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ARTICLE

Does Immunointensity Account for the Differences in Prognostic Significance of Bcl-2 Expression in Non-Small Cell Lung Cancer?

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Bcl-2 is an oncogenic protein that plays a central role in apoptosis. The association of Bcl-2 expression and prognosis in non-small cell lung cancer (NSCLC) is unclear, with some studies showing improved outcome whilst others show no survival advantage. We evaluated 178 surgically resected NSCLC specimens for Bcl-2 and p53 immunoexpression. Bcl-2 staining was present in 34.9% of cases (weakly staining 24.2%, strongly staining 10.7%), nuclear p53 in 43.3% and cytoplasmic p53 in 10.7%. There was no association between p53 and survival. Bcl-2 immunoexpression correlated with improved outcome (p=0.04). A sub-group of strongly Bcl-2 staining cases had a poor survival compared to those that stained weakly (p=0.01). The strongly staining cases had a similar survival to negative cases. Immunointensity may therefore account for the disparity in results regarding the prognostic significance of Bcl-2 demonstrated in previous studies. (Pathology Oncology Research Vol 6, No 2, 87–92, 2000)

Keywords: Bcl-2, apoptosis, prognosis, non-small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer death in Europe and the USA. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of cases. Neoplastic transformation is brought about through the activation of oncogenes or the inactivation of tumor suppressor genes. Bcl-2 is a member of a family of genes involved in the regulation of apoptosis. The Bcl-2 gene differs from conventional oncogenes as it can neither promote growth nor directly lead to cellular transformation. It is thought that Bcl-2 acts as a transmembrane protein and ion channel and as a docking protein.[11] Bcl-2 is capable of binding p53 and preventing its passage from the cytoplasm to the nucleus.[14,19] Increased levels of Bcl-2 expression prevent apoptosis from a wide range of insults including growth factor depletion, ionizing irradiation and chemotherapeutic regimes.[10,12,15] The association of Bcl-2 immunopositivity and prognosis in NSCLC remains controversial. Several studies have shown a good prognosis[3,7,9,16,18,22] whilst others have shown no survival advantage.[2,6]

An alteration in either the p53 gene or protein is the most common change in human malignancy. p53 causes arrest of the cell cycle at the G_1-phase, increases DNA repair time, decreases replicative DNA synthesis and induces apoptosis. Bcl-2 expression has been shown to inversely correlate with p53 expression in NSCLC in some studies[18,22] but not in others.[3,9]

This study sought to evaluate any interrelationships between Bcl-2 and p53 expression and to assess the impact of these factors on survival.

Methods

This is a retrospective study of 178 patients with stage I-IIIa non-small cell lung cancer who underwent surgical resection between 1991 and 1996. Those who died within 60 days of the operation were excluded to avoid the

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bias of peri-operative death. Minimal follow-up for surviving patients was 24 months. The clinicopathological features of the specimens were classified according to the WHO criteria[23] and the TNM staging system.[13]

4 µm thick formalin-fixed paraffin-embedded sections taken from the tumor periphery were mounted on silane-coated slides. Sections were dewaxed in xylene and rehydrated through graded alcohols. Antigen retrieval for Bcl-2 was carried out by placing sections for 12 minutes in 10 mM citrate buffer pH 6.0 in a microwave. Antigen retrieval for p53 was achieved by pressure cooking in 10mM citrate buffer pH 6.0 for 2 minutes. Endogenous peroxidase activity was blocked by placing sections in 2% hydrogen peroxide for 30 minutes. Sections were rinsed in deionised water and then tris buffered saline (TBS) containing 0.1% bovine serum albumin (BSA). To block non-specific staining slides were incubated in 20% appropriate serum for 10 minutes. Sections were incubated overnight at 4°C with the primary antibody. The antibodies used were Bcl-2 mouse monoclonal antibody (Dako, Wycombe, UK) clone 124 dilution 1 in 25 and p53 rabbit polyclonal antibody (Novocastra, Newcastle UK) dilution 1 in 800. Sections were washed in TBS then incubated sequentially with either biotinlyated rabbit anti-mouse IgG (Dako) at a dilution of 1 in 400 or biotinylated swine anti-rabbit IgG (Dako) at a dilution of 1 in 600 followed by streptavidin combined in vitro with biotinylated horseradish peroxidase at 1 in 1000 (Dako). The reaction product was developed using diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with haematoxylin then dehydrated through graded alcohols and mounted in resinous mountant. Known positive controls were included with each run and negative controls had the primary antibody omitted.

