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Bcl-2 expression in male breast carcinoma

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Abstract We have analysed the expression of bcl-2 protein retrospectively in 34 primary male breast carcinomas (MBC), using the monoclonal antibody bcl-2 in formalin-fixed, paraffin-embedded tissues. Bcl-2 expression was compared with tumour clinicopathological features, sex steroid hormone receptors, DNA content, p53 immunoreactivity and cell proliferative activity assessed by counts of the argyrophilic nucleolar organizer regions (AgNORs), the monoclonal antibody PC10 against proliferating cell nuclear antigen and the monoclonal antibody MIB-1. Most (28, or 82.3%) of the 34 cases of MBC were bcl-2 positive. No association was found with clinicopathological features of the tumours, although bcl-2 tended to be more frequently expressed in small tumours (P=0.09) and in cases without necrotic areas (P=0.1). Nor was any association found with hormone receptor status, p53 immunoreactivity, DNA content, cell proliferative activity or patient survival. In multivariate analysis, only proliferative activity (expressed by AgNOR counts) and p53 immunoreactivity had independent prognostic significance. Our results indicate that MBC differs from FBC in that in MBC bcl-2 protein is not related to an oestrogen-dependent transcription pathway and bcl-2 alone is not sufficient to induce increased proliferation. These characteristics, together with the high prognostic value of cell proliferation and the lack of prognostic significance for hormone receptor status, support the hypothesis that MBC is biologically different from FBC.

Key words Bcl-2 protein expression · Male breast carcinoma · Prognosis

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Introduction

Bcl-2 is a proto-oncogene that promotes cell growth by inhibiting apoptosis [18, 27]. Expression of bcl-2 protein allows cells to survive, which may facilitate the development of neoplasms [18]. Qualitative alterations of *bcl-2* gene were first detected in lymphomas, in which they have a pathogenetic and a prognostic role [31, 47]. Bcl-2 expression has also been investigated in nonlymphoid tissues, such as prostate [29], lung [32], thyroid [36], gastrointestinal [6], ovarian [15] and endometrial [7] tumours, and in soft tissue sarcomas [21].

Bcl-2 protein is expressed in 48–90% of female breast carcinomas (FBC) [1–3, 11, 12, 19, 23, 25, 28, 40, 41, 44, 45], more frequently in low-grade [1–4, 11, 23, 25, 40, 44, 45] and small tumours [41, 44], in node-positive patients [40], and in oestrogen receptor (ER)-positive [1–4, 11, 12, 14, 19, 28, 40, 41, 44, 45] and progesterone receptor (PGR)-positive cases [1, 3, 4, 11, 12, 14, 40, 44]. Low bcl-2 expression was found in p53-positive [2, 11, 12, 19, 23, 28, 41, 44] and highly proliferating tumours [4, 11, 19, 23, 41, 44]. Interestingly, bcl-2 immunopositivity was correlated with longer disease-free and overall survival [41, 44], especially in node-positive patients [14].

Bcl-2 protein has been investigated in only one series of male breast carcinoma (MBC), in which it was more frequently detected in ER-positive and p53-negative cases [46].

We have investigated the expression of bcl-2 protein retrospectively in 34 primary MBC, using immunohistochemistry in formalin-fixed paraffin-embedded tissues. Our aim was to assess whether bcl-2 protein was associated with clinicopathological features, sex steroid hormone receptors, p53 expression, DNA content, cell proliferative activity or patient survival.

Materials and methods

The study consisted of 34 MBC collected from the files of the pathology sections of the Department of Biomedical Sciences and Human Oncology of Turin University and S. Giovanni Hospital

