Virchows Arch (1998) 432:441–444 

ORIGINAL ARTICLE

**Rosella Silvestrini · Aurora Costa · Cosimo Lequaglie
Claudia Mochen · Silvia Veneroni · Monica Leutner
Gianni Ravasi**

# Bcl-2 protein and prognosis in patients with potentially curable non-small-cell lung cancer

Received: 5 August 1997 / Accepted: 11 December 1997

**Abstract** The bcl-2 proto-oncogene functions as a cell death suppressor, and its expression prolongs cell survival by blocking apoptosis. Data available on the clinical relevance of bcl-2 protein expression in patients with non-small-cell lung cancer (NSCLC) are controversial. We analysed the role of bcl-2 protein expression on 6-year relapse-free survival in 229 patients with stage I-IIIa NSCLC (101 squamous cell carcinomas and 128 adenocarcinomas) subjected to surgery, with curative intent. Immunohistochemical analysis was performed on archival material by using a monoclonal antibody anti-bcl-2 (clone 124). Bcl-2 protein expression, which was detected in 22% of the cases, was significantly related to stage, histology and grading, and was an indicator of clinical outcome. The probability of relapse-free survival at 6 years was longer for patients with bcl-2-positive tumours (74%) than for those with bcl-2-negative tumours (57%) ($P$=0.02). This finding was mainly evident for the subgroups of patients with stage IIIa tumours ($P$=0.05), squamous cell carcinoma ($P$=0.03) or moderately/poorly differentiated tumours ($P$=0.02). However, multivariate analysis by Weibull's regression model indicated that bcl-2 protein expression was not an independent prognostic risk factor in patients with curable NSCLC when the information provided by stage was available.

**Key words** bcl-2 expression · Operable lung cancer · Prognostic factor · Relapse-free survival

R. Silvestrini (✉) · A. Costa · C. Mochen · S. Veneroni
Oncologia Sperimentale C,
Istituto Nazionale per lo Studio e la Cura dei Tumori,
Via Venezian 1, I-20133 Milan, Italy

C. Lequaglie, G. Ravasi
Oncologia Chirurgica Toracica,
Istituto Nazionale per lo Studio e la Cura dei Tumori,
Milan, Italy

M. Leutner
Anatomia Patologica e Citopatologia,
Istituto Nazionale per lo Studio e la Cura dei Tumori,
Milan, Italy

## Introduction

Apoptosis, or programmed cell death, is a determinant of in vivo tumour growth and is largely responsible for continuous cell loss in many solid tumours [1, 12, 13, 17]. Defects in the cell death pathway are important in malignant transformation and may also influence response to treatment and long-term clinical outcome.

The bcl-2 gene encodes for a protein localized on the mitochondrial membrane, nuclear envelope, and endoplasmic reticulum [5]. Bcl-2 functions as a cell death suppressor; its expression prolongs cell survival by blocking apoptosis and appears to lead to neoplastic growth without affecting cell proliferation. Expression of the bcl-2 protein has been detected immunohistochemically in a large proportion of breast and prostate carcinomas [1, 4, 6, 17] and in a small proportion of lung, gastric and bladder carcinomas [7, 8, 12]. The relevance of bcl-2 protein expression on prognosis and response to treatment in different tumour types is still controversial. In particular, for non-small-cell lung cancer (NSCLC) some studies reported no prognostic relevance [3, 15, 16], while others showed a relation between bcl-2 protein expression and clinical outcome in subsets of patients with early disease [12], with squamous cell carcinoma [18] or in patient series including all stages [2]. However, in only one study [12] was the prognostic role of bcl-2 protein expression analysed in multivariate analysis.

In the present study, we analysed the role of bcl-2 protein expression in providing clinical relevant information in 229 patients with potentially curable NSCLC.

## Materials and methods

The case series included 229 patients recruited from February 1988 to June 1992 and operated on at the Istituto Nazionale Tumori of Milan, Italy. Patients had not previously undergone surgery or any radiation or systemic treatment. There were 202 men and 27 women, with a mean age of 62 years (range, 31–80 years). Staging was defined according to the International Staging System [10]. Preoperative staging included chest radiography, computer-