Sections were analysed in a blinded fashion by two independent observers and the results of the immunohistochemistry, tumor status and patient outcome correlated subsequently.

Statistical analysis was performed using SPSS for Windows version 9.0. Survival curves were plotted using the methods of Kaplan-Meier, and the log-rank test was used to assess the significance of statistical differences between groups. Chi squared-tests were used to assess associations between categorical variables. Inter-observer variation was assessed using Kappa statistics. The influence of clinicopathological factors including Bcl-2 and p53 on overall survival were assessed by the Cox proportional hazards regression model. Statistical significance was determined using a 5% significance level.

Table 1. **Prognostic significance of tumor variables**

Prognostic factor		*No.*	*%*	*log-rank survival*
No. patients		178		
Age (years)	mean	64.5 (SD 7.56)		
	median	66		p=0.54
	range	42-78		
Sex	male	125	70.2%	
	female	53	29.8%	p=0.18
Histology	squamous	111	62.4%	
	adenocarcinoma	53	29.8%	p=0.95
	large cell	14	7.9%	
Grade	well / moderate	90	50.6%	
	poorly differentiated	88	49.4%	p=0.29
T	1	33	18.5%	
	2	126	70.8%	p=0.22
	3	19	10.7%	
N	0	92	51.7%	
	1	54	30.3%	p<0.0001
	2	32	18.0%	
Stage	I	87	48.9%	
	II	49	27.5%	p=0.0001
	IIIa	42	23.6%	
Bcl-2	negative	116	65.2%	
	weak	43	24.2%	p=0.01
	strong	19	10.7%	
Nuclear p53	negative	101	56.7%	
	positive	77	43.3%	p=0.35

