ANTICANCER RESEARCH *23:* 565-568 (2003)

# Immunofluorometric Study of Bcl-2 and Bax Expression in Clinical Fresh Tumor Samples from Breast Cancer Patients

F. MARTÍNEZ-ARRIBAS[1], M.J. NÚÑEZ-VILLAR[1], A.R. LUCAS[1], J. SÁNCHEZ[1,2], A. TEJERINA[1] and J. SCHNEIDER[1,3]

[1]*Fundación Tejerina-Centro de Patología de la Mama, Madrid;* [2]*Universidad de Alcalá, Madrid; and* [3]*Universidad Rey Juan Carlos, Madrid, Spain*

**Abstract.** *Background: The balance between the expression of the antiapoptotic gene Bcl-2 and the proapoptotic gene Bax is considered a good indicator of the apoptotic activity of tumor cells. Bcl-2 and Bax expression seem also to individually play a prognostic role in breast cancer. Our aim was to study the expression of both genes in fresh breast cancer samples, and to correlate the obtained results with other available clinical and biological parameters of the tumors. Materials and Methods: Fresh tumor specimens from 86 breast cancer patients were studied by means of immunofluorocytometry for the expression of the apoptosis-associated Bcl-2 and Bax genes. Additionally, DNA-ploidy was also measured. Paraffin blocks corresponding to the same tumors were used for immunohistochemistry, to study the expression of hormone receptors (ER and PR), p53, c-erb-B2 and the Ki67 labelling index. Fourteen patients had been treated with four cycles of induction chemotherapy (cyclophosphamide, adriamycin and 5-fluorouracil), and separate statistical analyses were carried out both for the whole group, and for the 62 patients not having received any treatment whatsoever, in order to exclude any potential influence of the chemotherapeutic treatment on the expression of the studied antigens. Results: Bcl-2 expression correlated significantly with estrogen ($p = 0.002$) and progesterone ($p = 0.012$) receptor expression, as well as with c-erb-B2 ($p = 0.045$) expression in chemotherapy-naïve tumors, the correlation being completely lost if treated tumors were added to the study group. A high apoptotic index (Bcl2 / Bax < 0.5) correlated significantly with progesterone receptor expression ($p = 0.037$) and c-erb-B2 expression ($p = 0.018$), and this correlation was maintained, whether previously treated tumors were included into the study or not ($p = 0.038$ and $p = 0.027$, respectively). Bax expression did not correlate with any other clinical or biological parameters of the tumors, including Bcl-2 expression. Conclusion: Bcl-2 and Bax-expression can be easily determined in clinical breast cancer specimens by means of immunofluorocytometry. Bcl-2-expression signficantly correlates with hormone-receptor- and c-erb-B2-expression exclusively in previously untreated tumors. This, however, seems only to be the case when considering Bcl-2 expression in isolation, since a high apoptotic index, which considers the ratio of Bcl-2 versus Bax expression in the same tumor, seems not to be affected by the previous use of chemotherapy.*

Cell turnover in normal tissues is regulated by means of a balance between proliferation, on the one hand, and apoptosis on the other. Apoptosis is a phenomenon by which cells undergo programmed death after having gone through the normal process of senescence. Several genes are involved in the control of apoptosis (1), among which some exert a stimulatory or proapoptotic effect, and others the opposite, or an antiapoptotic one. Among the most studied are the proapoptotic gene Bax, and the antiapoptotic gene Bcl-2. The ratio of expression beween both is considered a useful parameter for estimating the apoptotic index of a given tissue or tumor: the lower the expression of Bcl-2 and the higher the expression of Bax, the more likely it is that apoptotic mechanisms are intact, *i.e.*, that cells die at their physiological rate, or even faster. This is of paramount importance in oncology, since tumor cells tend to immortalize themselves, or to acquire resistance against chemotherapeutic or radiotherapeutic treatment, by down-regulating or completely turning off apoptosis.