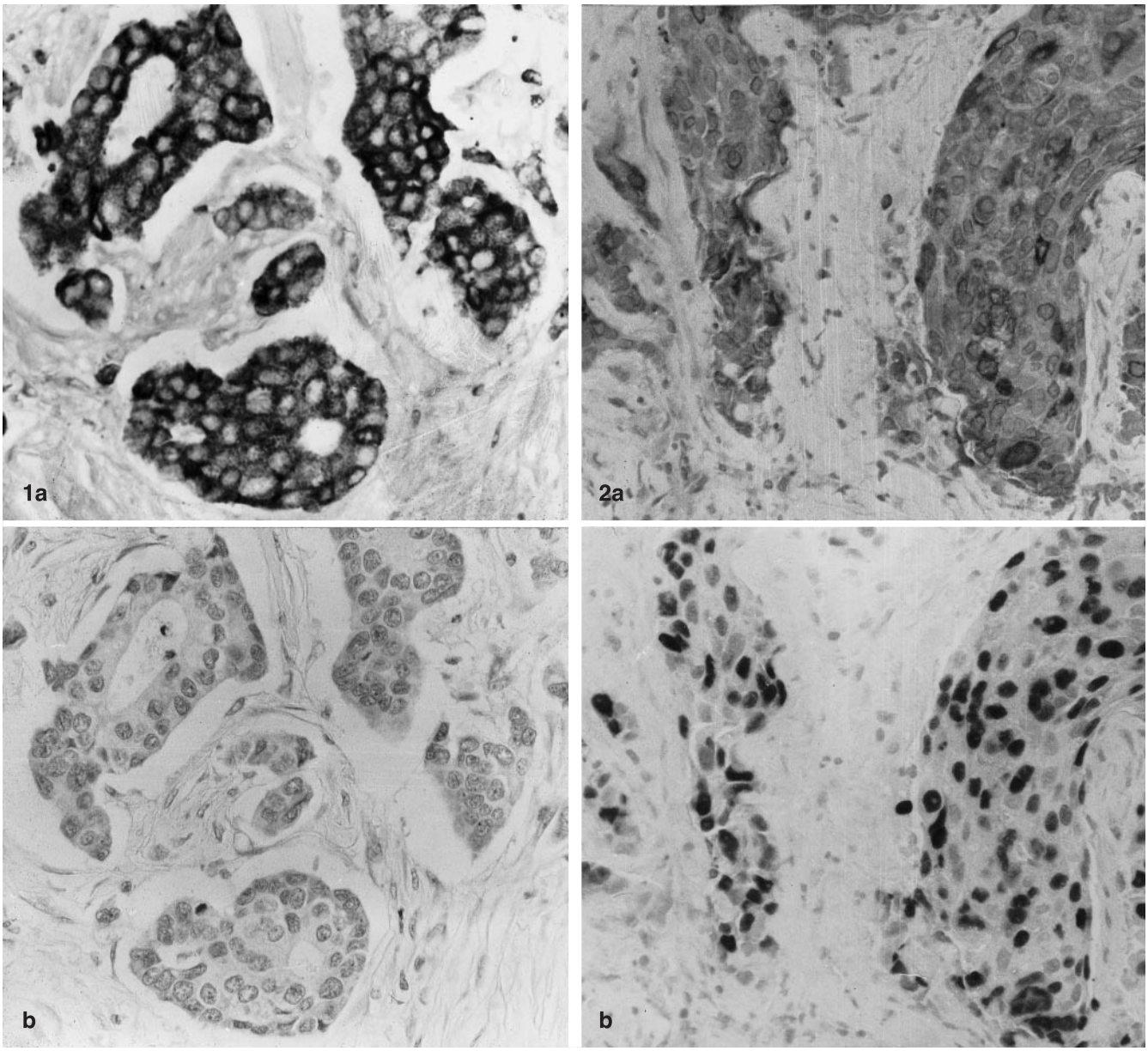

Fig. 1 Immunocytochemical detection of **a** bcl-2 protein and **b** oestrogen receptors (ER) in consecutive serial sections of grade 2 MBC. **a** Intense bcl-2 cytoplasmic immunoreactivity is present in almost all the neoplastic cells. LSAB immunoperoxidase, ×400. **b** No ER-positive nuclei are seen in the same field. ER-ICA immunoperoxidase, haematoxylin counterstain, ×400

Fig. 2 Immunocytochemical detection of **a** bcl-2 protein and **b** p53 protein in consecutive serial sections of grade 3 male breast carcinoma (MBC). Many neoplastic cells are positive for both antigens; bcl-2 immunoreactivity shows some variation in intensity from cell to cell. LSAB immunoperoxidase, ×400

