Pergamon

European Journal of Cancer Vol. 33, No. 8, pp. 1275–1280, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00124-X

Original Paper

Expression of Bcl-2 Protein in Human Primary Breast Carcinomas and its Correlation with Multifocality, Histopathological Types and Prognosis

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The Bcl-2 gene product prevents programmed cell death (apoptosis) and possibly promotes tumour development. This protein has mainly been demonstrated in the cytoplasm of various normal and neoplastic cells, including normal mammary epithelia and breast carcinomas. The aim of this retrospective study was to correlate the immunohistochemical expression of Bcl-2 protein with the multi-unifocality and the histology of the two main types of breast carcinoma. We used monoclonal antibody 124 to investigate Bcl-2 expression in paraffin sections of 62 primary breast carcinomas. Bcl-2 expression was associated mainly with this lobular carcinoma. High Bcl-2 protein positivity was found in this type, and was statistically significant in comparison to the level of Bcl-2 in ductal, NOS carcinomas (lobular versus ductal, NOS, $P < 0.0001$). In the entire group, including all histological types, Bcl-2 expression was higher in multifocal tumours ($P = 0.005$). Statistical significance ($P < 0.03$) was also found within the group of ductal, NOS cases, showing that Bcl-2 protein expression is associated with multifocality, irrespective of the histology of breast carcinomas. No definite association between Bcl-2 expression and prognosis was found. Our results suggest that Bcl-2 protein plays some role in the development of multifocality in breast carcinomas. © 1997 Published by Elsevier Science Ltd.

Key words: Bcl-2 protein, breast cancer, breast carcinoma, immunohistochemistry, oncogene

Eur J Cancer, Vol. 33, No. 8, pp. 1275–1280, 1997

INTRODUCTION

THE *BCL-2* gene, located at chromosome 18q21, was originally identified by chromosome analysis of the t(14;18) translocation in human follicular lymphoma [1]. The Bcl-2 gene product is a mitochondrial membrane protein, found also in the nuclear envelope and to a lesser degree in the cell membrane [2], that prevents programmed cell death (apoptosis) [3]. It can be detected by immunostaining in primary lymphoid follicles and in the mantle zone of secondary follicles that largely constitute the homing sites of long-lived memory B-cells [4]. It is also found in non-lymphoid haematopoietic precursors, such as the early identifiable myeloid cells [5]. Bcl-2 protein expression is not restricted to the haematolymphoid tissues only, but is also detected in several other tissues that are either long-lived pluripotent or hormone/growth factor dependent [6, 7]. Duct cells of exocrine glands as well as cells responsive to hormonal stimulation, such as uterine smooth muscle, endometrial glands, thyroid follicles, prostatic glands and breast lobules, have been reported to express Bcl-2 protein [6].

The detection of Bcl-2 protein in fetal epithelial germinative regions, in placental trophoblast, as well as endocrine cells and neurons, suggests that this protein may play a regulatory role in normal homeostasis of both developing and established tissues. Expression of Bcl-2 protein apparently serves to maintain cells in the tissues, by avoiding cellular senescence and death [6]. In addition, Bcl-2 protein is involved in morphogenesis by inducing interactions between epithelia and mesenchyme, contributing to the formation of

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Received 6 Jun. 1996; revised 1 Jan. 1997; accepted 14 Jan. 1997.

condensations of cells that are "commited" to develop more differentiated structures [6]. Bcl-2 has also been found to promote survival in response to a wide variety of cell stresses and cytotoxic chemicals, including heat shock, ionising radiation, excess calcium influx and a range of chemotherapeutic drugs [8, 9], rendering itself a very possible modulator in carcinogenesis and in cancer therapy.

Bcl-2 protein expression has been found in follicular non-Hodgkin's lymphomas with t(14;18) and in a variety of other B-cell lymphomas and Hodgkin's disease without the specific chromosome translocation [1, 4, 5]. Furthermore, expression of Bcl-2 protein has been reported in epithelial neoplasms of the thyroid [10], prostate [11], stomach [12] and pulmonary carcinomas [13]. It is believed that Bcl-2 protein promotes carcinogenesis, favouring effective exposure to mutagens by prolonging cell life [14]. However, its importance for tumour behaviour and patient survival is debatable [13, 15]. Although a fair number of studies on Bcl-2 expression in breast carcinoma have recently appeared in the literature, differences between unifocal and multifocal tumours in terms of Bcl-2 expression have not yet been explored.

