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The Small Variant of the Apoptosis-associated X-Chromosome *RBM10* Gene is Co-expressed with Caspase-3 in Breast Cancer

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

[1]*Fundación Tejerina-Centro de Patología de la Mama, Madrid;*
[2]*Departamento de Ciencias de la Salud-2, Universidad Rey Juan Carlos, Alcorcón (Madrid);*
[3]*Cancer Epidemiology Department, National Centre for Epidemiology, Madrid;*
[4]*Universidad de Alcalá, Alcalá de Henares, Madrid, Spain*

Abstract. *Background: There are very few studies on the final triggers of apoptosis, the caspases, in breast cancer. Materials and Methods: Caspase-3 expression was studied by means of reverse transcript polymerase chain reaction (RT-PCR) in a series of 108 previously untreated patients with breast cancer. Expression levels were correlated with those obtained using the same technique of the apoptosis-associated X-chromosome genes RBMX, RBM3, RBM10 small and RBM10 large variant; Bcl-2 and Bax; the angiogenesis-associated genes VEGF and CD105 (endoglin); hMAM and Nup88. The correlation with the expression of hormone receptors, c-erb-B2, mutant p53 and Ki-67, all measured by means of immunohistochemistry, was also studied, as well as that with standard clinical parameters such as histological type, tumor size, axillary metastasis and DNA-ploidy. Results: The only statistically significant correlations observed between caspase-3 mRNA expression and the parameters tested were a direct one with both the Bax (p=0.007) and the small variant of the X-chromosome RBM10 gene (p=0.018), and an inverse one with the angiogenesis-associated CD105 (endoglin) gene (p=0.044). Conclusion: These results indicate that very few genes are involved in the last steps of the apoptotic cascade in breast cancer, among them one of the X-chromosome RBM family. They also support the relatively unexplored link between apoptosis and angiogenesis.*

Cancer cells modulate the physiological process of apoptosis, or programmed cell death, to their own benefit. By down-regulating apoptosis, they are able to immortalise themselves, as well as to evade the effects of chemotherapy. In breast cancer apoptosis seems to play an important role (1, 2). However, virtually all studies carried out on apoptosis in breast cancer have disclosed a surprising paradox, in that overexpression of the most studied antiapoptotic gene in breast cancer, *Bcl-2*, is invariably associated with a better prognosis. Several explanations have been put forward to explain this contradictory finding (3). Among them, the fact that Bcl-2-expression is directly linked to the expression of the proapoptotic *Bax* gene, and it is ultimately the ratio between both which decides the fate of the cell. Furthermore, in a recent study, we were able to show that Bcl-2 expression, determined either at the mRNA or protein level, was associated in breast tumors in a significant way with hormone receptor expression, low proliferation, an absence of c-erb-B2 and p53 expression, small tumor size and low nuclear grade, thus defining a less aggressive phenotype (4). In contrast with the large number of existing studies on Bcl-2 expression in breast cancer, there are very few on the main triggers of apoptosis, the caspases, in this kind of tumor. The caspases (aspartate-specific cysteine proteases, hence their name) are the ultimate effectors upon which both apoptotic pathways, the intrinsic mediated by the mitochondria and the extrinsic mediated by cell death receptors on the membrane surface, converge. There are two groups of caspases, the first composed among other of the so-called initiator caspases 8, 9 and 10, the second of the so-called effector caspases, such as 2, 3, 6 and 7. These latter caspases degrade a number of cell proteins, and thus induce the morphological changes which define the characteristic appearance of apoptotic cells. One of the most studied caspases in breast cancer is caspase-3, and even in this instance the existing studies are relatively few. Most studies focused on the role of caspase-3 in resistance to chemotherapy, since it is known that many chemotherapeutic agents act by inducing apoptosis of the tumor cells, and these develop resistance by down-regulating propapototic genes, or activating antiapoptotic ones (5-8).

Correspondence to: Prof. J. Schneider, Universidad Rey Juan Carlos, Facultad de Ciencias de la Salud, Avenida de Atenas, S/N, 28922 Alcorcón, Madrid, Spain. e-mail: jose.schneider@urjc.es

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In the present study, we determined caspase-3 mRNA expression in a series of patients with untreated human breast cancer and correlated the expression with all available clinical and biological features in an attempt to gather some additional knowledge on the role of this gene in clinical samples of the tumor.

