bcl-2 and p53 Protein Expression in Non-Small Cell Lung Cancers: Correlation with Survival Time

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ABSTRACT
Apoptosis-related genes have received increasing attention in carcinogenesis, drug sensitivity, radiation sensitivity, and patient survival. bcl-2 and mutated p53 genes have been reported to inhibit apoptosis. To determine bcl-2 and p53 protein expression and their impacts on survival time in lung cancers, we studied 99 surgically resected, paraffin-embedded non-small cell lung cancer (NSCLC) specimens by immunohistochemical staining. The bcl-2 protein was expressed in 19.2% of NSCLCs. bcl-2-positive cases were found in 30.4% of stages I and II carcinomas in 36.8% of squamous cell carcinomas. Patients with bcl-2 expression survived longer than those without. p53 protein was found in 44.4%; there was no significant difference in survival time between patients with and without p53 expression. Patients who were both bcl-2 positive and p53 negative survived significantly longer than those who were bcl-2 negative or p53 positive. These results suggest that bcl-2 protein expression can be histologically specific and stage dependent, and that the bcl-2 protein expression is potentially valuable for prognosis in NSCLC, particularly in the early stages, when bcl-2 protein expression is considered with mutant p53 protein expression.

INTRODUCTION
bcl-2 is a proto-oncogene that was cloned from a human acute B-cell leukemia cell line with the t(14;18) chromosome translocation (1). Expression of the bcl-2 protein is not specific for the t(14;18) chromosomal translocation (2). Transfection of the bcl-2 gene into cultured lymphoma cells inhibited p53-mediated apoptosis (3). Therefore, the function of the bcl-2 gene is considered antiapoptotic (4). bcl-2 protein overexpression is detectable in the cells by immunohistochemical staining using monoclonal antibodies (5). Pezzella et al. (6) reported that bcl-2 protein expression was detected in NSCLC3 specimens at 20% by immunohistochemical staining, whereas overexpression of the bcl-2 protein correlated with good prognosis.

The tumor suppressor gene p53 has been extensively studied using human lung cancer specimens as well as cultured lung cancer cell lines (7, 8). Although available antibodies stain normal p53 protein as well as mutated p53 protein, immunohistochemical staining using monoclonal and polyclonal antibodies can detect the mutated p53 protein in the nuclei of the cancer cells (9, 10). Positive staining of the p53 protein in cancer cells correlates with poor prognoses in many malignant tumors, including breast, lung, and prostate cancer (11–13). The wild-type p53 protein prolongs cell cycle progression by increasing WAF1/CIP1 expression (14) and promotes DNA repair-related genes, including GADD45 (15). Interestingly, the wild-type p53 protein induces apoptosis in the cells treated with anticancer agents, radiation, and UV irradiation (16–18). Therefore, the p53 gene is considered one of the key genes in DNA repair in the cells and the removal of cells with DNA damage caused by apoptosis induction. A mutated p53 protein blocks the function of the wild-type p53 protein, possibly resulting in inhibition of apoptosis induction.

Simultaneous expression of bcl-2 and p53 proteins was studied by immunohistochemical staining using breast cancer and malignant lymphoma. In lymph node-negative breast cancer patients, the predictive role of bcl-2 protein expression on survival was mainly dependent on p53 expression (19). In high-grade B-cell lymphomas, cases with simultaneous expression of bcl-2 and p53 proteins survived for shorter periods than cases with expression of only one or neither protein (20). However, expression of bcl-2 and p53 proteins and their impact on patient survival are not clearly understood in lung cancers.

To determine the correlation of the simultaneous expression of these proteins with patient survival, we studied bcl-2 and p53 protein expression in surgically resected NSCLC specimens by immunohistochemical staining using monoclonal antibodies.

MATERIALS AND METHODS
Tissue Samples. Ninety-nine paraffin-embedded NSCLC specimens were culled from the surgical pathology files of the Department of Surgical Pathology at Asahikawa Medical College Hospital. All cases were clinically diagnosed in the First Department of Medicine and surgically resected at Asahikawa Medical College Hospital. The resected tissue was routinely fixed in buffered formalin and paraffin embedded. These specimens were reviewed by pathologists and classified in accordance with WHO classifications. All cases have complete follow-up records at our department.

