptavidin biotin (LSAB) method, Bcl-2 protein was detected using a mouse monoclonal antibody, Bcl-2/124 (Dako, Glostrup, Denmark), raised against a synthetic peptide sequence corresponding to amino acids 41 to 54 of Bcl-2 protein and recognizing the 26-kd protein by Western blotting.[33] Deparaffinized sections were treated in a microwave oven twice for 5 minutes in 10 mmol/L boiling citrate buffer (pH 6.0) to retrieve the antigenicity of Bcl-2 protein. To block endogenous peroxidase activity, the sections were incubated in 1% $H_2O_2$ in methanol for 40 minutes followed by incubations with the Bcl-2/124 antibody diluted 1:100 in phosphate-buffered saline (PBS) with 2% normal swine serum at 4°C overnight and then with the link antibody (biotinylated anti-rabbit and anti-mouse immunoglobulins) and finally with the streptavidin conjugated to horseradish peroxidase of the LSAB kit (Dako, Carpinteria, CA) for 15 minutes according to the instructions of the manufacturer. Peroxidase activity was developed in 0.02% 3,3′-diaminobenzidine with 0.01% $H_2O_2$ for 5 minutes. Slides were counterstained with hematoxylin, dehydrated, and mounted. Normal tonsil sections and infiltrating lymphocytes in most tumor tissues served as positive controls, whereas the Bcl-2 primary antibody was omitted to make the negative control. To determine the correlation between Bcl-2 protein expression and neuroendocrine differentiation in lung cancer, polyclonal general neuroendocrine markers including neuron-specific enolase (NSE; Dako, Denmark), protein gene product 9.5 (PGP-9.5[34]; Ultraclone, Cambridge, England), the $\alpha$-subunit of Go protein (Go-$\alpha$[35]; MBL, Nagoya, Japan), chromogranin A (CGA; Dako, Denmark) and antibodies against seven specific peptide hormones including adrenocorticotropic hormone (Dako, CA), calcitonin (Incstar, Stillwater, MN), pro-gastrin-releasing peptide[36] (a gift from Dr. K. Yamaguchi, National Cancer Center Research Institute, Tokyo, Japan), serotonin (Sera-Lab, Crawley Down, England), somatostatin (Dako, Denmark), human chorionic gonadotropin $\alpha$-subunit (ICN Biochemicals, Irvine, CA), and pancreatic polypeptide (a gift from Dr. R. F. Chance, Lilly Research Laboratories, Indianapolis, IN) were immunostained by the LSAB method on Bcl-2-positive pulmonary squamous cell carcinomas and adenocarcinomas as well as on all eight pulmonary neuroendocrine carcinomas. To examine neuroendocrine differentiation in the Bcl-2-negative squamous cell carcinomas and adenocarcinomas, CGA and Go-$\alpha$ stainings were performed and Bcl-2 staining results were rechecked in these cases. The staining procedures were the same as Bcl-2 staining but without antigen retrieval. For accurate assessment of the staining results of the general neuroendocrine markers, particularly of NSE and PGP-9.5, which usually showed high overall background or nonspecific staining, tumors expressing at least two of the general neuroendocrine markers or coexpressing general neuroendocrine marker(s) and specific peptide hormone(s) were defined as those with neuroendocrine differentiation. All stained sections were independently interpreted and rechecked by two of the authors (S.-X. Jiang and T. Kameya).

### Immunoblotting Analysis

To confirm the specificity of Bcl-2 antibody staining under the present experimental conditions, three SCLC cell lines, PC6 donated by the Department of Surgery, Tokyo Medical College, (Tokyo, Japan), and SEKI and Lu130 donated by the National Cancer Center Research Institute (Tokyo, Japan) and the fibroblast cell line, SFTY, purchased from JCRB (Tokyo, Japan), were subjected to immunoblotting analysis. Cultured cells were sonicated in PBS with 1 mmol/L phenylmethylsulfonyl fluoride, and the supernatants were collected after 5000× *g* centrifugation for 10 minutes. For each cell line, 20 $\mu$g of protein were electrophoresed on a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel, after the proteins had been denatured in SDS sample buffer, and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). The Bcl-2/124 antibody was used at a 1:200 dilution and a chemiluminescence reagent was employed according to manufacturer instructions (DuPont NEN, Boston, MA).

