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Down-regulation of *BRMS1* mRNA Expression in Breast Cancer is not Related to the Presence of Axillary Node Metastasis

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Abstract. *Background: BRMS1, recently identified in MDA-MB-435 breast cancer cells, seems to act as a potent antimetastatic gene in experimental tumor models. Materials and Methods: To verify if BRMS1 exerts its action in a similar way in clinical tumors, BRMS1-mRNA expression was investigated in a series of 107 human breast carcinomas and correlated with the presence or not of axillary node metastases. Additionally, BRMS1 expression was correlated with all available clinical (histologic variety, histologic and nuclear grade) and biological parameters (ploidy, expression of Ki67, hormone receptors, c-erb-B2 and p53), as well as with the expression of hMAM- and Nup88-mRNA, previously shown by us to correlate with very low and very high aggressiveness of breast cancer, respectively. Results: Down-regulation of BRMS1 expression in the tumors did not correlate with the presence of axillary node metastases. Furthermore, BRMS1 expression did not correlate with any other of the studied clinical or biological tumor parameters. Conclusion: In clinical breast cancers, down-regulation of BRMS1 expression seems not to mimic the very clear-cut antimetatstatic properties displayed in experimental tumor models.*

BRMS1 is a putative antimetastatic gene, located on chromosome 11, recently identified in MDA-MB-435 breast cancer cells (1). The same group subsequently showed that *BRMS1* transfection is able to suppress metastasis in melanoma cell lines (2). Thus, *BRMS1* seems to act as a potent antimetastatic gene in experimental tumor models.

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The present study aimed to verify whether *BRMS1* exerts its action in a similar way in clinical tumors. *BRMS1*-mRNA expression was tested in a series of human breast carcinomas and correlated with the presence or not of axillary node metastases, which are generally considered the first metastatic station of breast cancer. Additionally, *BRMS1* expression was correlated with all available clinical and biological parameters of the studied tumors, in an attempt to obtain information about the possible biological role of the *BRMS1* gene in human breast cancer.

Materials and Methods

We studied 107 breast carcinoma specimens, obtained from previously untreated patients operated upon at Fundación Tejerina, Madrid, Spain, for the expression of *BRMS1*-mRNA. Eighty-six were ductal infiltrating carcinomas, 18 lobular infiltrating carcinomas and the remaining 3 of the pure tubular type. The latter were grouped together, for statistical purposes, with the ductal infiltrating carcinomas, of which they constitute an especially well-differentiated subgroup. *BRMS1* expression was correlated with all available clinical (histologic variety, histologic and nuclear grade) and biological parameters (ploidy, expression of Ki67, hormone receptors, c-erb-B2 and p53), as well as with the expression of *hMAM*- and *Nup88*-mRNA, previously shown by us to correlate with very low and very high aggressiveness of breast cancer, respectively (3, 4).

RT-PCR. Total RNA was isolated from the tumor specimens using the Rneasy™ commercial kit (AMBION Inc., Austin, Texas, USA), according to the manufacturer's instructions. The RNA content was quantified in a spectrophotometer (GeneQuant *pro* RNA/DNA calculator®; Amersham Pharmacia Biotech, Uppsala, Sweden) 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 performed using a commercial one-step RT-PCR kit (AMBION Inc.) and the iCycler™ thermal cycler (BIORAD, Hercules, CA, USA). The primers were designed by means of the openly available Oligo Analizer 1.0.3. and Oligo Explorer 1.1.2. software, (www.uku.fi/~kuulasma/OligoSoftware).