1 The abbreviation used is: NSCLC, non-small cell lung cancer.
bcl-2 and p53 Protein Expression in NSCLC

Pathological typings were 56 adenocarcinomas, 38 squamous carcinomas, and 5 large cell carcinomas. Although clinical follow-up was available for all patients, three who died within 30 days after surgery (perioperative mortality) were excluded from the survival analysis. One patient died of heart failure, and two patients died of incomplete sutures. The 99 patients included in the present study had no other forms of cancer. The surgery was noncurative in 2 of the 33 stage I cases, 0 of the 13 stage II cases, 8 of the 45 stage III cases, and 6 of the 20 stage IV cases. In the stage IV cases, only lung biopsy was done. Pathological typings were 56 adenocarcinomas, 38 squamous cell carcinomas, and 5 large cell carcinomas. Although clinical follow-up was available for all patients, three who died within 30 days after surgery (perioperative mortality) were excluded from the survival analysis. One patient died of heart failure, and two patients died of incomplete sutures. The 99 patients included in the present study had no other forms of cancer. The surgery was noncurative in 2 of the 33 stage I cases, 0 of the 13 stage II cases, 8 of the 45 stage III cases, and 6 of the 20 stage IV cases. In the stage IV cases, only lung biopsy was done.

Histopatohological and Immunohistochemical Examination. Histopathological diagnosis was based on the conventional morphological examination of a H&E-stained slide of the paraffin-embedded material. Immunohistochemical staining was performed on the 4-μm-thick, formalin-fixed, paraffin-embedded, serial materials with monoclonal antibodies to the p53 protein (DO-7; DAKO Japan, Kyoto, Japan) and bcl-2 protein (clone 124; DAKO Japan Ref. 21). In the p53 staining, the monoclonal antibody was diluted 1:70 in PBS containing 2% BSA. In the bcl-2 staining, the monoclonal antibody was diluted 1:60 in PBS containing 2% BSA. After deparaffinization, the materials were treated for 10 min using microwaves in 1.24 M ZnSO4 for p53 staining and in 10 M sodium citrate for bcl-2 staining. Materials were incubated with 10% rabbit serum at room temperature, and in 10 M sodium citrate for bcl-2 staining. Bound antibodies were visualized with biotinylated rabbit antimouse IgG and streptavidin-horseradish peroxidase then detected by diaminobenzidine using a Histofine SAB-PO(M) kit (Nichirei Corp., Tokyo, Japan). Sections were counterstained with H&E. A colon adenocarcinoma section and a chronic tonsillitis paraffin-embedded section were used as positive controls for p53 and bcl-2 staining, respectively.

Clinical Data and Statistical Analysis. Results of the bcl-2 and p53 staining were analyzed in a blind fashion, although the study was unblinded for further analyses. Statistical analyses were done to examine the prognostic significance of p53 and bcl-2 protein expression in NSCLC. The Kaplan-Meier method was used to estimate survival possibility as a function of time, and survival differences were analyzed by the Cox-Mantel method. Factors potentially related to survival were analyzed by the Cox univariate proportional hazard model (22). Distributions of positive cases by histology and pathological stage were analyzed by the χ² test. Distributions of positive cases by sex and possibility of simultaneous expression of the p53 and bcl-2 proteins were analyzed using Fisher’s χ² test.

Table 1: Results of p53 immunostaining did not correlate with histology. More bcl-2-positive cases were observed in stages I and II than in stages III and IV (P < 0.05; Table 1).

Results of bcl-2 and p53 staining were analyzed in a blind fashion, although the study was unblinded for further analyses. Statistical analyses were done to examine the prognostic significance of p53 and bcl-2 protein expression in NSCLC. The Kaplan-Meier method was used to estimate survival possibility as a function of time, and survival differences were analyzed by the Cox-Mantel method. Factors potentially related to survival were analyzed by the Cox univariate proportional hazard model (22). Distributions of positive cases by histology and pathological stage were analyzed by the χ² test. Distributions of positive cases by sex and possibility of simultaneous expression of the p53 and bcl-2 proteins were analyzed using Fisher’s χ² test.

