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Immunohistochemical localization of Bcl-2 and Bax proteins in in situ and invasive duct breast carcinomas

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Abstract Bcl-2 and Bax proteins are coded by a family of genes that take part in the manteinance of the balance between cell proliferation rate and programmed cell death in multicellular organisms. The *Bax* gene acts as promoter of cell death by opposing the death protector effect of the *Bcl-2* gene. Expression of the Bcl-2 and Bax proteins has been investigated in 58 cases of duct carcinoma in situ (DCIS) and duct invasive and invasive lobular carcinomas (IC) of the breast. While both proteins were expressed at the same time in normal and benign epithelium, different staining patterns were observed according to the degree of differentiation of the neoplastic epithelium. In well-differentiated DCIS and grade I IC there was a predominance of Bcl-2 protein staining. Grade II lesions co-expressed both proteins. Poorly differentiated DCIS displayed a predominantly Bax protein staining pattern. Therefore, it appears that Bax protein expression, especially in DCIS, relates to more aggressive neoplasms while Bcl-2 protein expression is associated with less aggressive malignant lesions.

Key words *Bcl-2* · *Bax* · Breast carcinoma · Grading

Introduction

A great deal of interest has recently been focused on apoptosis or programmed cell death (PCD), which plays an important part in the regulation of tissue development, differentiation and homeostasis [1, 2, 18, 33]. The main product of the *Bcl-2* gene functions as a repressor of PCD without affecting cell proliferation [11, 15, 17, 23, 27]. The *Bcl-2* gene was first isolated at the chromosomal break point of t(14;18)-bearing follicular B-cell lymphomas [11, 17, 22–25]. This translocation frequently results in the overexpression of Bcl-2 protein [11, 17, 22, 24, 27]. However, this genetic abnormality is not the sole cause of Bcl-2 dysregulation in neoplastic cells [27]. A group of genes that have an extensive sequence homology with the *Bcl-2* gene has been recently described and named the *Bcl-2* gene family [2, 15, 16, 18, 23, 32, 34]. The most distinctive common feature of the members of this gene family is the fact that their expression modulates cell death [18]. The genes constituting this family can be subdivided into two functionally antagonistic groups: cell death suppressors, such as *Bcl-2*, and cell death promoters, such as *Bax*. These latter form heterodimers with *Bcl-2* and accelerate rates of cell death [15, 16, 23]. The ratio of Bcl-2 to Bax proteins determines whether a cell will survive or die following an apoptotic stimulus. When Bax protein expression predominates PCD is accelerated and the death suppressor activity of the *Bcl-2* gene product is overcome [2, 15, 16, 23]. The expression of Bcl-2 protein has been studied extensively in normal [11, 21, 28] and neoplastic human tissues, including malignant lymphomas [23, 26, 27], carcinomas of lung [27], prostate [6], and breast [3, 7, 10, 13, 14, 17, 29] and cutaneous malignant melanomas [5]. Recently the distribution of *Bcl-2* and *Bax* gene products in vivo has been investigated in mouse [15]. Both proteins are present in several normal epithelial tissues including small intestine, colon, breast, prostate, respiratory tract, skin [15] and thyroid [19].

In breast carcinoma the presence of Bcl-2 protein is usually associated with favourable clinicopathological features, such as presence of oestrogen and progesterone receptors, low proliferative activity, and low histological grade [3, 7, 10, 13, 14, 17, 29]. Some of these features are regarded as indicators of tumour differentiation. There have been only occasional reports about the relationship between Bcl-2 and Bax proteins expressed in

