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Immunohistochemical analysis of bcl-2 and p53 expression in breast carcinomas: their correlation with Ki-67 growth fraction

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Abstract We examined 59 breast cancers for p53 and bcl-2 protein expression by immunohistochemistry. The results were correlated with Ki-67 immunostaining. p53-negativity was noted in 40 cases and the remaining 19 tumours were p53-positive. Thirty-six tumours showed strong expression of bcl-2 and in 23 no staining for this protein was observed. We found statistically significant reverse correlation between expression of p53 and bcl-2 in majority of carcinomas: 31 cases were bcl-2 positive and p53-negative, and 14 tumours were bcl-2-negative and p53-positive. Six carcinomas showed no nuclear staining for Ki-67 and in the remaining 53 the percent of cancer cells positive for Ki-67 ranged from 1 to 60 (mean: 14.6). In these 53 cases we found that bcl-2-positive tumours were characterized by lower proliferation than bcl-2-negative tumours, the mean value of Ki-67 immunostaining being 10.7% and 23.0%, respectively. p53-negative tumours showed lower proliferation than p53-positive tumours: mean Ki-67 index was 10.2% and 23.9%, respectively.

We conclude that immunohistochemically detected p53 and bcl-2 proteins show a significant inverse relationship in majority of breast carcinomas and their expression correlates with tumour proliferation (Ki-67 immunostaining).

Key words Breast carcinoma · bcl-2 · p53 · Ki-67

Introduction

Breast carcinoma is a heterogeneous disease, and the prognosis depends first of all on the histological type of the tumour and the status of regional lymph nodes. Recently, to provide an objective evaluation of biological behaviour of this tumour, a number of molecular and cellular markers have been proposed, including DNA ploidy, oestrogen receptor, cathepsin D, or erbB-2. Many studies have focussed their attention on cell proliferation, using Ki-67 or proliferating cell nuclear antigen immunostaining, thymidine incorporation, mitosis counting or S-phase fraction. Neoplastic growth, however, does not depend exclusively on uncontrolled proliferation, but also on the cell loss, the latter being achieved by a special type of death called apoptosis [2, 5, 9, 18].

Apoptosis occurs spontaneously or may be induced by various anti-tumour agents; it is regulated by many oncogenes including *bcl-2, p53, c-myc, ras, ced-3, ced-4,* (for review, see [6, 8, 17, 18]). Both bcl-2 and mutated forms of p53 participate in cell immortalization. The *bcl-2* oncogene was discovered in lymphomas composed of B cells and it has been found in many benign and malignant tissues; benign epithelial cells of large and small ducts within the breast have abundant cytoplasmic bcl-2 staining [7]. In prostate carcinoma, bcl-2 expression correlated with progression of cancer from androgen dependence to androgen independence [10].

p53 expression was found to correlate with high morphological malignancy grades and tumour cell proliferation in breast cancer [1, 4, 16]. We present the correlation between bcl-2 and p53 expression with Ki-67 immunostaining in breast carcinomas.

Materials and methods

Fifty-nine breast carcinomas from patients treated at the Regional Oncological Hospital in Szczecin in 1992 and 1993 were included in this study. The patients were all women, ranging in age from 30 to 80 years (mean 52.0±11.6). Tumours were collected at surgery, snap frozen in liquid nitrogen and stored at −80° C until analysis. The parallel samples were fixed routinely in 10% buffered formalin (pH 7.0) for 24 h for routine histological evaluation. Histopathological diagnoses were performed according to standard criteria (World Health Organisation). Tumour size (measured in fresh, non-fixed material), lymph node status, histological grade (according to Bloom and Richardson criteria for ductal carcinomas) were recorded for each case. Oestrogen receptor status was not available for the majority of the patients.

For immunohistochemical determination of bcl-2, p53 and Ki-67, 4 μm thick frozen sections were mounted on 3-aminopropylo-

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trimetoxy-silane (Sigma, USA) coated slides, fixed in absolute cold acetone (–20° C; 30 min) and then air dried at room temperature (10 min). Sections were incubated with primary antibodies (bcl-2, Dako-124, Denmark; p53, Ab2, Oncogene Science, USA and Ki-67, Dako, Denmark, diluted 1:20, 1:40 and 1:10, respectively) for 60 min at room temperature in the humidified chamber. Staining was revealed with the streptavidin-biotin-peroxidase kit (Histostain-SP, Zymed Laboratory, San Francisco, USA). The sections were slightly counter stained with haematoxylin and mounted in glycergel (DAKO Denmark). Additionally, each section was incubated with a monoclonal antibody against cytokeratin (CK-2, Max Planck Institute, Goettingen, Germany).

