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Immunoreactivity for bcl-2 protein in malignant mesothelioma and non-neoplastic mesothelium

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Abstract Immunohistochemical study of bcl-2 protein immunoreactivity in human non-neoplastic mesothelium (44 cases) and in malignant mesothelioma (62 cases) using a murine monoclonal antibody (clone 124) showed cytoplasmic immunoreactivity for bcl-2 protein in five cases of malignant mesothelioma. Non-neoplastic mesothelium was not immunoreactive. Immunoreactivity for bcl-2 protein does not add useful prognostic information in malignant mesothelioma since survival times of bcl-2 positive and bcl-2 negative cases did not differ. Nevertheless, the detection of bcl-2 protein in malignant mesothelioma might be useful for the differentiation from reactive mesothelium.

Key words Bcl-2 protein · Mesothelioma · Pleura Immunohistochemistry · Prognosis

Introduction

The *bcl*-2 proto-oncogene was discovered at the chromosomal breakpoint of the t(14;18) (q32;q21) translocation found in human follicular lymphoma [16]. In this translocation the *bcl*-2 gene is brought under the control of the promoter of the immunoglobulin heavy chain gene. As a consequence, abnormally high levels of bcl-2 protein are produced [4, 16, 17]. The *bcl*-2 gene product is a 26 kDa protein located at the inner side of the mitochondrial membrane [7]. Its mode of action remains uncertain but the protein seems to protect cells from programmed cell death (apoptosis) [7] and is a normal constituent of different non-lymphoid organs and tissues including thyroid, skin, intestine, uterine smooth muscle and breast [11]. Immunoreactivity for the bcl-2 protein is present mainly in cell populations which are long lived and/or with high proliferating ability such as duct cells in exocrine glands, basal keratinocytes, cells at the bottom of colonic crypts, neurons and several embryonal tissues where it seems to play a role in maturation and terminal differentiation [11]. It has therefore been suggested that bcl-2 protein assists in the survival of stem cells while preventing the overaccumulation of differentiated cells. The bcl-2 protein is also expressed in non-lymphoid tumours including primitive neuroectodermal tumours, hormone therapy resistant prostate cancer and non-small cell lung cancer, suggesting a possible role of this protein in the genesis of these neoplasms [5,10, 14]. This prompted us to investigate the presence of bcl-2 protein in malignant mesothelioma and non-neoplastic mesothelium.

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Materials and methods

A total of 62 paraffin embedded mesothelioma tissue specimens comprising 47 epithelial mesotheliomas, 8 mixed mesotheliomas and 7 mesenchymal mesotheliomas were included in the study together with 44 paraffin embedded tissue specimens of pleura or pleural exudates with non-neoplastic mesothelium, 33 of which showed signs of hyperplasia. In all mesothelioma cases asbestos exposure was documented. All samples were fixed in an alcoholic formalin acetic acid solution. Additional histochemical stainings were performed including periodic acid-Schiff (PAS), PAS after diastase treatment, alcian blue and alcian blue after hyaluronidase treatment. Immunohistochemical staining for carcinoembryonic antigen (CEA), cytokeratin, vimentin and epithelial membrane antigen (EMA) was also performed employing standard immunohistochemical procedures.

For the detection of the bcl-2 protein, 5 μm thick sections were cut, dewaxed in xylene followed by rehydration in decreasing ethanol series, water and phosphate buffered saline (PBS) pH 7.4. Slides were immersed in methanol supplemented with 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase. After rehydration through graded ethanol and distilled water, sections were immersed in 10 mM citrate buffer (10 mM citrate monohydrate in distilled water, pH 6.0) and subjected to microwave treatment (750 W) for three times 5 min with a cooling peri-

od of 5 min between the sessions. This method makes it possible to perform immunohistochemistry on paraffin sections of formalin-fixed tissues with antibodies that apparently were only suitable for frozen sections [3, 8]. After cooling to room temperature, slides were removed to PBS and incubated with non-immune rabbit serum (diluted 1:20 in PBS; Dako, Denmark) to block the non-specific Fc receptor activity in tissue. The sections were incubated overnight at 4° C with the primary antibody anti-bcl-2 protein mouse monoclonal antibody (clone 124, isotype IgG_1; Dako) diluted 1:40 in PBS supplemented with 1% bovine serum albumin. This monoclonal antibody is directed against a synthetic peptide comprising the aminoacids 41–54 of the human bcl-2 protein (Pezella et al. 1990). It detects bcl-2 protein in western blotting experiments and in immunohistochemical techniques. Its efficacy has been proven on frozen sections and on paraffin sections [5, 9, 10, 11, 14]. The sections were then overlaid with biotinylated rabbit anti-mouse polyclonal antibody (Dako) diluted 1:40. Binding was detected by applying the avidin-biotin-peroxidase complex (Dako). Peroxidase was revealed by incubating the sections with a solution containing 3,3′diaminobenzidinetetrahydrochloride in 20 ml TRIS buffer pH 7.6 containing 0.03% hydrogen peroxide.

