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The X-chromosome *RBMX* Gene is Expressed in Mammary Carcinoma

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Abstract. *Background: A gene located on the q11.23 region of the male chromosome, RBMY, which plays a role in spermatogenesis, is down-regulated in testicular cancer. RBMY is a diverged X-Y shared gene. The corresponding X chromosome gene, RBMX, is located on Xq26. Materials and Methods: We studied fresh tissues from 122 infiltrating breast cancers (99 ductal infiltrating, 19 lobular infiltrating and 4 tubular carcinomas) for the expression of RBMX by means of differential RT-PCR (reverse transcription-polymerase chain reaction), using beta-actin as an internal control and normalization standard. The obtained results were compared with all available clinical and molecular data of the studied tumors (estrogen and progesterone receptors (ER & PR), c-erb-B2, p53, Ki67, DNA-ploidy, Bcl-2, VEGF, CD105 (endoglin), histologic variety, histologic and nuclear grade and axillary node invasion). Results: RBMX RT-PCR was successful in 120/122 instances. All 120 cases expressed RBMX. The only significant correlation found between RBMX expression and all the variables tested was an inverse one with CD105 (endoglin) mRNA-expression (r=-3063; p=0.0007). Conclusion: The X-chromosome RBMX gene is expressed in all breast cancers. The expression is inversely correlated with the expression of the angiogenesis-associated CD105 (endoglin) gene. The precise meaning of this association has still to be elucidated.*

In mammals, the sexual X and Y chromosomes are heteromorphic. They originate from an initial pair of autosomic chromosomes, the process having begun some 240-320 million years ago (1). During this evolution, the male Y chromosome, which originally was homologous to the X chromosome, has suffered what is known as "Y chromosome degradation", which is responsible for its lesser size (2). This same evolution has conserved gender-specific genes, such as the ones regulating the male phenotype and spermatogenesis (3). The X chromosome, in its turn, undergoes a specific phenomenon in females, known as "X-chromosome inactivation", by which one of both alleles is switched off at a very early stage of the embryonic development, in order to avoid a double expression of genes if compared to their male counterparts. Since the inactivation of one or the other allele occurs in a random fashion, adult females in the end are a mosaic of cells with either X allele inactivated (4). The outcome of this regulatory process is that most genes of the X chromosome are transcriptionally silenced, although alterations do occur and are generally associated with grave disorders, like the Wiskott-Aldrich, the Lesch-Nyhan or the Barth syndromes. Recently, alterations in the X-chromosome inactivation pattern have been associated with the BRCA1-mediated predisposition to develop ovarian cancer (5), and X-chromosome inactivation is also frequently encountered in young breast cancer patients (6). It seems all too logical that the female sexual chromosomes might be involved in the development of typically female tumors. On the other hand, one gene located on the q11.23 region of the male chromosome, *RBMY*, which plays a known role in spermatogenesis, is down-regulated in testicular cancer (7). *RBMY* is a diverged X-Y shared gene. The corresponding X chromosome gene, *RBMX*, is located on Xq26.

Based on this association of *RBMY* with testicular cancer, we studied the expression of its female homologue in a series of breast cancers, in order to investigate firstly, if *RBMX* is expressed in this kind of tumor and, secondly, if it is expressed, how relative expression levels correlate with the clinical and biological features of the tumors. This could give us a hint as to the possible role played by *RBMX* in breast cancer.

Materials and Methods

Fresh tissue from 122 breast cancers was processed for this investigation. The tissues were immediately snap-frozen or immersed in RNA-later® at the moment of intraoperative biopsy. The patients were operated upon at Fundación Tejerina, Madrid, Spain, and all had given

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Key Words: X-chromosome, *RBMX*, breast cancer.