Apoptosis has been studied in clinical breast cancer specimens and in experimental breast cancer cell models, though the results obtained have been somewhat paradoxical: in fact, high Bcl-2 expression, which in principle stands for a lower apoptotic rate, has been consistently correlated with a significantly better prognosis of the patients involved, and not with a worse one, as theoretically expected (2). This may be explained by the significant correlation found beween Bcl-2 expression (and to some extent also Bax expression) and hormone receptor positivity, low histological grade and absence of nodal metastases in human breast cancer (all defining a better prognosis), although strangely also with

*Correspondence to:* Prof. J. Schneider, Fundacion Tejerina, Calle José Abascal 40; 28003 Madrid, Spain. e-mail: schneider@teleline.es

*Key Words:* Cancer, breast, Bcl-2, Bax.

tumor aneuploidy (3), which is generally considered a negative prognostic factor in this kind of tumor. A recent study involving transfection experiments of the c-erb-B2 oncogene in MCF7 breast cancer cells (4) has evidenced a similarly puzzling association between biologically opposite features. In fact, c-erb-B2 transfectants showed down-regulation of Bcl-2 and consequently increased apoptosis, together with an up-regulation of p53, which also opposes c-erb-B2-mediated proliferation and enhances apoptosis. In order to veriyfy if these effects are reproduced in clinical breast cancer specimens, we carried out the present investigation. The tumors were studied for Bcl-2 and Bax expression, proliferation (DNA-ploidy and Ki67 labelling index), hormone receptor, mutant p53 and c-erb-B2 expression.

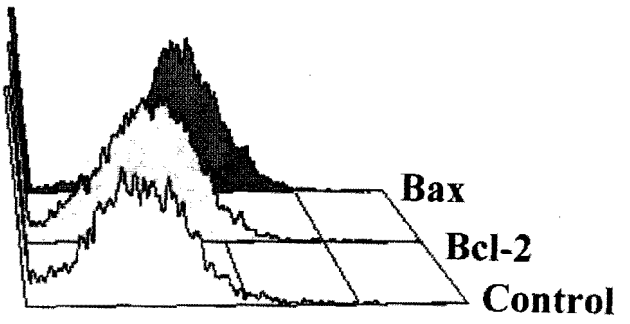


Figure 1. *Superimposed histograms of Bcl-2-, Bax-reactive and control tumor cells.*

## Materials and Methods

Fresh tumor specimens from 86 patients operated upon for breast cancer at Fundación Tejerina, Madrid, Spain, were studied by means of immunofluorocytometry for the expression of the apoptosis-associated Bcl-2 and Bax genes. Additionally, DNA-ploidy was also measured. Paraffin blocks corresponding to the same tumors were used for immunohistochemistry, to study the expression of hormone receptors (ER and PR), p53, c-erb-B2 and the Ki67 labelling index.

Fourteen patients had been treated with four cycles of induction chemotherapy (cyclophosphamide, adriamycin and 5-fluorouracil), and separate statistical analyses were carried out both for the whole group, and for the bulk of 62 patients not having received any treatment whatsoever, in order to exclude any potential influence of the chemotherapeutic treatment on the expression of the studied antigens.

*Flow cytometry.* Fresh tumors were finely minced with a scalpel blade, mixed with 2 ml of DNA-prep Stain reactant and 100 ml DNA-prep LPR reactant (both from Coulter Corporation, Miami, FL, U.S.A.), and incubated for 30 minutes at 37°C. The resulting mixture was then filtered through a 50 μm pore filter and was ready for DNA-ploidy and S-phase analysis.