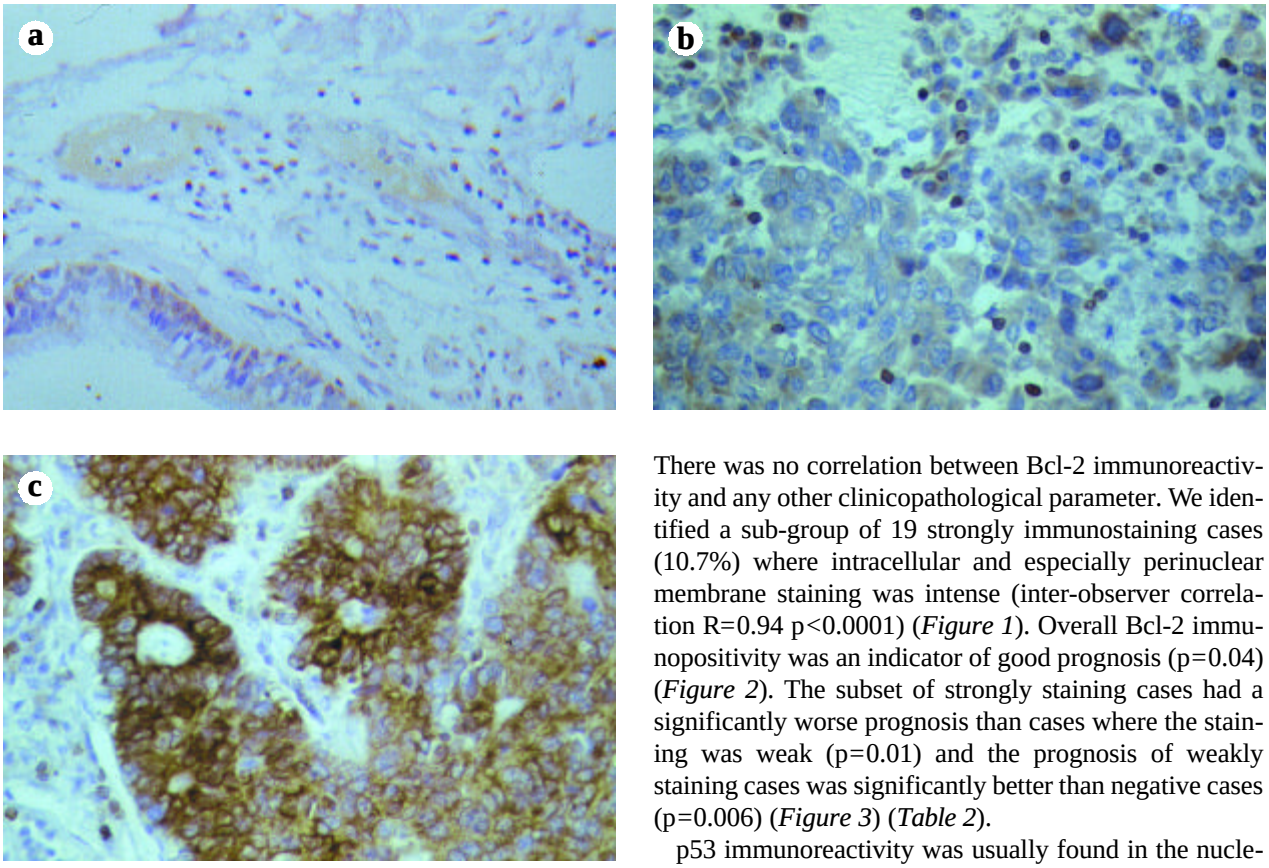


Figure 1. Immunohistochemical staining for Bcl-2 oncoprotein in non-small cell lung cancer. a) Basal respiratory epithelial staining b) Weak tumor cell staining c) Strong tumor cell staining. Positive staining in lymphocytes is used as an internal control.

Results

178 patients underwent resection with post-operative survival greater than 60 days. The follow-up ranged from 24–108 months (median 39.9 months). 98 (55.1%) subjects died from a recurrence of their primary lung cancer. Tumor spread to nodes was associated with poor prognosis (p=0.0002) and increasing nodal status was more significant (p<0.0001). No other clinicopathological finding, including grade, histology, age and sex, was associated with outcome (*Table 1*).

Lymphocytic staining was used as an internal control. Bcl-2 immunostaining was often detected in the basal layer of normal bronchial epithelium. Bcl-2 immunoreactivity was localised to the cytoplasm and the perinuclear region. Any case with >20% tumor cells showing immunostaining were evaluated as positive.[16] Bcl-2 positivity was detected in 62 out of 178 (34.9%) cases. Bcl-2 immunopositivity was more common in squamous cell carcinoma cases than in other histological types (p=0.01). There was no correlation between Bcl-2 immunoreactivity and any other clinicopathological parameter. We identified a sub-group of 19 strongly immunostaining cases (10.7%) where intracellular and especially perinuclear membrane staining was intense (inter-observer correlation R=0.94 p<0.0001) (*Figure 1*). Overall Bcl-2 immunopositivity was an indicator of good prognosis (p=0.04) (*Figure 2*). The subset of strongly staining cases had a significantly worse prognosis than cases where the staining was weak (p=0.01) and the prognosis of weakly staining cases was significantly better than negative cases (p=0.006) (*Figure 3*) (*Table 2*).

