Virchows Archiv (1995) 426:163–168

ORIGINAL ARTICLE

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bcl-2 protein in invasive ductal breast carcinomas

Received: 7 April 1994 / Accepted: 21 October 1994

Abstract The *bcl*-2 gene encodes a protein which inhibits programmed cell death (apoptosis). This protein was detected by immunohistochemical techniques in 48% of invasive ductal carcinomas of the breast. It was present in well-differentiated carcinomas with hormonal receptors, and proteins synthesized under the control of oestrogens: pS2, cathepsin D and ERD5. In contrast, bcl-2$^+$ carcinomas are less frequently positive for p53 and have a Ki67 score under the mean. bcl-2 protects cells against apoptosis. Accumulation of p53 protein, which is indicative of p53 mutation, would have the same effect; however, these two proteins seem inversely related, an inverse correlation observed by others in breast cancer cell lines and in lymphomas. Tumours positive for bcl-2 escape apoptosis and have worse prognosis but this is not what is found; survival at 5 years, and particularly the absence of recurrence during the first 5 years after surgery, seem to be associated with bcl-2 positivity. The bcl-2 protein seems only to be an important prognostic factor in women over 54 years of age. Moreover, p53$^-$ bcl-2$^+$ tumours have a better response to hormonal therapy than p53$^-$ bcl-2$^-$ tumours.

Key words *bcl*-2 · Apoptosis · Breast cancer

Introduction

bcl-2 protein is a mitochondrial membrane protein [17] which protects cells from programmed cell death (apoptosis) [12]. It has been studied in lymphoid tissues and in lymphomas [5, 26, 30, 32, 36] and in some of these tumours, the *bcl*-2 gene is translocated into the immunoglobulin heavy chain domain [1, 23]. This results in overexpression of the bcl-2 protein and extends B-cell life-span, an increase also demonstrated in cells in which the *bcl*-2 gene has been induced [20, 29, 33].

bcl-2 protein has also been shown to be expressed in epithelial cells and in carcinomas [13]. Epithelial cells that are positive for bcl-2 have a long life-span, they include basal cells in epithelium and in mucous membrane glands. They are often under hormonal control such as the epithelial cells of the prostate, breast and thyroid. Although bcl-2 may play an important role in carcinogenesis through its regulation of cell death [16, 34], studies dealing with bcl-2 in carcinomas are rare. We are aware of two; one on non-small-cell lung carcinomas [24], the other on prostate carcinomas [7].

Since we are engaged in the study of several markers in breast carcinomas [14, 15], we examined whether bcl-2 protein is expressed in these tumours. We sought a relationship between this protein and other clinicopathological characteristics of breast carcinomas and tried to establish the prognostic value of the bcl-2 protein.

Materials and methods

One hundred and ninety patients were studied. All were initially treated by mastectomy and axillary dissection. After surgery, 75 patients received endocrine treatment, 55 chemotherapy and 92 radiotherapy. In 70 patients, two or three types of treatments were combined. In order to have a homogeneous group for histological type, chosen cases all had invasive ductal carcinoma. The following indices were measured: size of the tumour, number of axillary metastases, and histological grade according to Bloom and Richardson [4]. One hundred and thirty-six patients had stage I or II disease, 24 stage III and 17 stage IV. The stage was not known in 13 patients. Clinical data were evaluated on a yearly basis, the last time on 1 June 1993. The observation period was 80–150 months. One-hundred and thirty-six patients were over 55 years of age and 54 patients were under 55 years. Eighty patients (42%) had disease recurrence during the 5 years after surgery and 49 of them (26%) died during this period.

To compare the various histological types, 12 infiltrating lobular carcinomas and 16 medullary carcinomas were also studied. We did not have follow-up for these cases.

bcl-2 protein was also sought in ten dysplastic lesions of the breast.

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For each case, fresh tissues were immediately frozen and kept at −80° C until immunological processing. We also obtained formalin-fixed paraffin-embedded tissues. The various antigens were immunostained according to the peroxidase-anti-peroxidase (PAP) method of Sternberger et al. [28] using diaminobenzidine as substrate. The incubation time in the antisera and in PAP was 30 min each separated by washing in TRIS-buffered saline (TBS). Mouse and rabbit PAP complexes (diluted 1:800 and 1:200) were obtained from Dakopatts, Copenhagen, Denmark, and from Sternberger Monoclonals, Baltimore, Mass., USA.

