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Positive Correlation Between the Expression of X-Chromosome *RBM* Genes (*RBMX, RBM3, RBM10*) and the Proapoptotic *Bax* Gene in Human Breast Cancer

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Abstract In a recent report, it has been postulated that the ubiquitous RBM proteins might constitute a novel family of apoptosis modulators. We measured the expression of the X-chromosome *RBM* genes (*RBMX*, *RBM3*, and *RBM10)* in 122 breast cancers by means of differential RT-PCR. Using the same method, we also studied the expression of the apoptosis-related genes *Bcl-2* and *Bax*. Markers of hormone dependence (estrogen and progesterone receptors), proliferation (Ki67 and DNA-ploidy), angiogenesis (VEGF and CD105), as well as oncogene (c-erb-B2), and tumor suppressor gene (p53) expression were also analyzed. The expression of all X-chromosome *RBM* genes was significantly associated with the expression of the proapoptotic *Bax* gene (*RBMX*, $P = 0.039$; *RBM3*, $P < 0.001$; *RBM10* large variant, $P < 0.001$; *RBM10* small variant, $P < 0.001$). Furthermore, the expression of both *RBM10* variants was significantly associated with the expression of the *VEGF* gene (large variant, $P = 0.004$; small variant, $P = 0.003$). We also found an association of borderline significance ($P = 0.05$) between the expression of *RBM3*, the large variant of *RBM10* and wild-type p53. Expression of the small *RBM10* variant, finally, was associated with high proliferation of the tumors (Ki67 $\geq 20\%$; $P = 0.037$). The expression of both *RBM10* variants seems to be interdependent to a significant degree ($r = 0.26$, $P = 0.006$). From these results, it seems that the X-chromosome, through its *RBM* genes, plays a formerly unknown role in the regulation of programmed cell death (apoptosis) in breast cancer. J. Cell. Biochem. 97: 1275–1282, 2006. © 2005 Wiley-Liss, Inc.

Key words: cancer; breast; RBMX; RBM3; RBM10; Bax; apoptosis

RNA-binding proteins (RBPs) seem to play a crucial role in the regulation of gene expression. They are known to be involved in pre-mRNA splicing and in posttranscriptional regulatory mechanisms [St. Johnston, 1995]. For this purpose, they bind reversibly to RNA through specific binding sites with a highly conserved aminoacid sequence, called "RNA-recognition motifs" (RRMs). Due to the presence of these sites in them, genes encoding RBPs are known as RNA-binding motif (RBM) genes, and are ubiquitous throughout the genome.

Sutherland et al. [2005] have recently postulated that RBM proteins might constitute a novel family of apoptosis modulators. Apoptosis, or programmed cell death, is a physiological event regulated by a wide variety of genes. Some of them seem to have functions uniquely related to apoptosis, whereas others, such as p53, have diversified roles. The main apoptotic pathway in breast cancer cells involves p53, as has been said, but also anti-apoptotic proteins like Bcl-2 and Bcl-x, and pro-apoptotic ones, like Bax and Bak [Kumar et al., 2000]. They all ultimately regulate the activation of another group of proteins, called "caspases" (from "cystein-aspartil-proteases"), which are the effective

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triggers of apoptosis. The two most widely studied apoptosis-related proteins in breast cancer are Bcl-2 and Bax [Krajewski et al., 1999]. Bcl-2 prevents apoptosis induced by a variety of stimuli, such as chemotherapy, radiotherapy, and cytotoxic cytokines, and is thus involved in resistance to therapy. Intriguingly, however, Bcl-2 overexpression has been consistently associated with a favorable prognosis of breast cancer patients. A possible explanation for this controversial finding has been attributed to the fact that Bcl-2 expression is regulated by estrogens. Therefore, tumors expressing Bcl-2 also express estrogen receptors, and the latter are an established marker of good prognosis in breast cancer. Alternatively, it has been postulated that Bcl-2, besides opposing apoptosis, has also intrinsic antiproliferative effects, which might explain the better prognosis associated with tumors overexpressing Bcl-2. Bax, in its turn, has functions, which are the complete opposite of those mediated by Bcl-2. Consequently, Bax downregulation has been related in metastatic breast cancer to shorter overall survival, shorter time to progression, and resistance to chemotherapy [Krajewski et al., 1999], all of which is consistent with its established pro-apoptotic role. Furthermore, Bax expression sensitizes breast cancer cells to chemotherapy, including such widely used compounds as epirubicin, cisplatin, and etoposide, as well as to radiotherapy. This sensitization is due to increased apoptosis [Kumar et al., 2000]. The possible implication of *RBM* genes in apoptosis, thus, is of great potential interest for the better understanding of such a fundamental process in the oncogenic chain.

