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Bcl-2 and Bax expression in thyroid tumours

An immunohistochemical and Western blot analysis

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Abstract Bcl-2 and Bax proteins, which are involved in repressing and promoting programmed cell death, respectively, have been investigated immunohistochemically and by Western blot analysis in a series of thyroid tumours. Three immunostaining patterns were identified. Benign lesions and well-differentiated thyroid carcinomas displayed a profile similar to that of normal follicular epithelium, in which Bcl-2 immunostaining was predominant. Thyroid carcinomas associated with an aggressive behaviour, such as the tall-cell variant of papillary carcinoma and the poorly differentiated carcinomas, co-expressed both proteins. Finally, anaplastic carcinomas expressed only the Bax protein. Western blot analyses revealed that the anti-Bcl-2 antibody recognized two bands, of molecular weights 21 kDa and 25 kDa. This was only seen in the tall-cell papillary carcinomas and in the anaplastic carcinomas.

Key words Bcl-2 · Bax · Thyroid carcinoma · Apoptosis

Introduction

Recently, both Bcl-2, which promotes cell survival, and Bax, which promotes cell death, have been considered as major factors in controlling the apoptotic pathways [5, 6, 9, 12, 16, 19]. Bax protein shows extensive amino acid homology with Bcl-2 and forms homodimers and heterodimers with Bcl-2 in vivo [12]. It has been suggested that the ratio of Bcl-2 and Bax proteins controls the relative susceptibility of cells to lethal stimuli [1, 12]. When Bcl-2 is present in excess cells are protected. However, when Bax is in excess and Bax homodimers are predominant, cells are susceptible to apoptosis.

The expression of Bcl-2 protein has been studied in several normal and malignant human tissues, including lymphomas and lung, breast and thyroid carcinomas. It appears to have a major role in the differentiation of epithelia [2, 9]. In follicular lymphomas, when there is the specific 14;18 translocation, Bcl-2 is increased two- to threefold [11]. In lung cancer, Bcl-2 is expressed in 25% of squamous cell carcinomas and 12% of adenocarcinomas [14]. Bcl-2 expression appears to be associated with less aggressive behaviour in both malignant lymphomas and lung tumours [13, 14]. A strong association between Bcl-2 expression and the presence of oestrogen receptors has been reported for breast carcinoma [4]. Similarly, the expression of Bcl-2 in thyroid carcinomas seems to relate to the degree of differentiation, being absent in anaplastic tumours [15]. It has also been shown that in medullary carcinomas of the thyroid bcl-2 is useful in identifying a subset of lesions with a more aggressive clinical course [20].

Recently the distribution of Bax and Bcl-2 in vivo has been described immunohistochemically in the mouse [7]. Bax and Bcl-2 proteins were expressed by several epithelia, including small intestine, colon, breast, prostate, respiratory tract and skin. In thyroid, Bax immunostaining was undetectable in follicular cells, while Bcl-2 was identified at high levels in these cells. To the best of our knowledge, studies on Bax expression in human benign and malignant thyroid neoplasms have not yet been reported. In order to evaluate the role of Bax protein and the Bcl-2/Bax ratio on thyroid tumours, we carried out an immunohistochemical and Western blot study using anti-Bcl-2 and anti-Bax antibodies. Our results indicate that the less aggressive malignant thyroid tumours express predominantly Bcl-2 protein, while the most aggressive thyroid cancers contain an excess of Bax protein.