(Turin, Italy) from 1972 to 1991. The mean age of the patients at diagnosis was 62 years (27–86 years). All underwent radical or modified radical mastectomy. A minimum follow-up of 8 years for censored (surviving) patients or follow-up to patient death was available for all the cases. The mean follow-up time was 71 months (range 1–216 months). Carcinomas were classified according to the World Health Organization [39] and staged pathologically according to International Union Against Cancer [16]. All were cases of invasive ductal carcinoma; 8 were stage pT1, 15 pT2 and 11 pT3–4; 20 were N0 and 14 N1–3. Histological grade was assessed according to Bloom and Richardson [5]: 21 tumours were grade 2 and 13, grade 3. Necrotic tumour areas were assessed on haematoxylin-eosin stained sections at ×400 magnification. Multiple samples from each case were fixed in 10% formalin and embedded in paraffin; serial sections from the same tissue blocks were cut for histology, DNA flow cytometry, sex hormone receptors, bcl-2, PCNA, MIB1 and p53 immunostaining and AgNOR staining.

For immunohistochemistry, sections 4 μm thick on poly-L-lysin coated slides were stained with specific monoclonal antibodies (MoAbs) using the labelled streptavidin biotin (LSAB) method (Dakopatts, Glostrup, Denmark) and diaminobenzidine as chromogen with light haematoxylin counterstaining. Slides taken to water were placed in a glass box filled with 10 mmol/l (pH 6.0) citrate buffer and subjected to microwave irradiation at 800 W for two periods of 5 min each. MoAb antihuman bcl-2 oncoprotein (Dako-bcl-2, 124; Dakopatts) at 1:40 dilution, p53-specific MoAb DO7 (Oncogene Science, Uniondale, N.Y.) at 1:75 dilution and MIB-1 MoAb (Immunotech, Marseille, France) at 1:100 dilution were then applied for 2 h at room temperature in a humidified atmosphere. For PCNA staining, MoAb PC10 (Dakopatts) at 1:200 dilution was applied for 2 h without mi-

crowave pretreatment. For hormone receptor staining, ER-ICA and PGR-ICA (Abbot Laboratories, North Chicago, Ill.) were used at kit dilution, after the procedure of Hiort et al. [17]. Positive tumour cells were independently quantified by two pathologists by evaluating at least 1,000 tumour cells from 10 randomly selected areas in each case, using a standard light microscope equipped with an ocular reticule (original magnification ×15) and a ×40 objective, ensuring that the whole section was scanned. In cases where intratumour heterogeneity of staining was found, the same number of areas with the highest percentage and with the lowest percentage of stained cells were examined. All the reactive nuclei (for ER, PGR, MIB-1, PCNA and p53 MoAbs) or cytoplasms (for bcl-2 MoAb) were considered positive, regardless of the intensity of the staining. When the scoring discrepancy was greater than 10% the inter observer disagreement was discussed and settled by means of a double-head microscope. For statistical analysis, cases were considered positive for bcl-2 if they showed immunoreactivity in more than 30% of the neoplastic cells, while for ER, PGR and p53 protein the cut-off value was nuclear staining of 10% neoplastic cells. For PCNA and MIB-1 immunoreactivity, the absolute percentage of the stained cells was recorded.

AgNOR staining was performed according to Ploton et al. [37] as previously described [33]. AgNORs were counted according to Crocker et al. [9], and the mean number per nucleus was calculated in each case.

For DNA flow-cytometry, sections 50 μm thick were processed according to Hedley et al. [13]. Twenty thousand events were acquired using a FAC Scan Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Cytograms were analysed using the Multicycle Software with debris-fitting algorithms and clumping model (Phoenix Flow Systems, San Diego, Calif.). Only histograms with a full peak coefficient of variation <7 were accepted. Histograms were grouped as diploid and aneuploid according to Joensuu and Klemi [22].

