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Bcl-2 Protein Expression Correlates with Better Prognosis in Patients with Advanced Non-small Cell Lung Cancer

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Abstract. *Background: Regulation of apoptosis is an important mechanism during the development of tumors including non-small cell lung cancer (NSCLC). The aim of this study was to assess the relationship between p53 and bcl-2 expression and various clinicopathological features and survival in patients with NSCLC. Materials and Methods: Histological specimens obtained from 120 patients with stage I-III NSCLC were examined immunohistochemically for p53 and bcl-2. Results: Positive immunostaining for p53 was observed in 50 and for bcl-2 in 35 patients. Tumors with lymph node metastasis were significantly more likely to be bcl-2-positive. However, there was no correlation between p53 immunostaining and clinicopathological parameters. Cox proportional hazard multiple regression analyses identified gender, N status and bcl-2 expression as independent prognostic factors. When advanced stage tumors or tumors with lymph node metastasis were analyzed, a more favorable survival was noted in patients with bcl-2-positive tumors than those with bcl-2-negative tumors. Conclusion: Bcl-2 protein expression correlates with better prognosis in patients with advanced NSCLC.*

Lung cancer is the major cause of cancer-related death worldwide. For treatment of non-small cell lung cancer (NSCLC), patients without distant metastasis mainly undergo surgical resection. Although the clinical outcome of these patients depends mainly on the disease stage, a discrepancy is sometimes observed in survival rate of patients with the same stage NSCLC. No biological parameter can predict the prognosis of these patients with NSCLC even in the present state of knowledge of the cellular and molecular mechanisms of NSCLC.

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The regulation of apoptosis is one of the important mechanisms during the development of tumors. The tumor-suppressor gene p53 functions by arresting the G1-phase of the cell cycle to give damaged DNA a chance for repair (1). If the damage cannot be repaired, p53 leads to apoptosis (2). Therefore, mutation of the p53 gene inhibits apoptosis and induces cellular proliferation. Mutations in the p53 gene have been found in many human cancers (3). Bcl-2 is a gene that inhibits p53-induced apoptosis (4). It is an oncogene that was discovered by genetic analysis of t(14;18) frequently observed in patients with B cell follicular lymphoma (5). Several studies have investigated the relationship between the expression of p53, bcl-2, the regulation of apoptosis and prognosis of patients with lung cancer (6-8), but such a relationship remains controversial. The aim of this study was to assess the relationship between p53, bcl-2 expression and clinicopathological features and prognosis of patients with NSCLC.

Materials and Methods

Patients. One hundred and twenty Japanese patients with NSCLC, who underwent surgical resection in the First Department of Surgery, Nagasaki University Hospital, Japan, between 1991 and 1995, were entered into this study. Informed consent was obtained from all patients. All specimens obtained from the patients were stained by hematoxylin-eosin and examined histopathologically, then classified according to the World Health Organization and TNM systems (9, 10). They included 54 squamous cell carcinomas and 66 adenocarcinomas. The patients were 87 men and 33 women, who ranged in age from 35 to 85 years (median, 68 years). According to the TNM stage, 27 patients had stage IA disease, 42 stage IB, 3 stage IIA, 19 stage IIB and 29 stage IIIA. The median follow-up period was 38.2 months.

Immunohistochemistry. Immunohistochemistry was performed using the LSAB method (DAKO LSAB kit, DAKO, Glostrup, Denmark) as follows. Serial sections (5-μm-thick) from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized and then heated in a microwave oven in 0.01 M citric acid buffer. The sections were treated with 3% hydrogen peroxide for 10 min. Normal goat serum was added dropwise onto the sections, which were treated overnight with primary

antibodies specific for p53 (DO-7, DAKO) and bcl-2 (clone 124, DAKO) at room temperature. Subsequently, the sections were reacted with the biotinylated secondary antibody and with peroxidase-labeled streptavidin. The sections were visualized using DAB (diaminobenzidine), followed by nuclear staining with hematoxylin. For anti-p53 antibody, known positive controls were used. The surrounding lymphocytes served as positive controls for anti-bcl-2 antibody. Simultaneous incubation of slides in which the primary antibody was omitted was used as a negative control for all antibodies. To determine the staining pattern, 10 fields were selected arbitrarily and 100 tumor cells/field, totaling 1,000, were counted. The p53 immunoreactivity was confined to the nuclei of tumor cells and tumor cells with more than 50% positive cells with either heterogeneous or homogeneous distribution were evaluated as positive. Sections were considered positive for bcl-2 when ≥10% of tumor cells were stained in the cytoplasm, while others were considered negative.