## Results

### Immunohistochemical Staining

The results of Bcl-2 staining are summarized in Table 1. Of the 111 SCLCs, 104 (93.7%) showed Bcl-2

Table 1. *Bcl-2 Staining Results in Lung Cancers and Six Extra-Pulmonary Small Cell Carcinomas*

| Histology | Number of cases | Positive/ negative | Diffusely/ focally positive* | Intensely/ weakly positive† |
|---|---|---|---|---|
| Small cell carcinoma | 111 | 104/7 | 93/11 | 73/31 |
| Oat cell type | 52 | 49/3 | 40/9 | 35/14 |
| Intermediate cell type | 59 | 55/4 | 53/2 | 38/17 |
| Squamous cell carcinoma | 64 | 23/41 | 14/9 | 13/10 |
| Poorly differentiated | 27 | 10/17 | 9/1 | 8/2 |
| Moderately to well differentiated | 37 | 13/24 | 5/8 | 5/8 |
| Adenocarcinoma | 65 | 4/61 | 3/1 | 3/1 |
| Poorly differentiated | 17 | 1/16 | 0/1 | 1/0 |
| Moderately to well differentiated | 48 | 3/45 | 3/0 | 2/1 |
| Neuroendocrine carcinoma | 8 | 8/0 | 8/0 | 8/0 |
| Typical carcinoid | 6 | 0/6 | 0/0 | 0/0 |
| Extra-pulmonary small cell carcinoma | 6 | 6/0 | 6/0 | 6/0 |

*Diffusely positive, more than 50% of tumor cells stained; focally positive, fewer than 50% of tumor cells stained.
†Intensely positive, staining intensity of tumor cells the same as or approximately comparable to that of internal lymphocytes; weakly positive, staining intensity of tumor cells much weaker than that of internal lymphocytes.

immunoreactivity (Figure 2). Staining intensity in some cases was heterogeneous among tumor cell populations, varying from area to area and even from cell to cell. Neither *bcl*-2 expression nor staining intensity differences could be detected for oat cell and intermediate cell type SCLCs (Wilcoxon test, $P = 0.8333$ and $P = 0.7984$). Staining in biopsy and surgical samples was stronger than in autopsy specimens. Of 111 SCLCs, only 7 failed to stain for Bcl-2. All were autopsy cases, including 3 oat cell and 4 intermediate cell type SCLCs, and infiltrating lymphocytes in the same sections did not stain for Bcl-2.

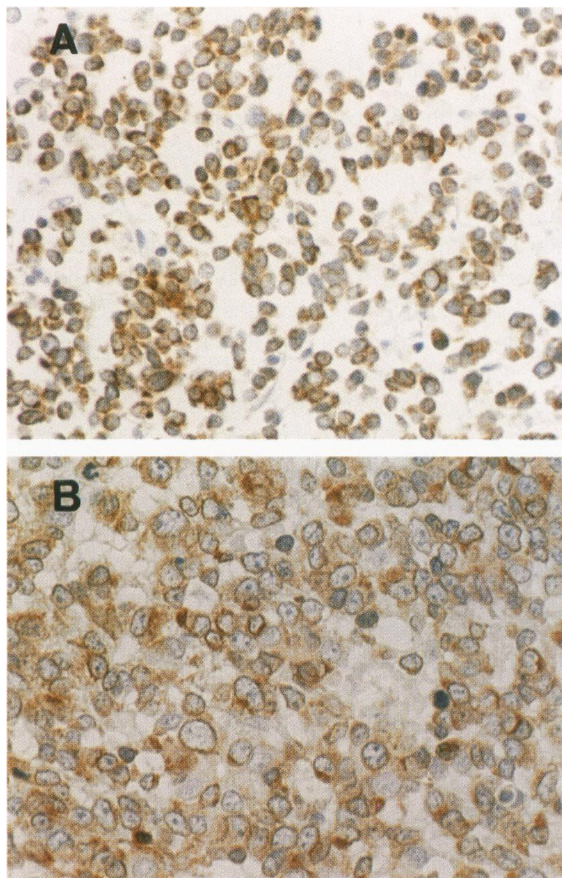


Figure 2. *Immunoreactivity for Bcl-2 in oat cell* (A) *and intermediate cell* (B) *type SCLCs. Most tumor cells show strong staining in cytoplasm. LSAB; magnification,* ×*400.*

All eight pulmonary neuroendocrine carcinomas and six extra-pulmonary small cell carcinomas showed diffuse and intense immunoreactivity for Bcl-2. Typical pulmonary carcinoid staining in all six cases was not seen.

