Bax Expression in Untreated Breast Cancer: An Immunocytometric Study of 255 Cases

F. MARTÍNEZ-ARRIBAS[1], E. MARTÍN-GARABATO[1], I. ZAPARDIEL[2], J. SÁNCHEZ[1,3], A.R. LUCAS[1], A. TEJERINA[1] and J. SCHNEIDER[1,4]

[1]*Research Unit, Fundación Tejerina – Centro de Patología de la Mama, Madrid;*
[2]*Obstetrics and Gynecology Department, Hospital Universitario Santa Cristina, Madrid;*
[3]*Pathology Department, Universidad de Alcalá, Madrid;*
[4]*Department of Health Sciences-2, Universidad Rey Juan Carlos, Madrid, Spain*

Abstract. *Background: Bax is one of the main effectors of apoptosis in breast cancer. However, in contrast with the antiapoptotic protein Bcl-2, which has been extensively studied in this tumor, there are relatively few clinical studies on the biological role of Bax in breast cancer. Materials and Methods: The expression of the apoptosis-related Bax gene was studied in a series of 255 previously untreated breast cancers by means of immuno-flow cytometry. Additionally, and by the same method, the expression of the Bcl-2, VEGF and Nup88 genes were also studied. As variables of the study for the final statistical analysis, the histological variety of the tumors, histological and nuclear grade, the expression of hormone receptors, p53, Ki-67 or c-erb-B2, axillary node invasion, tumor size and DNA-ploidy were also included. Results: The expression of the proapoptotic Bax protein was significantly associated with the expression of Nup88 ($p<0.0001$), VEGF ($p=0.0014$) and Bcl-2 ($p=0.0063$), all measured by the same method. An inverse correlation with c-erb-B2 expression, which almost attained statistical significance ($p=0.058$) was also registered. Conclusion: This study adds evidence to the little explored link between apoptosis and angiogenesis. Furthermore, it discloses a previously unreported relationship between Bax and Nup88 expression.*

Apoptosis is a physiological process which consists of programmed cell death after a definite number of mitoses. However, apoptosis not only regulates normal cell homeostasis, but is also involved in a number of other biological events, including organogenesis during embryonic development, elimination of genetically instable cells and removal of potentially harmful cells during bone marrow maturation (1). During tumor development, down-regulation of apoptosis also confers on cells such typical features of the oncogenic phenotype as resistance to chemotherapy and radiotherapy, to hypoxia and to lysis by T-killer cells (2). There are two main molecular pathways triggering apoptosis, an external one mediated by tumor necrosis factor (TNF) and Fas, and an intracellular one centered around the release of cytochrome *c* by the mitochondria. The Bcl-2 apoptosis-related, p53-dependent protein family is one of the main regulators of the mitochondrial apoptotic pathway and includes both apoptosis-promoting members, such as Bax and Bak, as well as inhibitors, such as Bcl-2 and Bcl-x. In breast cancer, Bax plays a pivotal role as a facilitator of apoptosis, its function being held in balance by the main antiapoptotic member of the family, Bcl-2 (3). Ultimately, it is the Bcl-2/Bax ratio which decides the fate of the cell. The ability of the tumor cells to evade apoptosis allows them to survive and multiply in a hostile environment, whereas the transfection-induced overexpression of Bax suppresses breast cancer xenograft growth in immunossuppressed mice (4).

In a previous report by our group in which Bcl-2 and Bax expression were studied by means of immuno-cytometry in breast cancer (5), we found no correlation between Bax expression levels and the available clinical and biological parameters of the tumors which could be tested. However, it being a pilot study, the series comprised a relatively low number of cases (86) and the available tumor features were relatively few. The Bax gene plays such a fundamental role in apoptosis that it is hardly imaginable that it lacks any biological link to other basic molecular processes in the breast tumor cell. With this in mind, we have extended our previous pilot study to a significantly larger number of samples, and have determined in them, together with Bax expression, the expression of several other genes previously studied in breast cancer at our laboratory.