Associations between bcl-2 positivity/negativity and the clinicopathological features of each tumour (ER, PGR, DNA ploidy and p53 expression) were assessed by the Yates-corrected Chi-square test. Associations between bcl-2 positivity/negativity and AgNOR counts, PCNA and MIB-1 scores were evaluated by one-way analysis of variance (ANOVA). Univariate survival analyses were based on the Kaplan-Meier product-limit estimates of survival distribution [24], and differences between survival curves were tested using the generalized Wilcoxon test. The relative importance of all the variables considered in the univariate analysis was estimated using the Cox proportional hazards regression model [8]. All data were processed with BMDP selected programs (7D, 4F, 1L, 2L) [10].

Results

Among the 34 MBC, 28 (82.3%) showed cytoplasmic immunoreactivity for bcl-2 in more than 30% of the neo-

Table 1 Bcl-2 expression in MBC according to clinicopathological features, hormone receptors, DNA ploidy, p53 expression and cell proliferative activity

Variable	N	Bcl-2 positive (%)	Bcl-2 negative (%)	*P*
Whole series	34	28 (82.3)	6 (17.6)	
Age (years)				
≤ 45	3	3 (100)	0 (0)	
46–70	20	17 (85)	3 (15)	
>70	11	8 (72.7)	3 (27.3)	0.48
Histological grade				
G2	21	18 (85.7)	3 (14.3)	
G3	13	10 (76.9)	3 (23.1)	0.84
T stage				
pT1	8	8 (100)	0 (0)	
pT2	15	10 (66.7)	5 (33.3)	
pT3–4	11	10 (90.9)	1 (9.1)	0.09
N stage				
N0	20	17 (85)	3 (15)	
N1–3	14	11 (78.6)	3 (21.4)	0.97
ER (%)				
≤ 10	19	16 (84.2)	3 (15.8)	
>10	15	12 (80)	3 (20)	1
PGR (%)				
≤ 10	17	13 (76.5)	4 (23.5)	
>10	17	15 (88.2)	2 (11.8)	0.65
DNA content				
Diploid	12	10 (83.3)	2 (16.7)	
Aneuploid	14	11 (78.6)	3 (21.4)	1
p53 immunoreactivity				
Negative	13	11 (84.6)	2 (15.4)	
Positive	21	17 (81)	4 (19)	1
Necrotic tumour areas				
Absent	23	21 (91.3)	2 (8.7)	
Present	11	7 (63.6)	4 (36.4)	0.1
AgNOR/cell (mean±SD)		8.04 ± 2.53	7.12 ± 1.91	0.34[a]
PCNA scores (mean±SD)		20.91 ± 8.64	17.79 ± 6.56	0.41[a]
MIB-1 scores (mean±SD)		24.11 ± 7.62	21.54 ± 5.93	0.44[a]

[a] ANOVA

Table 2 Correlation between clinicopathological features, bcl-2 and p53 expression, hormone receptors, cell proliferation indices and DNA ploidy with survival in MBC

Variable	*n*	Median (months)	5 year survival rate (%)	10 year survival rate (%)	*P*
Whole series	34	60	50	18	
Age (years)					
≤45	3	24	33	0	
46–70	20	73	60	21	0.28
>70	11	54	45	18	
Histological grade					
G2	21	77	67	21	
G3	13	33	23	11	0.008
T stage					
pT1	8	96	75	12	
pT2	15	41	40	13	0.43
pT3–4	11	57	45	27	
N stage					
N0	20	57	50	17	
N1–3	14	60	50	19	0.65
ER (%)					
≤ 10	19	60	53	21	
>10	15	41	47	-	0.5
PGR (%)					
≤ 10	17	77	70	25	
>10	17	39	29	11	0.08
DNA content					
Diploid	12	77	75	27	
Aneuploid	14	38	36	14	0.05
p53 immunoreactivity					
Negative	13	99	77	32	
Positive	21	39	33	9	0.007
AgNOR					
≤ 7.38	17	98	76	38	
>7.38	17	33	24	0	0.0001
PCNA					
≤ 18.25	18	77	67	36	
>18.25	16	33	31	0	0.001
MIB-1					
≤ 23.75	17	77	69	22	
>23.75	17	36	25	9	0.003
Bcl-2 immunoreactivity					
Negative	6	62	67	-	
Positive	28	57	46	19	0.49

plastic cells (Fig. 1a). Normal lymphocytes infiltrating the tumours or the peritumour tissues were present in most of the cases, and represented an effective internal control. A degree of staining heterogeneity within the neoplastic cells of immunoreactive cases was observed (Fig. 2a), but no difference between infiltrative margins and tumour centre was evident.