The aim of this study was to correlate the immunohistochemical expression of Bcl-2 protein with the presence of multi-/unifocality, and the histology of the two main types of breast carcinoma (lobular versus ductal, NOS), which are known to have different clinical behaviour.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissue samples of 62 serial archival cases of breast carcinoma were used. They were identified as multifocal (35 cases) and unifocal (27 cases) on the basis of clinical, radiological and mammographic findings as well as on pathological examination of the radical mastectomy specimens according to the standard sampling protocol [16]. The specimens were sliced at 1 cm intervals and sections taken from each quadrant. We found in 35 cases separate tumour foci in diverse quadrants or located at least more than 2 cm from the periphery of gross margin of the index tumour. These cases were characterised as multifocal carcinomas.

Haematoxylin and eosin (H&E)-stained sections of all cases were reviewed and classified according to the common histological criteria of the WHO classification. Histological subtyping of invasive lobular carcinomas was performed according to the criteria proposed by Fechner [17] and by Dixon and associates [18]. In addition, the histological grading based on tubular content, mitotic index and nuclear pleomorphism [19] was evaluated. DCIS (ductal carcinoma *in situ*) subclassification was performed in accordance with the criteria proposed by Holland and associates [20].

Paraffin sections of 5 μm thickness were immersed in citric acid buffer at pH 6.0 and incubated in a microwave oven at 700 W power, twice, for 5 min each time. Subsequently, the sections were immunostained by the ABComplex/HRP method (Dako, Denmark) applying a monoclonal antibody against Bcl-2 protein (MAb 124, Dako, Denmark) at a dilution of 1:10, for 2 h at room temperature. Sections stained by omitting the primary monoclonal antibody served as negative controls, while sections of follicular lymphomas were included as positive controls. The percentages of positive cells were estimated by counting over 2000 neoplastic cells in at least 10 random high-power fields. All patients had been followed for at least 10 years after mastectomy. The intensity of staining was semiquantitatively assessed as + = weak, ++ = moderate and + + + = strong.

The statistical analysis of the contingency table was based on the chi-square test or Fisher's exact test adapted to one or two sample proportion tests. The Bonferoni inequality test was used to adjust the probability level when two tests on the same data (e.g. 5 or 10 years survival) were calculated.

RESULTS

After reviewing the H&E stained sections, the 62 cases of breast carcinomas were classified as follows: invasive ductal carcinomas, NOS, 34 cases (including 2 cases of mixed carcinoma with predominant ductal component); invasive lobular carcinomas, 18 cases (including 8 cases of mixed carcinoma with predominant lobular component); other histological types, 5 cases (including 1 tubular, 2 colloid, 2 cribriform); and DCIS, 5 cases. In several cases of invasive ductal, lobular and mixed carcinomas, foci of DCIS, rarely foci of lobular carcinoma *in situ* (LCIS) and/or areas with microscopic features of cancerisation of lobules were recognised. According to the proposed new classification of Holland and associates, the 5 cases of DCIS were classified as well differentiated (1 case), intermediately differentiated (3 cases) and poorly differentiated (1 case).

Immunohistochemical positivity to Bcl-2 protein was shown to be cytoplasmic in neoplastic cells as well as in normal cells of the preserved mammary gland lobules (Figure 1). In 5 cases, nuclear positivity of Bcl-2 was also recognised in a fair number of neoplastic cells. 4 of these cases were multifocal and 1 unifocal. The relationship between positivity to Bcl-2 protein and multi-/unifocality of the 62 breast carcinomas is given in Tables 1 and 2. 11 out of the 35 (31%) multifocal cases and 18 out of the 27 (67%) unifocal cases (Table 1) were either negative or in low percentages (up to 2%) positive. Only 1 case showed positivity in up to 20% of the cells. However, this positivity was very faint. Therefore, we considered all these cases as practically negative. On the contrary, 24 of the 35 (69%) multifocal cases and only 9 of the 27 (33%) unifocal cases revealed Bcl-2 positive cells in more than 50% of cells. This difference between the unifocal and multifocal groups was