**Table 1** Tumour characteristics[a] (*RUL* right upper lobectomy, *RML* right middle lobectomy, *RLL* right lower lobectomy, *UB* upper bilobectomy, *RP* right pneumectomy, *LUL* left upper lobectomy, *LLL* left lower lobectomy, *LP* left pneumectomy)

| | Stage | | |
|---|---|---|---|
| | I | II | IIIa |
| pT | | | |
| 1 | 36 | 11 | 3 |
| 2 | 91 | 44 | 28 |
| 3 | – | – | 16 |
| Location | | | |
| RUL | 45 | 11 | 23 |
| RML | 7 | 6 | 2 |
| RLL | 23 | 6 | 1 |
| UB | 1 | – | – |
| RP | 1 | 2 | – |
| LUL | 35 | 18 | 11 |
| LLL | 11 | 9 | 7 |
| LP | 4 | 3 | 3 |

[a] Figures in body of figures show numbers of cases

**Table 2** Relationship between bcl-2 protein expression and pathological characteristics (*NSCLC* non-small-cell lung cancer)

| | No. of cases | bcl-2$^+$ NSCLC (%) | *P*-value[a] |
|---|---|---|---|
| Stage | | | |
| I | 127 | 28 | 0.008 |
| II/IIIa | 102 | 14 | |
| Histology | | | |
| Squamous cell carcinoma | 101 | 32 | 0.001 |
| Adenocarcinoma | 128 | 14 | |
| Grading | | | |
| Well differentiated | 32 | 9 | 0.006 |
| Moderately/poorly differentiated | 193 | 24 | |

[a] Chi-squared test

ized tomography or magnetic resonance of the whole body, cytological sputum examination, bronchofibreoscopy with brushing, and biopsy when possible. Pulmonary scan was performed when a pneumonectomy was suspected, and pulmonary function tests including spirometry and blood gas analysis were done in all patients. A total of 127 patients had stage I, 55 stage II and 47 stage IIIa cancers; 101 were squamous cell carcinomas and 128, adenocarcinomas. Tumour characteristics in terms of size and location are described in Table 1. The surgical procedure was sublobular resection in 6 cases, lobectomy in 173 cases, and pneumonectomy in 50 cases. A mediastinal lymphadenectomy was performed in all the cases.

Bcl-2 protein expression was determined on pathological material from surgical resection. Representative samples of the whole tumour were received from the Pathology Department within 60 min of surgery. Small fragments were picked from different areas (central and peripheral) of the tumour. Specimens were fixed for 6–10 h in buffered formalin and embedded in paraffin. Prior to immunostaining, freshly cut 4-µm histological sections were processed by a microwave antigen retrieval in 0.01 M citrate buffer (pH 6). The slides were then incubated for 1 h at room temperature in a humidified atmosphere with a 1:40 dilution of monoclonal mouse anti-human bcl-2 oncoprotein (clone 124; Dakopatts, Copenhagen, Denmark). After incubation, the specimens were processed by using the avidin–biotin peroxidase method (Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif.). The samples were then incubated in diaminobenzidine and hydrogen peroxide chromogen substrate for 5 min at room temperature, washed in tap water, counterstained with haematoxylin, and mounted with a permanent mounting medium. Staining without anti-bcl-2 monoclonal antibody was performed as a negative control. In most specimens, strong bcl-2 expression in the cytoplasm of normal epithelium and lymphocytes provided useful internal positive controls. Immunopositivity was considered if undoubt cytoplasmic staining was present in more than 1% of tumour cells. Semiquantitative measurements were performed independently by two observers, who were unaware of the clinical course.

The relationship between bcl-2 protein expression and pathological features was investigated using the Chi-square test. Relapse-free survival (RFS) was counted from the date of surgery to the date of first local or distant relapse, and the median follow-up was 66 months (range, 9–94 months). RFS probabilities were estimated by the Kaplan – Meier product – limit method. The role of each of the putative prognostic variables (univariate analysis) and their joint effects (multivariate analysis) were evaluated by using Weibull's regression model. Hazard ratios (HR) and their 95% confidence limits (CL) were determined by using the putative best prognostic category as reference.

**Table 3** Univariate analysis of 6-year relapse-free survival

| Variable | Probability (%) | Hazard ratio (95% CL) | *P*-value[b] |
|---|---|---|---|
| Disease stage | | | |
| I[a] | 76 | | |
| II | 46 | 5.7 (2.24–14.5) | 0.003 |
| IIIa | 36 | 13.4 (5.13–35.0) | 0.0001 |
| Histology | | | |
| Squamous cell carcinoma[a] | 61 | | |
| Adenocarcinoma | 60 | 1.0 (0.47–2.23) | 0.96 |
| Grading | | | |
| Well differentiated[a] | 58 | | |
| Moderately differentiated | 54 | 1.2 (0.38–3.63) | 0.78 |
| Poorly differentiated | 53 | 1.7 (0.49–5.73) | 0.41 |
| Bcl-2 expression | | | |
| Positive[a] | 74 | | |
| Negative | 57 | 3.7 (1.2–11.20) | 0.02 |