For Bcl-2/Bax-immunofluorocytometry, the tumor specimens were again minced with the use of a scalpel, as described above. From here on, however, the procedure varies considerably: the obtained tissue was mixed with 2 ml pepsin at 37°C for 30 minutes, in order to separate the cells from the surrounding connective tissue matrix; the resulting mixture was filtered through a 50 μm pore filter and centrifugued at 2,000 rpm for 10 minutes; the supernatant was eliminated and the obtained cell pellet fixed by resuspension in 1 ml 2% paraformaldehyde (pH 7.2) for 10 minutes at 4°C, followed by another 10 minutes at room temperature. After centrifugation (2,000 rpm for 10 minutes) the cells were resuspended in phosphate-buffered saline (PBS) and centrifugued again (same conditions), in order to wash off any residual formalin and resuspended in absolute methanol at –20°C for 5 minutes, to permeabilize the membrane; they were washed again with PBS (same conditions as above), centrifugued at 7,000 rpm for 5 minutes, and were now ready for incubation with the primary antibodies after resuspension in either PBS to attain the wanted antibody dilution, or in the provided antibody solution (see below).

The Bcl-2 antibody was the mouse monoclonal bcl-2/100/D5 from Nococastra, Newcastle, U.K., and was used at a concentration of 1:80, whereas the Bax antibody (clone 4F11) was purchased in ready-to-use prediluted presentation from Immunotech, Marseille, France. The cells were incubated with both antibodies for 1 hour at 4 °C. Afterwards, they were centrifugued at 7,000 rpm for 5 minutes, washed and resuspended in PBS, and incubated with the secondary, fluorochrome-coupled anti-mouse antibody (1:100) at 4 °C for 30 minutes. As an internal control, the cells were finally incubated with propidium iodide as described above for measuring DNA-ploidy, to select the cells effectively fixed, and to discard cell debris eventually reacting with the primary or secondary antibodies. This allows, additionally, for determining the percentage of cells specifically reacting with the primary antibody.

All measurements were carried out in a Coulter EPICS XL cytometer (Coulter Corporation, Miami, FL, U.S.A.). The analysis of the obtained histograms was carried out with the help of the MultyCycle DNA Cell Cycle Analysis software package (Phoenix Flow Systems, San Diego, CA, U.S.A.). The tumors were considered diploid when the DNA-index obtained was 1.0 and aneuploid for any diverging value, including tetraploid tumors, with a DNA-index of 2.0.

*Immunohistochemistry.* The immunohistochemical procedure routinely employed at our laboratory has been described extensively elsewhere (5). Briefly, 5-μm sections from archival paraffin blocks were mounted on poly-L-lysine-coated slides, deparaffinized, rehydrated, preincubated with blocking serum and subsequently incubated with the primary antibodies. The immunohistochemical kit was always the same, to ensure uniformity of results (Histostain-SP, Zymed, San Francisco, CA., U.S.A). The primary antibodies used were: NCL-CB11 (c-erb-B2), NCL-ER-6F11 (ER), NCL-p53-DO7 (p53), (all from Novocastra, Newcastle, U.K.); prediluted MIB1 (Ki67) and PR-2C5 (PR) from Zymed, San Francisco, CA, U.S.A.

The heat-induced antigen retrieval (HIER) technique developed at Zymed laboratories, as described on the data sheet accompanying their antibodies, was employed throughout. The incubation time was 1 hour for all antigens, at room temperature in a humid chamber. The tumors were considered positive for p53, ER and PR when more than 10% of tumor cell nuclei stained specifically and for c-erb-B2, when more than 10% tumor cell membranes showed distinct staining. The Ki67 labelling index was obtained directly as the percentage of stained tumor cell nuclei.

*Statistics.* Correlations between Bcl-2/Bax expression and all the tested biological parameters were calculated using the Spearman rank correlation test, after dichotomising the variables, with the help of the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, U.S.A.). Values were considered significant, when $p$ was $< 0.05$.