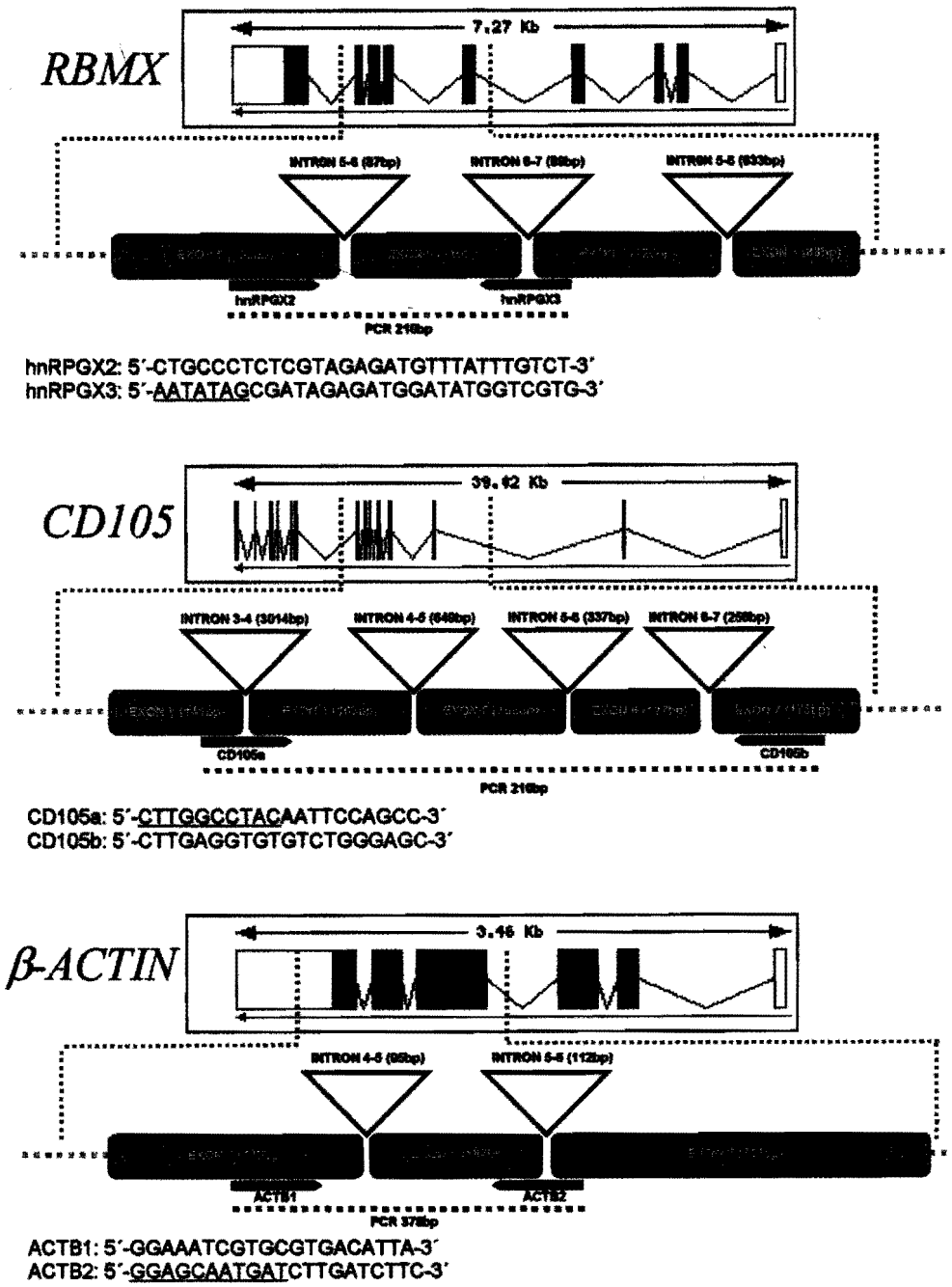

Figure 1. *Primer design to avoid amplification from contaminating genomic DNA.*

their informed consent for the research use of the obtained tissues prior to the operation. 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 the expression of the *RBMX, VEGF* and *CD105* (endoglin) genes by means of RT-PCR; estrogen and progesterone receptor (ER & PR), c-erb-B2, p53 and Ki67 expression by means of immunohistochemistry; DNA-ploidy by means of flow cytometry; and, finally, Bcl-2, Bax and CD105 (endoglin)-expression in a subset of 40 tumors by means of immuno-flow-cytometry. Clinical and pathological features (histologic variety, histologic and nuclear grade and axillary node invasion) were also included as variables of the study.