The results of immunostaining were analysed for statistical significance with Wilcoxon test and two sample analysis test, Statgraf 5.0 version.

Results

The results are summarized in Table 1. There were 50 infiltrating ductal carcinomas, 4 infiltrating lobular carcinomas, 4 medullary carcinomas and 1 metaplastic carcinoma. Thirty patients were axillary lymph node negative and 29 patients had lymph node metastases (1–29 positive lymph nodes, mean 3.5±7). The size of tumours ranged from 8 mm to 60 mm (mean 21.7±9 mm).

Of the 59 tumours 36 (61%) were positive for bcl-2. The bcl-2-positive tumours were characterized by strong, diffuse cytoplasmic staining (Fig. 1A). Some variability in intensity of staining was observed among different positive cases. Benign epithelial cells found at the periphery of some tumours had abundant cytoplasmic staining. Small lymphocytes were also very often positive for bcl-2.

The nuclei of 19 of the 59 carcinomas accumulated p53 (Fig. 2B). Normal cells were p53 protein-negative. There were 31 bcl-2-positive and p53-negative tumours, and 14 bcl-2-negative and p53-positive tumours. In 14 carcinomas both proteins were either positive (5 cases) or negative (9 cases). We found significant reverse correlation between p53 protein accumulation and bcl-2 (Wilcoxon test: $z=1.80413\times10^{-3}$).

In six cases there was no nuclear staining for Ki-67 antigen. In the remaining 53 cases the percent of cancer cells positive for Ki-67 ranged from 1 to 60, and the mean Ki-67 index was 14.6 (standard deviation=14.4; Figs. 1C, 2C). The bcl-2-positive tumours were characterized by lower proliferation than bcl-2 negative tumours: the mean value of Ki-67 index was 10.7 (±9.1) in the first group, and 23.0 (±18.0) in the second (Fig. 3). In the case of p53 we found just the reverse correlation: p53-positive tumours had a higher Ki-67 index (mean 23.9±20.5) and p53-negative tumours a lower Ki-67 index (mean 10.2±7.4; Fig. 4). The mean proliferation in bcl-2-positive and p53-negative tumours was 9.0 (±7.1), and in bcl-2-negative and p53-positive carcinomas was 25.3 (±22.1). The results were statistically significant according to the Wilcoxon test.

There was no correlation between accumulation of p53 and bcl-2 expression with lymph node status. Similarly, there was no correlation between the size of the primary tumour and p53 expression (mean value of tumour diameter for p53-positive tumours was 19.8 mm, and for p53-negative tumours was 18.7 mm; $P>0.05$) nor for bcl-2 (mean diameter of bcl-2-positive tumours was 17.5 mm and for bcl-2-negative tumours 20.7 mm, $P>0.05$). When p53 was related to tumour grade no significant correlation was found ($P>0.05$; $\chi^2=1.58$); however, bcl-2 expression was observed more often in low grade carcinomas then in high grade lesions ($P<0.05$; $\chi^2=5.11$).

Discussion

Pezzella et al. [12] have reported a significant inverse relationship between p53 and bcl-2 in non-Hodgkin's lymphoma, suggesting a possible relationship between the expression of these two proteins. Recently, Silvestrini et al. [13] found similar inverse relationship in breast carcinoma. A significantly higher fraction of bcl-2-positive cells was observed in p53-negative tumours: median values of bcl-2-positive cells were 70.0% and 11.3%, for p53-negative and p53-positive tumours, respectively. In the present study we have demonstrated bcl-2 expression in 61% and p53 expression in 32.2% of 59 cases of invasive breast carcinoma. Seventy-six percent of tumours (45 cases) showed an inverse correlation between these two proteins. In the remaining 14 carcinomas the stain-

Table 1 Tumour type, lymph node status, bcl-2, p53, and Ki-67 expression in 59 breast carcinomas (*LN* lymph node status)

