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The prevalence of BCL-2 immunoreactivity in breast carcinomas and its clinicopathological correlates, with particular reference to oestrogen receptor status

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Abstract BCL-2 protein plays a pivotal role in overriding programmed cell death (apoptosis), thus favouring a prolonged survival of normal and neoplastic cells. Expression of the *bcl*-2 gene has been documented in some human tumours (non-Hodgkin's lymphomas and prostatic adenocarcinomas), but findings in breast carcinomas have not been reported. We have used the monoclonal antibody 124 to investigate BCL-2 expression in 212 breast carcinomas, and to correlate it with the oestrogen (ER), progesterone (PR) and epidermal growth factor receptor (EGFR) status, and with other clinicopathological variables including tumour type, grade, stage, growth fraction (as evaluated by Ki-67 immunostaining), and p53 accumulation. Of the 212 carcinomas, 173 (81.6%) exhibited BCL-2 immunoreactivity in more than 25% of the neoplastic cells. BCL-2 immunoreactivity was strongly correlated with ER and PR expression ($P<0.00001$), with the lobular type ($P=0.012$) and with better differentiated neoplasms ($P=0.00003$), whereas it was inversely correlated with EGFR ($P<0.00001$), p53 ($P=0.0004$) and Ki-67 ($P=0.0002$) immunoreactivities. No association was found with tumour stage (T and N categories). We conclude that *bcl*-2 expression in breast cancers is related to the oestrogen-dependent transcription pathway.

Key words BCL-2 immunoreactivity
Breast carcinoma · Oestrogen receptors
Progesterone receptors · p53 accumulation

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Introduction

The *bcl*-2 proto-oncogene codes for a protein localized to the mitochondrial membrane (Hockenbery et al. 1990) effective in overriding programmed cell death (apoptosis; Vaux et al. 1988; Hockenbery et al. 1990; Nunez et al. 1990). *bcl*-2 gene activation has been documented in almost two-thirds of human follicular lymphomas of B-cell lineage (Pezzella et al. 1990a), where the t(14;18) reciprocal chromosomal translocation induces overproduction of BCL-2 mRNA and protein. In these cases, *bcl*-2 expression is most likely up-regulated by the influence of long-range transcription control elements associated with the immunoglobulin heavy chain locus (Cleary and Sklar 1985; Cleary et al. 1986).

The development of polyclonal (pAb) and monoclonal antibodies (mAb) to the BCL-2 protein, however, has allowed the documentation of *bcl*-2 expression in several normal embryonic and adult human cell types (Hockenbery et al. 1991; LeBrun et al. 1993; Lu et al. 1993), including some characterized by hyperplasia and involution (via apoptotic cell death) usually in response to cell-specific hormonal stimuli (Hockenbery et al. 1991). Furthermore, BCL-2 immunoreactivity has been demonstrated in follicular and diffuse lymphomas of B- and T-cell lineage even in the absence of any chromosomal translocation (Pezzella et al. 1990b, 1993a), in Hodgkin's disease (Bhagat et al. 1993; Doussis et al. 1993), in lung cancers (Pezzella et al. 1993b) and in prostatic adenocarcinomas (McDonnell et al. 1992; Colombel et al. 1993). These findings indicate that the 14;18 translocation is not necessary for the synthesis of an immunocytochemically detectable amount of BCL-2 protein.

Among the different normal human tissues investigated, BCL-2 protein has also been localized to the ductal epithelium of the breast (Hockenbery et al. 1991; Lu et al. 1993). The prevalence of BCL-2 immunoreactivity in human breast cancers, however, has not been assessed to date. Hyperplasia and involution of the mam-

mary glandular epithelium are regulated by oestrogens, and the anti-oestrogenic drug tamoxifen has been shown to induce apoptosis of hormone-responsive breast cancer cells (Knabbe et al. 1987; Kyprianou et al. 1991; Butta et al. 1992; Wilson et al. 1992). The question arises whether the trophic effects of oestrogen on the target mammary cells might be at least in part mediated by the up-regulation of *bcl*-2 gene expression. This would confer the selective advantage of prolonged survival.