**RESULTS**

**Immunohistochemical Staining.** Results of immunohistochemical staining were reviewed by three of the authors and scored positive or negative for the staining. Castle et al. (23) reported that 20–80% of cells stained bcl-2 positive in their study. In the present study, we defined the result as positive if there were more than 100 positive cells in 500 tumor cells in the bcl-2 staining. Reactive lymphocytes and histiocytes were not included as positive cells. In the p53 staining, the result was defined as positive if there were more than 100 positive cells in 500 tumor cells; i.e., staining was 20% or more. However, differentiation between positive and negative results was simple, because the cytoplasm for bcl-2 staining and the nuclei for p53 staining were stained well in many cells that were positive. This gives us a cutoff percentage of 20%; whereas other studies have used a value of 10%, we chose this higher value because it is consistent with that for bcl-2, and there was no incidence of between 10 and 20% nuclei staining in this study. There was no case of cytoplasmic staining without nuclear staining in the p53 study.

Nineteen specimens (19.2%) of 99 cases were positive for bcl-2 immunostaining. These 19 cases included 18 male and 1 female and 5 adenocarcinomas, 14 squamous carcinomas, and no large cell carcinoma. Twelve cases (36.4%) were positive in the 33 stage I cases; 2 (15.4%) were positive in the 13 stage II cases; 5 (11.1%) were positive in the 45 stage III cases, and 0 (0%) were positive in the 8 stage IV cases. Although statistically not significant, more bcl-2-negative cases were seen in female patients (P = 0.064). More bcl-2-positive cases were observed in squamous cell carcinomas than in adenocarcinomas (P < 0.01). More bcl-2-positive cases were observed in stages I and II than in stages III and IV (P < 0.05; Table 1).

Forty-four specimens (44.4%) of 99 cases were immunohistochemically stained for p53 protein. These 44 cases included 25 adenocarcinomas, 16 squamous carcinomas, and 3 large cell carcinomas. Their pathological stages were 11 (33.3%) stage I, 8 (61.5%) stage II, 19 (42.2%) stage III, and 6 (75%) stage IV. Results of p53 immunostaining did not correlate with histopathological types or pathological stages (Table 1).
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Prognostic Significance of p53 and bcl-2 Protein Expression. The Kaplan-Meier survival analyses demonstrated that cases with positive bcl-2 staining survived for a longer period than those with negative staining. When all patients were considered, the 2-year survival rate was 75.0%, the 5-year survival rate was 65.6%, and the median survival period was 50 months for those who were bcl-2 positive, cases whereas the figures were 49.0%, 33.0%, and 23 months, respectively, for those who were bcl-2 negative (Fig. 1A); the difference was statistically significant \((P < 0.05)\). When only stages I and II were considered, the 2-year survival rate was 92.3%, the 5-year survival rate was 80.8%, and the median survival period was 62 months for those who were bcl-2 positive, compared with respective figures of 71.9%, 49.5%, and 49 months for those who were bcl-2 negative (Fig. 1B); the difference was statistically significant \((P < 0.05)\).

Patients with positive p53 staining survived for shorter periods when all patients were considered. The 2-year survival rate was 61.3%, the 5-year survival rate was 41.1%, and the median survival period was 19 months for p53-negative cases, compared with 43.7%, 34.9%, and 27 months, respectively, for p53-positive cases (Fig. 2A). The difference was not statistically significant \((P = 0.105)\). When only stages I and II were examined, the 2-year survival rate was 84.6%, the 5-year survival rate was 57.9%, and the median survival period was 53 months for p53-negative cases, compared with 68.4%, 57.0%, and 48 months, respectively, for p53-positive cases (Fig. 2B); the difference was not statistically significant \((P = 0.363)\).