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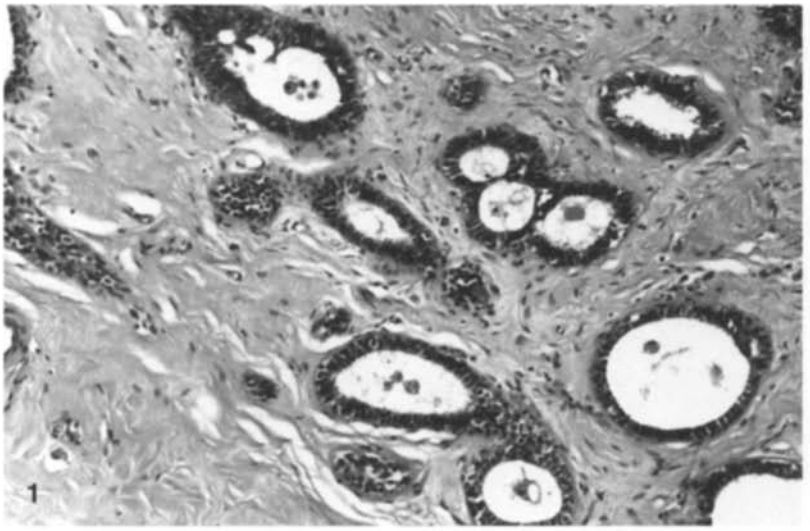