Of the 64 squamous cell carcinomas, 23 (36%) stained for Bcl-2. When *bcl*-2 expression was analyzed together with the extent of tumor differentiation, diffuse and/or intense immunoreactivity for Bcl-2 was observed much more often in poorly differentiated squamous cell carcinomas than in those differentiated moderately to well. Of the 13 intensely and 14 diffusely Bcl-2-positive cases, 8 and 9 were poorly differentiated, respectively (Table 1). In 2 poorly differentiated cases, Bcl-2 staining was positive only in undifferentiated cell regions without any indication of keratinization or intercellular bridges, whereas staining was negative in adjacent regions with keratinizing squamous cell differentiation except for some sporadic cells in transitional areas (Figure 3, A and B). In moderately to well differentiated squamous cell carcinomas, Bcl-2 immunoreactivity was located mainly in basaloid tumor cells but only slight or completely absent from cells more central to tumor nests (Figure 4).

Of the 65 adenocarcinomas, only 4 (6.2%) were positive for Bcl-2, and 3 of these were well differentiated with typical glandular histology (Figure 5A). The other one was poorly differentiated.

The frequency of Bcl-2 protein expression was much higher in SCLCs than squamous cell carcino-

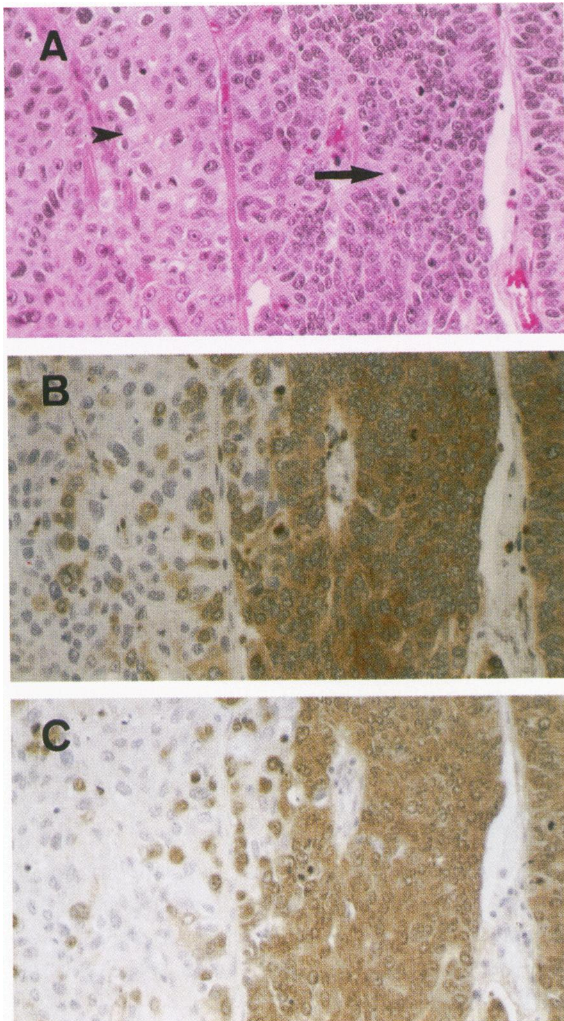


Figure 3. *Squamous cell carcinoma.* A: *The left part shows squamous cell differentiation* (arrowhead), *and the right part shows no feature of differentiation* (arrow). *Hematoxylin and eosin; magnification,* ×*200.* B: *Bcl-2 immunoreactivity in most cells of the undifferentiated region but only in sporadic cells in the differentiated area. LSAB; magnification,* ×*200.* C: *PGP-9.5 immunoreactivity and its same distribution with Bcl-2 staining on consecutive sections. LSAB; magnification,* ×*200.*