		Number	LN		bcl-2		p53		Ki-67
			+	–	+	–	+	–	(%)
Tumour type:									
ductal		50	28	22	30	20	16	34	13.7
lobular		4	1	3	4	0	0	4	5.3
medullary		4	0	4	2	2	3	1	27.2
metaplastic		1	0	1	0	1	1	0	6.0
LN	+	29			19	10	8	21	14.9
	–	30			17	13	11	19	13.9
bcl-2	+	36	19	17			5	31	10.7
	–	23	10	13			14	9	23.0
p53	+	19	8	11	5	14			23.9
	–	40	21	19	31	9			10.2

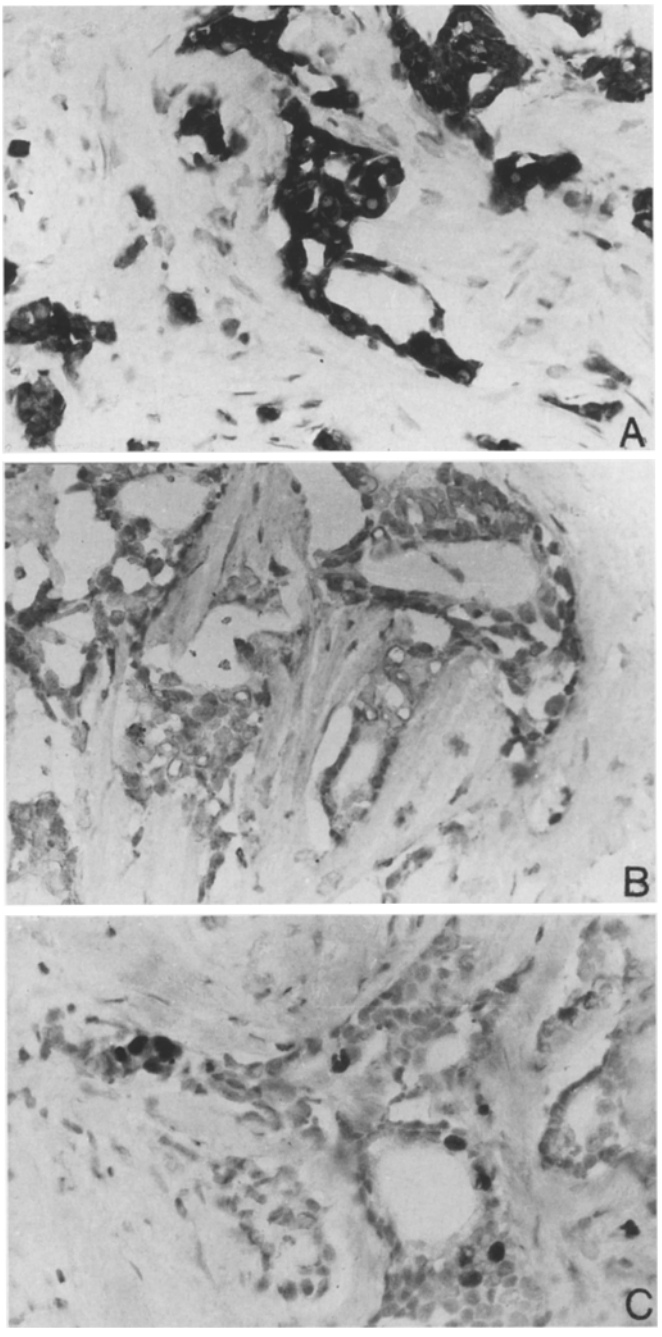

Fig. 1 Immunohistochemical staining for bcl-2 (**A**), p53 (**B**) and Ki-67 (**C**) antigens in infiltrating, low grade ductal carcinoma of the breast. **A** Strong positive bcl-2 staining in the cytoplasm of cancer cells. **B** Negative staining for p53 protein. **C** Reaction with Ki-67 is visible in only a few cancer cells. **A–C** × 400, streptavidin-biotin-peroxidase reaction with light haematoxylin counterstaining