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*

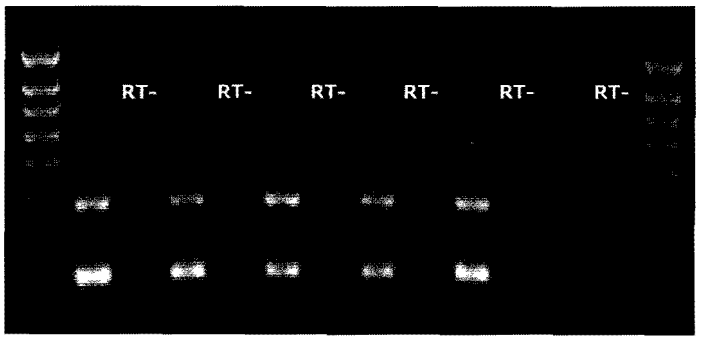

Figure 2. *RT-PCR of six breast cancers used in this study. The lanes RT- are reactions done without reverse transcription, demonstrating the lack of genomic DNA contamination in these RNA samples.*

Table I. *Primers and thermal cycler conditions used.*

Gene	Primer	Sequence (5'-3')	Product (bp)
RBMX	hnRPGX2	CTGCCCTCTCGTAGAGATGTTTATTTGTCT	210
	HnRPGX3	CACGACCATATCCATCTCTATCGCTATATT	210
CD105	CD105a	CTTGGCCTACAATTCCAGCC	542
	CD105b	CTTGAGGTGTGTCTGGGAGC	542
b-actin	ACTB1	GGAAATCGTGCGTGACATTA	378
	ACTB2	GGAGCAATGATCTTGATCTTC	378

Step	Temperature	Time (min)
Reverse transcription	50°C	30
Initial PCR activation step	95°C	15
PCR amplification		
Denaturation	94°C	0.5
Annealing	55°C	0.5
Extension	72°C	1
Number of cycles	35	
Final extension 72°C	10	

RNA/DNA calculator® from Amersham Pharmacia Biotech, Uppsala, Sweden) after 1:10 dilution in RNAase-free water and the 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 following software: Oligo Analizer 1.0.3. and Oligo Explorer 1.1.2., both available online at www.uku.fi/~kuulasma/OligoSoftware. When it was possible, the primers were designed to span an intron/exon boundary, in order to eliminate amplification from contaminating genomic DNA. In our case this was possible at least for one primer of each pair of primers used in this study (Figure 1). 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 (Figure 2). The primers used, as well as the conditions of the RT-PCR, are shown in Table I.

The relative abundance of a transcript in different samples can be estimated by semiquantitative, or relative RT-PCR, which has been described extensively elsewhere (8). It involves the inverse transcription of both the mRNA corresponding to the target gene and to a constitutive one into cDNA and subsequent amplification under identical conditions. Previously, we had adjusted the latter so that the PCR reaction was interrupted prior to the saturation phase in both cases. We found that at 35 extension cycles both saturation curves were still well within the ascending slope (Figure 3). The signal from the RT-PCR product is 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 *RBMX* and *CD105* with 0.6 mM of each of

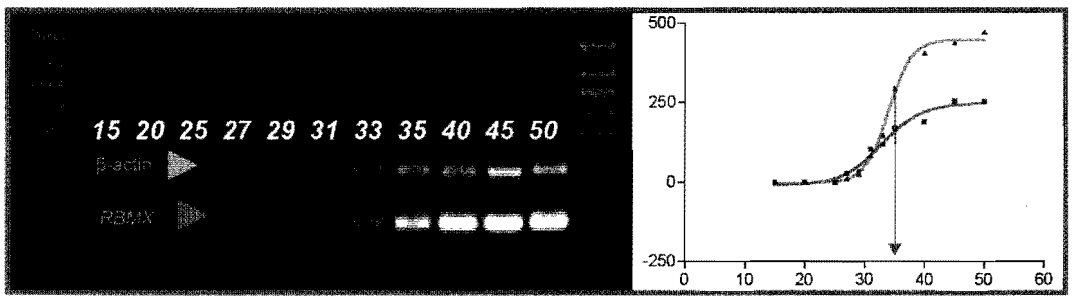

Figure 3. *Saturation curve for both the study and control gene. A number of cycles corresponding to the exponential phase of the reaction for both genes was chosen.*

