

Bcl-2 Expression in Breast Cancer: A Comparative Study at the mRNA and Protein Level

FERNANDO MARTÍNEZ-ARRIBAS¹, TERESA ALVAREZ^{1,2}, GABRIELA DEL VAL^{1,3}, ELENA MARTÍN-GARABATO¹, MARÍA-JOSÉ NÚÑEZ-VILLAR¹, RAUL LUCAS¹, JAIME SÁNCHEZ^{1,4}, ARMANDO TEJERINA¹ and JOSÉ SCHNEIDER^{1,5}

¹Fundación Tejerina-Centro de Patología de la Mama; ²Hospital Clínico San Carlos; ³Hospital de Móstoles; ⁴Hospital Universitario de Alcalá de Henares; ⁵Universidad Rey Juan Carlos, Madrid, Spain

Abstract. *Background:* Bcl-2 is one of the most important antiapoptotic genes. Although it facilitates the survival of tumor cells, its expression has been consistently associated with a better prognosis for breast cancer. Virtually all studies on Bcl-2 conducted in breast cancer have been carried out by means of immunohistochemistry. The aim of this study was to examine for the first time the expression of Bcl-2 in a series of human breast cancer, both at the mRNA and protein level. *Materials and Methods:* One hundred samples from previously untreated human breast cancers were used; Bcl-2 expression was determined both by means of immunohistochemistry using the bcl-2/100/D5 monoclonal antibody and differential RT-PCR. Additionally, the expression of hormone receptors (ER and PR), c-erb-B2, p53 and the proliferation-associated Ki-67 antigen were also studied by means of immunohistochemistry as part of the standard pathological workup. *Results:* Any degree of immunohistochemical staining correlated significantly and inversely with c-erb-B2 expression ($p=0.0008$), nuclear grade 3 ($p=0.0015$), a Ki-67 labeling index $>10\%$ ($p=0.02$) and tumor size ($p=0.048$), and in a direct fashion with estrogen ($p=0.0003$) and progesterone receptor expression ($p=0.0002$). mRNA expression of the Bcl-2 gene showed a significant inverse correlation with c-erb-B2 ($p=0.016$) and p53 ($p=0.014$) expression, as well as with a nuclear grade 3 ($p=0.006$), and a direct correlation with estrogen ($p=0.0004$) and progesterone ($p=0.001$) receptor expression, as well as with nodal invasion ($p=0.04$). *Conclusion:* The study of Bcl-2 expression in breast cancer by means of either immunohistochemistry or RT-PCR yields very similar results. In spite of its role opposing tumor cell death, Bcl-2 is associated with biological features of the tumors which define a better intrinsic prognosis, such as hormone receptor expression, low

proliferation and absence of c-erb-B2 and mutant p53 expression. This may in great part explain why Bcl-2 expression has been invariably found to correlate with a better prognosis of breast cancer.

Apoptosis is a physiological process following which normal cells die after a given number of replications. Tumor cells tend to interfere with this mechanism by activating genes which inhibit apoptosis, thus, achieving long-term survival or even immortality, as shown in experimental *in vitro* models (1, 2). One of the main genes limiting apoptosis is Bcl-2. Paradoxically, Bcl-2 expression has been consistently associated with a better prognosis of breast cancer patients, when, from a purely theoretical point of view, quite the contrary should be expected (1, 2).

Most studies of Bcl-2 expression in breast cancer have been carried out using immunohistochemical techniques (3-5). In this study Bcl-2 expression both at the mRNA and protein levels was examined in a series of breast cancers in order to exclude the possibility that the technique employed might account for the above-mentioned unexpected results.

Materials and Methods

One hundred samples from previously untreated patients operated upon for breast cancer at Fundación Tejerina - Centro de Patología de la Mama, Madrid, Spain, were studied. At the time of operation, one aliquot of the tumor was conserved in RNAlater® (QUIAGEN, Hilden, Germany) for further processing at the Molecular Biology laboratory of Universidad Rey Juan Carlos, Madrid, Spain, and the rest was formalin-fixed for routine processing at the Pathology laboratory of Fundación Tejerina - Centro de Patología de la Mama, Madrid, Spain.