[a] Computed by Weibull statistic
[b] Reference category

## Results

Bcl-2 reactivity was observed in 50 tumours (22%). Immunostaining was always localized in the cytoplasm of tumour cells, and the frequency of cells expressing bcl-2 protein varied widely from tumour to tumour. The frequency of bcl-2-positive tumours in stage I was twice that in stages II and IIIa (Table 2). A significantly higher frequency of bcl-2-positive cases was also found in squamous cell carcinoma than in adenocarcinoma, and in moderately/poorly differentiated than in well-differentiated tumours. No differences in bcl-2 expression were observed between stages II and IIIa or between moderately and poorly differentiated tumours. Bcl-2

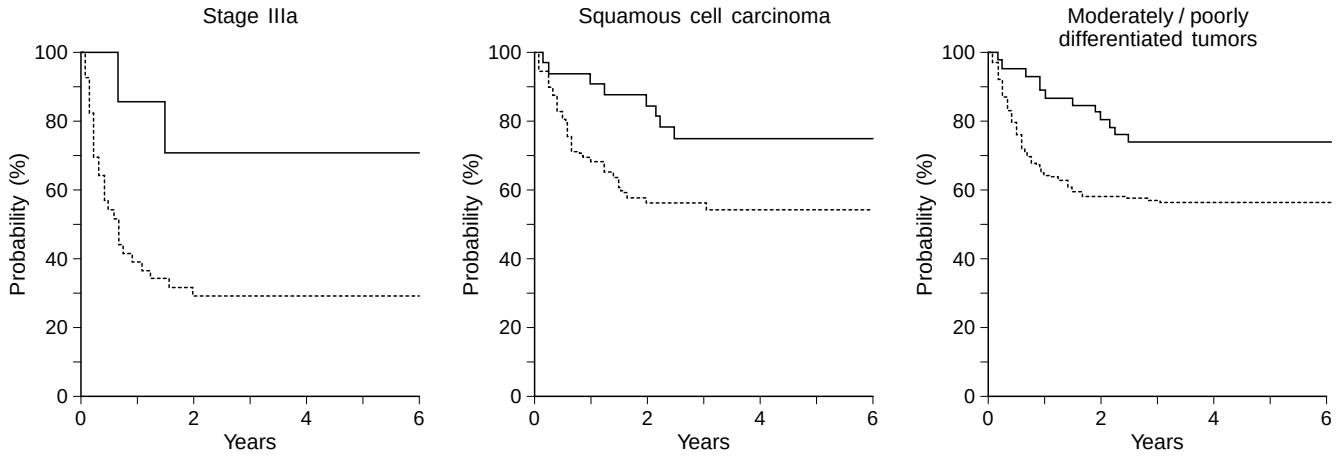


**Fig. 1** Relapse-free survival of patients with non-small-cell lung cancer as a function of bcl-2 protein expression (—— bcl-$2^+$ ---- bcl-$2^-$) in the subsets of patients with stage IIIa (bcl-$2^-$ vs bcl-$2^+$: HR=13.5, 95% CL=1–17.9; *P*=0.05), squamous cell carcinoma (bcl-$2^-$ vs bcl-$2^+$: HR=4.7, 95% CL=1.1–19.7; *P*=0.03), and moderately/poorly differentiated tumours (bcl-$2^-$ vs bcl-$2^+$: HR=4.3, 95% CL=1.3–14.3; *P*=0.02)

**Table 4** Multiple regression analysis of 6-year relapse-free survival

| Variable | Hazard ratio (95% CL) | *p*-value[b] |
|---|---|---|
| Initial model | | |
| Stage | | |
| II vs I[a] | 4.9 (1.9–12.41) | 0.001 |
| IIIa vs I[a] | 12.3 (4.74–32.09) | 0.0001 |
| Histology | | |
| Adenocarcinoma vs squamous cell carcinoma[a] | 1.1 (0.53–2.33) | 0.79 |
| Grading | | |
| Moderately/poorly vs well differentiated[a] | 1.1 (0.38–2.97) | 0.90 |
| Bcl-2 expression | | |
| Negative vs positive[a] | 2.6 (0.9–7.46) | 0.08 |
| Final model | | |
| Stage | | |
| II vs I[a] | 4.8 (1.9–12.2) | 0.0009 |
| IIIa vs I[a] | 12.2 (4.72–31.33) | 0.0001 |
| Bcl-2 expression | | |
| Negative vs positive[a] | 2.6 (0.94–7.4) | 0.065 |

[a] Reference category
[b] Computed by Weibull statistic

protein expression was unrelated to the gender or age of the patients.