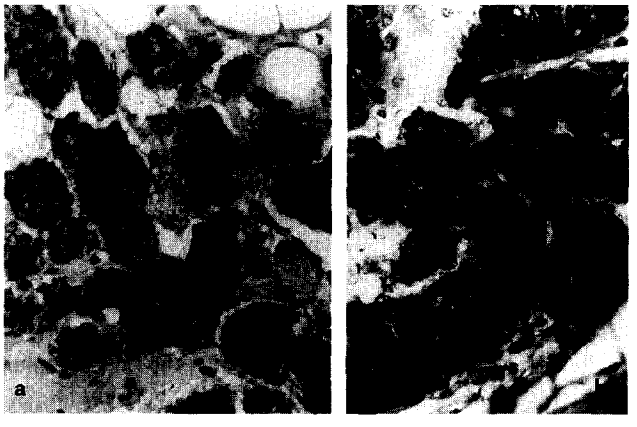

Figure 1. Cytoplasmic Bcl-2 protein positivity in a case of lobular (a) and tubular (b) breast carcinoma. ABComplex method for Bcl-2 protein, 400 ×.

Table 1. Expression of Bcl-2 protein in unifocal and multifocal groups

	Unifocal ($n = 27$)		Multifocal ($n = 35$)		All cases ($n = 62$)	
	Negative	Positive	Negative	Positive	Negative	Positive
Grade						
I ($n = 21$)	4	5	2	10	6	15
II ($n = 32$)	8	4	8	12	16	16
III ($n = 9$)	6		1	2	7	2
Total*	18	9	11	24	29	33

* The difference in positivity between the unifocal and multifocal groups was significant ($P = 0.005$).

statistically significant ($P = 0.005$) (Table 1). A high percentage of Bcl-2 positivity was seen in low grades ($P = 0.04$), particularly in the multifocal group, although in this group the difference was not statistically significant (Table 1).

The highest levels of positive cells and the strongest intensity of positive immunostaining were observed in lobular carcinomas and mixed-type carcinomas with predominant lobular component. The difference in Bcl-2 positivity between lobular/ductal NOS carcinomas was statistically highly significant ($P < 0.0001$). Cell positivity depended on histology rather than grading, with lobular carcinomas, as opposed to ductal NOS, having high levels of positivity irrespective of the histological grade (Table 2). Again the intragroup differences in terms of grading were not significant. Only 1 out of the 18 invasive lobular carcinomas was negative for Bcl-2 protein (Table 2). Positivity in more than 50% of the neoplastic cells was found in 17 cases. 13 of these cases were multifocal and 4 were unifocal. The staining intensity was moderate to strong. All 8 cases of mixed carcinoma with a predominant lobular component showed Bcl-2 positivity in more than 50% of the neoplastic cells. 5 of the cases were multifocal and 3 cases were unifocal.

28 cases of invasive ductal, NOS carcinomas (Table 2) showed Bcl-2 protein positivity in less than 20% of the neoplastic cells (21 cases were negative). 18 of the 28 cases were unifocal. Only 6 cases revealed >50% Bcl-2 positivity. The immunostaining intensity was weak to moderate. 5 of the 6 cases were multifocal. Multifocal ductal, NOS carcinomas had a significantly higher incidence of Bcl-2 positivity in neoplastic cells than unifocal tumours ($P < 0.05$, Fisher's exact test). The two cases that were classified as being of mixed histological type with predominant invasive ductal carcinoma showed lower than 20% cell positivity. As a rule, immunoreactivity was limited in the areas with a lobular component or with a cribriform *in situ* carcinoma. Areas with pure NOS appearance were mostly negative or weakly positive for Bcl-2 protein. They were positive only in the 6 cases with more than 50% immunoreactive neoplastic cells.

For the 5 cases of specific types of breast carcinoma and the 5 cases of DCIS, all had >50% Bcl-2 positive tumour cells. 6 cases were multifocal and 4 cases (1 colloid, 1 tubular, 1 invasive cribriform, 1 DCIS) were unifocal (Table 2).

No significant correlation was found between Bcl-2 expression and patient survival (either at 5 or 10 years), although the sample size was limited (Table 3) and did not permit a multivariate analysis.