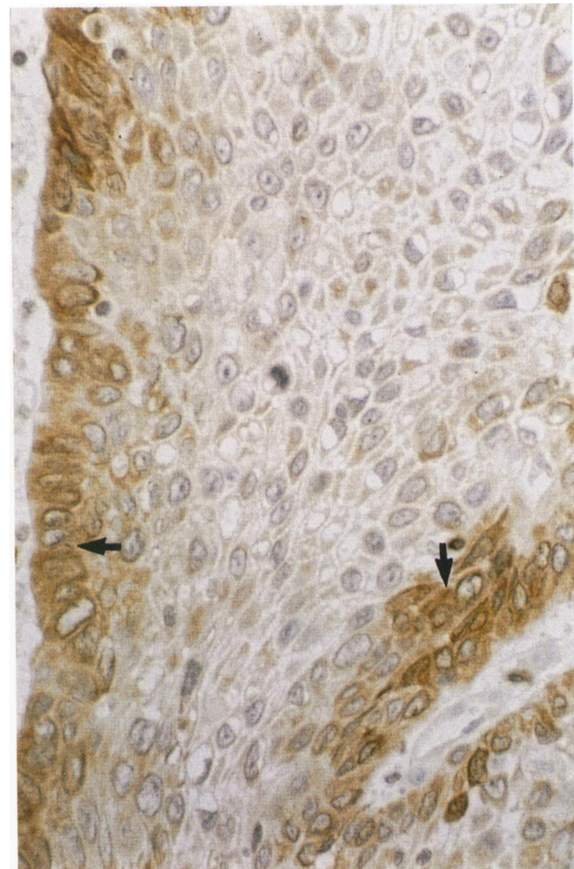

Figure 4. *Moderately differentiated squamous cell carcinoma. Bcl-2 immunoreactivity is evident in basaloid tumor cells* (arrow). *It is weak or absent in more differentiated areas. LSAB; magnification,* ×*200.*

mas or adenocarcinomas ($P < 0.0001$, respectively, Wilcoxon test). It was also significantly higher in squamous cell carcinomas than in adenocarcinomas ($P < 0.0001$, Wilcoxon test).

The immunohistochemical staining results of the 4 general neuroendocrine markers and the 7 specific peptide hormones in Bcl-2-positive squamous cell carcinomas and adenocarcinomas are presented in Table 2. Of the 23 Bcl-2-positive squamous cell carcinomas, 11 (47.8%) expressed multiple neuroendocrine markers. The mean number of neuroendocrine markers expressed per case was 4. Neuroendocrine marker expression in Bcl-2-positive squamous cell carcinomas was observed primarily in poorly or undifferentiated regions with intense Bcl-2 staining. Of the 11 neuroendocrine marker-positive squamous cell carcinomas, only 2 were moderately differentiated. In the 2 poorly differentiated cases that showed Bcl-2 staining only in undifferentiated cell areas, neuroendocrine marker expression also was confined mainly to the undifferentiated cell regions. On consecutive sections, the distribution of neuroendocrine marker immunoreactivity paralleled closely Bcl-2 staining (Figure 3C). Based on the present findings for Bcl-2 and neuroendocrine marker stainings in squamous cell carcinomas, Bcl-2-positive tumor cells could be subdivided into those with neuroendocrine differentiation features, usually demonstrating intense Bcl-2 staining, and basaloid tumor cells usually expressing weak to moderate Bcl-2 staining.

All four Bcl-2-positive adenocarcinomas expressed multiple neuroendocrine markers, the mean number of which was 6.6 per case. On consecutive sections, a surprisingly close correlation between neuroendocrine marker and Bcl-2 expression was observed. Neuroendocrine marker expression was confirmed in all cases to be present in Bcl-2-positive