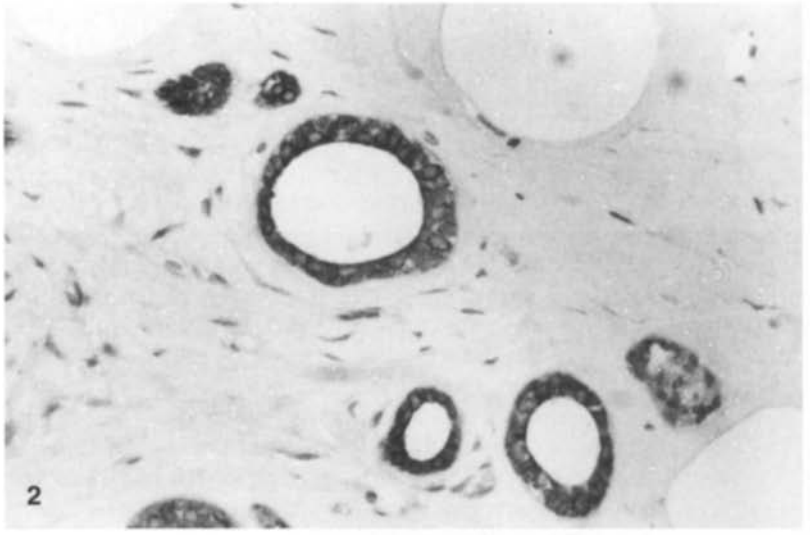

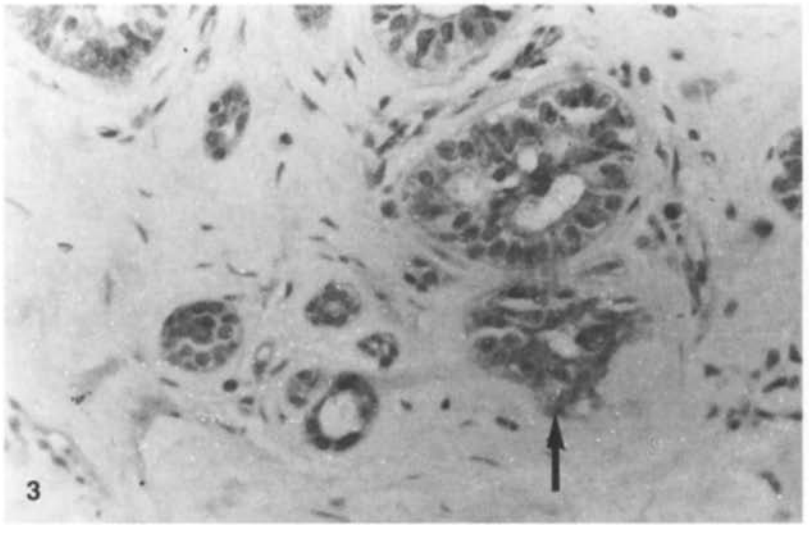

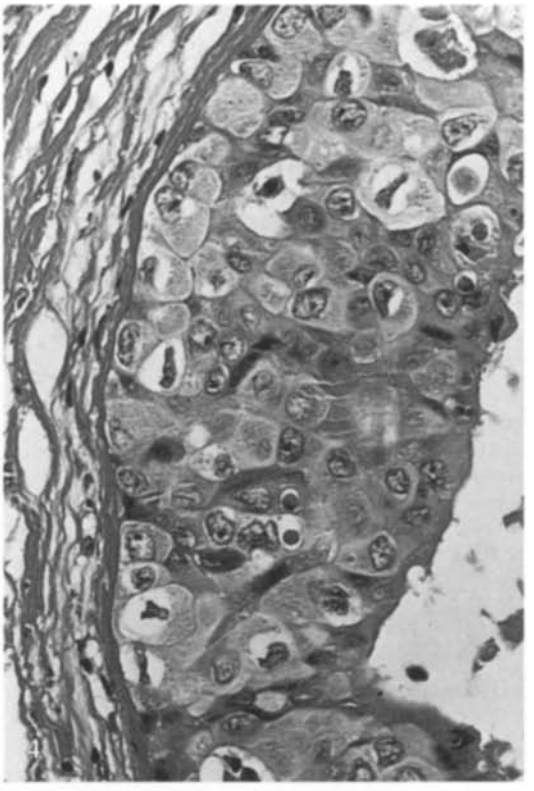

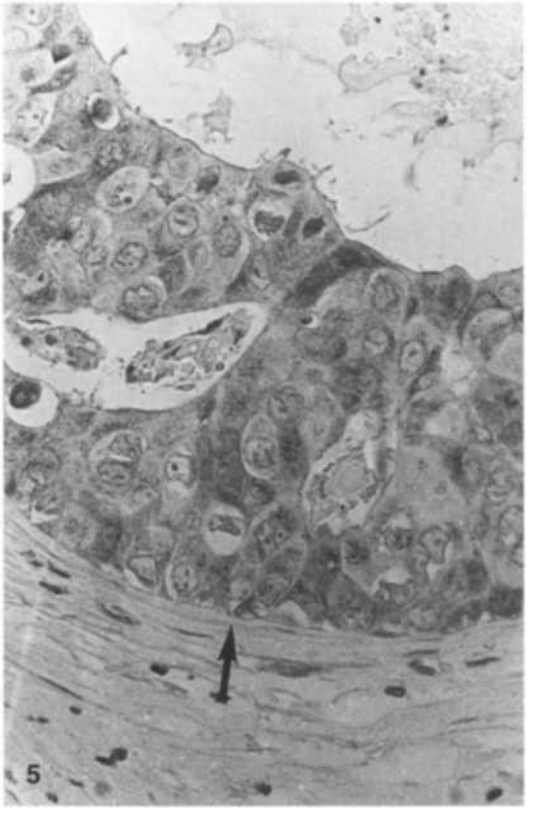

Fig. 1 Case 39: nonneoplastic epithelium. H&E, ×75

Fig. 2 Case 39: the epithelium is strongly positive with anti Bcl-2 antiserum. ABC peroxidase, ×10

Fig. 3 Case 39: weak positivity (*arrow*) of nonneoplastic epithelium is seen with anti-Bax antibody. ABC peroxidase, ×100)

Fig. 4 Case 47: poorly differentiated duct carcinoma in situ (DCIS). Neoplastic cells show irregular nuclei and prominent nucleoli, and no polarization is evident. H&E, ×350

Fig. 5 Case 47: the neoplastic cells show a distinctive granular cytoplasmic positivity (*arrow*). Anti-Bax ABC peroxidase, ×350

breast carcinoma [1, 16], which have mainly focused on invasive ductal carcinoma. This prompted us to investigate the relationship between the expression of these two proteins in in situ and invasive ductal breast carcinomas.