Statistical analysis. The correlations between clinicopathological features and expression of p53 and bcl-2 were evaluated by the χ^2 test or Fisher's exact test. Survival curves were calculated by the Kaplan-Meier method and statistical differences were evaluated by the log-rank test. Prognostic factors were analyzed using the Cox proportional hazards model. A p value of < 0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed on a personal computer with a statistical software package (StatView, version 5.0J, SAS Institute Inc., NC, USA).

Results

Seventy-one (59.2%) samples were positively immunostained for p53 while 35 (29.1%) were positively immunostained for bcl-2 (Table I). Bcl-2 expression in 24 out of 54 (44.4%) squamous cell carcinomas was significantly higher than in adenocarcinomas (11 out of 66, 16.6%, $p<0.001$, Table I). Tumors with lymph node metastasis and males had significantly higher expression of bcl-2 ($p<0.05$, $p<0.05$, respectively, Table I). However, there was no correlation between p53 immunostaining and clinicopathological parameters. There was no correlation between p53 expression and bcl-2 expression (data not shown).

Univariate survival analysis showed that tumor stage (T1 *vs.* T2-3; $p=0.037$) and nodal status (N0 *vs.* N1-2; $p=0.015$) correlated with survival (Table II, Figure 1), but the expression of either p53 or bcl-2 did not correlate with overall survival of patients with NSCLC. Cox proportional hazard multiple regression analysis revealed that gender, N status and bcl-2 expression were independent prognostic factors ($p=0.045$, $p=0.002$, $p=0.035$, respectively, Table II). To evaluate the prognostic significance of bcl-2 expression in advanced stage disease, the survival curves of patients with surgical stage 3 disease were compared with regard to bcl-2. Kaplan-Meier analysis and log rank test demonstrated that patient survival significantly correlated with bcl-2 expression. Moreover, when advanced stage tumors (T2/3) or tumors with histopathologically-confirmed lymph node metastasis (N1/2) were analyzed, patients with bcl-2-positive tumors had a more favorable survival than those with bcl-2-negative tumors ($p=0.014$ and 0.008, respectively, Figure 2).

Table I. *Relationship between expressions of bcl-2 and p53 and clinicopathological features in 120 cases of non-small cell lung cancer.*

	bcl-2 (%)			p53 (%)		
Variable	Positive	Negative	*P* value	Positive	Negative	*P* value
Age						
< 68 years	18 (32)	39 (68)	0.580	31 (54)	26 (46)	0.311
≥ 68 years	17 (37)	46 (73)		40 (64)	23 (36)	
Gender						
Male	30 (35)	57 (65)	0.038	56 (64)	31 (36)	0.060
Female	5 (15)	28 (85)		15 (45)	18 (55)	
Histology						
Adeno carcinoma	11 (17)	55 (83)	<0.001	35 (53)	31 (47)	0.131
Squamous	24 (44)	30 (56)		36 (67)	18 (33)	
T status						
T1	10 (30)	23 (70)	0.604	18 (55)	15 (45)	0.462
T2	23 (31)	52 (69)		44 (59)	31 (41)	
T3	2 (17)	10 (83)		9 (75)	3 (25)	
N status						
N0	18 (23)	60 (77)	0.046	45 (58)	33 (42)	0.654
N1-2	17 (40)	25 (60)		26 (62)	16 (38)	
Pathological stage						
Stage I	17 (25)	52 (75)	0.333	38 (55)	31 (45)	0.161
Stage II	9 (41)	13 (59)		17 (77)	5 (23)	
Stage III	9 (31)	20 (69)		16 (55)	13 (45)	

Discussion

Our immunohistochemical analysis revealed a significant correlation between oncoproteins and clinicopathological features and outcome of patients with NSCLC. The data showed that bcl-2-positive expression was more frequent in squamous cell carcinoma than in adenocarcinoma. These findings were consistent with other studies (11, 12). Further large-scale studies are necessary to define the relationship between bcl-2 expression and histological phenotype of NSCLC.