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Table 1 Histopathological characteristics of tumours and age of patients

Case no.	Histopathological type of tumour	Age of patient (years)	Size of tumour (cm)
1	Follicular adenoma	54	6
2	Follicular adenoma	72	3.5
3	Papillary carcinoma (classic)	39	2
4	Papillary carcinoma (classic)	44	1.8
5	Papillary carcinoma (follicular)	78	5
6	Papillary carcinoma (follicular)	40	2
7	Papillary carcinoma (tall cell)	51	2.5
8	Papillary carcinoma (tall cell)	56	1.5
9	Papillary carcinoma (tall cell)	73	2.5
10	Papillary carcinoma (tall cell)	65	3.5
11	Poorly differentiated carcinoma	80	2
12	Poorly differentiated carcinoma	67	3
13	Poorly differentiated carcinoma	50	5
14	Poorly differentiated carcinoma	80	4.8
15	Poorly differentiated carcinoma	35	2.9
16	Anaplastic carcinoma	73	5
17	Anaplastic carcinoma	67	2
18	Anaplastic carcinoma	63	8

Table 2 Immunohistochemical results (*NF* normal follicles, *NT* neoplastic tissue, % percentage of positive cells, *IHC Bcl-2, IHC Bax* immunohistochemistry, *WB BCl-1, WB Bax* Western blot analysis, *N.D.* not done)

Case no.	Tumour type	IHC Bcl-2		IHC Bax		WB Bcl-2	WB Bax
		NF %	NT %	NF %	NT %		
1	Follicular adenoma	100	100	1	0	25 kDa	21 kDa
2	Follicular adenoma	100	100	1	1	N.D.	N.D
3	Papillary carcinoma (classic)	100	70	1	20	25 kDa	21 kDa
4	Papillary carcinoma (classic)	100	80	1	2	25 kDa	21 kDa
5	Papillary carcinoma (follicular)	100	80	1	20	N.D.	N.D.
6	Papillary carcinoma (follicular)	100	90	1	2	N.D.	N.D.
7	Papillary carcinoma (tall cell)	100	50	1	70	25 kDa	21 kDa
8	Papillary carcinoma (tall cell)	100	60	1	100	N.D.	N.D
9	Papillary carcinoma (tall cell)	100	60	1	70	N.D.	N.D.
10	Papillary carcinoma (tall cell)	100	50	1	70	N.D.	N.D.
11	Poorly differentiated carcinoma	100	60	1	40	N.D.	N.D.
12	Poorly differentiated carcinoma	100	60	1	40	N.D.	N.D.
13	Poorly differentiated carcinoma	100	50	1	50	N.D.	N.D.
14	Poorly differentiated carcinoma	100	40	1	40	N.D.	N.D.
15	Poorly differentiated carcinoma	100	60	1	60	N.D.	N.D.
16	Anaplastic carcinoma	100	0	1	90	25 kDa 21 kDa	21 kDa
17	Anaplastic carcinoma	100	0	1	100	25 kDa 21 kDa	21 kDa
18	Anaplastic carcinoma	100	0	1	100	N.D.	N.D.

Materials and methods

Formalin-fixed, paraffin-embedded tissues of 18 cases of thyroid neoplasm, consisting of 2 follicular adenomas, 8 papillary carcinomas, 5 poorly differentiated carcinomas, and 3 anaplastic carcinomas, were used for the present study. The papillary carcinomas analysed included 2 cases of the classic papillary lesion, 2 cases of the follicular type, and 4 cases of the "tall cell" variant. Cases were selected from the surgical files of the Institute of Anatomic Pathology of the University of Bologna at Bellaria Hospital and Memorial Sloan-Kettering Cancer Center. Antigen retrieval, consisting in microwave-processing of tissue sections in citrate buffer (4 cycles of 5 min each at 650 W) [10], was applied to consecutive sections, which were stained immunohistochemically with a monoclonal antibody against Bcl-2 (1:100 dilution, Mab 124, Dako Spa, Milan, Italy) and a polyclonal antibody to Bax protein [7] (1:1000 dilution, produced and characterized in Dr. Reed's laboratory), respectively, using the avidin-biotin peroxidase method (Vector Laboratories, Burlingame, Calif.). Negative controls were performed by omitting the primary antibody. Lymph node and cerebellar tissues were used as positive controls for anti-Bcl-2 and anti-Bax antibodies, respectively. The scoring method used for assessing antigen expression was conducted by counting ten high power fields. The number of immunoreactive cells in both normal and neoplastic thyroid cases was expressed as the percentage of the total number of cells counted that were positive.