The current investigation was aimed at assessing the prevalence of BCL-2 immunoreactivity in a large series of human breast carcinomas, and at ascertaining its correlations with the expression of sex steroid hormone and epithelial growth factor receptors (EGFR), and with additional clinicopathological parameters, including tumour type, grade, stage and growth fraction (as evaluated by Ki-67 immunostaining; Gerdes et al. 1983), p53 accumulation and patient age at presentation.

Materials and methods

Formalin-fixed, paraffin-embedded tissue samples of 212 surgically removed invasive breast carcinomas were retrieved from the surgical pathology files of the institutions participating in this study. Only cases with immunocytochemically defined oestrogen (ER) and progesterone receptor (PR) status were selected. For all cases, the original pathological reports, which included type, grade (according to Elston and Ellis 1991) and pathological stage (according to the UICC recommendations) of the tumours, and age of the patients at presentation, were available. There were 170 ductal not otherwise specified (NOS), 27 lobular (including 18 classic variants, 6 mixed, 2 pleomorphic and 1 solid, according to Dixon et al. 1982) and 5 medullary, 4 mucinous, 3 tubular, and 3 mixed (ductal and lobular) carcinomas. Among the ductal NOS carcinomas, 18 tumours were grade I, 100 grade II, and 48 grade III (4 tumours were not graded, due to a predominant intraductal component). Ninety-six tumours were staged as pT1, 76 as pT2, 12 as pT3, and 24 as pT4. Four cases had tumour present at the margins of lumpectomy specimens and were staged pTx. Lymph node metastases were present in 101 cases; in 6 cases lymph node dissection was not performed. The mean age of the patients at presentation was 61 years (range 29–92 years). The haematoxylin and eosin-stained slides of all cases were reviewed, as were frozen tissue sections immunostained for ERs and PRs, using commercially available reagents in kit form (ER-ICA and PgR-ICA assay, Abbott, North Chicago, Ill., USA). Frozen tissue sections immunostained with the Ki-67 mAb (Dakopatts, Glostrup, Denmark), and routinely fixed and embedded sections immunostained for EGFR (31G7 mAb, Triton, Alameda, Calif., USA) and p53 protein (pAb 1801 mAb, Oncogene Science, Manhasset, N.Y., USA) were also available for revision in 54, 149 and 197 cases, respectively.

For immunocytochemistry, consecutive serial sections were cut from a representative paraffin block of each case, and immunostained overnight for localization of BCL-2 protein (using 1:200 dilution of the 124 mAb, Dakopatts), according to an indirect avidin-biotin procedure, as previously reported (Doglioni et al. 1987).

Negative control sections for specificity were obtained by substituting the immunoglobulin fraction of nonimmune mouse serum for the specific primary mAb, and they were consistently unstained. Normal lymphocytes infiltrating the tumours or the peritumour tissues were present in several cases and represented a built-in positive control for BCL-2 immunostaining.

In selected cases ($n=50$), ER were localized also in the paraffin sections (Hiort et al. 1988) to allow comparison of the number and distribution of the ER and BCL-2 immunoreactive cells. Double immunostaining experiments for the simultaneous localization of ER and BCL-2 in the same formalin-fixed, paraffin-embedded tissue sections were performed additionally in 10 of the above cases, according to a previously detailed staining procedure (Doglioni et al. 1990).

Immunocytochemical results were scored as the percentage of cells exhibiting definite immunoreactivity over at least 2000 neoplastic cells in ten or more randomly selected high-power fields ($\times 400$). For statistical analysis, cases were considered positive for BCL-2 and EGFR if they showed immunoreactivity in more than 25% of the neoplastic cells, while for ER, PR and p53 protein the cut-off value was nuclear staining of 10% neoplastic cells. For Ki-67 immunoreactivity, the absolute percentage of the stained cells over at least 2000 neoplastic cells was recorded.