The numbers of histological types were as follows: ductal infiltrating 76, lobular infiltrating 20 and tubular 4. Other, less frequent types were excluded from the study. Of the 100 tumors, 61 were T1, 27 T2, 4 T3, and the remaining 19 were multifocal, diffuse tumors involving several quadrants of the breast, according to the standard TNM classification. Axillary nodal invasion was registered in 43 (43.0%) of the patients. Of them,

Correspondence to: Prof. J. Schneider, Fundación Tejerina, Calle José Abascal 40, 28003 Madrid, Spain. e-mail: jose.schneider@urjc.es

Key Words: Breast, Bcl-2, immunohistochemistry, mRNA.

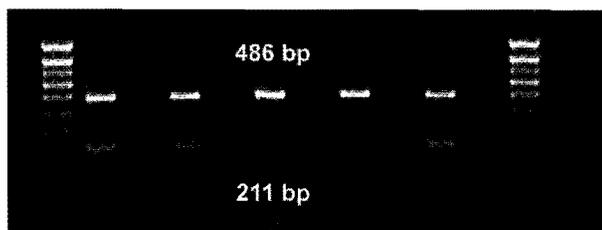


Figure 1. Detection of Bcl-2 mRNA expression by means of RT-PCR. The low-density band (211 bp) corresponds to the target gene Bcl-2, whereas the high density band (486 bp) corresponds to the beta-actin internal control. The empty lanes are plain PCR controls to exclude the amplification of an eventual pseudogene.

20 had between one and three nodes involved, 12 patients had between 4 and 10 metastatic nodes and the remaining 11 had more than 10 affected nodes.

Immunohistochemistry. The protocols used were those standard at our laboratories, and have been extensively described in previous papers (6-7). For the present study, the bcl-2/100/D5 monoclonal antibody (Novocastra, Newcastle, UK) was used at a dilution of 1:50 after pretreatment of the slides with citrate buffer in a pressure boiler ("HIER" technique). The incubation was carried out for 1 h at room temperature in a humid chamber. Normal breast tissue reactive for Bcl-2 was used as positive controls, and duplicates from the tumors studied in each batch, for whom all steps were carried out in parallel exactly in the same way, but omitting the first antibody were used as negative controls (Figure 2). The slides were also routinely processed for hormone receptors (ER and PR), c-erb-B2, p53 and Ki-67. All the procedures employed were exactly the same as described in other papers by our same group, and the reader is referred to them for details (6-8). For the evaluation of the Bcl-2 immunohistochemical reaction, the semiquantitative scale we developed was used, which grades the tumors from 0 to 6 depending on the number of tumor cells stained and the intensity of the reaction in relation to the positive control (9). The tumors were considered ER-, PR-, c-erb-B2- and p53-positive when more than 10% of the cells showed specific staining, in agreement with our previous studies (6-9). In the case of p53, this degree of staining has previously been found to be almost invariably associated with expression of the mutant protein (10). The Ki-67-score was expressed directly as the percentage of reactive tumor cells.

Differential RT-PCR. For the study of Bcl-2-mRNA expression the differential RT-PCR technique described by our group in previous papers (11-13) was used. It consists of the concomitant reverse transcription and subsequent amplification of the study gene and a constitutive gene under exactly the same conditions in the same tube. The quotient study gene/constitutive gene of the corresponding band densities expressed in arbitrary units gives a relative measure of the expression of the target gene (Figure 1). The primers used and the conditions applied are shown in Table I.

The results are comparable with those obtained using the same technique in the same samples in previous studies corresponding to the expression of the *hMAM* (11) and *Nup88* (12) genes.

Table I. Sequence of primers and conditions used.

Primer	Sequence 5' - 3'	Amplicon size	Annealing temperature
bcl-2a	CCCTGTGGATGACTGAGTAC	211 bp	54 °C
bcl-2b	GCATGTTGACTTCACTTGTG		
AC 1	GACCCAGATCATGTTTGAG	486 bp	55 °C
AC 2	GAGTTGAAGGTAGTTTCGTG		

Process	Time	Temperature
RT-PCR		
Inverse transcription	30 min	50 °C
Activation prior to PCR	15 min	95 °C
PCR		
Denaturalization	1min	95 °C
Annealing	30 sec	55 °C
Extension	1 min	72 °C
No. of cycles		34 cycles
Final extension	10 min	72 °C

Table II. Correlation of Bcl-2 expression with clinical and biological variables of the tumors. Immunohistochemistry: any degree of staining. RT-PCR cut-off: 25th percentile. Spearman's rank correlation test.