In the present series stage, but not histology or grading, was a significant indicator of 6-year RFS, with a risk of relapse about 6 times higher for stage II and 13 times higher for stage IIIa than for stage I tumour patients (Table 2). In contrast, no difference in probability of RFS was observed between patients with squamous cell carcinoma and those with adenocarcinoma, or between patients with well-differentiated and those with poorly or moderately differentiated tumours. Bcl-2 protein was an indicator of RFS, with a risk of relapse 3.7 times higher for patients with tumours not expressing it than for those with bcl-2-expressing tumours (Table 3).

The prognostic role of bcl-2 protein expression was analysed as a function of stage, histology and grading. Bcl-2 expression was not indicative of RFS in stage I (HR=2.2, 95% CL: 0.4–12.4; *P*=0.39) or in stage II (HR=1.1, 95% CL: 0.25–4.52; *P*=0.92), whereas it was an important discriminant of RFS in stage IIIa patients (Fig. 1). Again, bcl-2 expression was a discriminant of RFS in the subgroup of patients with squamous cell carcinoma or with moderately or poorly differentiated tumours (Fig. 1).

Multiple regression analysis of the joint prognostic effect of the biological, and pathological factors (Table 4) showed, in the initial and final models, only stage as independent indicator of 6-year RFS, whereas bcl-2 protein expression appeared to lose its prognostic relevance.

## Discussion

NSCLC accounts for most lung diseases. About one third of cases are treated by surgery, but the long-term outcome is unsatisfactory even in patients who undergo potentially curative resection [9]. As for other tumour types, numerous biological markers have been investigated as putative indicators of prognosis, but the results are often controversial. Our investigation analysed the potential of bcl-2 protein expression to predict clinical outcome of NSCLC patients subjected to curative surgery.

In our study, bcl-2 was expressed in about 20% of cases. Such data are in agreement with the results published by Pezzella et al. [12] and Ritter et al. [16], but not with those of Fontanini et al. [2], who observed some expression of bcl-2 in most NSCLC. Such a discrepancy could be ascribed to the different criteria used in defining bcl-2-positive lesions. Moreover, we confirmed the reported [12] significant relationship between bcl-2 expression and stage, histology and grading. Stage I tumours were more frequently bcl-2 positive than stage II

and IIIa tumours, suggesting a loss of control of programmed cell death with progression of the disease. The more frequent detection of bcl-2 protein expression in moderately and poorly differentiated than in well-differentiated tumours has led to the supposition that bcl-2 may be an indicator of clinical outcome in differentiated tumours [12]. This hypothesis is not supported by our results, which showed a prognostic discriminant power of bcl-2 protein expression within moderately/poorly differentiated but not within well-differentiated tumours.

In our study, bcl-2 protein expression emerged as an indicator of a favourable long-term prognosis. The association between bcl-2 expression and favourable prognosis has been consistently reported for various human tumour types [6, 11, 14, 17], but the explanation of this finding may vary with the tumour histology. In particular, in NSCLC, bcl-2 expression was not inversely related to p53 expression (R. Silvestrini, personal communication) as observed in breast cancer [17]. Considering the decreased frequency of tumours with bcl-2 expression in more advanced stages, a hypothesis that we need to verify experimentally is whether the final event of apoptosis (as seen in necrosis) is the release of proteases, which may favour metastatic cell spread. Prognostic relevance of bcl-2 protein expression in NSCLC has been reported for stage I–II patients [12] and also in two studies including stage I–IIIb patients [2] and stage I–III squamous cell carcinomas [16]. In our study, bcl-2 expression was associated with a favourable prognosis in patients with stage IIIa tumours and only suggestive of such a clinical outcome in patients with stage I tumours, possibly because of the small number of events and the relatively short follow-up. Moreover, in the subgroup of patients with squamous cell carcinoma we confirmed the prognostic relevance of bcl-2 previously reported by Pezzella et al. [12] and by Volm and Mattern [18] in smaller series of patients.

Multivariate analysis confirmed pathological stage as a robust prognostic indicator, whereas bcl-2 protein expression did not appear to predict risk of relapse. However, within subsets of patients homogeneous for tumour stage, bcl-2 expression was predictive of clinical outcome mainly in stage IIIa patients.