Statistical analyses were performed using the Fisher's exact test for 2×2 contingency tables, the chi-squared test for larger tables, and the Mann-Whitney two sample test as unpaired non-parametric test. Two-tailed P values <0.05 were considered statistically significant.

Results

Non-neoplastic breast tissue was present in 80 cases. BCL-2 immunoreactivity was consistently detectable in approximately 60% of the epithelial cells lining the mammary ducts and lobules. The staining was cytoplasmic and mostly confined to the luminal cells, the myoepithelial cell component being unreactive or very weakly stained (Fig. 1).

Among the 212 breast carcinomas studied, 173 (81.6%) showed cytoplasmic immunoreactivity for BCL-2 in more than 25% of the neoplastic cells (Fig. 2a). Neoplastic cells were interpreted as positively stained if staining intensity was comparable to that of the immunoreactive normal cell counterpart. A certain

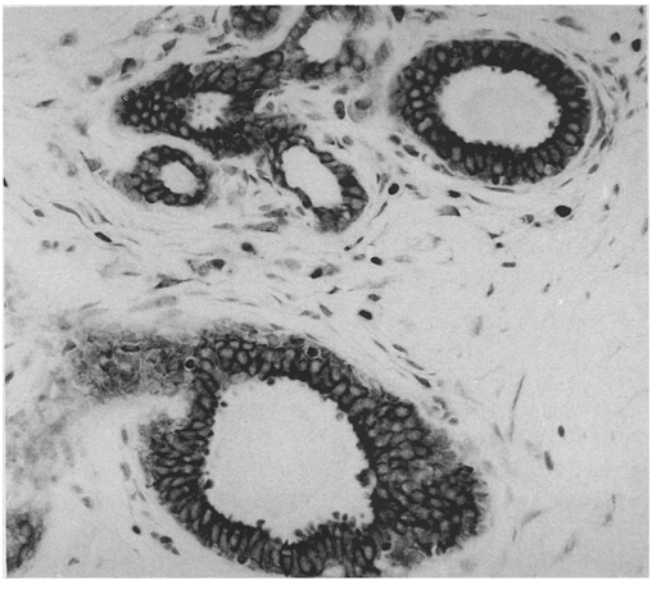

Fig. 1 Immunoreactivity for BCL-2 protein in non-neoplastic breast tissue, $\times 400$