We were the first to report that one of the *RBM* genes, *RBMX*, located on the X-chromosome at Xq26, is expressed in human breast cancer [Gómez-Esquer et al., 2004a]. *RBMX* is the homologue of the Y-chromosome *RBMY* gene. They now constitute a pair of shared X/Y genes, which were conserved after evolutionary X–Y divergence. According to our just cited pilot study, *RBMX* overexpression in breast cancer is inversely related to the expression of the angiogenesis-related *CD105* (endoglin) gene. Two other genes belonging to the RBM family, *RBM3* and *RBM10*, are located on the X-chromosome. They are both in close proximity on the short arm of the chromosome at Xp11.23. Interestingly, *RBM3*, despite being located on the "female" half of the male sexual chromosome pair, seems to be essential for spermatogenesis. In fact, normal levels of expression of the RBM3 protein are only found in Sertoli cells of normally developing testes, as opposed to cryptorchid testes [Danno et al., 2000]. A certain degree of environmental cold is necessary for spermatogenesis, and in this context it is interesting to note that RBM3 is one of the first cold-shock proteins to have been described [Danno et al., 1997]. RBM3 has also been involved in cancer, where it seems to play a role in tumor progression as revealed in an experimental melanoma model [Baldi et al., 2003]. The functional role of RBM10, finally, is at present completely unknown [Sutherland et al., 2005].

In the present investigation we have completed the study of all described X-chromosome *RBM* genes in breast cancer. Besides elucidating if all of them are expressed in mammary carcinoma, we have also studied the correlation of *RBMX, RBM3*, and *RBM10* expression with that of genes related with apoptosis in breast cancer, to explore their possible involvement in apoptosis, as suggested by Sutherland et al.

MATERIALS AND METHODS

We studied 122 breast cancers, which were immediately snap-frozen or immersed in RNAlater® at the moment of surgery. The patients were operated upon at Fundación Tejerina, Madrid, Spain, and all had previously given their informed consent for the research use of the obtained tissues. The histologies were as follows: 99 ductal infiltrating, 19 lobular infiltrating, and 4 tubular (which were grouped together with the ductal infiltrating carcinomas for statistical purposes).

In each tumor sample, we studied by means of differential RT-PCR the expression of *RBMX*, *RBM3*, and *RBM10*, *Bcl-2*, *Bax*, of the angiogenesis-related genes *VEGF* and *CD105* (endoglin), of the *hMAM* gene, shown by us in a previous study [Núñez Villar et al., 2003] to be associated to lower aggrressiveness of breast cancer, and the *Nup88* gene, also shown by us [Agudo et al., 2004] to define a highly aggressive tumor phenotype. Estrogen and progesterone receptor (ER and PR), c-erb-B2, mutant p53, and Ki67 expression were studied by means of immunohistochemistry, and DNA-ploidy by means of flow-cytometry. Clinical and pathological