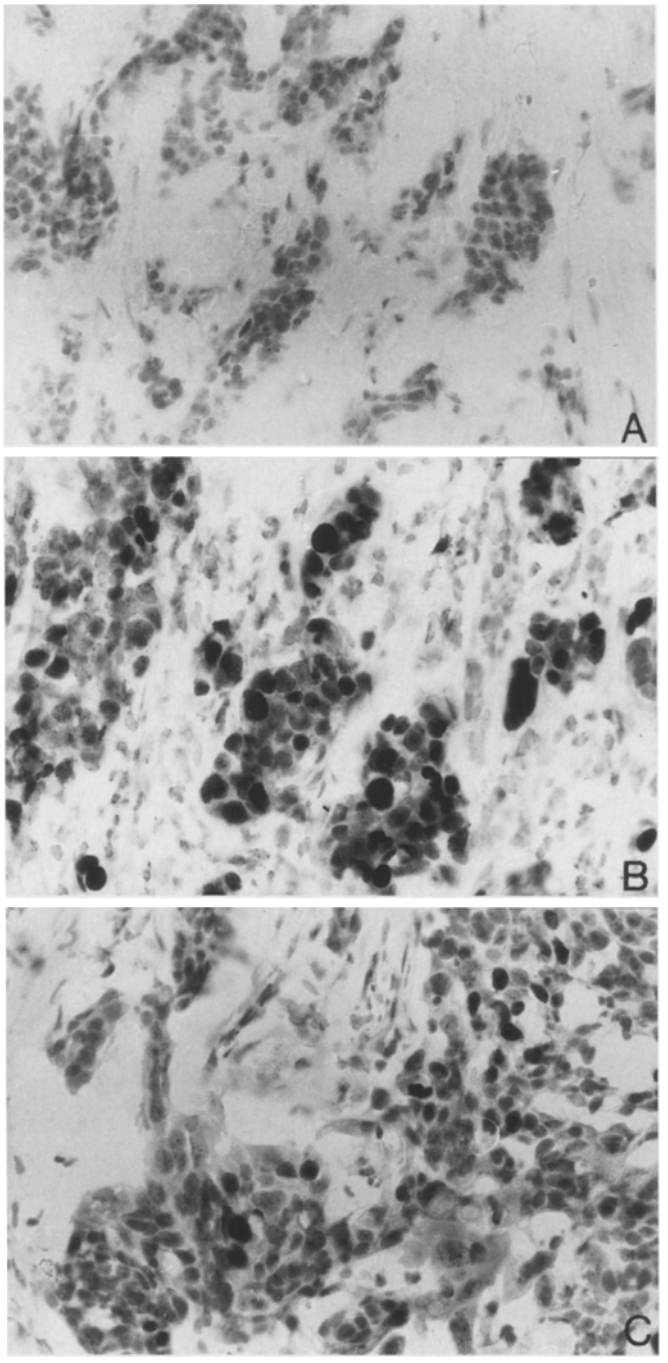

Fig. 2 Immunohistochemical staining for bcl-2 (**A**), p53 (**B**) and Ki-67 (**C**) antigens in infiltrating high grade ductal carcinoma of the breast. **A** Negative staining for bcl-2. **B** Strong positive staining for p53 protein. **C** Strong positive reaction with Ki-67 is visible in many cancer cells. **A–C** × 400, streptavidin-biotin-peroxidase reaction with light haematoxylin counterstaining

ing for both proteins was either positive (5 cases) or negative (9 cases). Another finding of interest was the demonstration of correlation between proliferation, estimated on the basis of immunoreactivity for Ki-67 antigen, and expression of bcl-2 or p53. Tumours positive for p53 were characterized by high proliferation rates, similar to bcl-2-negative tumours (the mean value of Ki-67 index was 23.9% and 23.0%, respectively). However, p53-negative tumours showed lower proliferation rates resembling those in bcl-2-positive tumours (10.1% and 10.7%, respectively). Silvestrini et al. [13] found bcl-2 expression in 70% of breast carcinomas (node negative tumours only) and noted higher median numbers of bcl-2-positive cells in lesions with low tritiatedthymidine-la-