The specificity of the immunohistochemical reactions was controlled as follows: firstly by omitting the first antibody; secondly by substituting the anti-bcl-2 antibody for an unrelated monoclonal antibody of the same isotype IgG_1 in the same concentration but directed against an unrelated antibody (monoclonal mouse anti-human CD 68 antibody, isotype IgG_1, Dako).

Sections of a follicular lymphoma were used as positive controls [4, 16, 17]. Counterstaining was with Mayer's haematoxylin for 5 min.

The Fisherman's exact test was used for statistical analysis.

Survival data were available in 52 patients with mesothelioma and survival time was determined from the date of diagnosis until last follow-up or death. Patient actuarial survival curves were constructed using the Kaplan-Meier method with statistical significance determined by the log-rank test.

Results

Sixty-two specimens from patients with a malignant mesothelioma and 44 specimens of non-neoplastic mesothelium were studied including 33 cases with signs of hyperplasia.

All mesotheliomas exhibited alcian blue positivity and no neutral mucins were found. Immunohistochemically all were immunoreactive for cytokeratin, vimentin and EMA. No CEA positivity was found.

Positive control sections treated with anti-bcl-2 antibody showed strong cytoplasmic immunoreactivity in most lymphoid cells in the follicles of a follicular lymphoma. There was a strong fine brown granular cytoplasmic immunoreactivity for bcl-2 protein in five mesotheliomas (Fig. 1). In some of these mesotheliomas only scattered foci of neoplastic mesothelial cells situated in the immediate vicinity of vessels were bcl-2 protein immunoreactive. In other mesotheliomas more than 75% of the neoplastic cells were immunoreactive for bcl-2 protein. The results of the immunohistochemical stainings are summarized in Table 1. No statistically significant differences were found between the various mesothelioma subtypes ($P>0.5$). In the remaining mesothelioma cases as well as in all cases with non-neoplastic mesothelium no immunoreactivity for bcl-2 protein was detected. Scattered stromal lymphocytes in neoplastic and non-neoplastic mesothelial tissues were immunoreactive and served as internal positive controls for the anti-bcl-2 antibody.

The median actuarial survival as determined by the Kaplan-Meier method was 1.45 months for mesothelioma patients with bcl-2 immunoreactive cells and 6.87 months for patients without bcl-2 immunoreactive cells ($P=0.09$; fig. 2).