the two *RBMX* and *CD105* specific primers and 0.22 mM of each beta-actin-specific primer. The sequence verification of the RT-PCR products was carried out on an automated sequencer ABI PrismTM 377 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 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 Acquisition 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 and has also been described elsewhere (8). 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, USA) 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, UK; prediluted MIB1 (Ki67) and PR-2C5 (progesterone receptor) from Zymed. 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 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, Ki67 and p53) was straightforward, since specimens positive for ER, PR or p53 always showed specific staining in more than 10% of tumor cells. The Ki67 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.

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 (9). It was first 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, 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). Bcl-2 and Bax immunofluorocytometry was carried out as described in a previous paper (10). CD105 (endoglin) expression was studied in an analogous manner, using the CD105 monoclonal antibody from Beckman Coulter, USA at a 1<.10 dilution.

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, 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 all other qualitative variables, the *RBMX*/ β-actin band density quotient, expressed in arbitrary units, was divided into "high" and "low" values, using 2 different cut-offs (the median value and the 66th percentile). Afterwards, using these two cut-offs, *RBMX* expression was correlated with all other biological and clinical variables in a univariate model (Table II). The statistical analysis was performed using the STATA statistical package (Stata Corporation, College Station, TX, USA).

Results

Of the 122 tumor samples studied, inverse transcription and amplification of the *RBMX* gene was successfully attained in 120. In the other two cases, neither amplification of the study gene nor of the internal control gene was possible, so that we attribute this negative result to poor conditions of the RNA, despite the quality controls we had carried out to exclude RNA degradation after its extraction.

The only significant correlation we obtained between *RBMX* expression and all the variables tested was an inverse one with *CD105* mRNA expression (Table II). This was maintained throughout the statistical analysis, regardless of where the cut-off point between high and low expression was put (median value of the distribution or 66th percentile), and especially evident when both variables, both continuous, were compared directly by means of Spearman's test ($r=-3063$; $p=0.0007$).

Table II. *Correlation of RBMX expression with biological and clinical variables.*

Variable	Num	Median	RBMX P25-P75	*p*-val[1]	n (%)	RBMX > 1.21 (Percentile 66) OR[2]	95% CI[2]	*p*-val[2]
Histology								
Ductal+ tubular	101	1.21	1.04-1.41	0.191	37 (37%)	1.00		
Lobular	19	1.10	0.94-1.31		4 (21%)	0.47	0.14- 1.52	0.206
Histologic grade								
1+2	80	1.13	0.99-1.33 0.021		22 (28%)	1.00		
3	40	1.28	1.06-1.53		19 (48%)	2.43	1.10- 5.35	0.028
Nuclear grade								
1+2	71	1.24	1.04-1.39	0.964	25 (35%)	1.00		
3	49	1.17	1.00-1.44		16 (33%)	0.91	0.42- 1.97	0.813
Positive nodes								
No	65	1.19	1.01-1.39	0.669	23 (35%)	1.00		
Yes	52	1.25	1.01-1.46		18 (35%)	0.99	0.46- 2.12	0.979
Ploidy								
Aneuploid	64	1.19	1.04-1.40	0.889	21 (33%)	1.00		
Diploid	56	1.21	0.98-1.44		20 (36%)	1.11	0.52- 2.35	0.792
ER								
Negative	33	1.16	1.01-1.33	0.404	8 (24%)	1.00		
Positive	86	1.21	1.02-1.42		32 (37%)	1.82	0.73-4.51	0.197
PR								
Negative	61	1.16	1.04-1.39	0.603	20 (33%)	1.00		
Positive	59	1.21	1.01-1.42		21 (36%)	1.10	0.52-2.34	0.797
c-erbB-2								
Negative	95	1.21	1.01-1.39	0.915	33 (35%)	1.00		
Positive	25	1.14	1.04-1.44		8 (32%)	0.90	0.35- 2.30	0.823
p53								
Negative	99	1.20	1.03-1.41	0.833	34 (34%)	1.00		
Positive	21	1.20	1.01-1.40		7 (33%)	0.97	0.36- 2.63	0.953
ki67								
≤ 20%	74	1.20	1.01-1.39	0.610	24 (32%)	1.00		
> 20%	46	1.21	1.04-1.47		17 (37%)	1.25	0.58- 2.69	0.576
h-MAM[3]								
≤ 206	61	1.27	1.02-1.40	0.769	22 (36%)	1.00		
> 206	59	1.17	1.03-1.44		19 (32%)	0.82	0.39- 1.75	0.609
Endoglin[1]								
≤ 1	78	1.27	1.06-1.47	0.001	32 (41%)	1.00		
> 1	42	1.06	0.92-1.27		9 (21%)	0.40	0.17- 0.95	0.038