Materials and methods

Fifty-eight cases of breast carcinoma were obtained from the files of the Department of Radiology and Anatomic Pathology of the University of Bologna at Bellaria Hospital and from the consultation files of one of us (V.E.). These were 28 cases of pure duct carcinoma in situ (DCIS), 6 cases of pure invasive duct carcinoma (DCI), 2 cases of classical lobular carcinoma and 22 cases of DCIS and DCI present in the same section. Appropriate blocks (one from each case) from 10% buffered formalin-fixed tissues were selected. After dewaxing in xylene and rehydrating through graded alcohols, sections were immersed in citrate buffer (0.01 M, pH 6), treated in a microwave oven (four cycles for 5 min each at 750 W) and cooled to room temperature. Then sequential tissue sections were incubated with monoclonal antibody against Bcl-2 protein (1:10, Mab 124, DAKO Spa, Milan, Italy) and polyclonal antibody to Bax protein (rabbit, 1:1000 from Dr. J.C. Reed) for 60 min at room temperature [15]. The immunostaining was carried out using the indirect immunoperoxidase avidin–biotin complex method (Vector Laboratories, Burlingame, Calif., USA). In negative controls the primary antibodies were omitted. Sections from a lymph node and a poorly differentiated thyroid carcinoma known to be positive for Bcl-2 and Bax antibodies, respectively were used as positive controls.

All slides were independently reviewed by two observers (N.K. and V.E.). In situ carcinomas were classified according to the recently proposed criteria by Holland et al. [12], and invasive components were graded according to the Elston and Ellis modification [8] of Bloom and Richardson's method. Immunoreactivity of both Bcl-2 and Bax proteins was detected as cytoplasmic granular staining. Positive cases were those that had clear-cut positive staining in at least 5% of the total cellular constituents. Inter-observer disagreement was discussed and settled by means of a double-head microscope.

For statistical analysis the $\chi 2$ test was used.

Results

Bcl-2 and Bax protein immunoreactivity was detected both in normal and neoplastic breast epithelium and in foci of epithelial hyperplasia associated with the neoplastic lesions (Figs. 1–3). The distribution of types and grades of DCIS and invasive carcinoma (IC) is shown in Table 1. Two of the ICs were lobular carcinoma of the classical type. Forty (69%) and 39 (67%) of 58 carcinomas were Bcl-2 and Bax protein positive, respectively. The distribution of Bcl-2 and Bax protein immunoreactivity in DCIS and IC is shown in Table 2. In poorly differentiated DCIS Bax protein positivity was prominent (81%; Figs. 4–6), while in contrast Bcl-2 protein expression was prominent in well-differentiated DCIS (Figs. 7, 8), appearing positive in all cases (100%). In intermediately differentiated DCIS high levels of immunoreactivity were observed with both Bcl-2 (85%) and Bax (69%) antibodies. Similar features were seen for Bcl-2 immunoreactivity when the IC component was present. Strong positivity (100% of cases) was observed in grade I and II IC, while only 48% positivity was seen in grade III IC.

Table 1 Type and grade distribution of DCIS and IC (*DCIS* duct carcinoma in situ, types I, II, III, *IC* invasive carcinoma)

Type		Grade		
		I	II	III
DCIS	50	11	13	26
IC	30	2	11	17

Table 2 Bcl-2 and Bax immunoreactivity in DCIS and IC, related to grade

Type	Grade			
	I	II	III	Total
DCIS				
Bcl-2 +	11/11(100%)	11/13(85%)	12/26(46%)	34/50(68%)
Bax +	3/11(27%)	9/13(69%)	21/26(81%)	33/50(66%)
IC				
Bcl-2 +	2/2(100%)	11/11(100%)	8/17(48%)	21/30(70%)
Bax +	1/2(50%)	8/11(73%)	10/17(58%)	19/30(63%)