Materials and Methods

One hundred and eight breast cancer samples, which were immediately snap-frozen or immersed in RNA-later® at the moment of surgery, were studied. The patients were operated upon at Fundación Tejerina, Madrid, Spain, and all had given their informed consent for the research use of the obtained tissues prior to the operation. The histologies were as follows: 88 ductal infiltrating, 16 lobular infiltrating and 4 tubular. Other less common varieties were excluded from the study. According to the TNM staging system, 71 tumors were T1, 32 T2, 3 T3 and one was an unclassifiable diffuse tumor composed of minute foci encompassing the whole breast. Axillary node invasion was present in 44 cases (40.7%).

In each tumor sample, the expression of the following genes was studied by means of differential RT-PCR: the X-chromosome genes *RBMX*, *RBM3* and *RBM10* (small and large variant), shown by us in a previous study to be related with apoptosis in breast cancer (9); *Bcl-2*, *Bax*; the angiogenesis-related genes encoding the vascular endothelial growth factor (*VEGF*) and *CD105* (endoglin); and *hMAM* and *Nup88*, shown also by our group to be associated with a significantly lower and higher biological aggressiveness of breast cancer, respectively (10, 11). By means of immunohistochemistry, the expression of estrogen and progesterone receptors (ER, PR), c-erb-B2, mutant p53 and K i67 were studied. By means of flow cytometry, DNA-ploidy was studied. Clinical and pathological features (histological type, histological and nuclear grade, and axillary node invasion) were also included as variables of the study.

Reverse transcription polymerase chain raction (RT-PCR). RNA was extracted from the tumor specimens using the Rneasy™ commercial kit (AMBION Inc., Austin, Texas, USA), according to the manufacturer's instructions. The total RNA content was immediately quantified in a spectrophotometer (GeneQuant pro RNA/DNA calculator® from Amersham Pharmacia Biotech, Uppsala, Sweden) after 1:10 dilution in RNAase-free water and its integrity was checked by means of denaturing agarose-gel electrophoresis and ethidium bromide staining. The RNA was frozen at –80°C until further use. The RT-PCR reaction was carried out in a thermal cycler (iCycler™, BIORAD, Hercules, CA, USA) using a commercial one-step RT-PCR kit (AMBION Inc.). The primers were selected using the Oligo Analizer 1.0.3. and Oligo Explorer 1.1.2. software, both available online at www.uku.fi/~kuulasma/OligoSoftware. The primers were designed to span several intron/exon boundaries in the case of the study genes in order to eliminate amplification from contaminating genomic DNA. Moreover, a second tube with the same components, save for the reverse transcriptase, was run in parallel with each reaction, in order to exclude an amplification of a pseudogene from contaminating DNA. The primers used, as well as the conditions of the RT-PCR, are shown in Table I and Figure 1.

The relative abundance of a transcript in different samples can be estimated by semiquantitative, or relative RT-PCR, which has been described extensively elsewhere (9-12). It involves the inverse transcription of the mRNA corresponding both to the target gene and to a constitutive one into cDNA and their subsequent amplification under identical conditions. Previously, the latter had been adjusted, so that the PCR reaction was interrupted prior to the saturation phase in both cases. At 30 extension cycles in the case of caspase-3, both saturation curves were still well within the ascending slope. The primers and conditions for the study of the *RBMX, RBM3, RBM10* (small and large variant), *VEGF, CD105, hMAM* and *Nup88* genes were exactly the same as previously described (9-12). The signal from the RT-PCR product was normalized to the signal from an internal control (in our case beta-actin) included in all samples and amplified at the same time as the target cDNA. This internal control transcript is usually more abundant than the transcript under study and this difference in abundance can lead to preferential amplification of the internal control and, in some cases, prevent amplification of the target RT-PCR product. This problem can be overcome by reducing the internal control primer concentration. In our system we optimized *caspase-3* with 0.6 μM of each specific primer and 0.15 μM of each beta-actin-specific primer. The sequence verification of the RT-PCR products was carried out on an automated ABI Prism™ 377 sequencer and 3730 DNA Analyzer (Applied Biosystems, CA, USA).

Table I. *Reagents employed in the differential PCR performed to determine caspase-3 expression in breast cancer.*

Component	Volume (μL)	Final concentration
RNA TEMPLATE	1	0.5 ng/μL
Caspase-2 start primer	2	0.6 μM
Caspase-2 end primer	2	0.6 μM
Beta-actin start primer	0.5	0.15 μM
Beta-actin end primer	0.5	0.15 μM
5X buffer	4	1 X
dNTPs	0.8	400 μM of each
Enzyme mix	0.8	-
RNAse-free water	8.4	-
total volume	20	-

The amplification products were resuspended in loading buffer with 10% bromophenol blue and run at 100 V in a 1.5-2% agarose gel prepared in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) with 0.5 μg/ml ethidium bromide. The bands were visualized in a UV transilluminator and analyzed by means of the LabWorks™ Image Acqisition and Analysis software package from Ultra-Violet Products, Ltd., Cambridge, U.K. The quotient of the study to the control band densities gives a numerical value in arbitrary units which reflects the relative expression level of the studied gene in each tumor.