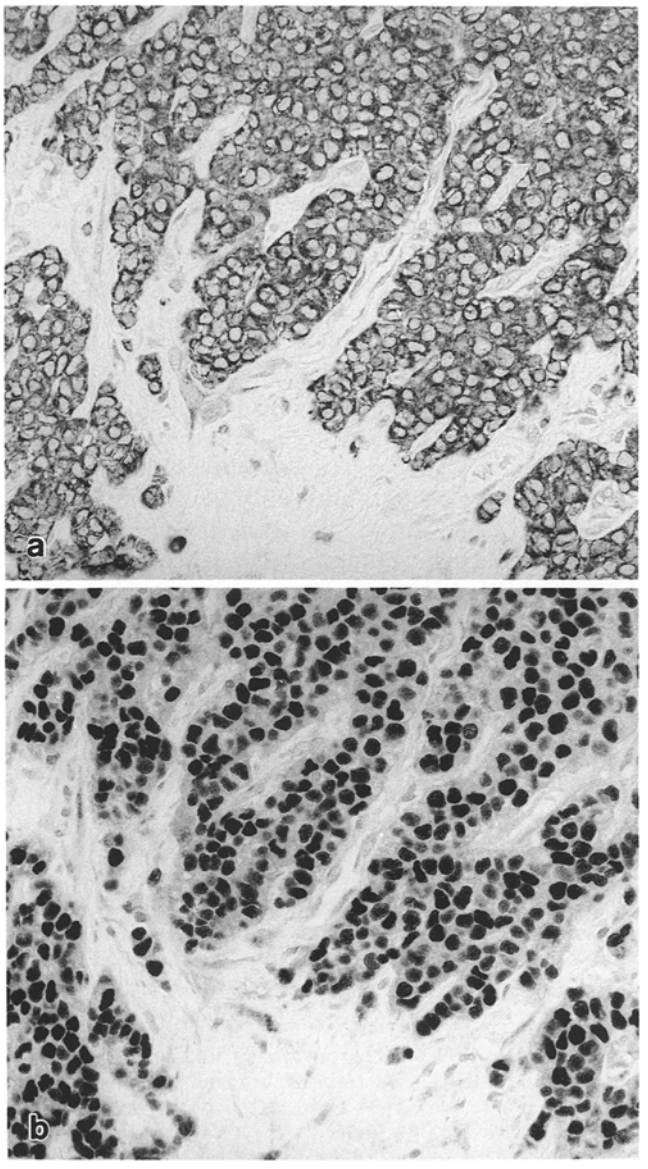

Fig. 2 Immunocytochemical detection of BCL-2 protein (**a**) and oestrogen receptors (**b**) in consecutive serial sections of a ductal not otherwise specified carcinoma of the breast. The identical neoplastic cells are simultaneously immunoreactive for both antigens. ×400

degree of staining heterogeneity within the neoplastic cell population of immunoreactive cases was commonly observed, but no consistent increase in definite neoplastic areas (infiltrative margins vs tumour centre) was detectable.

As shown in Table 1, BCL-2 immunoreactivity showed a strong positive correlation with ER and pR immunostaining ($P < 0.00001$). In most (120 of 164) cases immunoreactive for both BCL-2 and ER, the percentage and distribution of stained cells were very similar for either antigen (Fig. 2); furthermore, in double immunostaining experiments, almost 90% of the stained cells exhibited simultaneous immunoreactivity for both antigens. In the remaining 44 cases, BCL-2 expressing cells outnumbered ER-immunoreactive neoplastic cells, and ER immunoreactivity was never encountered in epithelial cells lacking BCL-2 immunostaining.

Table 1 Summary of the immunostaining results and clinicopathological correlations (*NOS* not otherwise specified, *n.s.* not significant)

Clinicopathological features	BCL-2 immunoreactivity		*P* value
	positive	negative	
Oestrogen receptor			
positive	164	2	<0.00001
negative	9	37	
Progesterone receptor			
positive	132	2	<0.00001
negative	41	37	
Epidermal growth factor receptor			
positive	11	16	<0.00001
negative	120	2	
p53 positive	41	21	0.0004
negative	119	16	
Ductal NOS carcinomas	135	35	0.025
Lobular carcinomas	27	0	(0.012*)
Other types	11	4	
Tumour grade I	18	0	
II	88	12	0.00003
III	29	19	
Tumour stage pT1	82	14	
pT2	58	18	n.s.
pT3	10	2	
pT4	21	3	
Lymph node metastases			
present	83	18	n.s.
absent	86	19	

**P* value calculated for ductal vs lobular carcinomas

BCL-2 immunoreactivity was also significantly more prevalent in lobular (both in the classic variant and in the other varieties) than in ductal NOS tumours ($P = 0.012$) and in better differentiated neoplasms ($P = 0.00003$), whereas it was inversely correlated with EGFR ($P < 0.00001$), p53 ($P = 0.0004$) and Ki-67 ($P = 0.0002$) immunoreactivity. In the 3 mixed (ductal and lobular) carcinomas, BCL-2 immunoreactivity was detected in either component. No association was found between BCL-2 immunoreactivity and tumour stage (T and N categories) or age of patients at presentation.

Discussion

The current study documents a high prevalence (81.6%) of BCL-2 immunoreactivity in human breast carcinomas. The finding is consistent with the invariable immunoreactivity for this oncoprotein exhibited by the majority of normal breast epithelial cells. BCL-2 protein produces a significant extension of cell survival, and extended cell survival may be considered a key event ei-

ther in cell transformation or in tumour growth (Korsmeyer 1992).