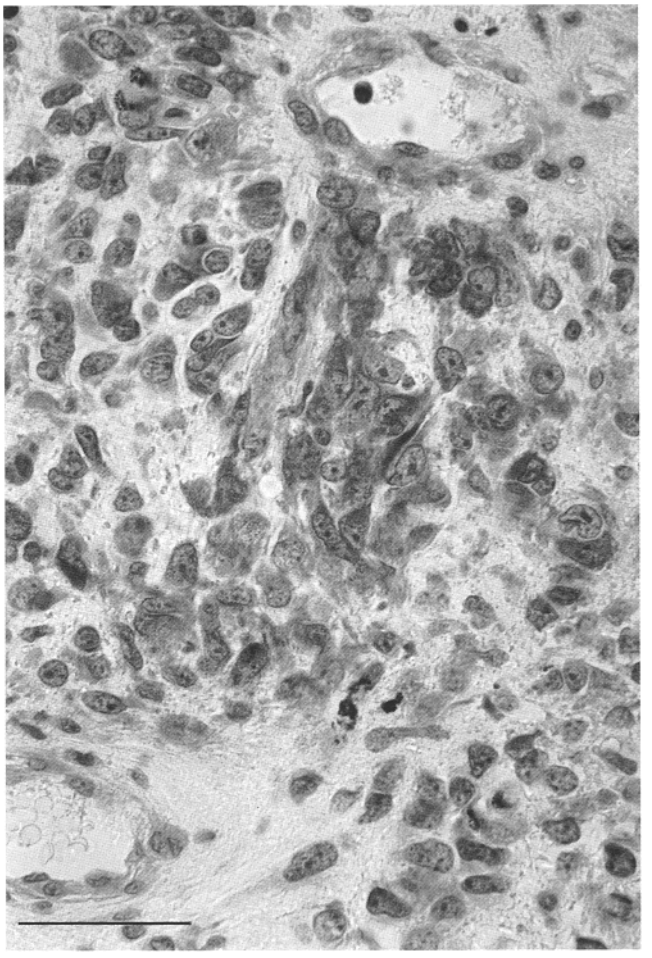

Fig. 1 Neoplastic mesothelial cells of an epithelial mesothelioma displaying strong granular cytoplasmic immunoreactivity for bcl-2 protein in the vicinity of a vessel (avidin-biotin-complex-immunohistochemical technique; magnification ×450; *bar*= 50 μm)

Table 1 Immunoreactivity for bcl-2 protein in malignant mesothelioma and non-neoplastic mesothelium

Immunoreactive mesothelial cells	0%	1–5%	5–25%	50–75%	>75%
Epithelial type	45		1		1
Mesenchymal type	5	1	1		
Mixed type	7			1	
All mesotheliomas	57	1	2	1	1
Non-neoplastic mesothelium	44				

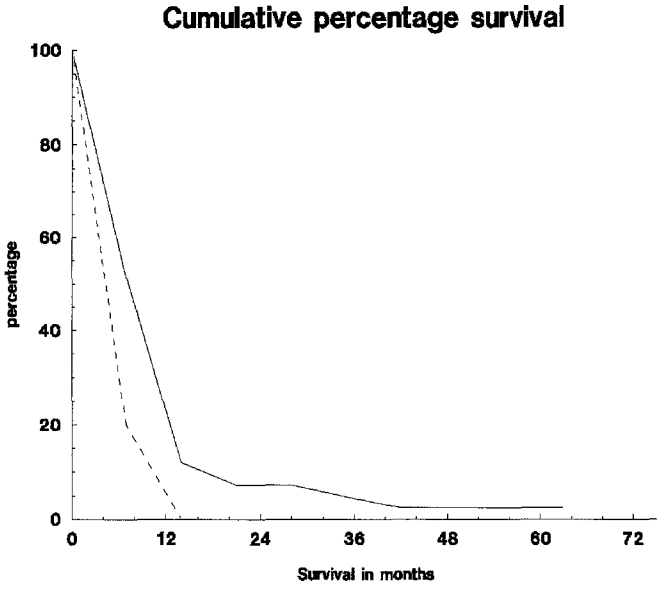

Fig. 2 Actuarial survival comparing mesothelioma cases (n=5) with bcl-2 immunoreactive cells (- -) to mesothelioma cases (n=47) without bcl-2 immunoreactive cells (–). There is no statistically significant difference between the two groups (P=0.09, log-rank tests)

Discussion

Immunoreactivity for the bcl-2 protein was found in approximately 10% of the mesotheliomas in our series. It is very unlikely that this immunoreactivity for bcl-2 protein would be the result of a t(14;18)(q32;q21) translocation since this translocation has not been described in mesothelioma [6, 15]. However, some lymphomas overexpress bcl-2 protein without the t(14;18) translocation indicating that this cytogenetic abnormality is not the sole cause of bcl-2 protein disregulation in neoplastic cells [9]. The bcl-2 protein is not restricted to lymphoid neoplasms but has also been found in reactive lymphoid tissue and in non-lymphoid tissues [7]. Some authors state that several types of long lived cells, like muscle and other mesodermal cells, do not always show bcl-2 expression and that bcl-2 protein is restricted to those portions of tissues characterized by apoptotic cell death [7]. Other authors suggest that several mechanisms of apoptosis exist that are not affected by the *bcl-2* gene and it is clear that apoptosis can be prevented by other genes [1]. This may explain why, in our study, bcl-2 immunoreactivity was not detected in non-neoplastic mesothelium. This result might be useful for the distinction between neoplastic and reactive mesothelium, for which no specific markers exist [2]. Other authors suggest that there is a relationship between bcl-2 protein expression and hormone or growth factor dependent cell proliferation as the protein is expressed in breast, thyroid, and prostatic epithelium [5, 11]. Malignant mesothelioma is known to express the B-chain of platelet derived growth factor (PDGF) and receptors for epidermal growth factor and PDGF [12, 13, 18].