Variable	Immunohistochemistry		RT-PCR	
	r	p	r	p
ER	0.36	0.0003	0.36	0.0004
PR	0.37	0.0002	0.34	0.001
p53	-0.12	0.28	-0.27	0.014
c-erb-B2	-0.34	0.0008	-0.25	0.016
Ki-67	-0.23	0.02	-0.09	0.37
Histologic grade 3	-0.16	0.17	-0.22	0.06
Nuclear grade 3	-0.28	0.0015	-0.32	0.006
Tumor size	-0.21	0.048	-0.20	0.07
Nodal invasion	-0.05	0.63	0.21	0.04

Statistics. For comparative purposes with qualitative variables, the study gene/beta-actin band density quotient, expressed in arbitrary units, was divided into "high" and "low" values, using the 25th and 75th percentile together with the median value as different cut-offs. Afterwards, using the chosen final cut-off, the corresponding relative expression level was correlated with all other biological and clinical variables (Table II). Continuous variables were compared with each other directly by means of Pearson's correlation test if they showed a Gaussian distribution, otherwise comparison was by means of Spearman's test. The statistical analysis was performed using the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, USA). Values were considered significant when p-value was <0.05.

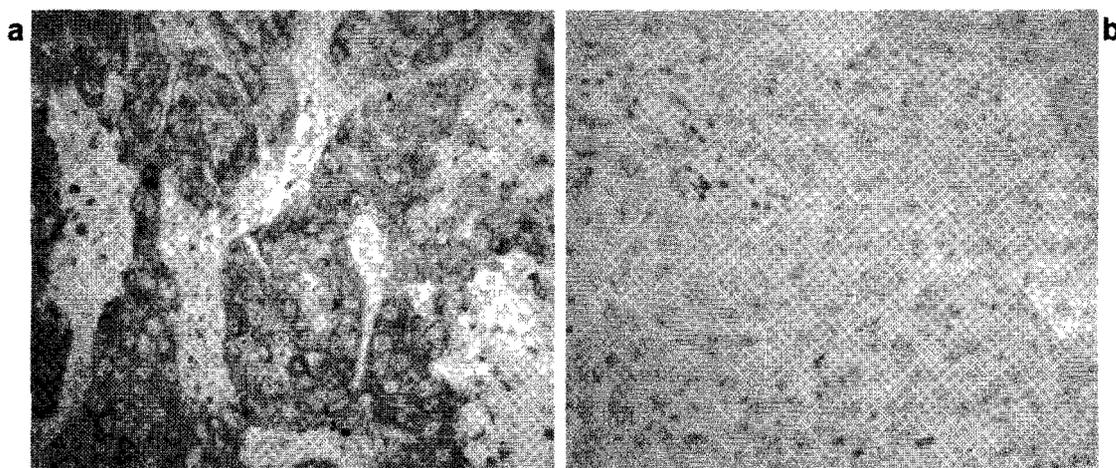


Figure 2. Immunohistochemical detection of Bcl-2 expression. Strptavidin-biotin-peroxidase x250. a) Cytoplasmic reactivity in virtually all tumor cells. b) Negative control of a.

Results

Of the 100 samples tested, 96 yielded interpretable results by means of immunohistochemistry and 93 by means of RT-PCR. The causes for failure were lack of large enough tumor areas in the case of very small T1 tumors analyzed by means of immunohistochemistry, and RNA-degradation in the case of uninformative RT-PCR reactions.

Of the 96 samples which were evaluable by means of immunohistochemistry, 42 did not show any Bcl-2-reactivity in the tumor tissue, although in most of them a clear reactivity was demonstrable in normal breast glandular tissue, which served as an excellent internal control, in addition to the external control used in every tested batch of tumors. Of the 54 reactive samples, 48 attained a score of 3 or higher according to our semiquantitative scale, so that the interpretation was straightforward (Figure 1).

Any degree of immunohistochemical staining correlated significantly and inversely with c-erb-B2 expression ($p=0.0008$), nuclear grade 3 ($p=0.0015$), a Ki-67 labeling index $>10\%$ ($p=0.02$) and tumor size ($p=0.048$), and in a direct fashion with estrogen ($p=0.0003$) and progesterone receptor expression ($p=0.0002$).