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

Key Words: Breast cancer, Bax, Nup88, immunocytometry.

Table I. *Correlation of Bax expression measured by means of immunofluorocytometry and continuous variables. Spearman's rank correlation test.*

Variable	n	r	*p*-Value
Bcl-2	135	0.24	**0.0063**
VEGF	232	0.22	**0.0014**
Nup88	215	0.34	**<0.0001**
DNA index	255	0.03	0.59
Ki-67	254	0.02	0.75
Tumor size	247	–0.09	0.17

Table II. *Correlation of Bax expression measured by means of immunofluorocytometry and dichotomic variables. Bax cut-off: median value. Wilcoxon's test.*

Variable	n	*p*-Value
p53	254	0.89
c-erb-B2	254	0.058
Estrogen receptors	253	0.46
Progesterone receptors	254	0.97
Axillary nodes	242	0.97
Histologic grade 3	214	0.79
Nuclear grade 3	214	0.66
Ductal *vs.* lobular histology	255	0.27

Materials and Methods

The expression of the apoptosis-related *Bax* gene was studied in a series of 255 previously untreated patients with breast cancer, operated upon at the Fundación Tejerina-Centro de Patología de la Mama, Madrid, Spain. Of them, 227 were ductal infiltrating carcinomas, 23 lobular infiltrating carcinomas and 5 tubular tumors, which are a special form of extremely well-differentiated ductal carcinomas, and for this reason were grouped with the latter for statistical purposes. Other, less frequent histological varieties of breast cancer were excluded from the study.

According to TNM stage, 194 were classified as T1, 51 as T2, 53 as T3 and the remaining 8 were diffuse tumors encompassing the whole breast.

Bax expression was studied in the tumors by means of immuno-flow cytometry, peformed as described below. Additionally, and by the same method, the expression of the *Bcl-2*, vascular endothelial growth factor (*VEGF*) and *Nup88* genes were also studied.

Flow cytometry. The technique employed has been described previously (5). Briefly, fresh tumors were finely minced with a scalpel blade, mixed with 2 ml of DNA-prep Stain reactant and 100 μl DNA-prep LPR reactant (both from Coulter Corporation, Miami, FL, USA), and incubated for 30 min at 37°C. The obtained tissue was mixed with 2 ml pepsin at 37°C for 30 min 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 min; the supernatant was eliminated and the obtained cell pellet fixed by resuspension in 1 ml 2% paraformaldehyde (pH 7.2) for 10 min at 4°C, followed by another 10 min at room temperature. After centrifugation (2,000 rpm for 10 min), the cells were resuspended in phosphate-buffered saline (PBS) and centrifuged again (same conditions) in order to remove any residual formaline, and resuspended in absolute methanol at –20°C for 5 min to permeabilize the membrane. The cells were washed again with PBS (same conditions as above), centrifugued at 7.000 rpm for 5 min, and were then ready for incubation with the primary antibody after resuspension in either PBS to attain the wanted antibody dilution, or in the provided antibody solution (see below).

The Bax monoclonal antibody (clone 4F11) was purchased in ready-to-use prediluted form from Immunotech, Marseille, France. The Bcl-2 monoclonal was the bcl-2/100/D5 antibody from Novocastra, Newcastle, U.K., used at 1:80 dilution. VEGF was detected by means of the rabbit polyclonal AB1442 antibody from Chemicon, Temecula, CA, U.S.A., used at 1:100 dilution. Nup88-expression, shown by our group previously to be associated with a biologically more aggressive tumor phenotype in breast cancer (6), was studied using the Nup88 monoclonal antibody from BD Biosciences, Erembodegem, Belgium, at 1:100 dilution. The cells were incubated with the antibodies for 1 h at 4°C. Afterwards, they were centrifugued at 7.000 rpm for 5 min, washed and resuspended in PBS, then incubated with the secondary, fluorochrome-coupled anti-mouse antibody (1:100) at 4°C for 30 min. As an internal control, the cells were finally incubated with propidium iodide to select the cells effectively fixed and to discard cell debris eventually reacting with the primary or secondary antibodies. This allows, additionally, for the percentage of cells specifically reacting with the primary antibody to be determined. This percentage, in its turn, reflects the relative level of expression of the studied protein in that particular sample.