Finally, all sections were washed in TBS and stained for 10 min in the diamino hydrogen peroxide substrate (40 mg 3–3′ diaminobenzidine in 100 ml TBS containing 35 µl H_2O_2). Nuclei were weakly stained with haematoxylin.

In control slides, the first antiserum was replaced by normal rabbit serum or a supernatant from a hybridoma without specificity for the tissues examined. To select the best methodology on formalin-fixed and paraffin-embedded tissues, 20 cases positive for bcl-2 on frozen tissues were studied using two different technical variations: First the antibody incubation was preceded by enzymatic digestion: protease VII (Sigma 5255 1 mg/1.3 ml phosphate BS) was employed for 10 min at 37° C and second antibody incubation was preceded by treatment with microwaves in citrate buffer according to a method [10] modified from that of Shi et al. [27]. The second method was preferred.

Tumours were defined as positive if one third of their cells were bcl-2$^+$.

All of the antisera employed in the first step are listed in Table 1. For progesterone receptors (PR), oestrogen receptors (ER), androgen receptors (AR) and p53, cases were considered positive if even only rare cells were labelled. For ERD5, pS2 and cathepsin, tumours were considered positive if one-third of their cells were labelled [14, 15]. For Ki67, ten tumour fields were randomly selected. One hundred tumour cells were counted per high-power field (1000×), using a 10×10 eye piece grid and a cell-counter. Any nuclear staining (weak or strong) was considered positive. The average number (of ten fields) of positive cells was multiplied by 0.1 to obtain a tumour score. A mean Ki67 index was then calculated from the total number of scores. Tumours were then separated into two categories (positive and negative) based upon this index. In another study, different cut-off values were expressly chosen in an attempt to best correlate the different variables with clinical outcome. In the present study, the cut-off was selected at the Ki67 mean.

Non-immune serum from swine and swine antirabbit IgG serum were obtained from Dakopatts.

Goat anti-mouse immunoglobulins was obtained from Sternberger Monoclonals.

Monoclonal Clonab LN-C, used as control, was obtained from Biotest Diagnostics, Dreieich, Germany.

Chi-square and Fisher's exact test were used for univariate analysis. Survival analysis was performed using the life-table method.

Results

Forty-three tumours were less than 2 cm diameter, 70 were 2–2.9 cm and 74 were larger than 3 cm. In three cases, the tumour limits were not well defined and the size was therefore not measured.

Sixty tumours were classified as grade I, 78 as grade II and 52 as grade III. Eighty-five cases had no axillary metastases, 53 cases had one to three axillary metastases and 46 cases had more than three axillary metastases. In six cases the axillary contents were not removed.

The usual prognostic variables studied in our series are summarized in Tables 2, 3.

Ninety-one carcinomas (48%) were positive for bcl-2. The staining intensity and the number of positive cells were variable. Tumours were defined as positive if one-third of their cells were bcl-2$^+$. Generally the positivity was cytoplasmic. Several cases showed a mosaic staining pattern with an alternation of negative and positive cells (Fig. 1a, d). In other cases, positive cells were only present at the periphery of the invading tumour (Fig. 1b). In some cases, bcl-2 positivity was localized on the nuclear membrane and in the nucleoli (Fig. 1c). In many cases with lymphocytic infiltration, the lymphocytes were generally bel-2$^+$. This was particularly marked when the tumour cells were bcl-2$^-$ (Fig. 2a, b).

For comparison, 12 infiltrating lobular and 16 medullary carcinomas were studied. Six lobular carcinomas (50%) and 4 medullary carcinomas (25%) were positive. The difference between the percentage of positive medullary carcinomas and that of the other histological types almost attained statistical significance (P=0.06). In dysplastic lesions, the luminal cells were slightly positive in half of the cases and the myoepithelial cells were always negative.

Correlation of bcl-2 positivity with clinicopathological characteristics

There was no relationship between bcl-2 positivity and patient age, tumour size, or the presence of axillary metastases. Significant results are seen in Table 4.