A double immunostaining assay was performed in 1 case (tumour 8) to evaluate the potential co-expression of Bcl-2 and Bax proteins on tumour cells. Diaminobenzidine was used as the substrate to unveil anti-Bax immunostaining, while nitro-blue tetrazolium was utilized to reveal anti-Bcl-2 reactivities.

For Western blotting, frozen tissue from 6 of the 18 cases studied was available. These included 1 follicular adenoma, 2 papillary carcinomas of the classic type, 1 papillary carcinoma of the tall-cell type, and 2 anaplastic carcinomas. Tissues were solubilized in

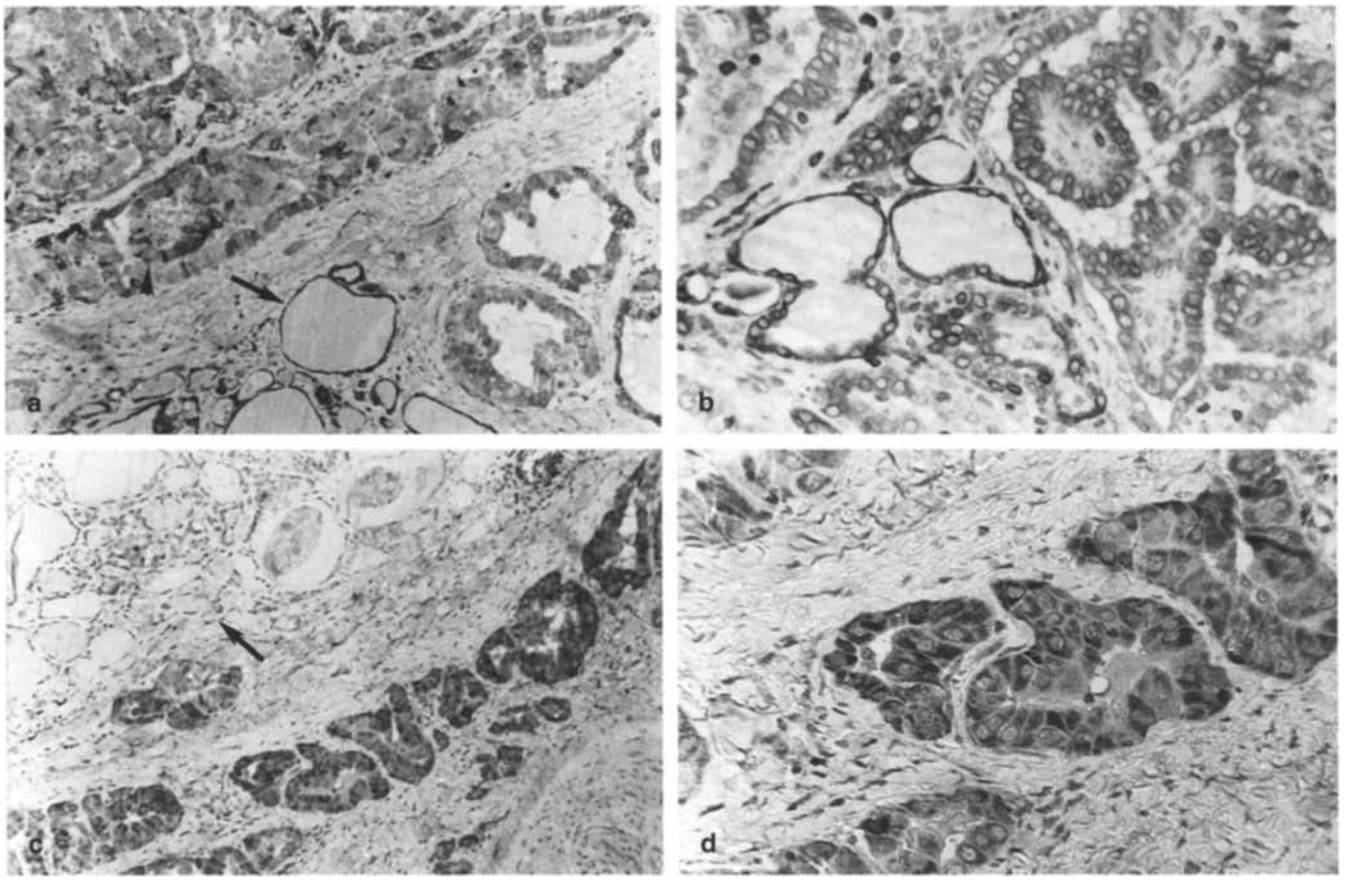

Fig. 1 **a** Case 7: thyroid papillary carcinoma, tall-cell type. The anti-Bcl-2 antibody immunostained the majority of normal follicular cells (*arrow*) and approximately 50% of the neoplastic cells (*arrowheads*). × 125. **b** Case 4: thyroid papillary carcinoma, classic type. Anti-Bcl-2 antibody immunostained approximately 90% of the neoplastic cells. × 210. **c** Case 9: thyroid papillary carcinoma, tall-cell type. Anti-Bax antibody immunoreacted with the majority of neoplastic cells. Note that normal follicular epithelium is unstained (*arrow*). × 100. **d** Case 9: thyroid papillary carcinoma, tall-cell type (higher magnification). Anti-Bax antibody immunostaned 70% of neoplastic cells in this lesion. × 350

sodium dodecyl sulphate (SDS) sample buffer (2% SDS, 200 mM 2-mercaptoethanol). In each case, 100 µg of solubilized proteins was run on a SDS 12.5% polyacrylamide gel [8] and transferred to a nitrocellulose filter [18]. After microwave treatment in citrate buffer (4 cycles of 5 min each at 650 W), anti-Bcl-2 and anti-Bax antibodies were incubated at 1:1000 dilution utilizing the avidin–biotin peroxidase method.

Results