Immunohistochemistry. The immunohistochemical technique employed was that standardly used at our laboratory, and has also been described elsewhere (4, 11). Briefly, 5 μm paraffin sections were mounted on poly-L-lysine coated slides for heat-induced epitope retrieval ("HIER" technique) in citrate buffer. The same, commercially available streptavidin-biotin-peroxidase kit (Histostain-SP, Zymed, San Francisco, CA, USA) was used throughout the whole procedure to ensure uniformity of results. The antibodies employed were as follows: NCL-CB11 (c-erb-B2),

Beta-actin start	GACCCAGATCATGTTTGAG	486 bp
Beta-actin end	GAGTTGAAGGTAGTTTCGTG	
Caspase-3 start	GGCGTGTCATAAAATACCAG	220 bp
Caspase-3 end	AGTAGCGTCAAAGGAAAAGG	

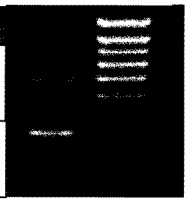

Figure 1. *Primer sequence used to amplify the beta-actin and caspase-3 reverse transcript. Ladder: 100 bp increments from the bottom up.*

NCL-ER-6F11 (estrogen receptor), NCL-p53-D07 (p53), all from Novocastra Laboratories, Newcastle, UK; prediluted MIB1 (Ki-67) and PR-2C5 (progesterone receptor) from Zymed, San Francisco, CA, USA The incubation time was 1 h at room temperature in a humid chamber for all antibodies, which, apart from the prediluted MIB1-Ki-67 solution, directly used as supplied, were employed at the following dilutions: NCL-CB11 (c-erb-B2), 1:40; NCL-ER-6F11 (ER), 1:100; NCL-p53-D07, 1:100. The evaluation of nuclear staining patterns (ER, PR, Ki-67 and p53) was straightforward, since specimens positive for ER, PR or p53 always showed specific staining in more than 20% of tumor cells. This is a particularly important point regarding *TP53* expression, since it has been convincingly shown that this range of positive staining almost invariably reflects the expression of mutant p53 protein (13, 14). The Ki-67 labelling index was expressed as the percentage of reactive tumor cells. The tumors were considered c-erb-B2-positive when more than 10% of cells showed specific membrane staining.

Flow-cytometry. The procedure was always carried out on fresh tumor tissue, which was kept in phosphate-buffered saline (PBS) at 4°C for less than 24 h after having been obtained, according to protocols previously described by us (10). Tissue was first 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 resulting mixture was then filtered through a 50 μm pore filter and was ready for cytometric analysis 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, USA). 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.

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 median value 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). Continuous variables were compared with each other directly by means of Pearson's correlation test if they showed a Gaussian distribution, and otherwise by means of Spearman's test (Table III). 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.

Table II. *Association of caspase-3 mRNA expression with dichotomic variables. Caspase-3 cut-off: median value of the distribution. Wilcoxon's test.*

Variable	N	Caspase-3 median value	p25-p75	p-value
Histology				
Ductal	88	1.77	1.26-2.67	
Lobular	16	1.97	1.73-2.80	
Tubular	4	2.42	1.42-2.86	0.259
Histologic grade				
1&2	56	1.84	1.25-2.88	
3	30	1.62	1.30-2.25	0.574
Nuclear grade				
1&2	44	1.81	1.23-2.86	
3	43	1.78	1.31-2-67	0.770
Axillary nodes				
Negative	63	2.05	1.38-2.90	
Positive	43	1.77	1.26-2.53	0.199
Ploidy				
Euploidy	54	2.08	1.37-2.89	
Aneuploidy	54	1.68	1.28-2.54	0.077
Estrogen receptors				
Positive	80	1.84	1.26-2.73	
Negative	27	1.88	1.46-2.73	0.556
Progesterone receptors				
Positive	58	1.67	1.25-2.71	
Negative	50	2.03	1.46-2.73	0.130
c-Erb-B2				
negative	88	1.91	1.27-2.80	
positive	20	1.78	1.36-2.29	0.687
p53				
Negative	93	1.88	1.33-2.74	
Positive	15	1.79	1.28-2.67	0.783
Ki-67				
≤20%	66	1.93	1.28-2.81	
>20%	42	1.79	1.30-2.60	0.671