The prognostic value of bcl-2 was established according to death and to disease recurrence at 5 years (Table

Table 1 Antibodies used in this study (*ER* oestrogen receptors, *PR* progesterone receptors, *AR* androgen receptors)

Antiserum	Specificity	Dilution	Tissue	Pretreatment	Source
Monoclonal	Bcl-2	1:200	Fixed	Microwaves	Dakopatts, Copenhagen, Denmark
Monoclonal	Cathepsin D	1:50	Frozen	–	Transbio, Paris, France
Monoclonal	pS2	1:5	Fixed	Proteolytic digestion	CIS Bioindustries, Gif-sur-Yvette, France
Monoclonal	ERD5	1:20	Fixed	–	Amersham, Buckinghamshire, UK
Monoclonal	ER	Kit	Frozen	–	Abbott Laboratories, North Chicago, USA
Monoclonal	PR	Kit	Frozen	–	Abbott Laboratories
Monoclonal	AR	1:10	Frozen	–	Sanbio, Uden, Holland
Monoclonal	Ki67	1:40	Frozen	–	Dakopatts
Monoclonal 1801	p53	1:4000	Frozen	–	Cambridge Research Biochemical, Northwick, UK
Monoclonal 240	p53	1:400	Frozen	–	Oncogene Science, Manhasset, N.Y., USA
Monoclonal 421	p53	1:100	Frozen	–	Oncogene Science
Polyclonal rabbit CM_1	p53	1:2000	Fixed	–	Novocastra, Newcastle-upon Tyne, UK

Table 2 Prognostic value of usual parameters with regard to survival

Clinicopathological characteristics		Number of survivors at 5 years	Number of deaths at 5 years	*P* value
Age	<54 years	41	13	0.44
	>54 years	100	36	
Tumour size:	<2 cm	38	5	0.02
	2–2.9 cm	54	16	
	>2.9 cm	47	27	
Axillary metastases:	0	75	10	<0.001
	1–3	40	13	
	>3	21	25	
Stage:	I	65	8	<0.001
	II	46	17	
	III	13	11	
	IV	6	11	
Grade:	I	49	11	0.21
	II	57	21	
	III	35	17	
ER	+	103	33	0.56
	–	38	15	
PR	+	68	15	0.04
	–	73	33	
Ki 67	+	50	26	0.03
	–	90	23	
p53	+	47	24	0.05
	–	94	25	
pS2	+	64	19	0.46
	–	76	29	
Cathepsin D	+	89	30	0.76
	–	51	19	

Table 3 Prognostic value of usual parameters with regard to recurrence

Clinicopathological characteristics		Number of disease-free survivors	Number of cases recurring at 5 years	*P* value
Age	<54 years	27	27	0.28
	>54 years	76	60	
Tumour size:	<2 cm	33	10	0.003
	2–2.9 cm	37	33	
	>2.9 cm	31	43	
Axillary metastases:	0	60	25	<0.001
	1–3	29	24	
	>3	10	36	
Stage:	I	51	22	<0.001
	II	31	32	
	III	10	14	
	IV	3	14	
Grade:	I	34	26	0.79
	II	40	38	
	III	29	23	
ER	+	75	61	0.77
	–	28	25	
PR	+	50	33	0.16
	–	53	53	
Ki 67	+	36	40	0.13
	–	66	47	
p53	+	38	33	0.88
	–	65	54	
pS2	+	40	43	0.13
	–	62	43	
Cathepsin D	+	66	53	0.59
	–	36	34	