The histopathological characteristics and some demographic data of this group of patients with thyroid neoplasms are summarized in Table 1. Table 2 summarizes the immunohistochemical results obtained in the present study. Anti-Bcl-2 antibody immunostained all thyroid carcinomas with the exception of the three anaplastic cases (Table 2). Positivity was detected in the cytoplasm of the cells composing normal follicles surrounding the lesions as well as the neoplastic areas (Fig. 1A). The staining pattern was that of a finely granular reactivity scattered throughout the cytoplasm. While all neoplastic cells of follicular adenomas showed positive staining, papillary carcinomas of both classic and follicular types displayed an heterogeneous profile. The percentage of positive cells in these cases ranged from 70% to 90%, the tall-cell type varying from 50% to 60% (Fig. 1B). Poorly differentiated carcinomas also displayed an heterogeneous pattern of staining, the proportion of neoplastic cells that were immunoreactive ranging from 40% to 60%.

In the tall-cell type of papillary carcinomas, as well as in all cases of poorly differentiated and anaplastic carcinomas, anti-Bax antibody stained between 40% and 100% of cells (Table 2, Fig. 1C, D). The pattern of staining was finely granular, and mainly detected on the apical portion of the neoplastic cells in the more differentiated areas. In the anaplastic carcinomas the immunostaining was intense and homogeneous. However, all the other cases studied, including the well-differentiated and the benign lesions, showed a decreased staining profile, with a Bax-positive phenotype in 1–20% of tumour cells in these neoplasms. Normal thyroid tissue was found to exhibit scattered positive follicular and parafollicular cells.