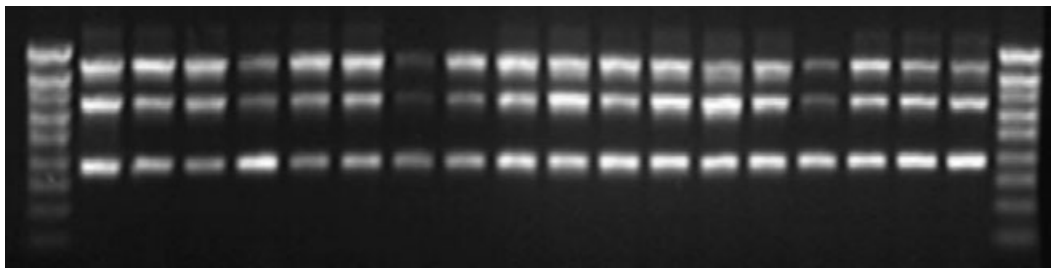

Fig. 1. RT-PCR of 18 representative patient samples. Both splicing variants of the *RBM10* gene were studied in a single experiment, using a set of primers bridging the variable region of the c-DNA. *RBM10* large isoform, 901 bp; *RBM10* small isoform, 670 bp; beta-actin internal control, 378 bp.

features (histologic variety, histologic and nuclear grade, and axillary node invasion) were also included as variables of the study. For establishing the statistical correlations between the expression of all studied genes, we included the results from our previous study on *RBMX* expression carried out on those same tumor samples, and already published [Gómez-Esquer et al., 2004]. The transcription of the *RBM10* gene results in two splicing variants of different size. We designed a set of primers encompassing the variable *RBM10* c-DNA region and adjusted the conditions in order to obtain on the same gel the two bands corresponding to the different transcription products. Thus, in a single experiment we could study at the same time the expression of both *RBM10* variants by each tumor (Fig. 1).

RT-PCR

RNA was extracted from the tumor specimens using the Rneasy™ commercial kit (AMBION, Inc., Austin, TX), 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 its further use. The RT-PCR reaction was carried out in a thermal cycler (iCycler™, BIORAD, Hercules, CA) using a commercial one-step RT-PCR kit (AMBION, Inc.). 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 (Fig. 2). The primers used, as well as the conditions of the RT-PCR, are shown in Table I.

The primers and conditions for the study of the *VEGF*, *CD105*, *hMAM*, and *Nup88* genes were exactly the same as previously described [Núñez Villar et al., 2003; Agudo et al., 2004;

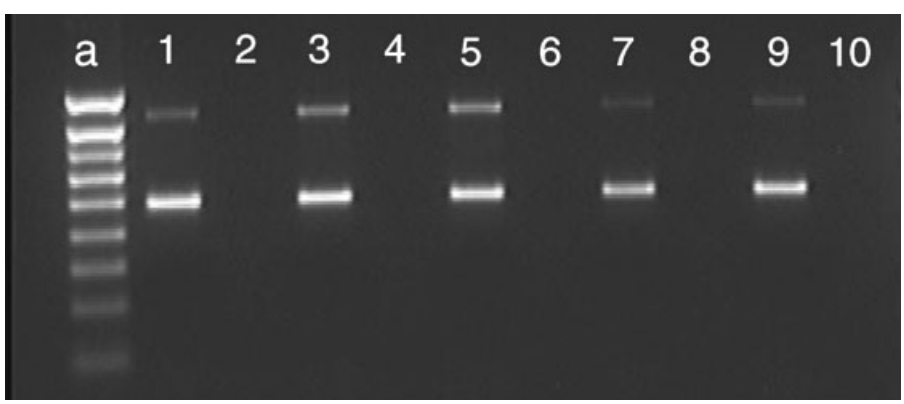

Fig. 2. Odd lanes, RT-PCR; even lanes, DNA contamination controls, omitting the inverse transcription, a = size ladder. High-weight band, *RBM3* (874 bp); low-weight band, beta-actin (486 bp).