When Bax protein staining was studied in the IC component it appeared positive in about 60% of all cases (Figs. 9, 10). When the two markers were analysed statistically no significant difference was seen for Bcl2 between in situ and invasive forms. In contrast, an inverse statistically significant relationship was seen between Bcl2 and Bax when type of DCIS (P=0.001) and grade of IC (P=0.01) were taken in consideration. Bcl-2 and Bax protein immunoreactivity in the different components of the cases studied is shown in Table 3. No relevant difference was seen among the single groups when compared for each stain (DCIS with and without IC component). One of the 2 lobular carcinomas (both grade II) displayed immunoreactivity with both markers and the other case stained with Bcl-2 but not with Bax. Both components in the mixed cases (DCIS and IC) stained at the same time with anti-Bcl-2 and anti-Bax. In 2 cases Bax immunoreactivity was positive in the IC component while it was negative in DCIS. One case displayed immunoreactivity with Bcl-2 antiserum in the IC component, while the DCIS counterpart was distinctly negative. In the same case Bax immunostaining gave opposite results, being positive in DCIS and negative in IC.

Discussion

Recently Holland et al. [12] have proposed a new classification for DCIS in which there is a range extending from well to poorly differentiated types. Bobrow et al. [4] have shown that well-differentiated DCIS are frequently progesterone receptor positive and have a low proliferative index. In contrast, poorly differentiated DCIS are seldom positive with antiserum antiPR, while they posses a high proliferative index. In addition, Eu-