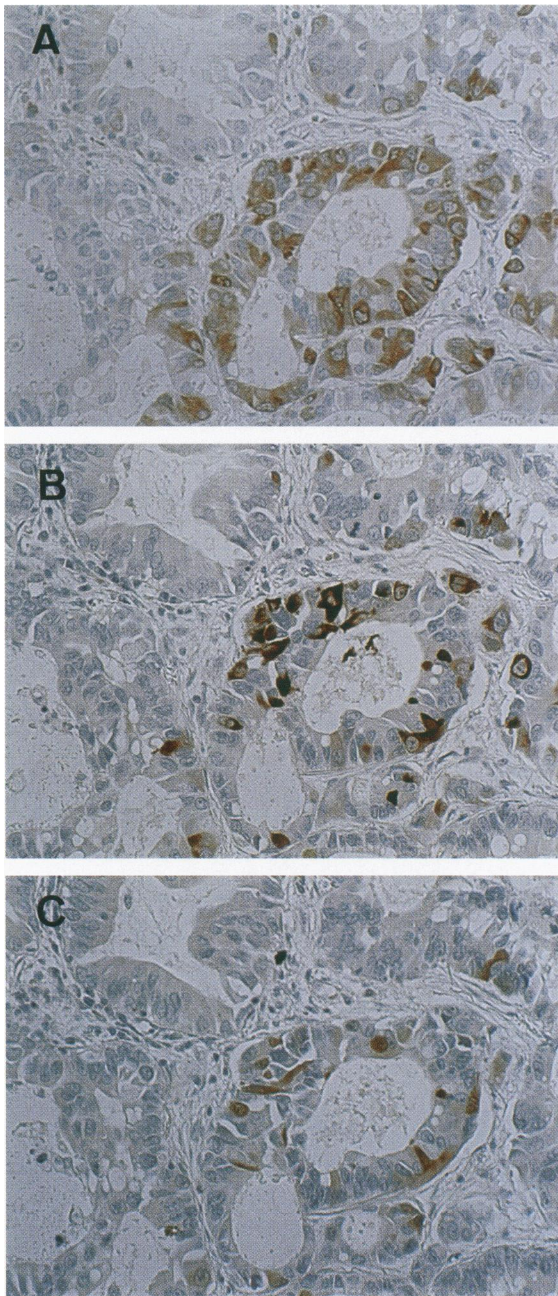


**Figure 5.** *Well differentiated tubular adenocarcinoma. On consecutive sections, locations of immunoreactivity for CGA* (**B**) *and pro-gastrin-releasing peptide* (**C**) *are strictly confined to Bcl-2-positive regions* (**A**). *LSAB; magnification,* ×*200.*

regions (Figure 5). The distributions of immunoreactivity for different neuroendocrine markers did not always overlap, but Bcl-2 staining covered nearly all regions that were positive for neuroendocrine markers.

The staining results of neuroendocrine markers in eight pulmonary neuroendocrine carcinomas are summarized in Table 3. Every case showed staining for multiple neuroendocrine markers. The mean number of neuroendocrine markers expressed per case was seven.

Of the 41 Bcl-2-negative squamous cell carcinomas and the 61 Bcl-2-negative adenocarcinomas, 2 and 9 cases, respectively, showed positive staining for CGA and/or Go-$\alpha$ in sparsely scattered tumor cells (much fewer than 1% of total tumor cells). Based on these findings, we rechecked Bcl-2 staining in the corresponding cases and found very few and scattered Bcl-2-positive cells in the 2 squamous cell carcinomas and 3 of the 9 adenocarcinomas, but we failed to find any Bcl-2-positive cell in the remaining 6 adenocarcinomas.

### *Immunoblotting Analysis*

As shown in Figure 6, a discrete band at 26 kd was found in all three SCLC cell lines, Lu130, SEKI, and PC6. In these lines, Bcl-2 protein content was approximately the same, although somewhat lower in the PC6 cell line. In the fibroblast cell line, SFTY, no 26-kd Bcl-2 protein could be detected.

## *Discussion*

Lung cancer tumorigenesis may possibly proceed in multiple steps and be due to multiple interrelated genetic events.[37,38] *bcl*-2 is an oncogene that can contribute to malignancy by blocking apoptosis and synergizing with classical oncogenes.[1,15–17,39,40] In the present study, a surprisingly high incidence of *bcl*-2 expression was noted in SCLCs. The negative staining in 7 cases may possibly have been due to antigenic loss by postmortem change, unsuitable fixation, or embedding because of the simultaneous negativity for Bcl-2 in infiltrating lymphocytes in the same sections. The present results are similar to two recent reports showing 12 of 14 SCLC cell lines and 15 of 23 SCLCs to express *bcl*-2.[11,28] The positive rate of Bcl-2 staining of their 23 SCLCs is somewhat lower than that of the present study, but the Bcl-2 staining of infiltrating lymphocytes in their 8 negative cases remains to be elucidated, and the discrepancy, to some extent, may be attributable to fixation or embedding. Because of the oncogenic potential of *bcl*-2 and its extremely high incidence of expression in SCLCs, the results here indicate *bcl*-2 to possibly be importantly involved in the tumorigenesis of SCLC. *bcl*-2 may function in tumorigenesis either by blocking apoptosis to increase the possibility of SCLC progenitor cell participation in secondary genetic events resulting in an increase in tumor mass or