All measurements were carried out in a Coulter EPICS XL cytometer (Coulter Corporation). The analysis of the obtained histograms was carried out with the help of the MultiCycle DNA Cell Cycle Analysis software package (Phoenix Flow Systems, San Diego, CA, U.S.A.).

Statistics. Continuous variables were compared with each other directly by means of Spearman's rank correlation test (Table I). For comparative purposes with qualitative variables, Bax expression values were divided into "high" and "low", using the median as cut-off. Afterwards, using this cut-off, the corresponding relative expression level was correlated with all other biological and clinical variables in a univariate model using Wilcoxon's test (Table II). 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 was <0.05.

Results

The results are summarised in Tables I and II. From them, it can be seen that expression of the proapoptotic Bax protein, measured by means of immuno-flow cytometry was significantly associated with the expression of Nup88 (p<0.0001), VEGF (p=0.0014) and Bcl-2 (p=0.0063), all measured by the same method. An inverse correlation with c-erb-B2 expression, which almost attained statistical significance (p=0.058), was also registered.

Discussion

In the results we present here, the strongest correlation between Bax expression and the expression of all other proteins tested was found with Nup88 expression. Nup88 is a nucleoporin which forms part of the nuclear pore complex, where it interacts strongly with another nucleoporin, Nup214 (also known as CAN), forming the CAN/Nup complex, and was first identified by Bastos *et al.* (7) and Fornerod *et al.* (8). Our group, in its turn, was the first to report shortly afterwards that Nup88 is overexpressed by tumor cells (9), and in a further study carried out on clinical tumor samples, that its overexpression is associated with a significantly higher biological and clinical aggressiveness of breast cancer (6). These findings were reproduced in collaboration with researchers from Linköping University (Sweden) in human melanoma and colon cancer as well (10, 11). Despite all these coincidental data, the precise role of Nup88 in tumor biology is still obscure. There have been hints, already in the pioneering investigations concerning this protein, pointing towards some kind of link with apoptosis. Bonten *et al.* (12) found that overexpression of CAN/Nup overexpression induces, among other things, apoptosis in myeloid precursor cells. More recently, Shu *et al.* (13) reported that deguelin, a naturally occurring rotenoid, strongly influences the normal apoptotic process of Jurkat cells by means of a specific interaction with Nup88. Our own group also found a correlation, albeit an inverse one, between Nup88 overexpression and apoptosis in human colon cancer (11). All in all, mounting evidence is accumulating to support the notion that nucleocytoplasmic transport may be involved in normal and tumor cell apoptosis, and in this context nucleoporins seem to play a fundamental role. Our present results suggest that one of them, Nup88, may be directly involved in apoptosis in breast cancer through a correlation with the expression of the proapoptotic Bax protein.

We also found a significant correlation between the expression of Bax and the angiogenic protein VEGF. The possible connection between apoptosis and angiogenesis has been recently studied by several groups, among which our own one. In fact, we recently reported that the expression of some members of the *RBM* gene family located on the X-chromosome, *RBM10l* and *RBM10s*, is concomitantly related to the expression of the *Bax* gene and the *VEGF* gene (14). The expression of another member of the family, *RBMX*, furthermore, was inversely related to the expression of another angiogenesis-involved gene, *CD105* (endoglin) (15).

Finally, Bax expression was directly related to the expression of one of the main antipoptotic genes, *Bcl-2*. This is not surprising, since it is well known that in breast cancer the balance between the expression of both genes is an important regulator of apoptosis. In a previous pilot study using the same experimental approach (16), we did not find any correlation between the expression of both genes reported now. However, on that occasion, we studied far fewer tumor samples (only 86 cases), including a significant proportion of previously treated ones. It is now well known that chemotherapeutic treament significantly acts on apoptosis and that interactions with Bcl-2 play a prominent role in this mechanism of action. We are now convinced that this fact largely explains our findings reported in that previous paper and that the present ones, obtained from a much larger series of chemotherapy-naïve tumors are those which most accurately reflect the expression of apoptosis-related genes in untreated breast cancer.