p53 immunoreactivity was usually found in the nucleus of tumor cells and occasionally in the cytoplasm. 43.3% (77/178) demonstrated nuclear positivity and 10.7% (19/178) cytoplasmic reactivity. Both nuclear and cytoplasmic staining was demonstrated in 11 cases (6.2%). No correlation was found between p53 and T status or grade. Cases with N_2 disease were more likely to be p53 negative (p=0.01). Both p53 and nuclear p53 immunoreactivity were more frequent in squamous cell

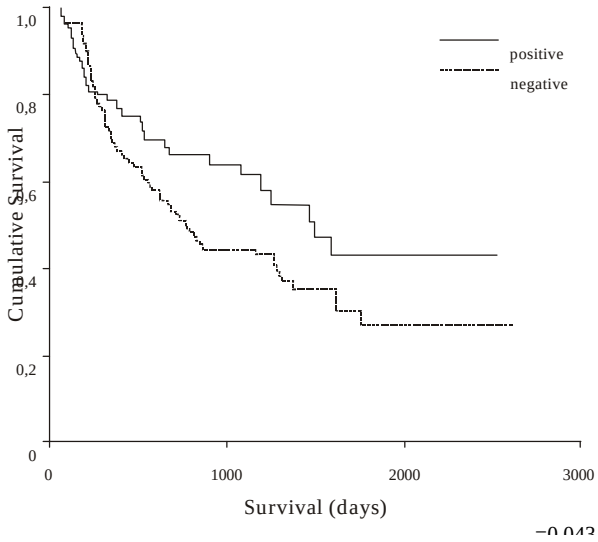


Figure 2. Kaplan–Meier survival plot for Bcl-2 expression.

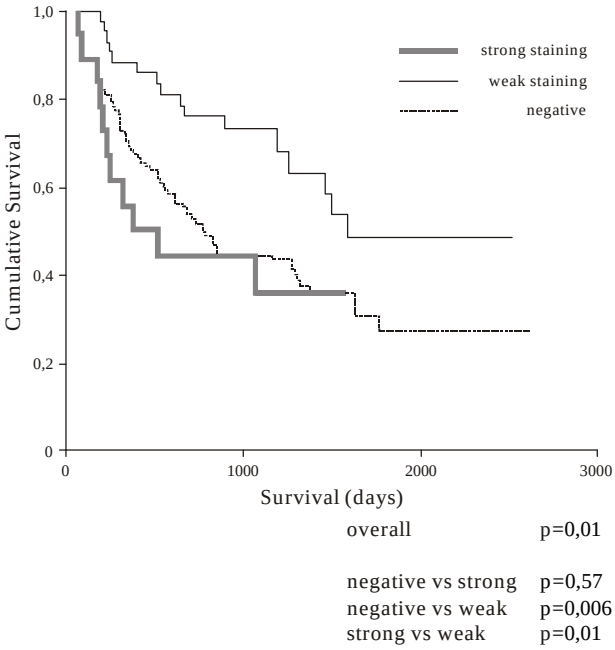


Figure 3. Kaplan–Meier survival plot for Bcl-2 immunointensity

carcinoma than in other histological subtypes (p=0.003 and <0.001 respectively). Neither nuclear p53 nor cytoplasmic p53 reactivity correlated with survival (p=0.35 and p=0.31 respectively).

Of 62 Bcl-2 positive tumors, 30 (48.4%) showed nuclear p53 expression compared to 47 out of 116 Bcl-2 negative tumors (40.5%) (NS).

Cox proportional hazards regression analysis was used to define biological markers with independent predictive value with respect to cancer-specific survival (*Table 3*). The most significant independent prognostic factor was nodal status. The addition of Bcl-2 immunointensity produced a statistically significant improvement in the model.