TABLE I. Primer Sequence and Thermal Cycler Conditions

Gene	Primers	Sequence (5′-3′)	Product (bp)
RBM10	R10A	AGGGCAAGCATGACTATGA	901
	R10B	GTGGAGAGCTGGATGAAGG	670
RBM3	R3A	CAGCAGTTTCGGACCTATC	874
	R3B	AGTACAGATGGGGTTTCAC	
β-actin 1	AC1	GACCCAGATCATGTTTGAG	486
	AC2	GAGTTGAAGGTAGTTTCGTG	
β-actin 2	AC3	GGAAATCGTGCGTGACATTA	378
	AC4	GAAGATCAAGATCATTGCTCC	
Step		Temperature (°C)	Time (min)
RBM10 / β-actin			
Reverse transcription		50	30
Initial PCR activation step		95	15
PCR amplification			
Denaturation		94	0.5
Annealing		51	0.5
Extension		72	1
Number of cycles		34	
Final extension		72	7
RBM3 / β-actin			
Reverse transcription		50	30
Initial PCR activation step		95	15
PCR amplification			
Denaturation		94	0.5
Annealing		53	0.5
Extension		72	0.5
Number of cycles		29	
Final extension		72	7

Gómez-Esquer et al., 2004b]. The sequence verification of the RT-PCR products was carried out on an automated ABI PrismTM 377 sequencer and 3730 DNA Analyzer (Applied Biosystems).

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 1× 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 an 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/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 the standard one at our laboratory. Briefly, 5 μm paraffin sections were mounted on poly-L-lysine coated slides for heat-induced epitope retrieval (HIER technique) in citrate buffer. We used the same, commercially available streptavidin-biotin-peroxidase kit (Histostain-SP, Zymed, San Francisco, CA) throughout the whole procedure, to ensure uniformity of results. The antibodies employed were as follows: NCL-CB11 (c-erb-B2), NCL-ER-6F11 (estrogen receptor), NCL-p53-D07 (p53), all from Novocastra Laboratories, Newcastle, U.K.; prediluted MIB1 (Ki67), and PR-2C5 (progesterone receptor) from Zymed, San Francisco, CA. The incubation time was 1 h at room temperature in a humid chamber for all antibodies, which apart from the prediluted MIB1-Ki67 solution, which was directly used as supplied, were employed at following dilutions: NCL-CB11 (c-erb-B2), 1:40; NCL-ER-6F11 (ER), 1:100; NCL-p53-D07, 1:100.

Flow-Cytometry

The procedure was carried out always 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 [Núñez Villar et al., 2003]. It 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), 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).

TABLE II. Correlation of RBMX, RBM3, RBM10 Large and Small Variant Expression Levels With Continuous Variables; Spearman's Rank Correlation Test

	RBMX		RBM3		RBM10 large		RBM10 small	
Variable	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
h-MAM	0.00	0.960	−0.04	0.665	−0.06	0.497	−0.12	0.210
Nup88	0.07	0.422	0.03	0.732	−0.06	0.508	0.09	0.329
CD105 (endoglin)	−0.31	0.001	−0.12	0.193	0.08	0.400	<0.01	0.999
VEGF	0.16	0.080	0.00	0.982	0.27	0.004	0.28	0.003
Bcl2	0.12	0.236	−0.11	0.292	0.19	0.063	0.05	0.622
Bax	0.19	0.039	0.34	<0.001	0.36	<0.001	0.47	<0.001
RBMX	—	—	0.03	0.720	0.13	0.154	0.02	0.795
RBM3	0.03	0.720	—	—	0.05	0.621	0.11	0.272
RBM10 large	0.13	0.154	0.05	0.621	—	—	0.26	0.006
RBM10 small	0.02	0.795	0.11	0.272	0.26	0.006	—	—

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). 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

The correlation between continuous variables was assessed by means of Spearman's correlation test (Table II).

The relationship of RBM gene expression with qualitative, dichotomic variables, was studied dividing the gene/beta-actin band density quotient, expressed in arbitrary units, into "high" and "low" values, using the median value as cutoff. Afterwards, using this cutoff, Wilcoxon's test was applied in a univariate model (Table III).

The statistical analysis was performed using the STATA-8 (Stata, College Station, TX) biostatistical package. Values were considered significant when P was <0.05.