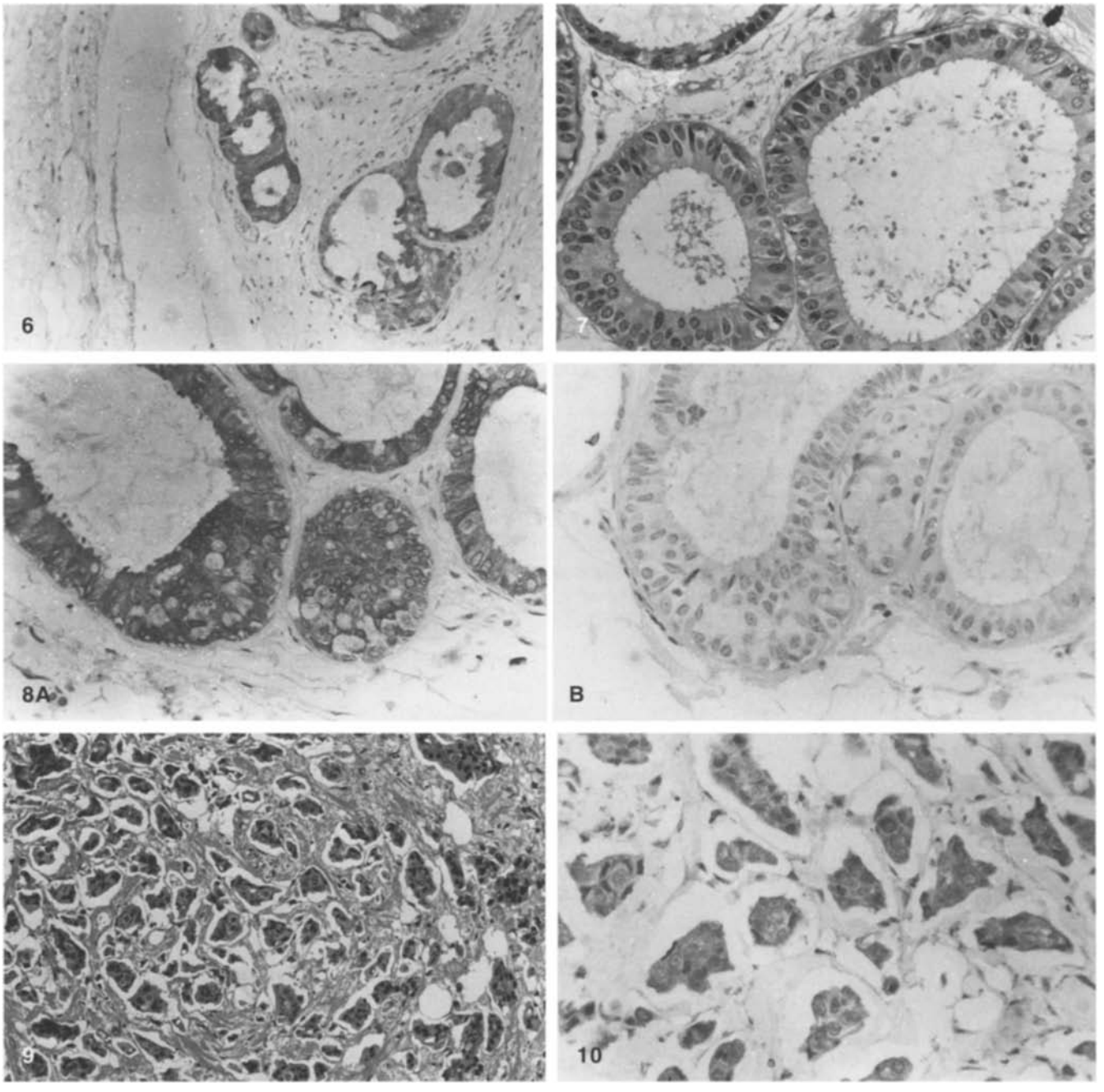

Fig. 6 Case 47: anti-Bax antibody stains the neoplastic cells of this poorly differentiated DCIS intensely, which shows clinging structure. ABC peroxidase, ×75

Fig. 7 Case 54: well-differentiated DCIS. Nuclei are monotonous, and the neoplastic cells show distinctive polarization. H&E, ×75)

Fig. 8A, B Case 54: **A** The neoplastic cells are immunoreactive with anti-Bcl-2 antibody, while **B** anti-Bax antibody gives consistent negative reactivity. ABC peroxidase, ×175

Fig. 9 Case 44: invasive micro-papillary [30] duct carcinoma. H&E, ×100

Fig. 10 Case 44: most of the cells are immunostained by anti-Bax antibody. ABC peroxidase, ×175

sebi et al. [9] have shown that in persons bearing poorly differentiated DCIS the risk of developing an invasive carcinoma is 9 times that in the normal population. However, well-differentiated DCIS involve a very low risk of invasion. Bcl-2 antibody stained all well-differentiated DCIS and most (85%) intermediately differentiated DCIS, but only 46% of poorly differentiated DCIS were positive. In contrast, with Bax antibody only 27% of well-differentiated DCIS and most (81%) poorly differentiated DCIS reacted positively. IC was stained with Bcl-2 antibody in all cases of grade I and II lesions and in 48% of grade III lesions, while Bax antibody consistently stained about 60% of cases in different grades. With the exception of 3 cases the staining with the two markers used was consistent between the two compo-

Table 3 Bcl-2 and Bax immunoreactivity when every component is studied separately (*DCIS*, predominant DCIS with invasive subset, *IC***, IC with minor DCIS component)

Type	Grade			
	I	II	III	Total
DCIS				
Bcl-2 +	8/8(100%)	7/8(88%)	4/12(33%)	19/28(68%)
Bax +	3/8(38%)	5/8(63%)	11/12(92%)	19/28(68%)
DCIS *				
Bcl-2 +	3/3(100%)	4/5(80%)	8/14(57%)	15/22(68%)
Bax +	0/3(0%)	4/5(80%)	10/14(71%)	14/22(64%)
IC **				
Bcl-2 +	2/2(100%)	7/7(100%)	7/13(54%)	16/22(73%)
Bax +	1/2(50%)	5/7(71%)	9/13(69%)	15/22(68%)
IC				
Bcl-2 +	0/0(0%)	4/4(100%)	1/4(25%)	5/8(63%)
Bax +	0/0(0%)	3/4(75%)	1/4(25%)	4/8(50%)