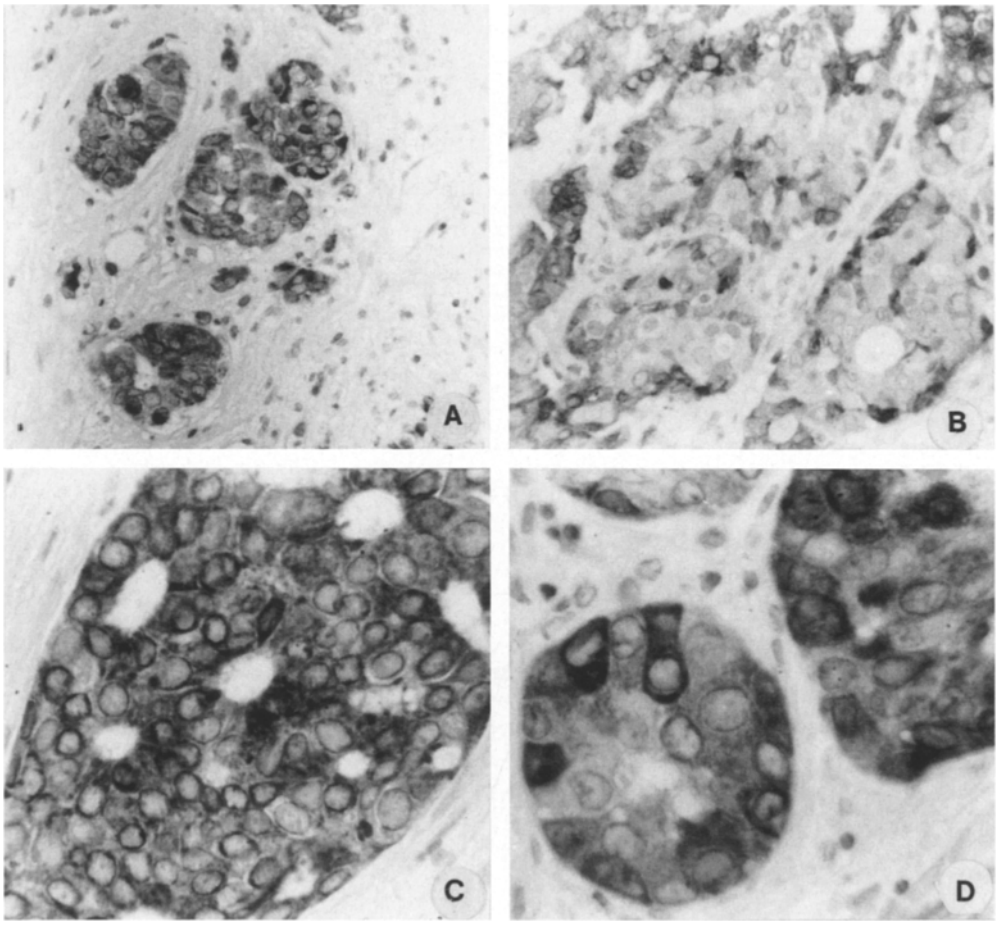

Fig. 1a–d Immunohistochemical detection of blc-2. **a**, **d** Cytoplasmic positivity with alternating negative and positive cells, (**a** ×250; **d** ×600). **b** The bcl-2+ cells are at the periphery of the tumour, ×250. **c** Bcl-2 positivity on nuclear membrane and in nucleoli, ×600

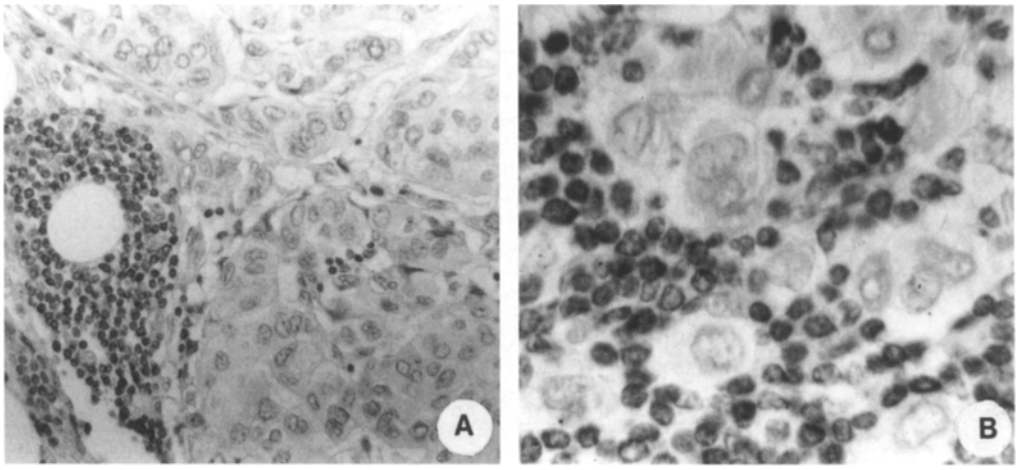

Fig. 2a, b Two bcl-2⁻ carcinomas with bcl-2⁺ lymphocytic infiltration. **a** Case number 345, ×250; **b** case number 13, ×600

Table 4 Relationship between bcl-2 and clinicopathological characteristics

Clinicopathological characteristics		Number of bcl-2⁻ cases	Number of bcl-2⁺ cases	*P* value
Grade I or II		65	73	0.018
Grade III		34	18	
AR	+	48	58	0.044
	−	47	31	
ER	+	57	79	<0.001
	−	41	12	
PR	+	33	50	0.003
	−	65	41	
Ki 67	+	50	26	0.001
	−	48	65	
p53	+	46	25	0.006
	−	53	66	
Cathepsin D	+	55	64	0.043
	−	43	27	
pS2	+	34	49	0.006
	−	64	41	
ERD 5	+	36	48	0.023
	−	63	43	