Double immunostaining performed in case 8 demonstrated that certain areas possessed neoplastic cells that co-expressed Bcl-2 and Bax proteins. However, other ar-

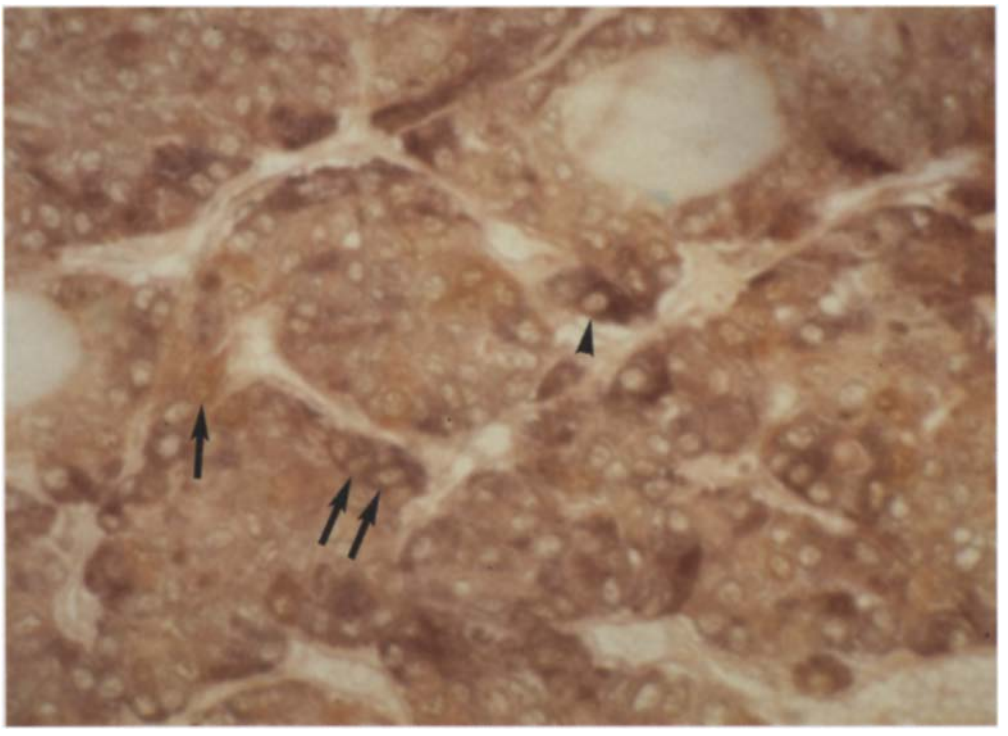

Fig. 2 Thyroid papillary carcinoma, tall-cell type. Double immunostaining using diaminobenzidine substrate for anti-Bax antibody and nitro-blue tetrazolium for anti-Bcl-2 antibody (*dark blue* to *black reaction*). Some cells immunoreacted only with anti-Bax (*arrow*), and others revealed only Bcl-2 (*arrowheads*), while some others immunoreacted with both antibodies (*double arrow*). × 350

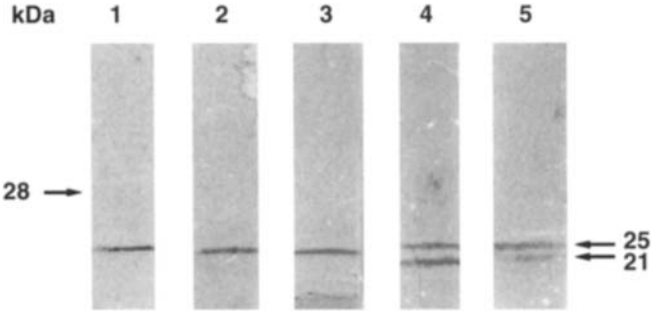

Fig. 3 Western blot analysis of SDS solubilized proteins. Anti-bcl-2 antibody. *1* Follicular adenoma (case 1); *2* papillary carcinoma, classic type (case 3); *3* papillary carcinoma, classic type (case 4); *4* papillary carcinoma, tall-cell type (case 7); *5* poorly differentiated carcinoma (case 11). Anti-Bcl-2 antibody recognized two bands of molecular weight, 21 kDa and 25 kDa, in tall-cell papillary and anaplastic carcinomas

eas revealed a mutually exclusive expression pattern, in which Bcl-2 positive neoplastic cells lacked Bax staining, while cells expressing Bax were Bcl-2 negative (Fig. 2).