The bcl-2-positive immunostaining was observed more frequently in NSCLC with lymph node metastasis than those without such metastasis. Holmgren *et al.* (13) studied dormant lung metastasis and growing metastasis in mice. They found that the proliferative activity of tumor cells was not different in the two types of metastases, but tumor cells of dormant metastasis exhibited a higher incidence of apoptosis. Furthermore, Manfred *et al.* (14) analyzed 215 NSCLCs by flow cytometry and immunohistochemistry for proliferative cell nuclear antigen (PCNA), cyclin A, several apoptotic factors and bcl-2. They demonstrated that the proliferative activity of tumors did not correlate with lymph

Table II. *Univariate and multivariate Cox regression analyses of prognostic factors of non-small cell lung cancer.*

Variables	Univariate analysis HR (95%CI)*	*P* value	Multivariate analysis HR (95%CI)*	*P* value
Age				
< 68 years	1		1	
≥ 68 years	1.69 (0.91-3.14)	0.097	1.69 (0.90-3.17)	0.105
Gender				
Female	1		1	
Male	1.81 (0.84-3.92)	0.130	2.34 (1.02-5.38)	0.045
Histological type				
Squamous cell	1		1	
Adenocarcinoma	0.94 (0.51-1.72)	0.838	1.36 (0.68-2.69)	0.386
T status				
T1	1		1	
T2-3	2.27 (1.05-4.92)	0.037	1.86 (0.79-4.36)	0.156
N status				
N0	1		1	
N1-2	2.14 (1.16-3.93)	0.015	3.11 (1.54-6.30)	0.002
Bcl-2 expression				
Negative	1		1	
Positive	0.68 (0.34-1.39)	0.290	0.42 (0.19-0.94)	0.035
p53 expression				
Negative	1		1	
Positive	0.99 (0.54-1.84)	0.985	0.76 (0.40-1.45)	0.403

*HR, hazard ratio; CI, confidence interval.

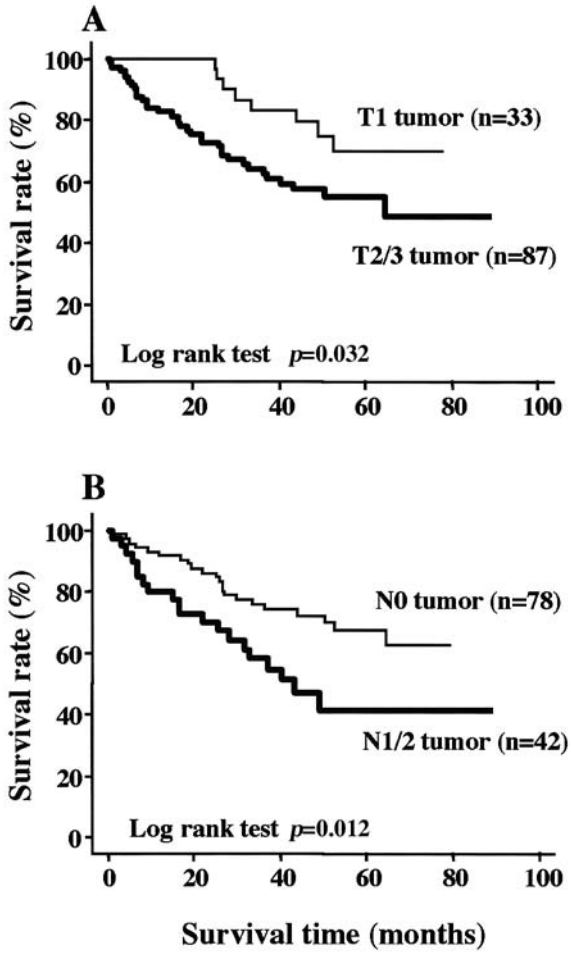

Figure 1. *(A) Overall survival of 120 patients with tumors of stage T1 and T2/3 non-small cell lung cancer. (B) Overall survival of 120 patients with lymph node-positive and -negative non-small cell lung cancer.*

node involvement, whereas apoptotic factors correlated with lymph node metastasis in NSCLC. These results suggested that a reduced apoptotic response might be associated with prolonged survival of lymphatic metastatic tumor cells in a different environment from the primary lesion.