Results

The results are summarized in Tables II and III. As can be seen from them, the only statistically significant correlations observed between caspase-3 mRNA expression and all of the parameters tested were a direct one with both the *Bax* (p=0.007) and the small variant of the X-chromosome

Table III. *Association of caspase-3 mRNA expression with continuous variables. Spearman's rank correlation test.*

Variable	n	r	*p*-value
Bcl-2	90	0.077	0.471
Bax	100	0.267	**0.007***
RBMX	105	0.110	0.280
RBM3	108	0.031	0.758
RBM10 large	102	0.005	0.960
RBM10 small	100	0.235	**0.018***
VEGF	102	0.161	0.105
CD105 (Endoglin)	104	–0.198	**0.044***
H-MAM	108	–0.155	0.109
Nup88	106	–0.035	0.717

*Statistically significant.

RBM10 gene (p=0.018), and an inverse one with the *CD105* (endoglin) gene (p=0.044).

There was a tendency towards a higher caspase-3 expression in tumors with a diploid DNA content, if compared to aneuploid tumors, which however did not reach statistical significance (p=0.077).

Discussion

Despite its central role in the triggering of apoptosis, there are very few studies of caspase-3 expression in breast cancer. O'Donovan *et al.* (15) measured caspase-3 mRNA levels as we have done in breast cancer in a series of similar size to ours. However, they did not correlate their mRNA findings with the expression levels of other genes or with the clinical and biological features of their tumors. Instead, they studied caspase-3 expression additionally at the protein level by means of Western blotting and found no correlation whatsoever with tumor size, tumor grade, nodal status or steroid receptor status. The only significant correlation they found was with ductal histology, if compared to lobular histology. Although protein expression does not always reflect mRNA expression due to posttranscriptional regulation, the results reported by O'Donovan *et al.* and ours are very similar. We too found no correlation with the parameters studied by them, with the only exception of the correlation they also detected with ductal histology. To partly explain this discrepancy, it must be considered that their series included only 6 lobular carcinomas (*vs.* 67 ductal ones), whereas we were able to compare mRNA levels of 16 lobular carcinomas to those of 92 ductal infiltrating carcinomas. It must be borne in mind, furthermore, that strictly speaking, the mRNA studied by us corresponds not to caspase-3, but to procaspase 3, which is metabolically processed by the cell to the ultimate effector protein, caspase-3. On the other hand, we studied the mRNA expression of a number of genes involved in apoptosis and angiogenesis in our tumors, and found a direct significant correlation with Bax and *RBM10s* expression, something not reported before. This result seems interesting, since in a previous study by our group, we were able to show that all the members of the X-chromosome *RBM* gene family are coexpressed with Bax in breast cancer (9). That only one of them, the small variant of *RBM10*, is coexpressed with caspase-3, further downstream in the apoptotic cascade, immediately prior to the triggering of apoptosis, may indicate that it could play a key role in this last step of the pathway. It is remarkable in this respect that both the caspase-3 gene and all of the X-chromosome *RBM* genes are coexpressed with Bax, however only one of the *RBM* genes is coexpressed with caspase-3. This points towards a direct relationship between both genes, independent of their common relationship with *Bax*, further upstream.

We also found a significant inverse correlation between caspase-3 expression and the expression of a gene involved in angiogenesis, *CD105* (endoglin). This result is in agreement with that reported previously by Li *et al.* (16), who described an antiapoptotic effect of CD105 in endothelial cells submitted to hypoxic stress. The relatively unexplored link between angiogenesis and apoptosis has been recently supported by a number of reports (17, 18), among which our own on expression of the X-chromosome *RBM* genes in breast cancer (9, 12). In fact, in these studies, besides the reported significant coexpression with Bax, we also found a significant inversely associated coexpression of *RBMX* with *CD105*, as well as a significant direct association of both splicing variants of *RBM10* with *VEGF*.

In conclusion, caspase-3 mRNA is coexpressed in breast cancer with *Bax* mRNA and *RBM10s* mRNA. An inverse correlation with the expression of the angiogenesis-related gene *CD105* (endoglin) also exists, which further supports the relationship at the molecular level between such different, and even opposed, biological processes as angiogenesis and apoptosis.

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