The best degree of correlation in expression levels between both techniques was obtained for a cut-off at the 25th percentile of the distribution obtained by means of RT-PCR ($p<0.05$). This low threshold explains well why any degree of positivity registered by means of immunohistochemistry seems to be biologically relevant. Using this cut-off for dichotomization and comparison with qualitative variables, mRNA expression of the Bcl-2 gene showed a significant inverse correlation with c-erb-B2 ($p=0.016$) and p53 ($p=0.014$) expression, as well as with a

nuclear grade 3 ($p=0.006$), and a direct correlation with estrogen ($p=0.0004$) and progesterone ($p=0.001$) receptor expression. Surprisingly, it also showed a positive correlation with nodal invasion ($p=0.04$).

All these results are summarized in Table II. Additionally, Bcl-2 mRNA expression was significantly and directly associated with hMAM mRNA expression ($p<0,5$), and inversely with Nup88 mRNA expression ($p<0.01$). As shown in our previous studies (11, 12), hMAM, in its turn, is associated with a significantly better prognosis for breast cancer, whereas Nup88 mRNA expression is an independent predictor of a significantly worse prognosis.

Discussion

The results obtained by either method, immunohistochemistry or RT-PCR, are very similar, as shown in Table II. This fully validates the previously reported results by other groups using immunohistochemistry and indicates that during the translational process from mRNA to protein there occur few, if any, changes in the final expression of the Bcl-2 gene. Since the interpretation of the immunohistochemistry is rather straightforward, as has also been shown, and any level of reactivity seems to have biological consequences, this method, being cheap and simple, appears to be the one of choice for its routine use in the clinic.

Our results show an association between expression of the Bcl-2 gene and favorable biological and clinical features of the tumors: small tumor size, low nuclear grade, hormone receptor expression, absence of c-erb-B2 and mutant p53 expression, and low proliferation (inverse correlation with Ki-67 expression). This explains why Bcl-2 expression has been consistently associated with a better prognosis for breast cancer in previous reports (1-3, 5, 14) despite the

conceptual paradox represented by its putative role as a facilitator of tumor cell survival. In particular, the very strong association between Bcl-2 and hormone receptor expression found by us is likely to have a prognostic power that overshadows all other possible prognostic factors. The dependence between hormone receptor and Bcl-2 was reported by Park *et al.* (5) for invasive ductal carcinoma using immunohistochemistry, and shortly afterwards by us for all common types of breast cancer using immunofluorocytometry (7). Knowlton *et al.* hypothesized that Bcl-2 might slow cell proliferation independently from its antiapoptotic effect, and showed this to be indeed the case in an experimental tumor model (15). This is confirmed by our present finding of an inverse correlation between Bcl-2 and Ki-67 expression. Silvestrini *et al.* found a significant inverse correlation between Bcl-2 and mutant p53 expression in node-negative breast cancer, which did not hold its prognostic power in a multivariate model (3). Their cited association was also found in our study using RT-PCR. Furthermore, when carrying out a sub-analysis stratified by nodal invasion, we obtained a very similar result to the one reported by Silvestrini *et al.* (3), since in our case the inverse correlation between Bcl-2 and mutant p53 expression was also mainly at the expense of node-negative tumors ($r=-0.34$; $p=0.014$) if compared to their node-positive counterparts ($r=-0.12$; $p=0.49$). Bukholm *et al.* (4) found a significant inverse correlation between Bcl-2 determined by means of immunohistochemistry and nodal invasion. This association was not found in our study using immunohistochemistry, but surprisingly, the opposite was found by means of RT-PCR. This unexpected result is devoid of a plausible explanation, and merits further investigation.

Conclusion

The study of Bcl-2 expression in breast cancer by means of either immunohistochemistry or RT-PCR yields very similar results. In spite of its role opposing tumor cell death, Bcl-2 is associated with biological features of the tumors, which define a better intrinsic prognosis, such as hormone receptor expression, low proliferation and absence of c-erb-B2 and mutant p53 expression. This may in great part explain why Bcl-2 expression has been invariably found to correlate with a better prognosis for breast cancer.