As shown in Table 1, no association was found between bcl-2 immunoreactivity and clinicopathological features of the tumours, AgNOR counts, PCNA and MIB-1 scores, ER (Fig. 1a, b) or PGR, DNA ploidy and p53 immunostaining. Only a trend was found for the association between bcl-2 and tumour stage, the small, pT1 cases all being positive as against 66.7–90.9% of pT2-pT3–4 cases (P=0.09). Bcl-2 protein also tended to be more frequently expressed in tumours without necrotic areas (91.3%) than in those with necrotic areas (63.6%, P=0.1). In cases simultaneously immunoreactive for bcl-2 and p53 protein, most cells were positive for both antigens (Fig. 2a, b).

At the time of analysis, 27 patients (79.4%) had died of the disease and 7 (20.6%) were alive. The mean follow-up for censored patients was 143 months. The median survival of the whole series was 60 months (1–216). The overall 5- and 10-year survival rates were 50% and 18%, respectively. As shown in Table 2 and Fig. 3, bcl-2 immunoreactivity was not associated with survival: the median survival was 62 months for bcl-2-negative, compared with 57 months for bcl-2-positive cases (P=0.49). Histological grade (P=0.008), AgNOR counts (P=0.0001), PCNA scores (P=0.001), MIB-1 scores (P=0.003) and p53 immunoreactivity (P=0.007) each had a strong prognostic

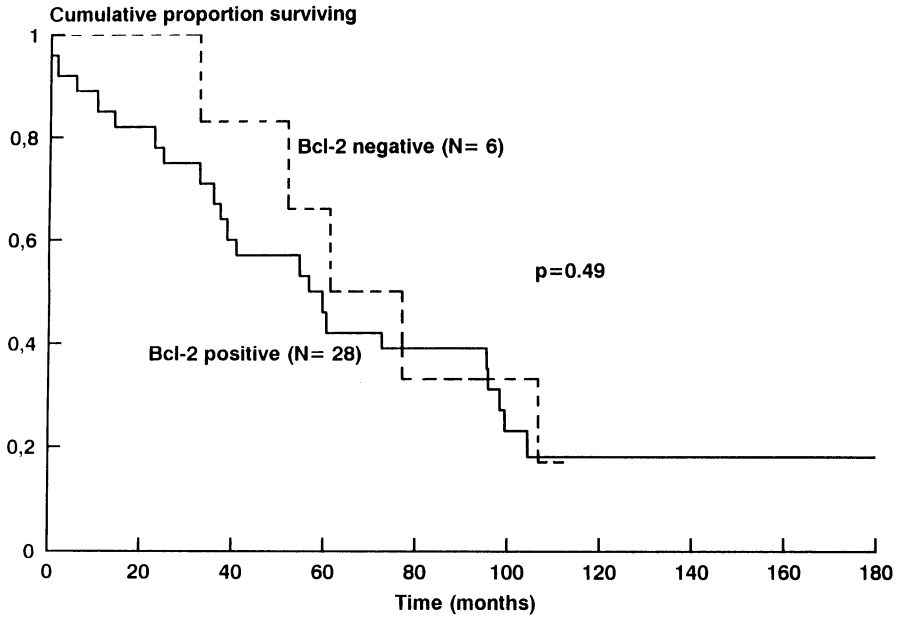

Fig. 3 Kaplan-Meier survival curves for MBC, categorized according to bcl-2 immunopositivity

value. DNA content also was associated with survival (P=0.05). A trend was found for PGR status (P=0.08).