Discussion

The results of our study show Bcl-2 immunoreactivity to correlate with a favourable outcome in NSCLC. (p=0.04). This is in agreement with several studies[3,7,9,16,18,22] whilst others have not demonstrated a survival advantage.[2,6] The different findings may be in part due to various staining techniques and inconsistent or ill-defined criteria for Bcl-2 positivity. Some studies used frozen tissue,[9,18] the APPAP technique[7,9,18] or the Bcl-2 monoclonal antibody clone 100[3,9,18]. The defining criteria for immunopositivity varied between >0.5% (median value)[3] and >20%[16] of tumor cells staining and in two studies the criteria was not clear.[9,18]

Two previous studies in NSCLC performed a semi-quantitative intensity scoring system for Bcl-2 reactivity.[3,6] In this regard we observed a subset of strongly staining cases with a significantly worse prognosis compared to the more weakly immunopositive cases (p=0.01). The outcome for strongly Bcl-2 staining cases was similar to those that showed no immunoreactivity. Bcl-2 immunointensity was shown to be an independent prognostic factor (p=0.015). Although one study in NSCLC demonstrated intense Bcl-2 immunostaining to be associated with the squamous cell histological subtype,[3] we are unaware of any study in NSCLC showing the intensity of Bcl-2 immunoreactivity to have any influence on outcome.

Bcl-2 is a member of a family of genes involved in the regulation of apoptosis. The Bcl-2 gene differs from conventional oncogenes as it neither promotes growth nor directly leads to cellular transformation. The Bcl-2 gene codes for an integral membrane protein localised to the cytoplasm on the outer nuclear and mitochondrial membranes and the endoplasmic reticulum where it may be involved in the control of calcium flux and protein translocation.[11] Bcl-2 inhibits apoptosis and therefore, theoretically, Bcl-2 over-expression should favour the malignant process and result in a poor outcome. Paradoxically Bcl-2 expression is associated with improved survival in various solid tumors including breast and lung cancer.[3,7,9,16,18,21,22]

Weak Bcl-2 staining is present in the basal cells of normal bronchial epithelium.[9,22] A study of dysplastic bronchial epithelium has shown increasing Bcl-2 expression as the dysplasia becomes more severe associated with loss of basal cell staining whilst Bcl-2 expression is reduced in overt NSCLC.[22] A similar finding has been demonstrated in cervical intraepithelial neoplasia (CIN) where Bcl-2 immunoexpression is more frequently seen in CIN-3 than CIN-1 and -2 lesions[20] with reduced expression found in cervical squamous cell carcinoma.[4] These observations suggest that the loss of Bcl-2 expression could be a late event in tumorigenesis, occurring as the malignant process evolves.[9,20-22] This is in keeping with the finding that the majority of tumors in our study were Bcl-2 negative in contrast to the high levels of expression in dysplasia reported in previous studies. The weakly staining tumors, which have a good prognosis, show an immunointensity similar to that of basal respiratory epithelial cells raising the possibility that these tumors have not lost their inherent Bcl-2 expression. Loss of Bcl-2 expression may indicate more severe molecular dedifferentiation resulting in a more aggressive phenotype.

In contrast, our strongly staining cases, which have a significantly worse prognosis compared to weakly staining cases (p=0.01), may represent Bcl-2 amplification or up-regulation. This may be in response to other tumor-

Table 2. **Relationships with Bcl-2 expression**

Prognostic factor	*Bcl-2 expression*			*Bcl-2 immunointensity*			
	neg	*pos*	χ^2	*neg*	*weak*	*strong*	χ^2
	(no. cases)			*(no. cases)*			
Age							
below median	58	26		58	19	7	
above median	58	36	p=0.30	58	24	12	p=0.51
Sex							
male	81	44		81	27	17	
female	35	18	p=0.87	35	16	2	p=0.11
Histology							
squamous	64	47		64	33	14	
adenocarcinoma	43	10	p=0.01	43	8	2	p=0.02
large cell	9	5		9	2	3	
Grade (differentiation)							
well/moderate	62	28		62	22	6	
poor	54	34	p=0.29	54	21	13	p=0.21
Stage							
I	57	30		57	21	9	
II	29	20	p=0.47	29	14	6	p=0.82
IIIa	30	12		30	8	4	
T							
1	19	14		19	10	4	
2	84	42	p=0.59	84	28	14	p=0.79
3	13	6		13	5	1	
N							
0	61	31		61	22	9	
1	31	23	p=0.24	31	16	7	p=0.56
2	24	8		24	5	3	
Nuclear p53							
negative	69	32		69	25	7	
positive	47	30	p=0.31	47	18	12	p=0.18