Very recently other tumours such as non-small cell lung cancers [10], primitive neuroectodermal tumours [14] and hormone resistant prostate cancer [5] have been found to be bcl-2 protein immunoreactive. Our results are comparable with the results obtained in non-small cell lung cancer where 5 of 42 (12%) adenocarcinomas and 20 of 80 (25%) squamous cell cancers were immunoreactive for bcl-2 protein [10]. However, in these cancers bcl-2 protein immunoreactivity was correlated with a better prognosis in contrast to primitive neuroectodermal tumours and some high grade malignant lymphomas where bcl-2 immunoreactivity seems to confer an ominous prognosis [9, 14]. By contrast, bcl-2 protein immunoreactivity does not add prognostic information in follicular lymphomas [17], or as shown in the present study, in malignant mesothelioma. One might argue that due to the low number of bcl-2 immunoreactive cases no statistical significance (P=0.09) was reached, although the median actual survival of both groups differed (1.45 months versus 6.87 months). The presence of bcl-2 protein in non-small cell lung cancer invalidates the use of the detection of this protein for distinguishing between malignant mesothelioma and pleural metastasis of non-small cell lung cancer.

We conclude that a fraction of the malignant mesotheliomas in our series are bcl-2 protein immunoreactive in contrast to non-neoplastic mesothelium which is not immunoreactive. Immunoreactivity for the bcl-2 protein does not add prognostic information in our series but may be of value for the discrimination between neoplastic and reactive mesothelium.

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References

1. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X. Nuñez G, Thompson CB (1993) *bcl*-x, a *bcl*-2 related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597–608
2. Brown RW, Clark MG, Tandon AR, Allred DC (1993) Multiple marker immunohistochemical phenotypes distinguishing malignant pleural mesothelioma from pulmonary adenocarcinoma. Hum Pathol 24:347–354
3. Cattoretti G, Becker MH, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J (1992) Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168:357–363
4. Cleary ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19–28
5. 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
6. Hagemeijer A, Versnel M, Van Drunen E, Moret M, Bouts M, Kwast TH van der, Hoogsteden A (1990) Cytogenetic analysis of malignant mesothelioma. Cancer Genet Cytogenet 47:281–285

7. Hockenberry DM, Zutter M, Hickey W, Nahm M (1991) BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88:6961–6965
8. Khan G, Gupta RK, Coates PJ, Slavin G (1993) Epstein-Barr virus infection and *bcl-2* proto-oncogene expression. Separate events in the pathogenesis of Hodgkin's disease? Am J Pathol 143:1270–1274
9. Pezzella F, Tse AG, Cordell JL, Pulford KA, Gatter KC, Mason DY (1990) Expression of the *bcl-2* oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137:225–232
10. Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnil MS, Pierce CB, Harris A, Gatter KC, Mason DY (1993) bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med 10:690–694
11. Qi-Long L, Poulsom R, Wong L, Hanby AM (1993) bcl-2 expression in adult and embryonic non-haematopoetic tissues. J Pathol 169:431–437
12. Ramael M, Segers K, Buysse C, Van den Bossche J, Van Marck E (1991) Immunohistochemical distribution patterns of epidermal growth factor receptor in malignant mesothelioma and non-neoplastic mesothelium. Virchows Arch [A] 419:171–175
13. Ramael M, Buysse C, Van den Bossche J, Segers K, Van Marck E (1992) Immunoreactivity for the β-chain of the PDGF-receptor in malignant mesothelioma and non-neoplastic mesothelium. J Pathol 167:1–4
14. Swanson PE, Humphrey PA, Dehner LP (1993) Immunoreactivity for bcl-2 protein in peripheral primitive neuroectodermal tumors. Appl Immunohistochem 1:182–187
15. Taguchi T, Jhanwar SC, Siegfried JM, Keller SM, Testa JR (1993) Recurrent deletions of specific chromosomal sites in 1p, 3p, 6q, and 9p in human malignant mesothelioma. Cancer Res 53:4349–4355
16. Tsujimoto Y, Croce CM (1986) Analysis of the structure, transcripts and protein products of *bcl-2*, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214–5218
17. Tsujimoto Y, Ikegaki N, Croce CM (1987) Characterization of the protein product of *bcl-2* the gene involved in follicular lymphoma. Oncogene 2:3–7
18. Versnel M, Hagemeijer A, Bouts M, Van der Kwast Th, Hoogsteden H (1988) Expression of c-*sis* (PDGF-B-chain) and PDGF-A chain in ten human malignant mesothelioma cell lines derived from primary tumours and metastatic tumours. Oncogene 2:601–605