Simultaneous Expression of p53 and bcl-2 Proteins. Six specimens were positively stained for both bcl-2 and p53; 13 cases were bcl-2 positive and p53 negative; 38 cases were bcl-2 negative and p53 positive; and 42 cases were both bcl-2 and p53 negative. Results of the \(\chi^2\) test revealed that the difference was not significant \((P = 0.307; \text{Table 2})\). When only stages I and II were considered, 4 cases were bcl-2/p53 positive, 12 cases were bcl-2 positive/p53 negative, 15 cases were bcl-2 negative/p53 positive, and 17 cases were bcl-2/p53 negative. The difference was not statistically significant using the \(\chi^2\) test \((P = 0.213)\).

Patients' survival time was analyzed according to being bcl-2/p53 positive, bcl-2 positive/p53 negative, bcl-2 negative/p53 positive, and bcl-2/p53 negative. When all patients were considered, cases who were bcl-2 positive/p53 negative survived for

### Table 2

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<th></th>
<th>bcl-2 positive</th>
<th>bcl-2 negative</th>
<th>Total</th>
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<tr>
<td>p53 positive</td>
<td>6</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>p53 negative</td>
<td>13</td>
<td>42</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
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<td>99</td>
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\(a\) \(P = 0.307\) by \(\chi^2\) test.
longer periods than those who were bcl-2/p53 negative, followed by those who were bcl-2/p53 positive and those who were bcl-2 negative/p53 positive (Fig. 3A). The differences in survival time between those who were bcl-2 positive/p53 negative, bcl-2 negative/p53 positive, bcl-2/p53 negative, and bcl-2/p53 positive were statistically significant ($P < 0.05$). When only stages I and II were examined, cases who were bcl-2 positive/p53 negative survived longer than those who were bcl-2/p53 negative, indicating the prognostic value of bcl-2 expression. The differences in survival time between those who were bcl-2 positive/p53 negative, bcl-2 negative/p53 positive, and bcl-2/p53 negative were statistically significant ($P < 0.05$; Fig. 3B).

Relative Risks Contributing to Survival Time. Results of Cox proportional hazard model analyses are summarized in Table 3. Cox proportional hazards regression analysis revealed that the disease stage was a significant factor. The risk ratio of death was 2.29 among patients who were bcl-2 negative versus those who were bcl-2 positive ($P = 0.054$) and 1.56 among patients who were p53 positive versus those who were p53 negative ($P = 0.087$).

DISCUSSION

In the present series of resected NSCLC, the results show that bcl-2 expression is detectable in about 20% of cases, and there is a correlation with pathological variables, which provides prognostic information for overall survival probabilities. Immunohistochemical staining for Bcl-2 stained cells in 19.2% of NSCLC specimens and 30.4% in stages I and II. The bcl-2 protein was found in 36.8% of squamous cell carcinomas, 8.9% of adenocarcinomas, and 0% of large-cell carcinomas. Abnormal bcl-2 expression occurred more frequently in squamous cell carcinoma than in other types of lung cancer. These results confirmed the previous results using stage I and II NSCLC by
In human lung cancers, bcl-2 was apparently lost in the more advanced cancers (25). In contrast, in prostate cancers, bcl-2 expression was observed in the more advanced cancers (26). In NSCLCs, we speculate that bcl-2 deregulation is an early event in carcinogenesis and follows the takeover of bcl-2-positive cells by the rapidly growing bcl-2 negative cells. Examinations using squamous dysplasia and metaplasia of the bronchus, which are considered premalignant neoplasms, including a lung adenoma (31), whereas germline mutation of humans also causes different types of neoplasms (32, 33). This evidence strongly suggests that p53 mutation is one of the key phenomena in carcinogenesis in lung cancers, and it may have some role in maintaining the malignant character of the cancer. bcl-2 seems to play a protective role over a wider range of apoptotic inducers than does mutant p53 (34). Inhibition of apoptosis by mutant p53 or bcl-2 may play an important role in carcinogenesis. However, explanations are not simple, because different pathways of apoptosis induction exist (35), and information is still limited. More studies are needed to understand roles of apoptosis induction in the carcinogenesis of NSCLC.

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