In conclusion, This study adds evidence to the little explored link between apoptosis and angiogenesis. Furthermore, it discloses a previously unreported relationship between Bax and Nup88 expression.

References

1 Kumar R, Vadlamudi RK and Adam L: Apoptosis in mammary gland and cancer. Endocrine-Related Cancer *7*: 257-269, 2000.

2 Reed JC: Bcl-2 and the regulation of programmed cell death. J Cell Biol *124*: 1-6, 1994.

3 Reed JC: Balancing cell life and death: apoptosis and breast cancer. J Clin Invest *97*: 2403-2404, 1996.

4 Bargou RC, Wagener C, Bommert K, Mapara MY, Daniel PT, Arnold W, Dietel M, Guski H, Feller A, Royer HD and Dörken B: Overexpression of the death-promoting gene *bax-alpha* which is down-regulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. J Clin Invest *97*: 2651-2659, 1996.

5 Martinez-Arribas F, Nuñez-Villar MJ, Lucas AR, Sanchez 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.

6 Agudo D, Gómez-Esquer F, Martínez-Arribas F, Núñez-Villar MJ, Pollán M and Schneider J: *Nup88* mRNA overexpression is associated with high aggressiveness of breast cancer. Int J Cancer *109*: 717-720, 2004.

7 Bastos R, Ribas DP, Enarson M, Bodoor K and Burke B: Nup84, a novel nucleoporin that is asociated with CAN/Nup214 on the cytoplasmic face of the nuclear pore complex. J Cell Biol *137*: 989-1000, 1997.

8 Fornerod M, Boer J, Van Baal S, Morreau H and Grosveld G: Interaction of cellular proteins with the leukemia-specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN. Oncogene *13*: 1801-1808, 1996.

9 Martínez N, Alonso A, Moragues D, Pontón J and Schneider J: The nuclear pore complex protein Nup88 is overexpressed by tumor cells. Cancer Res *59*: 5408-5411, 1999.

10 Zhang H, Schneider J and Rosdahl I: Expression of p16, p27, p53 and Nup88 proteins in matched primary and metastatic melanoma cells. Int J Oncol *21*: 43-48, 2002.

11 Emterling A, Skoglund J, Arbman G, Zhang H, Schneider J, Evertson S, Carstensen J and Sun XF: Clinicopathological significance of Nup88 expression in patients with colorectal cancer. Oncology *64*: 361-369, 2003.

12 Boer J, Bonten-Surtel J and Grosveld G: Overexpression of the nucleoporin CAN/Nup214 induces growth arrest, nucleocytoplasmic transport defects, and apoptosis. Mol Cell Biol *18*: 1236-1247, 1998.

13 Shu W, Chen Y, Wu Q, Li R and Cui G: Deguelin represses both the expression of nucleophosmin and some nucleoporins: Nup88 and Nup214 in Jurkat cells. Biol Pharm Bull *31*: 27-32, 2008.

14 Martínez-Arribas F, Agudo D, Pollán M, Gómez-Esquer F, Díaz-Gil G, Lucas R and Schneider J: Positive correlation between the expression of X-chromosome *RBM* genes (*RBMX, RBM3, RBM10*) and the proapoptotic Bax gene in human breast cancer. J Cell Biochem *97*: 1275-1282, 2006.

15 Gómez-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.

16 Martínez-Arribas F, Núñez-Villar MJ, Lucas AR, Tejerina A and Schneider J: Immunfluorometric study of Bcl-2 and Bax expression in clinical fresh tumor samples from breast cancer patients. Anticancer Res *23*: 565-568, 2003.

Received April 10, 2008
Revised June 10, 2008
Accepted June 16, 2008