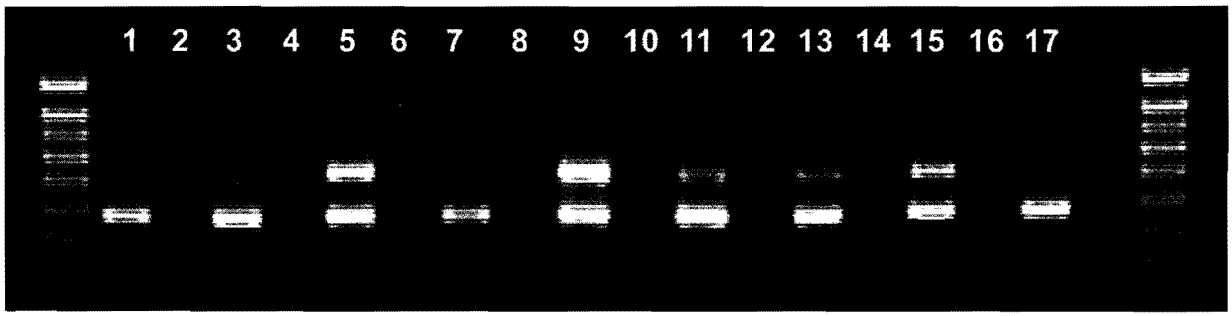

Figure 1. *Reverse transcriptase polymerase chain reaction (RT-PCR) of BRMS1-mRNA corresponding to nine breast cancers of this study. The even-numbered lanes represent reactions done using the same RNA as in the preceding odd-numberd lane, but without reverse transcription, demonstrating the lack of genomic DNA contamination in these RNA samples. The lower weight band corresponds to the internal control (beta-actin). Absence of detectable BRMS1-mRNA expression in tumors 1, 3 and 7.*

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

Gene	Primer Sequence (5'-3')		Product (bp)
BRMS1	*BRMS1*a	CTGCCCTCTCGTAGAGATGTTTATTTGTCT	538
	*BRMS1*b	CACGACCATATCCATCTCTATCGCTATATT	538
β-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

Whenever 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 every pair used in this study. As a control for genomic contamination, the same reactions were performed in the absence of reverse transcriptase (Figure 1). The primers used, as well as the conditions of the RT-PCR, are shown in Table I. The primers and conditions corresponding to the *hMAM* and *Nup88* genes were exactly the same as described in previous publications (3, 4).

The relative abundance of a transcript in different samples can be estimated by means of semiquantitative, or relative RT-PCR. 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. 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 was overcome by reducing the internal control primer concentration. In our system, we optimized *BRMS1* with 0.6 μM of each specific primer and 0.3 μM of each beta-actin-specific primer. The sequence verification of the RT-PCR products was carried out on an automated

Table II. *Analysis of the correlation between BRMS1-mRNA expression and the rest of the clinical and biological variables.*

Variable	n	*BRMS1* level Median	P25-P75	*p*-value[1]	Association with *BRSM1* >0 n (%)	OR[2]	95% CI[2]	*p*-value[2]
Histology								
Ductal	89	0	0.00-0.40	0.963	34 (38%)	1		
Lobular	18	0	0.00-0.55		7 (39%)	1.03	0.36-2.91	0.956
Histologic grade								
1+2	47	0	0.00-0.55	0.676	18 (38%)	1		
3	36	0	0.00-0.61		15 (42%)	1.15	0.47-2.79	0.756
Nuclear grade								
1+2	40	0	0.00-0.64	0.573	18 (45%)	1		
3	44	0	0.00-0.22		15 (34%)	0.63	0.26-1.53	0.308
Positive nodes								
No	62	0	0.00-0.53	0.892	25 (40%)	1		
Yes	42	0	0.00-0.40		15 (36%)	0.82	0.37-1.85	0.636
Aneuploidy								
No	57	0	0.00-0.52	0.815	22 (39%)	1		
Yes	50	0	0.00-0.37		19 (38%)	0.98	0.45-2.13	0.95
ER								
Negative	30	0	0.00-0.04	0.24	7 (23%)	1		
Positive	76	0	0.00-0.54		33 (43%)	2.52	0.97-6.59	0.059
PR								
Negative	55	0	0.00-0.63	0.429	23 (42%)	1		
Positive	52	0	0.00-0.25		18 (35%)	0.74	0.34-1.61	0.444
c-erbB2								
Negative	84	0	0.00-0.46	0.991	33 (39%)	1		
Positive	23	0	0.00-0.63		8 (35%)	0.82	0.31-2.16	0.694
p53								
Negative	87	0	0.00-0.52	0.692	32 (37%)	1		
Positive	20	0	0.00-0.34		9 (45%)	1.41	0.53-3.76	0.497
Ki67								
≤20%	70	0	0.00-0.52	0.647	26 (37%)	1		
>20%	37	0	0.00-0.45		15 (41%)	1.15	0.51-2.61	0.731
h-MAM[3]								
≤206	54	0	0.00-0.22	0.44	18 (33%)	1		
>206	53	0	0.00-0.63		23 (43%)	1.53	0.70-3.36	0.285
Nup88[3]								
≤2	57	0	0.00-0.59	0.669	24 (42%)	1		
>2	50	0	0.00-0.28		17 (34%)	0.71	0.32-1.56	0.39