The major finding of the multivariate survival analysis conducted in the present study was that bcl-2 expression, but not p53 expression, was associated with a better prognosis of patients with NSCLC. Although some studies have reported that bcl-2 expression is associated with a good prognosis of NSCLC (15- 18), several studies have reported that such expression had no prognostic value (12, 19, 20). Interestingly, our univariate survival analysis by log rank test did not reveal a significant correlation between bcl-2 expression and prognosis. These findings suggested that the different background features of patients with NSCLC influenced the results. In addition, these conflicting results may be due to differences in the antibody used for immunohistochemistry, cut-off value of number of positively-stained tumor cells and intratumoral heterogeneity of samples. More recently, one meta-analysis also revealed that patients with bcl-2-positive tumors had a significantly better survival than those with bcl-2-negative tumors (21). These results strongly suggest that bcl-2 expression is an important prognostic factor in patients with NSCLC.

The majority of previous studies focused on early-stage NSCLC, therefore little is known about advanced NSCLCs. The present study found significant correlations between bcl-2 expression and better prognosis of patients with lymph node-positive NSCLC and T2/3 stage NSCLC. This finding seems to be in contradiction with the role of bcl-2 oncoprotein in inhibiting the process of apoptosis. The role of bcl-2 in enhancing prognosis of NSCLC patients remains unclear at this stage. Apoptosis depends on a balance between various apoptotic factors and anti-apoptotic factors. Therefore, various other factors might contribute to the survival of these patients. Nevertheless, further analysis is necessary to define the role of bcl-2 with regards to survival of patients with NSCLC.

In conclusion, we demonstrated that the expression of bcl-2 was associated with better outcome in patients with NSCLC, especially those with advanced tumors including those with lymph node involvement. Clinical use of bcl-2 immunostaining could help in clinical decision making with respect to the follow-up interval of surveillance for recurrence and additional adjuvant therapeutic approaches after surgery for patients with NSCLC.

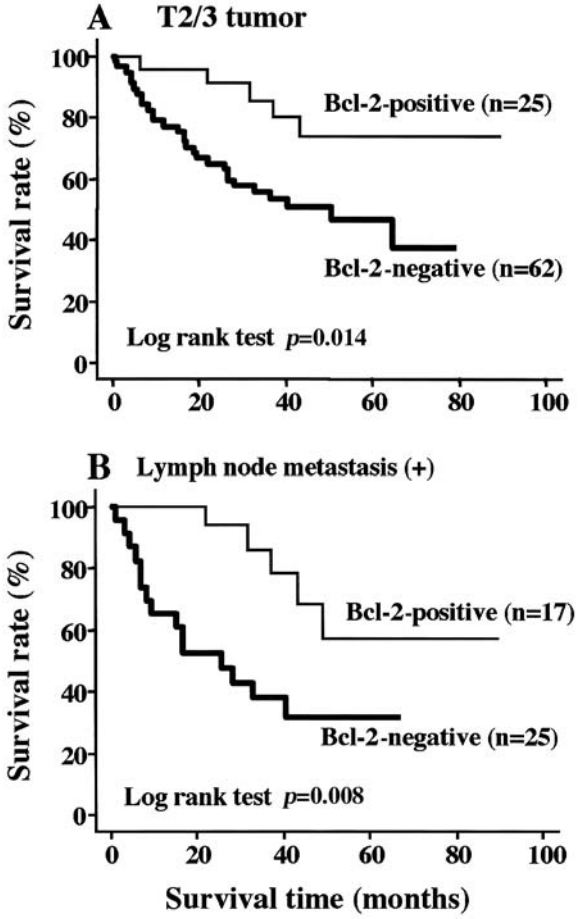

Figure 2. *(A) Overall survival by bcl-2 expression in 87 patients with tumor stage T2/3 non-small cell lung cancer. (B) Overall survival of 42 patients with lymph node-positive non-small cell lung cancer by bcl-2 expression.*

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