## Results

A series of 86 human breast cancers was studied for the expression of the apoptosis-associated Bcl-2 and Bax genes, as

Table I. *Correlation of absolute and relative Bcl2- and Bax-expression with other clinical and biological parameters of human breast cancer. Analysis of the whole group (N = 86), of which 14 had been previously treated with induction chemotherapy.*

| Parameter | Bcl2-positive | Bax-positive | Bcl2 / Bax < 0.5 |
|---|---|---|---|
| Axillary nodes | n.s. | n.s. | n.s. |
| Histological grade | n.s. | n.s. | n.s. |
| Nuclear grade | n.s. | n.s. | n.s. |
| Ki67 > 20% | n.s. | n.s. | n.s. |
| DNA-ploidy | n.s. | n.s. | n.s. |
| Estrogen receptors | n.s. | n.s. | n.s. |
| Progesterone receptors | n.s. | n.s. | $p = 0.038$ |
| c-erb-B2 | n.s. | n.s. | p = 0.027 |
| p53 | n.s. | n.s. | n.s. |

Table II. *Same analysis as Table I, but considering previously untreated tumors (N = 72).*

| Parameter | Bcl2-positive | Bax-positive | Bcl2 / Bax < 0.5 |
|---|---|---|---|
| Axillary nodes | n.s. | n.s. | n.s. |
| Histological grade | n.s. | n.s. | n.s. |
| Nuclear grade | n.s. | n.s. | n.s. |
| Ki67 ≥ 20% | n.s. | n.s. | n.s. |
| DNA-ploidy | n.s. | n.s. | n.s. |
| Estrogen receptors | $p = 0.002$ | n.s. | n.s. |
| Progesterone receptors | $p = 0.012$ | n.s. | $p = 0.037$ |
| c-erb-B2 | $p = 0.045$ | n.s. | $p = 0.018$ |
| p53 | n.s. | n.s. | n.s. |

well as for their relative expression in the same tumor, a ratio between both inferior to 0.5 being considered indicative of apoptotic activity. Bcl-2 and Bax expression were considered positive when more than 1% reactive cells were detected by immunofluorocytometry (Figure 1).

Bcl-2 expression correlated significantly with estrogen and progesterone receptor expression, as well as with c-erb-B2 expression in chemotherapy-naïve tumors, the correlation being completely lost if treated tumors were added to the study group (Tables I & II).

A high apoptotic index (Bcl-2/Bax < 0.5) correlated significantly with progesterone receptor- and c-erb-B2 expression, and this correlation was maintained, whether previously treated tumors were included into the study or not (Tables I & II).

Bax expression "*per se*" finally did not correlate with any other clinical or biological parameters of the tumors.

## Discussion

In the present study we have found, firstly, that Bcl-2 expression in clinical tissue samples from breast cancer patients correlates significantly with both hormone-receptor and c-erb-B2 expression, and that this correlation gets lost when including chemotherapy-treated tumors into the study. Kandouz *et al.* (6) studied breast cancer cell cultures and has already reported that Bcl-2 expression is modulated by the existing hormonal antagonism existing between estrogens (estradiol) and progestins. It is therefore not surprising that there exists a significant association between hormone-receptor and Bcl-2 expression in our solid tumor samples. Furthermore, Eissa *et al.* (3), in a previous report involving breast cancer specimens analogous to the ones studied by us, also found Bcl-2 expression to be strongly associated to both estrogen receptor expression and aneuploidy of the tumors. This latter result represents the only discrepancy between our own findings and theirs. In fact, Eissa *et al.*, in agreement with us, were also unable to establish any correlation between Bcl-2 expression and either nodal status, differentiation of the tumors or mutant p53 expression by them. As to the disagreement regarding tumor DNA-ploidy, this may lie in the different methodology employed for determining Bcl-2 expression, or to the positivity threshold used, since otherwise both series are virtually identical. Our results are validated by the fact that we additionally used the Ki67 labelling index to determine the proliferative rate of the tumors, and no association was found with Bcl-2 expression. In a previous study (7), we found both Ki67 and DNA ploidy to strongly correlate with each other, and since then we have always used both parameters together as an internal control.