Table 5 Prognostic value of bcl-2 with regard to survival

Clinicopathological characteristics	Number of survivors at 5 years	Number of deaths at 5 years	*P* value
Bcl-2⁺	72	19	0.09
Bcl-2⁻	69	30	
AR⁺ bcl-2⁺	25	6	0.05
AR⁺ bcl-2⁻	30	17	
Ki67⁻ bcl-2⁺	56	9	0.04
Ki67⁻ bcl-2⁻	34	14	
Age >54 years bcl-2⁺	55	14	0.07
Age >54 years bcl-2⁻	45	22	

5; Fig. 3). The difference in survival between the patients with bcl-2⁻ or bcl-2⁺ tumours was not significant (P=0.09). However, when the tumours were grouped according to the presence of AR, Ki 67 negativity, p53 negativity, or patient age over 54, the difference between bcl-2⁺ and bcl-2⁻ tumours was greater (Table 5). Disease recurrence at 5 years in patients with bcl-2⁺ tumours was

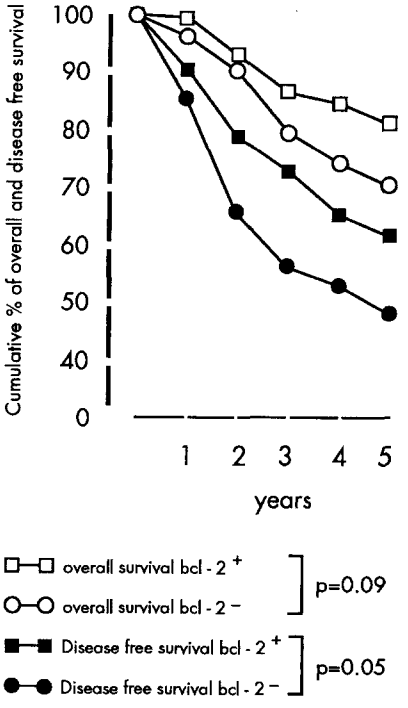

Fig. 3 Percentage of overall survival and disease free survival during the 5 years after surgery according to bcl-2 immunohistochemical findings

Table 6 Prognostic value of bcl-2 with regard to recurrence

Clinicopathological characteristics	Number of disease-free survivors	Number of cases recurring at 5 years	*P* value
Bcl-2⁺	56	35	0.05
Bcl-2⁻	47	52	
ER⁺ bcl-2⁺	49	30	0.04
ER⁺ bcl-2⁻	26	31	
Ki67⁻ bcl-2⁺	43	22	0.04
Ki67⁻ bcl-2⁻	23	25	
Cathepsin D⁺ bcl-2⁺	41	23	0.03
Cathepsin D⁺ bcl-2⁻	25	30	
pS2⁻ bcl-2⁺	30	11	0.01
pS2⁻ bcl-2⁻	32	32	
Age >54 years bcl-2⁺	45	24	0.02
Age >54 years bcl-2⁻	31	36	

statistically different from that of patients with bcl-2$^-$ (P=0.05). This difference was even greater when subgroups were made with ER$^+$ tumours, Ki 67$^-$ tumours, cathepsin D$^+$ tumours, pS2$^-$ tumours, and also when only patients over 54 years of age were considered (Table 6). It must be emphasized that bcl-2 positivity did not modify the prognosis of ER$^-$, Ki 67$^+$, AR$^-$, p53$^+$, p53$^-$ or of small tumours (less than 2 cm).

Of the 75 patients receiving endocrine therapy, 35 were treated with success. No correlation could be established between bcl-2 and the response to therapy even in the ER$^+$ bcl-2$^-$ and ER$^+$ bcl-2$^+$ groups. However, in the group of p53$^-$ tumours, the bcl-2$^+$ tumours responded better to hormonal therapy than bcl-2$^-$ tumours. Of the 33 p53$^-$ bcl-2$^+$, 19 responded well to therapy. In comparison, of the 23 p53$^-$ bcl-2$^-$ tumours, only 8 were responsive to hormonal therapy (P=0.07).

Discussion

Forty-eight percent of breast carcinomas were positive for bcl-2 protein with immunohistochemical techniques, possibly due to the persistence of bcl-2 expression during oncogenesis. Breast epithelium has phases of proliferation, secretion and apoptosis which are under hormonal, and probably bcl-2, control [2, 9, 18].We have detected, like Hockenbery et al. [13], bcl-2 protein in epithelial cells of large and small breast ducts. This protein may be expressed in breast carcinomas secondary to a genetic modification, and in lymphomas, the classic aberration found is at (14; 18) translocation [1, 3, 6, 23, 31]. This translocation has never been described in breast carcinomas, and it is important to note that bcl-2 protein has been overexpressed in lymphomas without this translocation [22]. It is probable that in several tumours, overexpression of bcl-2 is secondary to other causes.