DISCUSSION

Apoptosis, which plays a central role in both normal cell cycle and tumour biology, is controlled by a family of related proteins. Bcl-2 protein is the main representative of this family, and its role in preventing apoptosis is well known. However, alternative apoptotic pathways have also been found, which are regulated by Bcl-2-like proteins. Some of these proteins, such as Bcl-xl, Mcl-1, Murine A1, Ced 9 and Bag, inhibit apoptotic cell death, whereas others, such as Bcl-2xs, Bax, Bad and Bak accelerate apoptosis [21, 22].

A number of recent studies have drawn attention to Bcl-2 expression in breast carcinomas [2, 15, 21, 23]. However, limited information on the correlation between Bcl-2 expression and histology has been published. Furthermore, no data regarding Bcl-2 expression in multifocal tumours have thus far appeared in the literature. Our current study shows that one of the strongest features influencing Bcl-2 protein expression is histology, as lobular carcinoma is significantly more frequently immunoreactive for this oncoprotein compared to ductal, NOS carcinoma. This is in keeping with previous observations by other authors [24]. In the literature, the intensity of Bcl-2 staining in breast carcinomas has been inversely related to tumour grade [24–27], necrosis

Table 2. Expression of Bcl-2 protein in different histological types of breast carcinoma

	NOS ($n = 34$)		Lobular ($n = 18$)			
Grade	Negative	Positive	Negative	Positive	Specific types	DCIS
I	6 (2M-4U)	3 (2M-1U)		3 (2M-1U)	5 (2M-3U)	4 (4M-0U)
II	15 (7M-8U)	2 (2M-0U)	1 (1M-0U)	13 (10M-3U)		1(0M-1U)
III	7 (1M-6U)	1 (1M-0U)		1 (1M-0U)		
Total	28	6	1	17	5	5
			$P < 0.0001$			

U, unifocal; M, multifocal.

Table 3. Correlation of Bcl-2 expression to 5- and 10-year survival

	5-year survival		10-year survival	
	Negative	Positive	Negative	Positive
Unifocal	11/18	9/9*	8/18	8/9
Multifocal	6/11	18/24	6/11	12/14
All cases	17/29	27/33	14/29	20/33

*A P value of 0.029 was obtained, but this was not considered of statistical significance since another analysis had already been applied on these data and the P value should be double according to Bonferoni correction.

[23, 28], mitotic index [27], apoptotic index [27], EGFR [23, 28] and c-erbB-2 expression [15]. In our study, a tendency but non-significant association between Bcl-2 protein expression and favourable histological grade was found. It is believed that the regulation of Bcl-2 protein is hormone dependent, since its expression shows an inverse relationship to oestrogen and progesterone receptors [15, 27]. Bcl-2 immunostaining has also been found to be a more accurate predictor of response than oestrogen receptor status [28].

An inverse relationship between p53 and Bcl-2 protein expression has been found in the majority of breast carcinomas [29, 30]. Moreover, it has been suggested that the predictive role of Bcl-2 protein on the survival of women with breast carcinoma is mainly dependent on p53 expression [24, 31]. Additionally, p53 (−)/Bcl-2 (+) breast tumours have shown a better response to hormonal therapy than p53 (−)/Bcl-2 (−) tumours [32].

In an extensive recent study of 441 women with node-negative breast carcinomas, van Slooten and associates [2] confirmed the positive correlation between Bcl-2 expression and oestrogen/progesterone receptors or low tumour grade. In the same study, high Bcl-2 expression was found to be negatively correlated with p53, c-erbB-2, high Ki-67, mitotic index and large tumour size. On these grounds, it was concluded that the Bcl-2 expression is generally associated with favourable clinicopathological features [24].