The significant association we have established in our series of untreated solid breast cancers between Bcl2- and c-erb-B2 expression also corroborates a similar previous study using experimental MCF7 breast cancer cells. In it, Kumar *et al.* (8) reported that overexpression of c-erb-B2 resulted in up-regulation of Bcl-2. An interesting new finding of our study was that all the above-mentioned correlations disappeared when including into the analysis those tumors having been subject to chemotherapeutic treatment. This may just reflect a selective vulnerability of Bcl-2-expressing cells to chemotherapy, something that is not altogether surprising if we consider that one of the main mechanisms of

chemotherapy is precisely to induce the apoptosis of tumor cells, and a main step in this process might be the inacivation of a major antiapoptotic gene, like Bcl-2.

Bax-expression, on the other hand, did not correlate with any of the studied tumor parameters, and this is also in agreement with previous findings reported for experimental breast cancer cell systems (2,9).

A high apoptotic index, finally, as represented by a Bcl-2 / Bax ratio lower than 0.5, correlated again with c-erb-B2 and progesterone receptor expression, but intriguingly not with estrogen receptor expression. This correlation, furthermore, was maintained throughout the study, irrespective of the inclusion or not of previously treated tumors into the analysis (Tables I&II).

In conclusion, Bcl-2 and Bax-expression can be easily determined in clinical breast cancer specimens by means of immunofluorocytometry. Bcl-2 expression significantly correlates with hormone-receptor and c-erb-B2 expression of previously untreated tumors. This, however, seems only to be the case when considering Bcl-2 expression in isolation, since a high apoptotic index, which considers the ratio of Bcl-2 *versus* Bax expression in the same tumor, seems not to be affected by the previous use of chemotherapy. Larger studies are necessary to verify if the study by means of immunofluorocytometry of apoptosis-related markers has significant predictive or prognostic implications in human breast cancer.

## References


1 Steller H: Mechanisms and genes of cellular suicide. Science *267:* 1445-1448, 1995.

2 Krajewski S, Krajewska M, Turner BC, Pratt C, Howard B, Zapata JM, Frenkel V, Robertson S, Ionov Y, Yamamoto H, Perucho M, Takayama S and Reed JC: Prognostic significance of apoptosis regulators in breast cancer. Endocr-Relat Cancer *6:* 29-40, 1999.

3 Eissa S, Labib R, Khalifa A, Swelam N, Khalil F and El-Shenawi AM: Regulators of apoptosis in human braest cancer. Clin Biochem *32:* 321-326, 1999.

4 Huang CG, Hobbs S, Walton M and Epstein RJ: Dominant negative knockout of p53 abolishes ErbB2-dependent apoptosis and permits growth acceleration in human breast cancer cells. Br J Cancer *86:* 1104-1109, 2002.

5 Schneider J, Pollán M, Jiménez E, Ruibal A, Lucas AR, Núñez MI, Sánchez J and Tejerina A: Histologic grade, Ki67 and CD44 are predictors of axillary lymph node invasion in early (T1) breast cancer. Tumor Biol *20:* 319-330, 1999.

6 Kandouz M, Siromachkova M, Jacob D, Chretien M, Therwath A and Gompel A: Antagonism between estradiol and progestin on Bcl2-expression in breast-cancer cells. Int J Cancer *68:* 120-125, 1996.

7 Martinez-Arribas F, Núñez MJ, Piqueras V, Lucas AR, Sánchez J, Tejerina A and Schneider J: Flow-cytometry *vs.* Ki67 labelling index in breast cancer: a prospective evaluation of 181 cases. Anticancer Res *22:* 295-8, 2002.

8 Kumar R, Mandal M, Lipton A, Harvey H and Thompson CB: Overexpression of HER2 modulates bcl-2 and bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin Cancer Res *2:* 1215-1219, 1996.

9 van Slooten HJ, van de Vijver MJ, van de Velde CJ and van Dierendonck JH: Loss of Bcl-2 in invasive breast cancer is associated with high rates of cell death, but also with increased proliferative activity. Br J Cancer *77:* 789-796, 1998.


*Received August 23, 2002*
*Accepted November 4, 2002*