Western blot studies revealed a single band at 25 kDa in all tissues tested with anti-Bcl-2 antibody, corresponding to the expected molecular weight of the product encoded by the *BCL2* gene. Nevertheless, in 2 cases of tall-cell papillary carcinoma and 2 anaplastic lesions, anti-Bcl-2 also immunoreacted with a 21 kDa band, coinciding with the molecular weight of the Bax protein (Fig. 3). In all the cases analysed, anti-Bax antibody recognized a single 21-kDa band.

Discussion

Data from this study on Bcl-2 and Bax expression in benign and malignant thyroid tissues reveal three patterns of immunostaining. Follicular adenomas and certain types of papillary carcinoma (mainly classic and follicular variants) showed a Bcl-2-positive phenotype in most of the proliferating elements. Anti-Bax antibody reacted only with a low percentage of neoplastic cells in these cases. However, we observed that both proteins were expressed by the majority of neoplastic cells in the papillary carcinomas of the tall-cell type and in poorly differentiated carcinomas. Finally, the three anaplastic carcinomas studied had a Bax-positive/Bcl-2-negative phenotype.

The phenotypes described above for neoplastic thyroid lesions appear to correlate with their corresponding differentiation stages. It appears that benign lesions and well-differentiated carcinomas exhibit a pattern similar to that of normal follicular epithelium, in which Bcl-2 expression predominates. However, the tall-cell papillary carcinomas and poorly differentiated neoplasms studied were characterized by co-expression of both proteins. The tall-cell papillary tumours are regarded as a variant of papillary thyroid carcinomas with more aggressive behaviour than those of the classic type [17]. Finally, the anaplastic carcinomas analysed were found to express Bax but not Bcl-2 proteins.

Western blot analyses showed that anti-Bcl-2 and anti-Bax antibodies recognize a 25-kDa and a 21-kDa band, respectively. In addition, a second 21-kDa band was detected by the anti-Bcl-2 antibody in the 2 tall-cell

papillary carcinomas and in the 2 anaplastic carcinomas analysed. The presence of the 25-kDa band, corresponding to the molecular weight of the Bcl-2 protein, and of the 21-kDa band, coinciding with the molecular weight of the Bax protein, in all tumours tested, including tumours that were immunohistochemically Bcl-2 or Bax negative, is probably related to the superior sensitivity of the western blot. The second 21-kDa band unexpectedly detected by the anti-Bcl-2 antibody, which corresponds to the identification of the Bax protein, was only noted in the cases where the Bax staining pattern predominated on immunohistochemistry. This phenomenon might be due to the identification of a phosphorylated form of Bcl-2, as has been observed in some cell types (J. Reed, personal communication). It could also be the result of an excessive amount of Bax protein detectable with anti-Bcl-2 antibodies. A cross-reactivity of the anti-Bcl-2 antibody with Bax protein cannot be completely excluded, but is less likely in view of the immunohistochemical results. Recently Branet et al. [3], like us, reported Bax negativity in normal follicles of thyroid. In contrast, most of their cases of papillary carcinomas were positive with anti-Bax antiserum. Branet et al. [3] did not use the microwave antigen retrieval procedure as in the present study, and this method might make the immunohistochemical staining more selective, preventing a cross-reaction with the phosphorylated form of Bcl-2. Finally in the paper of Branet et al. [3], the data on the different subtypes of papillary and follicular carcinomas were not given and it is difficult to exclude a possible selection bias in their cases.

In agreement with the results observed in the present study, Bcl-2 immunostaining has been recently reported in well-differentiated, but low to undetectable in poorly differentiated and anaplastic thyroid carcinomas [12]. Furthermore, this study also related Bcl-2 expression to degree of tumour cell differentiation [12].