1 Kruskal-Wallis test
2 Odds Ratio and 95% Confidence Interval of the association between each variable and a RBMX level greater than 1.21 (corresponding to the 66th percentile)
3 Cut-off used in a previous work
Significant correlations between continuous variables (Spearman correlations): ENDOGLIN & RBMX
Spearman's rho=-0.3063
p-value=0.0007

Discussion

The only X-chromosome gene so far studied in relation to human breast cancer has been the androgen receptor gene. This is the first report about the expression of another X-chromosome gene in breast cancer. The Y-chromosome analogue of RBMX, RBMY, has been studied in relation to a typically male tumor, *i.e.*, testicular cancer, and shown to be down-regulated in this kind of tumor (7). In this report by Lifschitz-Mercer *et al.*, down-regulation of RBMY was associated with a higher incidence of "*in situ*" carcinoma, *i.e.*, it seemed to play a role at the earliest stages of tumor development, prior to the stage of overt invasive neoplasia. Following our results on the *RBMY* analogue, *RBMX*, it is not altogether clear which role, and at which stage of tumor development, this gene might play in breast cancer. The only significant (albeit, very clear) association we found between overexpression of the gene and all variables tested was an inverse one with *CD105* (endoglin) expression. *CD105* (endoglin), is induced by hypoxia in activated endothelial cells, where it acts as a receptor for transforming growth factor (TGF) beta1 and beta3 (11). Endoglin expression has been studied in breast cancer and has been identified as an independent prognostic marker (12,13). Moreover, CD105 circulating levels in the plasma of breast cancer patients have also been studied and shown to correlate with the presence of metastasis (14). However, despite its proven prognostic potential, the precise role of CD105 in breast cancer is currently less well defined than that of the most extensively studied angiogenesis-related gene, *VEGF*. We have recently studied the mRNA expression of both genes in a large series of breast cancers (unpublished results) and found that, whereas VEGF expression was associated with a number of expected features of the tumors (proliferation, ploidy, absence of hormone receptors), *CD105* expression was not The obtained results produced more questions than answers as to the possible biological role of *CD105* in breast cancer. However, through studies at the protein level, it has been convincingly shown that expression of the *CD105* gene product, endoglin, is directly associated with neoangiogenesis and with a significantly worse prognosis of the patients showing the highest levels of expression. In this sense, *RBMX* seems to share with its Y-chromosome analogue the feature of being opposed to the development of the tumor phenotype, since neoangiogenesis is one of the most important initial features of this process. Since *RBMX* was expressed in all breast cancer samples studied and showed this clear relationship with the expression of another gene, *CD105*, which plays a still not completely defined, but apparently important role in breast cancer, further studies along this line seem to be worthwhile.

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