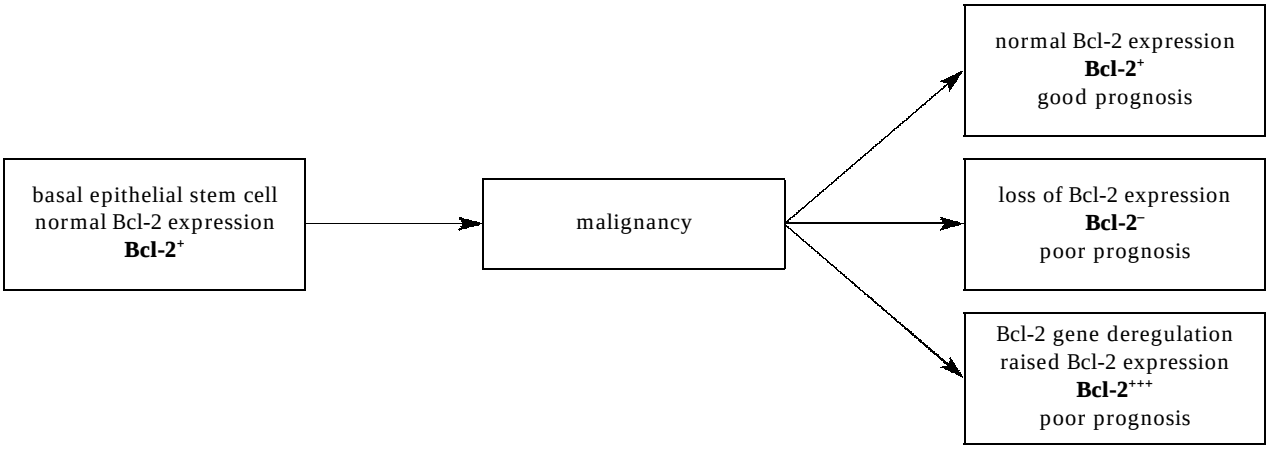
related poor prognostic markers; for instance *c-myc* regulated proliferation[5] or hypoxia.[8] Furthermore Bcl-2 transfection has been shown to enhance malignant transformation and to induce the expression of transcription activator protein-1 (AP-1).[1] Tumors with strong Bcl-2 immunostaining may therefore activate this pathway facilitating tumor growth and invasion.

We found no correlation between p53 and Bcl-2 expression, consistent with other studies.[3,9,22] A previous study has shown apoptosis occurring independently of Bcl-2 and p53 expression in NSCLC.[17] This suggests the effects of Bcl-2 and p53 may be altered by other oncogene products or regulators of apoptosis.

In conclusion the prognostic role of Bcl-2 immunoreactivity in NSCLC remains controversial. We found that strongly staining tumors had a worse outcome than tumors that stained weakly. Strongly staining cases may represent amplification or up-regulation whereas weak

Table 3. **Multivariate analysis**

Factor		*Hazard ratio*	*95% confidence interval*	*p-value*
Nodal status				
	0	1.00		0.0001
	1	1.60	0.97–2.63	
	2	3.04	1.82–5.07	
Bcl-2 expression				
	negative	1.00		0.015
	weak	0.48	0.28–0.82	
	strong	1.27	0.66–2.44	
Grade				
	well/moderate	1.00		0.23
	poor	1.28	0.85–1.94	



Proposed pathway for Bcl-2 expression in non-small cell lung cancer

staining may represent normal levels of Bcl-2 expression. Further studies should emphasise the different intensity and localisation of staining as this may in part account for the disparate results in prognosis from previous studies.

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