As mentioned by Pezzella et al. [24], the accumulation of bcl-2 could be due to a post-transcriptional dysregulation. Our study did not allow us to determine which mechanism is responsible for accumulation of bcl-2 protein in breast carcinomas. The percentage of bcl-2$^+$ tumours is low in Ki67$^+$ carcinomas and in carcinomas of histological grade III. The bcl-2 protein is also expressed with markers associated with biological differentiation such as ER, PR and AR. It is also associated with proteins that are under hormonal control such as pS2, cathepsin D, and ERD5. In normal tissues, bcl-2 has also been described in cells which are regulated by hormonal stimuli [13]. It is present more often in carcinomas which do not have p53 protein accumulation. For example medullary carcinomas are almost always positive for p53 protein [14] but rarely positive for bcl-2.

The correlation between p53 and bcl-2 proteins is surprising. The latter protects the cells against apoptosis; p53 protein is necessary for induction of apoptosis by exogeneous or endogeneous factors [19, 35]. As accumulation of p53 protein indicates a mutated protein with loss of normal function, presence of p53 would be associated with absence of apoptosis. Therefore one would expect a relationship between positivity for bcl-2 and accumulation of p53 protein. This is not the case. This intriguing point was also observed in non-Hodgkin's lymphomas [25] and in Hodgkin's disease [8]. Moreover, an inverse correlation between the expression of bel-2 and p53 proteins has been found in several human breast cancer cell lines [11]. It could be that this relationship is due to down-regulation of bcl-2 by mutant p53. This has been demonstrated by transfection of MCF7 cultures [11]. Therefore although the relationship between p53 and bcl-2 seems strong, the consequences of the interaction are not always predictable.

The prognosis of p53$^+$ tumours is poor when compared with bcl-2$^+$ tumours; the presence of bcl-2 protein tends to confer better prognosis, as judged by overall survival at 5 years. This is an unexpected result since bcl-2 protects cells against apoptosis and one might expect that cells expressing bcl-2 would escape apoptosis, live longer and therefore confer a worse prognosis on the tumour. This is not the case; other factors act on tumours, and those that have evolved over a long period of time are not necessarily aggressive. In our series presence of bcl-2 is associated with Ki67 negativity (Ki67 score under the mean). Moreover, the prognostic value of bcl-2 for disease recurrence at 5 years is significant (P=0.05) and in patients over 54 years of age, has a still better prognostic value (P=0.07 for overall survival and P=0.02 for disease-free survival).

The bcl-2 protein is associated with molecules of prognostic value such as ER, PR, cathepsin D and pS2. Thus, it might be argued that our results on bcl-2 are due to the association of bcl-2 with proteins with good prognosis. This is not the case since ER, pS2 and cathepsin D have no prognostic value in our series, and that only the association ER$^+$ PR$^+$ had independent predictable value. Our results concerning prognosis are similar to those of Pezzella et al. [24] on non-small cell lung carcinomas. However, the results of Colombel et al. [7] and McDonnell et al. [21] on prostate carcinomas are different. In these carcinomas, bcl-2 seems to be associated with high grade tumours refractory to hormonal therapy. The expression of bcl-2 could make it possible for cancer cells to survive in an androgen-deprived environment. In our cases, it was not possible to show whether or not bcl-2$^+$ ER$^+$ carcinomas were independent of hormonal stimuli. After endocrine treatment, the percentage of therapeutic success was the same in bcl-2$^-$ and bcl-2$^+$ tumours. However, when subgroups are made according to p53 and bcl-2 positivity, it seems that bcl-2 confers on p53$^-$ tumours the possibility of a good response to hormonal therapy. It could be, as suggested by Haldar et al. [11], that subgroups of breast cancers based on bcl-2 and p53, will identify breast cancers with different prognosis. However, the groups of patients in our study were too small in number to make such as assumption. Both bcl-2 and p53 should be studied together with several different oncogenes in both infiltrative carcinomas, and in carcinoma in situ.

Acknowledgements Dr. Jean Hurlimann has the financial support of the Ligue vaudoise contre le cancer, Lausanne. The authors thank MM Bertholet, S. Laurini, M. Correvon, S. Burki and J. Maillardet for their technical assistance as well as G. Perriard for her help in the preparation of the manuscript.

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