nents. Therefore it seems that in DCIS, Bcl-2 and Bax staining is inversely correlated with the degree of differentiation of the tumour, as also found in invasive thyroid carcinomas [19]. In contrast, in the invasive component only the Bcl-2 protein expression appeared to be consistently decreased in grade III IC, whereas Bax expression was consistently present in about 50% of the cases, independently of grade. Bcl-2 expression is usually correlated with oestrogen and progesterone receptor positivity, low proliferative activity and low histological grade [3, 7, 13, 14, 17, 29], and is usually lacking in p53- [13, 17, 29, 31] , EGFR- [7, 17] and c-erbB-2- [17] positive tumours. This is in keeping with the data reported by Bobrow et al. [4], who found low Ki-67 and high progesterone receptor positivity in well-differentiated DCIS as opposed to poorly differentiated DCIS. These had significantly low progesterone receptor scores and high Ki-67-positive cell counts. An inverse relationship has also been seen between Bcl-2 immunoreactivity and proliferative index in DCIS by Siziopikou et al. [31].These findings suggest that Bcl-2 protein positivity is a feature of less aggressive, better differentiated breast carcinomas.

Bax, which is a member of *Bcl-2* gene family, acts as a promoter of cell death, by opposing the death protector effect of *Bcl-2* [2, 15, 16, 18, 23]. In normal breast epithelium both Bcl-2 and Bax proteins are expressed [1, 15, 16], indicating an active antagonism between Bcl-2 and Bax proteins [15]. This antagonism is clearly evident in the present cases of DCIS, in which a clear inverse relationship has been seen in relation to the differentiation of the various tumour types, with the highest value for Bax protein expression being seen in poorly differentiated DCIS. Krajewski et al. [16] have reported Bax immunoreactivity in 98% of their DCIS cases. This probably comes about because most if not all the DCIS cases in Krajewski's series were poorly differentiated, as their patients all had advanced breast cancer, a finding in keeping with the present data. In addition, Krajewski et al. [16] also found (as in the present paper) marked reduction in immunostaining when the invasive component of advanced breast cancer was evaluated. The *Bax* gene promoter region contains four motifs with homology to consensus p53-binding sites. Regardless of whether there exists an obligatory requirement for stimulation of *Bax* gene expression in all types of cells in which p53 has been shown to induce apoptosis, it seems reasonable to propose that p53-mediated elevations in Bax protein levels would at least render cells more susceptible to apoptotic cell death [20]. Admittedly, p53 was not considered here, but nevertheless, future studies will probably clarify whether *Bax* is a primary-response gene for p53 and is involved in a p53-regulated patway for induction of apoptosis. Sixty-three percent of cases of IC in the present series were immunostained by Bax antiserum. The positivity was 58% for grade III IC. This is in keeping with 34% positivity found by Krajewski et al. [16], who studied predominantly high-grade tumours all selected from women with metastatic breast carcinoma. In the present cases positive correlation was seen in grade III IC between Bcl-2 and Bax immunostaining; this correlation was also found by Krajewski et al. [16]. A similarly low Bax protein expression was seen mainly in cell cultures by Bargou et al. [1], who suggested that the apoptosis-promoting gene *Bax* is down-regulated in some breast cancers. In these, the constituent cells where the *Bax* gene is not expressed are resistant to apoptosis.

We conclude that Bcl-2 protein expression predominates in well-differentiated, and Bax protein expression in poorly differentiated, high-grade carcinomas in the early stages of breast carcinoma (in situ lesions). As the tumour progresses to a more aggressive form and loses its differentiation, Bcl-2 and Bax expression become coordinate and decrease. These changes can be attributed to other unknown genetic modifications in different stages of breast carcinogenesis. In poorly differentiated pure DCIS, predominance of Bax protein expression accelerates cell death. In well-differentiated DCIS the predominance of Bcl-2 protein expression probably contributes to the more protracted behaviour of the lesions [9]. Similar expression of both proteins in both components of mixed cases where DCIS and IC are found and, conversely the loss of expression in pure IC, may be the result of altered mechanisms of PCD in advanced forms of breast carcinomas, as suggested by Krajewski et al. [16]. Alterations in PCD and expression of oncogenes are probably two of several factors that contribute to the evolution of a breast lesion from DCIS to IC.

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