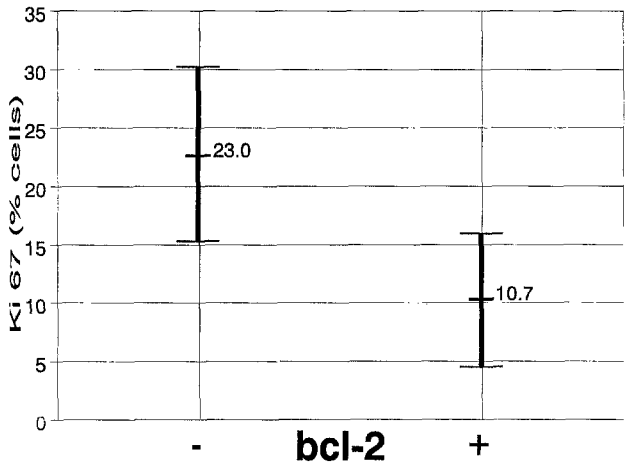

Fig. 3 Ki-67 staining (%) in bcl-2-negative (–) and bcl-2-positive (+) breast carcinomas (*bars* = 95% confidence intervals for means)

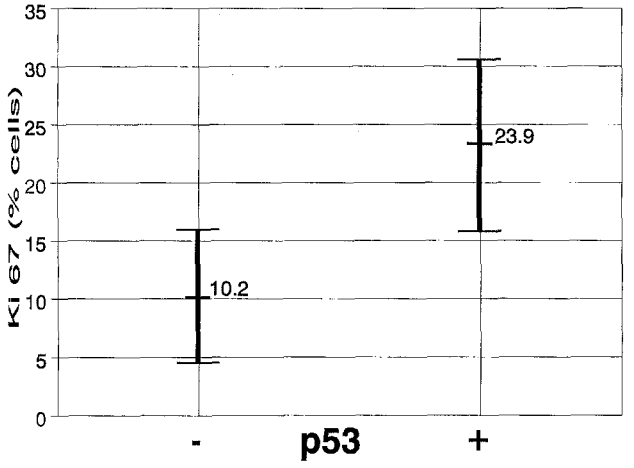

Fig. 4 Ki-67 staining (%) in p53-negative (–) and p53-positive (+) breast carcinomas (*bars* = 95% confidence intervals for means)

belling index when compared with tumours with high thymidine incorporation (27.1% and 10.6%, respectively). p53 is expressed in 22%–45% of breast carcinomas [3, 11, 15]. Cattoretti et al. [3] noted strong positive correlation between p53 and Ki-67. Spandidos et al. [14] did not observe any correlation between p53 and histological type or tumour grade. They noted, however, that a significantly higher number of p53 staining specimens overexpressed the *ras* gene. In the study of Trudel et al. [16] p53 expression correlated with poorly differentiated breast cancers and neu expression. Ostrowski et al. [11] noted that p53 expression showed a significant relationship to high tumour grade. Immunohistochemically detected p53 protein was found to be an independent marker of shortened survival and was seen more often in familial than in sporadic carcinomas [15]. Nevertheless bcl-2 did not have an independent prognostic value when compared with other biological prognostic factors [13] (Silvestrini et al. 1994).

We conclude that immunohistochemically detected p53 and bcl-2 proteins showed a significant inverse relationship in the majority of breast carcinomas and their expression correlates with tumour proliferation (Ki-67 immunostaining). The low proliferation rates of bcl-2-positive tumours is somewhat surprising. bcl-2 in organized epithelium is restricted to stem cells and proliferation zones [8] and we expected higher proliferation in bcl-2-positive tumours. bcl-2 expression prevents a cell from entering apoptosis and a mutant version of *p53* gene product acts much like bcl-2 [6, 19]. In other words, both proteins lead to cell immortalization, allowing to accumulation of chromosomal defects and acquisition of a more malignant phenotype. Later the cells with "fixed" genetic damage proliferate leading to development of tumours [2]. Malignant cells expressing bcl-2 or p53 fail to undergo apoptosis and may inappropriately enter the cell cycle instead of dying. Leoncini et al. [9] found that bcl-2 was significantly more often expressed in lymphomas with low than in those with high apoptotic index (evaluated by number of apoptotic bodies). It may be the case that in bcl-2-negative tumours the products of other oncogenes undertake the role of *bcl-2* in cell immortalization. Further studies are required to find if bcl-2-negativity due to its correlation with high proliferation and whether p53-positivity is a marker of poor prognosis.

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