RESULTS

For the present study, tumor material from 122 breast cancer patients was available, of which an aliquot was conserved either deep-frozen or in RNA-later® for genomic studies, and the rest embedded in paraffin for immunohistochemistry. Retrotranscription and amplification of *RBMX* was successfully achieved in 120 instances. For *RBM3*, *RBM10* large variant and *RBM10* small variant, the success rate was 111, 116, and 113, respectively. The failures were due most possibly to RNA-degradation, since two repetitions of the reactions yielded the same results. In particular, both the target sequences and the internal control could not be retrotranscribed and amplified. Furthermore, *Bcl-2*, *Bax*, *Nup88*, *hMAM*, *VEGF*, and *CD105* were successfully studied by means of RT-PCR in all cases, which were informative for the *RBM* genes.

The expression of all X-chromosome *RBM* genes was significantly associated with the expression of the proapoptotic *Bax* gene (*RBMX*, $P = 0.039$; *RBM3*, $P < 0.001$; *RBM10* large variant, $P < 0.001$; *RBM10* small variant, $P < 0.001$). Furthermore, the expression of both *RBM10* variants was significantly associated with the expression of the *VEGF* gene (large variant, $P = 0.004$; small variant, $P = 0.003$). We also found an association of borderline significance ($P = 0.05$) between the expression of *RBM3*, the large variant of *RBM10* and wild-type p53. Expression of the small *RBM10* variant, finally, was associated with high proliferation of the tumors (Ki67 $\geq 20\%$; $P = 0.037$).

The expression of both *RBM10* variants seems to be interdependent to a significant degree ($r = 0.26$, $P = 0.006$).

In the case of immunohistochemistry, we used the following cutoffs for positivity: the evaluation of nuclear staining patterns (ER, PR, Ki67, 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 [Schneider et al., 1994; Schmitt et al., 1998]. The Ki67

TABLE III. Correlation of RBMX, RBM3, RBM10 Large and Small Variant Expression Levels With Qualitative (dichotomic) Variables; Wilcoxon's Test

	RBMX			RBM3			RBM10 large			RBM10 small		
Variable	Median	P25–P75	*P*-value	Median	P25–P75	*P*-value	Median	P25–P75	*P*-value	Median	P25–P75	*P*-value
Histology			0.137			0.748			0.811			0.451
Ductal	1.21	1.04–1.43		0.28	0.19–0.46		1.18	0.93–1.45		1.15	0.98–1.41	
Other	1.10	0.94–1.31		0.32	0.14–0.48		1.21	0.97–1.34		1.13	0.97–1.29	
Histological grade			0.068			0.784			0.613			0.546
1 + 2	1.16	1.00–1.38		0.28	0.19–0.46		1.18	0.98–1.41		1.11	0.96–1.31	
3	1.28	1.06–1.53		0.30	0.21–0.47		1.20	0.90–1.54		1.14	0.97–1.43	
Nuclear grade			0.536			0.828			0.235			0.675
1 + 2	1.27	1.05–1.44		0.29	0.22–0.46		1.22	1.00–1.55		1.11	0.97–1.39	
3	1.17	1.00–1.44		0.28	0.18–0.47		1.16	0.87–1.42		1.15	0.94–1.41	
Nodal invasion			0.669			0.412			0.973			0.427
No	1.19	1.01–1.39		0.30	0.20–0.46		1.15	0.94–1.45		1.15	0.99–1.42	
Yes	1.25	1.01–1.46		0.27	0.13–0.45		1.22	0.95–1.43		1.13	0.94–1.35	
Aneuploidy			0.889			0.106			0.299			0.194
Yes	1.19	1.04–1.40		0.31	0.22–0.48		1.17	0.92–1.35		1.17	0.98–1.42	
No	1.21	0.98–1.44		0.27	0.14–0.42		1.22	0.99–1.55		1.05	0.96–1.31	
ER			0.404			0.794			0.760			0.100
Negative	1.16	1.01–1.33		0.30	0.18–0.40		1.15	0.96–1.36		1.25	0.94–1.57	
Positive	1.21	1.02–1.42		0.28	0.17–0.47		1.20	0.94–1.45		1.13	0.97–1.31	
PR			0.603			0.503			0.102			0.222
Negative	1.16	1.04–1.39		0.28	0.15–0.41		1.16	0.92–1.35		1.20	1.00–1.43	
Positive	1.21	1.01–1.42		0.30	0.19–0.47		1.22	1.00–1.61		1.07	0.97–1.31	
c-erb-B2			0.915			0.527			0.936			0.971
Negative	1.21	1.01–1.39		0.29	0.19–0.47		1.17	0.94–1.44		1.14	0.97–1.41	
Positive	1.14	1.04–1.44		0.27	0.16–0.40		1.22	1.04–1.30		1.14	0.87–1.39	
p53			0.833			0.054			0.047			0.389
Negative	1.20	1.03–1.41		0.30	0.19–0.47		1.20	0.97–1.52		1.15	0.98–1.40	
Positive	1.20	1.01–1.40		0.23	0.13–0.29		1.05	0.83–1.31		1.13	0.78–1.38	
Ki67			0.610			0.727			0.948			0.037
<20	1.20	1.01–1.39		0.29	0.19–0.47		1.17	0.92–1.44		1.06	0.93–1.35	
>20	1.21	1.04–1.47		0.27	0.17–0.45		1.20	0.97–1.37		1.25	1.05–1.47	