Because BCL-2 immunoreactivity is a feature of normal epithelial cells undergoing hyperplasia and involution under hormonal control (Hockenbery et al. 1991), the current investigation was aimed at ascertaining any possible correlation of BCL-2 immunoreactivity with the sex steroid hormone and EGFR status of breast carcinomas. Our findings document a strong positive correlation between BCL-2 and ER and PR immunoreactivities; furthermore, the identical normal and neoplastic cells are simultaneously immunoreactive for BCL-2 and ER, as revealed by double staining experiments. Also, the prevalence of BCL-2 immunoreactivity is significantly higher in lobular carcinomas, in better differentiated and low-cycling tumours, and in tumours lacking EGFR and p53 immunostaining. These findings suggest that BCL-2 immunoreactivity might be a feature of a less aggressive subset of breast carcinomas, as has been recently shown in squamous cell carcinomas of the lung (Pezzella et al. 1993b). Studies of large series of cases with long-term follow-up may document, with multivariate analysis, whether *bcl*-2 expression alone actually has prognostic relevance.

The very close association between BCL-2 and ER immunoreactivities indicates that up-regulation of *bcl*-2 gene expression is likely to be related to the oestrogen-dependent transcription pathway. Accordingly, in oestrogen-dependent breast cancers (and also in normal breast epithelium), the trophic hormonal effects may well include, apart from the promotion of cell proliferation, the induction of prolonged cell survival due to the inhibition of programmed cell death. The lack of immunocytochemically detectable BCL-2 protein in high-grade and high-cycling breast carcinomas suggests that tumour growth in these cases is preferentially sustained by proliferative stimuli. If the inhibition of apoptosis also plays a significant role in these neoplasms, then factors other than *bcl*-2 up-regulation must be effective. The inverse relationship between BCL-2 immunoreactivity and p53 accumulation may be of particular interest in this respect. Indeed, wild-type p53 protein (which is synthesized in low amounts and has a very short half-life, to the point that it is not immunocytochemically detectable; Rogel et al. 1985) not only inhibits cell proliferation (Lane 1992), but has been also shown to induce apoptosis in a leukaemic cell line (Yonish-Rouach et al. 1991). Following gene mutation, an abnormal p53 protein is synthesized, which accumulates within the cell and becomes detectable immunocytochemically (Cattoretti et al. 1988; Finlay et al. 1988; Bartek et al. 1991; Bosari et al. 1992). It is tempting to speculate that loss of function of the mutated p53 protein might confer on the breast cancer cells a double growth advantage, because the uncontrolled proliferation is combined with a reduced cell death rate. Interestingly, a significant inverse relationship between BCL-2 protein expression and p53 accumulation has also been documented recently in non-Hodgkin's lymphomas (Pezzella et al. 1993a).

The data presented herein may also have therapeutic implications. Tamoxifen is widely used in the treatment of oestrogen-dependent breast cancers, and it is now under investigation as a chemopreventive agent (Fisher 1992; Jordan et al. 1992). Our findings suggest that the effectiveness of this drug in inducing apoptosis of oestrogen-dependent cancer cells (Knabbe et al. 1987; Kyprianou et al. 1991; Butta et al. 1992; Wilson et al. 1992) might also be mediated by decreased *bcl*-2 gene expression, which is no longer up-regulated by oestrogens. Further studies are now in progress to evaluate the rate of BCL-2 expression in hormone-dependent breast cancer cell lines in response to different oestrogen concentrations, and to the administration of tamoxifen. These studies will allow us to ascertain whether BCL-2 immunoreactivity of breast carcinomas is predictive of a therapeutic response to anti-oestrogenic therapy. Furthermore, a better knowledge of the actual role of BCL-2 oncoprotein in sustaining breast cancer growth may result in the development of new therapeutic regimens, combining the effects of tamoxifen with that of apoptosis-inducing chemotherapeutic agents (Hickman 1992).

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