Table 2. *Expression of Neuroendocrine Markers in Bcl-2-Positive Squamous Cell Carcinomas and Adenocarcinomas*

| Histology | Number of cases | Positive cases | | | | | | | | | | | Any 2 neuroendocrine markers positive |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NSE | CGA | PGP-9.5 | Go-α | CT | PP | pGRP | 5-HT | ACTH | HCG-α | SS | |
| Squamous cell carcinoma | 23 | 8 | 5 | 9 | 8 | 4 | 0 | 2 | 0 | 3 | 2 | 3 | 11 |
| Poorly differentiated | 10 | 7 | 4 | 7 | 6 | 4 | 0 | 2 | 0 | 2 | 2 | 2 | 9 |
| Moderately to well differentiated | 13 | 1 | 1 | 2 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 2 |
| Adenocarcinoma | 4 | 3 | 3 | 4 | 4 | 4 | 0 | 2 | 0 | 2 | 3 | 2 | 4 |
| Poorly differentiated | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| Moderately to well differentiated | 3 | 3 | 2 | 3 | 3 | 3 | 0 | 2 | 0 | 1 | 2 | 2 | 3 |

CT, calcitonin; PP, pancreatic polypeptide; pGRP, pro-gastrin-releasing peptide; 5-HT, serotonin; ACTH, adrenocorticotropic hormone; HCG-α, human chorionic gonadotropin α-subunit; SS, somatostatin.

by being involved in cell differentiation as discussed below.

In normal tissue, *bcl*-2 may have a function in the pathway to terminal cell differentiation in addition to merely protecting cells from apoptosis. In embryonic kidney, skin, and cartilage, *bcl*-2 expression is restricted to cells at an early stage of transition from undifferentiated stem cells to committed precursor cells.[5] In the adult, *bcl*-2 expression in selective basal compartments of epithelia may be related to cell differentiation and development.[13] Neurons in the adult central nervous system are cells with long life spans, and *bcl*-2 expression is confined to only a few specific neurons. But this expression is extensive in neurons of the developing central nervous system. In developing brain, there are two different populations of cells expressing *bcl*-2, one of proliferating stem cells and another of postmitotic cells. Bcl-2 protein content is much greater in postmitotic differentiating cells than in stem cells, indicating its expression to be essential not only for inhibiting apoptosis but also neuronal differentiation.[18] The peripheral nervous system and amine/peptide hormone-associated endocrine system are closely related embryologically and anatomically. The high frequency of *bcl*-2 expression has been found in these systems at immature or developing stages as well as their tumors.[4,5,18–22] The cellular heterogeneity of neuroblastoma is quite evident. When the neuroblastoma cell line, SKNSH, expressing an intermediate level of Bcl-2 protein, was subcultured into neuronal-like SY5Y and epithelial-like SHEP sublines, the former expressed a high level of Bcl-2 protein, whereas in the latter, no Bcl-2 protein could be detected,[19] suggesting the expression of this oncogene protein to be correlated with neuronal differentiation. Ben-Ezra et al[11] studied *bcl*-2 expression in eight SCLC cell lines and found that only one cell line, H82, did not express *bcl*-2, but H82 was a variant SCLC cell line with fewer neuroendocrine features. In the present study, the high incidence of *bcl*-2 expression in lung cancers with neuroendocrine features was observed (104/111 SCLCs, 8/8 pulmonary neuroendocrine carcinomas, and 3/3 SCLC cell lines). All 6 extra-pulmonary small cell carcinomas showed intense and diffuse Bcl-2 staining. A close correlation was also noted between Bcl-2 and neuroendocrine marker expression in squamous cell carcinomas and adenocarcinomas of the lung; 11 (47.8%) Bcl-2-positive squamous cell carcinomas and four (100%) Bcl-2-positive adenocarcinomas expressed multiple neuroendocrine markers. On consecutive sections, it is a significant finding that Bcl-2 and neuroendocrine marker immu-

Table 3. *Neuroendocrine Marker Staining Results in Eight Neuroendocrine Carcinomas*

| Case | NSE | CGA | PGP-9.5 | Go-α | CT | PP | pGRP | 5-HT | ACTH | HCGα | SS | total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | − | + | + | + | − | − | − | − | − | − | 4 |
| 2 | + | + | + | + | + | − | + | + | + | + | + | 10 |
| 3 | + | + | + | − | + | + | + | − | − | + | + | 8 |
| 4 | + | + | + | + | + | − | + | + | − | + | − | 8 |
| 5 | + | + | + | + | + | − | + | − | − | + | − | 7 |
| 6 | + | + | + | + | + | − | + | − | + | + | − | 8 |
| 7 | + | − | + | + | − | − | − | − | − | − | − | 3 |
| 8 | + | + | + | + | + | − | − | − | + | + | + | 8 |
| Total 8 | 8 | 6 | 8 | 7 | 7 | 1 | 5 | 2 | 3 | 6 | 3 | |

CT, calcitonin; PP, pancreatic polypeptide; pGRP, pro-gastrin-releasing peptide; 5-HT, serotonin; ACTH, adrenocorticotropic hormone; HCG-α, human chorionic gonadotropin α-subunit; SS, somatostatin.