labeling 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.

The results of the statistical analyses, contemplating all studied variables, are summarized in Tables II and III.

DISCUSSION

To our knowledge, neither the expression of *RBM3* and *RBM10* nor the correlation of X-chromosome RBM gene expression and apoptosis has been studied before in breast cancer. In particular, as noted recently by Sutherland et al. [2005], the possible role of *RBM10* was completely unknown up to now.

As can be seen from our results, the expression of the X-chromosome *RBM* genes correlates in a highly significant way with the expression of the *Bax* gene, which is a central member of the caspase apoptotic pathway. This association seems to be an independent feature of each gene, since their expression is not interrelated, with the exception of both *RBM10* variants (Table III). This notion is further reinforced by the fact that *RBM10* is expressed independently from *RBM3*, despite lying close together in the same region of the X-chromosome.

Furthermore, the expression of *RBM3* and the large variant of *RBM10* was also significantly associated with the expression of wild-type *p53*, another member of the caspase apoptotic pathway.

Interestingly, the expression of both *RBM10* variants was also significantly associated with the expression of the angiogenesis-related gene *VEGF*. In our previous study on *RBMX* expression in breast cancer [Gómez-Esquer et al., 2004a], we found it to be inversely associated with the expression of another gene involved in angiogenesis, *CD105* (endoglin). This seems to indicate that the expression of X-chromosome genes in human breast cancer is not only related to apoptosis, as suggested by Sutherland et al., but possibly also, to some extent, with angiogenesis.

In any case, the hypothesis ventured by Sutherland et al. that the *RBM* genes might constitute a novel family of apoptosis modulators seems to be fully validated by our results.

It is interesting, furthermore, to note that all members of the wider RBM family studied up to date in human tumors, including the genes subject of this study, seem in some way or another to oppose the development of the tumor phenotype. In fact, *RBM8A* and *RBM8B* codify for almost identical isoforms of the BOV-1 protein, which in its turn binds to *OVCA1*, a candidate tumor suppressor gene in ovarian cancer [Salicioni et al., 2000], and *RBMY* is downregulated in testicular cancer [Lifschitz-Mercer et al., 2000]. *RBMY*, as has been mentioned in the "Introduction," is the Y-chromosome homologue of the *RBMX* gene studied by us in breast cancer, whose expression correlates inversely with the expression of *CD105*, a gene involved in a typical feature of oncogenic development such as angiogenesis.

In conclusion, the expression of all X-chromosome *RBM* genes correlates significantly with the expression of genes involved in apoptosis (*Bax*, *p53*). Additionally, the expression of some of them correlates significantly with the expression of genes (*VEGF*, *CD105*) involved in angiogenesis, another typical feature of oncogenic activation. These are novel findings that open the door for a better understanding of both the role of the X-chromosome, which was up to now marginal, and of the *RBM* genes, in breast cancer.

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