Since the ratio Bcl-2/Bax seems to be more relevant than the expression of Bcl-2 protein to the control of apoptosis [1], the identification of Bcl-2/Bax ratios in thyroid carcinoma might give more insight into the biology of these tumours. Benign tumours and indolent malignant tumours contain an excess of Bcl-2 protein and an amount of Bax protein only detectable by Western blot. More aggressive malignant tumours, such as the tall-cell variant of papillary carcinoma and poorly differentiated lesions, express both proteins either on Western blot or at the immunohistochemical level. The more aggressive form of thyroid neoplasms, the anaplastic carcinomas, contain an excess of Bax protein as revealed by immunohistochemistry. In addition, in cases in which Bax protein is present in significant amounts, anti-Bcl-2 antibodies also detect a 21-kDa band when used for Western blot analysis.

These results suggest that a spectrum of Bcl-2 and Bax phenotypes are associated with distinct types of thyroid tumours. An excess of Bcl-2 appears to be associated with benign and less aggressive malignant tumours, while predominance of Bax relates to more aggressive thyroid neoplasms. Bcl-2 and Bax proteins appear to be balanced in malignant thyroid tumours of intermediate aggressiveness. However, the relationship of these phenotypes with a more or less hostile clinical course remains to be clarified.

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References

1. Barinaga M (1994) Cell suicide by ice, not fire. Science 263:754–756
2. Bosman FT, Visser BC, Oeveren J van (1996) Apoptosis: pathophysiology of programmed cell death. Pathol Res Pract 192:676–683
3. Branet F, Brousset P, Krajewski S, Schlaifer D, Selves J, Reed JC, Caron P (1996) Expression of the cell death-inducing gene *bax* in carcinomas developed from the follicular cells of the thyroid gland. J Clin Endocrinol Metab 81:2726–2730
4. Doglioni C, Dei-Tos AP, Laurino L, Chiarelli C, Barbareschi M, Viale C (1994) The prevalence of Bcl-2 immunoreactivity in breast carcinoma and its clinicopathological correlates, with particular references to oestrogen receptor status. Virchows Arch 424:47–51
5. Hockenbery DM, Zutter M, Hickey 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. Korsmeyer SJ (1992) Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood 80:879–886
7. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed J (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol 145:1323–1336
8. Laemmli UK (1997) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680–685
9. Lu Q-L, Abel P, Foster CF, Lalani E-N (1996) Bcl-2: role in the epithelial differentiation and oncogenesis. Hum Pathol 27:102–110
10. Munakata S, Hendricks JB (1993) Effect of fixation time and microwave oven heating time on retrieval of the Ki-67 antigen from paraffin-embedded tissue. J Histochem Cytochem 41: 1241–1246
11. Ngan BY, Chen-Levy Z, Weiss LM, Warnke RA, Cleary ML (1988) Expression in non-Hodgkin's lymphoma of the Bcl-2 protein associated with the t(14;18) translocation. N Engl J Med 318:1638–1644
12. Oltvai Z, Milliman C, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619
13. Pezzella F, Morrison H, Jones M, Gatter KC, Lane D, Harris AL, Mason DY (1993) Immunohistochemical detection of p53 and Bcl-2 proteins in non-Hodgkin's lymphoma. Histopathology 22:39–44
14. Pezzella F, Turley H, Kuzu I, Tungekar MF, 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
15. 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
16. Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6

17. Sobrino-Simoes M (1995) Tumours of thyroid: a brief overview with emphasis on the most controversial issues. Curr Diagn Pathol 2:15–22
18. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
19. Vaux DL (1993) Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci USA 90:786–789
20. Viale G, Roncalli M, Grimelius L, Graziani D, Wilander E, Johansson H, Bergholm U, Coggi G (1995) Prognostic value of bcl-2 immunoreactivity in medullary thyroid carcinoma. Hum Pathol 26:945–950