The problem of Bcl-2 upregulation seems to be complex. It can be caused by chromosome translocations (and gene rearrangements) as is the case with t(14;18) in follicular and other B-cell lymphomas [1]. Post-transcriptional causes may also influence Bcl-2 protein expression [4, 13]. Although a positive relationship between Bcl-2 expression and hormone stimuli has been inferred not only in the mammary gland and its carcinomas but also in other glandular structures such as the thyroid follicles, a negative correlation has been documented in some instances. Namely, in normal and carcinomatous cells of the prostate gland, Bcl-2 protein expression has been associated with androgen insensitivity [11]. Moreover, Bcl-2 protein expression has been related positively to stem cells and negatively to differentiated cells [6]. Bcl-2, as a suppressor of apoptosis, plays a significant role in organ morphogenesis and regeneration by assisting the survival of stem cells and preventing the overaccumulation of differentiated cells [33], and by promoting induction processes between parenchyma and stromal cells [6, 7, 34]. After completion of mammary gland morphogenesis, Bcl-2 protein disappears from the basal cells of the fetal breast and appears in a heterogeneous distribution in the luminal cells of the infant and adult breast [34]. It is possible that the presence of Bcl-2 protein in neoplastic cells of breast carcinomas may not merely reflect the degree of cell differentiation and hormone responsiveness, but may also be a constitutive feature of the cell type component of each carcinoma. Accordingly, in neuroblastomas, where Bcl-2 expression represents a constitutive element of neuroblasts and neurons, it is present in all histological types irrespective of their degree of differentiation [23, 35].

In our study, the histology of breast (lobular versus ductal, NOS) carcinomas seems to be the most critical feature influencing the expression of Bcl-2 protein irrespective of the histological grade. The statistically significant high Bcl-2 positivity in lobular carcinomas, in contrast to ductal, NOS carcinomas, was unrelated to the histological grade. However, it is known that the lobular type has been characterised as a small uniform cell tumour with low nuclear grade [36]. There are papers concerning various malignant neoplasms, such as renal cell tumours [37] and medullary thyroid carcinomas [10], that report no clear relationship between grading and level of Bcl-2 expression. We think that immunohistochemical positivity to Bcl-2 protein must favour the diagnosis of lobular against ductal, NOS carcinoma in any morphologically doubtful case. The neoplastic cells of all examined DCIS (cribriform, comedo-like) and specific histological types of invasive breast carcinomas (cribriform, colloid, tubular) appear strongly Bcl-2 positive, similarly to invasive lobular carcinomas. It seems that other features such as Bcl-2 and p53 expression could be used to complement the morphological classification of breast carcinomas. An inverse relationship to Bcl-2 protein expression has been documented for p53 not only in breast carcinomas [15, 23] but also in neoplasms of other sites [38]. It can be assumed that as Bcl-2 protein counteracts apoptosis and extends cell life, it gives long-lived neoplastic cell clones the opportunity to spread within the breast via ducts or other methods. Regarding lobular carcinomas, it has been assumed that multiple carcinomatous foci could be multicentric arising from different cell clones [39, 40]. In that case, it could be said that the presence of Bcl-2 protein prolongs the life of different cell clones at different breast sites and gives them the opportunity to undergo multistep carcinogenesis. Multistep mutagenesis on long-lived Bcl-2 positive cell clones has been offered as a possible explanation for the growth of some types of lymphomas [33] and other tumours [41].

The importance of Bcl-2 protein expression for tumour prognosis is debatable as the role of that protein on carcinogenesis and neoplastic cell growth seems to be complicated. The *BCL-2* oncogene has been shown to synergise and/or antagonise other oncogenes such as *P53* and *C-MYC* [24, 38]. The results of the interaction between the *BCL-2* oncogene and the mutant varieties of other oncogenes remain to be solved [23, 38, 42]. Although Bcl-2 protein upregulation promotes drug resistance, other ways to chemoresistance seem to exist as well [43–45]. For the time being, immunohistochemical positivity to Bcl-2 protein has been associated with a better prognosis in non-small cell lung carcinomas, and especially in the group of squamous cell carcinomas [13]. In non-Hodgkin's malignant lymphomas, Bcl-2 positivity has no significance for overall survival. However, it seems that a lack of Bcl-2 expression and a high apoptotic

index may be adverse prognostic factors independent of histological grade [46]. In contrast, the Bcl-2 oncoprotein does not appear to be one of the factors influencing prognosis in neuroblastomas [43]. Others have described heterogeneous expression of Bcl-2 in less differentiated neuroblastomatous cells being absent from cells expressing N-myc [35]. In our present study, Bcl-2 expression in breast carcinomas does not seem to be correlated to 5 or 10 years' survival irrespective of the unifocality/multifocality.

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