When all the variables were introduced in the Cox model, only AgNOR counts (x^2=7.95; P=0.005; hazard ratio: 3.17) and p53 immunoreactivity (x^2=3.5; P=0.05; hazard ratio: 2.61) retained independent prognostic significance.

Discussion

Bcl-2 protein was expressed in 82.3% of MBC: this rate is similar to that found in several series of FBC [1, 11, 12, 28], but it is higher that that found by some investigators [2, 3, 19, 25, 40, 41, 45], especially Weber-Chappuis et al. [46], who also reported a significantly higher expression of bcl-2 in male than in female breast carcinomas. No significant association between bcl-2 expression and histological tumour grade was found, which is a point of difference from most FBC [1–4, 11, 23, 25, 40, 44, 45] but in accordance with other series [12, 14, 28]. No association was found between bcl-2 expression and tumour size, in contrast to some reports on FBC [41, 44], but in agreement with others [11, 12, 14, 23, 28]. Finally, no correlation was found with node status, as in most FBC [11, 12, 14, 23, 28] but in contrast to the findings of Sierra et al. [40]. These conflicting results may be due in part to different scoring procedures: indeed, bcl-2 staining was variously interpreted as positive when tumour cells showed distinct cytoplasm staining in more than 5% of cells [3, 25, 40, 45], 25% [2, 11] or 30% [41, 46]. We used the value of 30% bcl-2 positive cells as a cut-off because this value has provided the most significant prognostic information in large series of patients with FBC [41].

We did not find any positive correlation between bcl-2 and ER or PGR status, unlike reports of investigations in FBC [1–4, 11, 12, 14, 19, 28, 40, 41, 44, 45]. This suggests that bcl-2 expression in MBC is not related to an oestrogen-dependent transcription pathway [11], and also cannot be regarded as an oestrogen-regulated protein [28, 43].

Again in contrast to FBC, we did not find an inverse relationship between bcl-2 and p53 overexpression [2, 11, 12, 19, 23, 28, 41, 44] or cell proliferative activity [4, 11, 19, 23, 41, 44]. This suggests that in MBC, as in soft tissue sarcomas, *bcl-2* oncogene alone is not sufficient to induce increased proliferation [21] and, in marked contrast to other oncogenes, *bcl-2* does not confer a proliferative advantage upon cells that express it [27]. On the contrary, in MBC the cell proliferation seems more likely to be related to p53 tumour suppressor gene: indeed a strong linear correlation was found between p53 expression and AgNOR counts and both PCNA and MIB-1 scores [35].

Nevertheless, an association between the expression of bcl-2 protein and ER-positive and p53-negative MBC has recently been reported by Weber-Chappuis et al. [46]. The discrepant results may be partly due to case selection: in our series there were no G1 carcinomas and 38,2% of all cases were G3, while the corresponding figures were 48% and 15% in the series of Weber-Chappuis et al. It is well known that well-differentiated FBC are more often ER positive [30] and p53 negative [20] than are poorly differentiated ones. Moreover, different antibodies that recognize various epitopes of p53 protein were used under different conditions: DO7MoAb after microwave irradiation in our series, CM1 or PAb 1801 without antigen retrieval in that of Weber-Chappuis et al. [46].

Finally, we did not find a correlation between bcl-2 expression and patient survival, which has been described in a few reports on FBC [14, 41, 44]. This is not surprising, since bcl-2 was not associated with any of the known prognostic features, such as tumour stage, histological grade and proliferative activity. However, in some series of FBC, bcl-2 was significant only in short-term

follow-up [23] and did not appear to be an independent variable in multivariate analysis [23, 41, 44]. Moreover, bcl-2 was not associated with survival in other reports, mainly in node-negative patients [1, 2]. At present, it remains to be defined whether a weak bcl-2 expression, regardless of treatment, is an indicator of biological aggressiveness [42].

In conclusion, bcl-2 protein is frequently expressed in MBC, but is not associated with any prognostic clinicopathological features. Such characteristics, together with the lack of prognostic significance of hormone receptor status (as in other MBC series [26, 38]) and the high prognostic value of cell proliferative activity, as previously reported [34, 35], lend further support to the hypothesis that MBC are biologically different from FBC.

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