References

- 1 Kumar R, Vadlamudi RK and Adam L: Apoptosis in mammary gland and cancer. *Endocrine-Related Cancer* 7: 257-269, 2000.
- 2 Krajewski S, Krajewska M, Turner BC, Pratt C, Howard B, Zapata JM, Frenkel V, Robertson S, Ionov Y, Yamamoto H, Peruch M, Takayama S and Reed JC: Prognostic significance of apoptosis regulators in breast cancer. *Endocrine-Related Cancer* 6: 29-40, 1999.
- 3 Silvestrini R, Veronesi S, Daidone MG, Benini E, Boracchi P, Mezzetti M, Di Fronzo G, Rilke F and Veronesi U: The Bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. *J Natl Cancer Inst* 86: 499-504, 1994.
- 4 Bukholm IR, Bukholm G and Nesland JM: Reduced expression of both Bax and Bcl-2 is independently associated with lymph node metastasis in human breast carcinomas. *APMIS* 110: 214-220, 2002.
- 5 Park SH, Kim H and Song BJ: Down regulation of bcl2 expresion in invasive ductal carcinomas is both estrogen- and progesterone-receptor dependent and associated with poor prognostic factors. *Pathol Oncol Res* 8: 26-30, 2002.
- 6 Schneider J, Lucas R, Sánchez J, Ruibal A, Tejerina A and Martín M: Modulation of molecular marker expression by induction chemotherapy in locally advanced breast cancer: Correlation with the response to therapy and the expression of MDR1 and LRP. *Anticancer Res* 20: 4373-4378, 2000.
- 7 Martínez-Arribas F, Núñez MJ, Piqueras V, Lucas Ar, Sánchez J, Tejerina A and Schneider J: Flow-cytometry vs. Ki-67 labeling index in breast cancer: a prospective evaluation of 181 cases. *Anticancer Res* 22: 295-298, 2002.
- 8 Martínez-Arribas F, Núñez MJ, Lucas AR, Sánchez J, Tejerina A and Schneider J: Immunofluorometric study of Bcl-2 and Bax expression in clinical fresh tumor samples from breast cancer patients. *Anticancer Res* 23: 565-568, 2003.
- 9 Schneider J, Pollan M, Tejerina A, Sanchez J and Lucas AR: Accumulation of uPA-PAI-1 complexes inside the tumour cells is associated with axillary nodal invasion in progesterone-receptor-positive early breast cancer. *Br J Cancer* 88: 96-101, 2003.
- 10 Schneider J, Rubio MP, Rodríguez-Escudero FJ, Seizinger BS and Castresana JS: Identification of p53 mutations by means of SSCP (single strand conformation polymorphism) analysis in gynaecologic tumors: comparison with the results of immunohistochemistry. *Eur J Cancer* 30A: 504-508, 1994.
- 11 Núñez-Villar MJ, Martínez-Arribas F, Pollán M, Lucas AR, Sánchez J, Tejerina A and Schneider J: Elevated mammaglobin (h-MAM) expression in breast cancer is associated with clinical and biological features defining a less aggressive tumour phenotype. *Breast Cancer Res* 5: R65-R70, 2003.
- 12 Agudo D, Gomez-Esquer F, Martinez-Arribas F, Núñez-Villar MJ, Pollan M and Schneider J: Nup88-mRNA overexpression is associated with high aggressiveness of breast cancer. *Int J Cancer* 109: 717-720, 2004.
- 13 Gomez-Esquer F, Agudo D, Martínez-Arribas F, Núñez-Villar MJ, Pollán M and Schneider J: The X-chromosome *RBMX* gene is expressed in mammary carcinoma. *Cancer Genomics & Proteomics* 1: 39-44, 2004.
- 14 O'Driscoll L, Cronin D, Kennedy SM, Purcell R, Linehan R, Glynn S, Larkin A, Scanlon K, McDermott EW, Hill AD, O'Higgins NJ, Parkinson M and Clynes M: Expression and prognostic relevance of Mcl-1 in breast cancer. *Anticancer Res* 24: 473-482, 2004.
- 15 Knowlton K, Mancini M, Creason S, Morales C, Hockenberry D and Anderson BO: Bcl-2 slows *in vitro* breast cancer growth despite its antiapoptotic effect. *J Surg Res* 76: 22-26, 1998.

Received September 13, 2006

Revised November 3, 2006

Accepted November 20, 2006