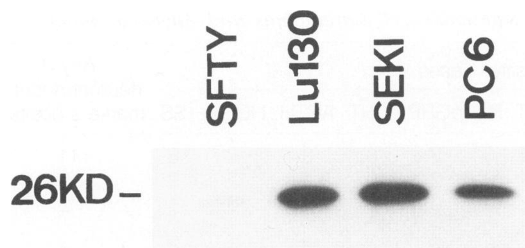


**Figure 6.** *Western blotting of protein extracted from three SCLC cell lines, Lu130, SEKI, and PC6, and the fibroblast cell line SFTY stained with the Bcl-2/124 monoclonal antibody. All three SCLCs show a discrete 26-kd band. SFTY shows no 26-kd protein.*

noreactivity distributions closely paralleled each other. It thus follows that Bcl-2 protein expression is very much differentially regulated in lung cancer histological types and may quite likely be closely associated with neuroendocrine differentiation. Accordingly, Bcl-2 might be usable as a neuroendocrine marker in lung cancers and possibly also in neural-crest-derived tumors.[20] In the present study, very few and scattered cells showed CGA and/or Go-$\alpha$ staining but no Bcl-2 staining in 6 adenocarcinomas. We think that the sparsely scattered neuroendocrine marker-positive cells may be too few to be taken as an index of neuroendocrine differentiation in these cases.

Although the present data show a close correlation between tumor neuroendocrine differentiation and *bcl*-2 expression, it should be noted that six typical (well differentiated) carcinoids in the present study did not stain for Bcl-2. *bcl*-2 expression may possibly be associated with poorer differentiation and more malignant phenotypes of neuroendocrine tumors but not with completely differentiated neuroendocrine cells and well differentiated neuroendocrine tumor cells. In the present study, all eight pulmonary neuroendocrine carcinomas that stained diffusely and intensely for Bcl-2 showed some extent of neuroendocrine immaturity. Our observations are in accordance with the findings that the levels of Bcl-2 protein in neuroblastoma are heterogeneous in undifferentiated cells, highest in more differentiated but still immature cells as seen in immature ganglionic cells at intermediate stages of neuronal differentiation, and lowest or totally absent in the most differentiated cells similar to mature ganglionic cells.[41] Additional study should be conducted on a larger number of pulmonary as well as extra-pulmonary typical (well differentiated) and atypical (poorly differentiated) carcinoid tumors, in comparison with non-neoplastic neuroendocrine cells at embryonic and adult stages.

Although close parallelism was noted between Bcl-2 and neuroendocrine marker expression, 12 Bcl-2-positive squamous cell carcinomas did not stain for neuroendocrine markers, 11 of which were moderately to well differentiated. In these squamous cell carcinomas, Bcl-2 immunoreactivity was heterogeneous in distribution and stronger in cells situated in basal areas of tumor nests. It was much weaker or negative in areas more central to the nests. Basaloid tumor cells may thus possess to some extent the features of normal basal cells that are not related to neuroendocrine cells, and *bcl*-2 may provide a survival advantage to stem cells.[4,5] Based on the results of Bcl-2 and neuroendocrine marker stainings, the 23 Bcl-2 positive squamous cell carcinomas shown in Table 2 could be roughly included in one category as poorly differentiated with either focal or total neuroendocrine features. The present study shows that at least some cases diagnosed as poorly differentiated squamous cell carcinoma with intense and diffuse immunoreactivity for Bcl-2 may actually have been neuroendocrine carcinomas but misdiagnosed by conventional light microscopic examination owing to poor differentiation. Pulmonary neuroendocrine carcinomas differing from SCLCs and carcinoids and indistinguishable from non-SCLCs by light microscopy have been reported.[27,42] A second category was moderately to well differentiated squamous cell carcinomas without neuroendocrine features, showing weak to moderate Bcl-2 staining usually of basaloid tumor cells.

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