In conclusion, our results in this large series of patients confirm the association between bcl-2 protein expression and a good prognosis in specific subgroups of patients with potentially curable NSCLC. Further efforts are needed to assess the relevance of bcl-2 expression, alone and in association with other biomarkers, as an indicator of response to different treatments to improve the management of patients with lung cancer.

**Acknowledgements** This work was supported by grants from the Associazione Italiana Ricerca Cancro (AIRC), Milan, Italy. The authors thank B. Johnston for editing and R. Vio for typing the manuscript.

## References

1. Colombel M, Symmans F, Gil S, O'Toole KM, Chopin D, Benson M, Olsson CA, Korsmeyer S, Buttyan R (1993) Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. Am J Pathol 143: 390–400
2. Fontanini G, Vignati S, Bigini D, Mussi A, Lucchi M, Angeletti CA, Basolo F, Bevilacqua G (1995) Bcl-2 protein: a prognostic factor inversely correlated to p53 in non-small-cell lung cancer. Br J Cancer 71:1003–1007
3. Gaffney EF, O'Neill AJ, Staunton MJ (1994) Bcl-2 and prognosis in non-small-cell lung carcinoma. N Engl J Med 330: 1757–1758
4. Gee JMW, Roberson JFR, Ellis IO, Willsher P, McClelland RA, Hoyle HB, Kyme SR, Finlay P, Blamey RW, Nicholson RI (1994) Immunocytochemical localization of bcl-2 protein in human breast cancer and its relationship to a series of prognostic markers and response to endocrine therapy. Int J Cancer 59:1–6
5. Hockenbery DM, Zutter M, Hichey W, Nahm M, Korsmeyer SJ (1991) Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88:6961–6965
6. Joensuu H, Pylkkanen L, Toikkanen S (1994) Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol 145:1191–1198
7. Lauwers GY, Scott GV, Hendricks J (1994) Immunohistochemical evidence of aberrant bcl-2 protein expression in gastric epithelial dysplasia. Cancer 73:2900–2904
8. Lu Qi-L, Abel P, Foster CS, Lalani EL-N (1996) Bcl-2: role in epithelial differentiation and oncogenesis. Hum Pathol 27: 102–110
9. Minna JD, Pass H, Glatstein E, Ihde DC (1989) Lung cancer. In: DeVita VT, Rosenberg S, Hellmann S (eds) Principles and practice of oncology. Lippincott, Philadelphia, pp 591–705
10. Mountain CF (1986) A new international staging system for lung cancer. Chest 89 [suppl]:225S–233S
11. Öfner D, Riehemann K, Maier H, Riedmann B, Nehoda H, Tötsch M, Böcker W, Jasani B, Schmid KW (1985) Immunohistochemically detectable bcl-2 expression in colorectal carcinoma: correlation with tumor stage and patient survival. Br J Cancer 72:981–985
12. Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, Harris A, Gatter KC, Mason DY (1993) Bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med 329: 690–694
13. Pilotti S, Collini P, Rilke F, Cattoretti G, Del Bo R, Pierotti MA (1994) Bcl-2 protein expression in carcinomas originating from the follicular epithelium of the thyroid gland. J Pathol (Lond) 172:337–342
14. Piris MA, Pezzella F, Martinez-Montero JC, Orradre JL, Villuendas R, Sanchez-Beato M, Cuena R, Cruz MA, Martinez B, Garrido MC, Gatter K, Aiello A, Delia D, Giardini R, Rilke F (1994) p53 and bcl-2 expression in high-grade B-cell lymphomas: correlation with survival time. Br J Cancer 69: 337–341
15. Rao SK, Krishna M, Woda BA, Savas L, Fraire AE (1996) Immunohistochemical detection of bcl-2 protein in adenocarcinoma and non-neoplastic cellular compartments of the lung. Mod Pathol 9:555–559
16. Ritter JH, Dresler CM, Wick MR (1995) Expression of bcl-2 protein in stage T1N0M0 non-small cell lung carcinoma. Hum Pathol 26:1227–1232
17. Silvestrini R, Veneroni S, Daidone MG, Benini E, Boracchi P, Mezzetti M, Di Fronzo G, Rilke F, Veronesi U (1994) The bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. J Natl Cancer Inst 86:499–504
18. Volm M, Mattern J (1995) Increased expression of bcl-2 in drug resistant squamous cell lung carcinomas. Int J Oncol 7:1333–1338