[1]Kruskal-Wallis test.
[2]Odds Ratio, 95% Confidence Interval and significance of the association.
[3]Cut-off values obtained from previous studies (see References).
ER: Estrogen receptors; PR: progesterone receptors.

sequencer (ABI PrismTM 377 and 3730 DNA Analyzer, Applied Biosystems, Foster City, CA, USA).

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 (Ultra-Violet Products, Ltd., Cambridge, UK). 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 been extensively described elsewhere (5). 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), 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, 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-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 20% of tumor cells. The Ki67 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, as previously described (3, 4). The tumors were 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, Miami, FL, USA).

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. The expression or not by the tumors of *BRMS1* was correlated with all other biological and clinical variables in a univariate model (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

Of the 107 tumors studied, 40 (37.4%) expressed *BRMS1* and 67 (62.6%) did not. We were also able to study 9 normal breast tissues adjacent to the tumors. All of them expressed *BRMS1*, and the expression was maintained in 7 and absent in 2 corresponding tumors.

As can be seen from Table I, *BRMS1* expression did not correlate with any one of the studied clinical or biological tumor parameters. In particular, down-regulation of *BRMS1* expression in the tumors did not correlate in any way with the presence of axillary node metastases, as could have been expected from the previous experimental models.

Discussion

This is the first study of *BRMS1* expression in human breast cancer and, to our knowledge, in any solid human tumor. Our results seem to indicate that *BRMS1* mRNA expression is down-regulated in approximately two-thirds of human breast cancers, but that this down-regulation bears no relevance to the occurrence of axillary node metastases. What other function *BRMS1* might have in this context is also obscure, since we found no correlation with any other clinical or molecular feature of the tumors tested. In fact, we studied a wide range of molecular features covering various fundamental aspects of oncogenic activation, and none showed a relationship with the expression of the *BRMS1* gene. We also correlated *BRMS1* expression with that of two genes recently shown by us to be very closely associated with a relatively indolent and a very aggressive breast cancer phenotype, *hMAM* and *Nup88*, respectively, and were also unable to find any association. This may seem surprising at first sight, given the properties shown by the *BRMS1* gene in experimental models, but from past experience with a very similar gene, in which our group actively participated, it is not. In fact, *nm23*, also a putative antimetastatic gene, was initially studied in a very similar, if not identical, experimental system as *BRMS1*, where it displayed comparable features. Subsequent studies in clinical tumor samples, however, gave very different and, sometimes, quite opposing results. We were the first to report that *nm23*, far from predicting the absence of metastasis, was indeed associated with a significantly worse outcome in ovarian cancer (5). This finding was afterwards corroborated by several other studies, some of them in breast cancer (6, 7), a tumor where *nm23* had shown a striking antimetastatic activity in cell culture systems (8).

Experimental cell culture systems are a landmark of basic cancer research, but it must be borne in mind that they represent not less, but also not more, than the behavior of isolated tumor cells. Since host-tumor cell interactions play such a fundamental role in oncogenic development, it is not surprising that certain genes play quite different roles in cancer cells when these grow in their natural context as part of clinical tumors. Indeed, these real-life tumors are composed of many other elements besides just tumor cells, and grow in a constant interplay with all the environmental features provided by the host. Our present results seem to demonstrate, once more, this fundamental difficulty inherent in translational research.

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