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mRNA Expression of the Putative Antimetastatic Gene *BRMS1* and of Apoptosis-related Genes in Breast Cancer

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Abstract. *Breast cancer metastasis suppressor-1 (BRMS1) is a putative antimetastatic gene. However, results relating its expression to the prognosis of breast cancer are still controversial, and all studies carried out to date have failed to show a relationship between the expression of BRMS1 and axillary lymph node metastasis in breast cancer. It has been recently suggested that BRMS1 may exert its physiological role through the modulation of apoptosis. In order to test this hypothesis, we studied the expression of BRMS1 and genes known to be directly related with apoptosis in human breast carcinoma. The expression of mRNA corresponding to BRMS1, BCL2, BAX, CASP3 and the apoptosis-related X-chromosome RNA binding motif (RBM) genes (RBMX, RBM3, RBM10 small and large variant) was studied by means of differential RT-PCR in 94 samples obtained from previously untreated patients with breast carcinoma. A significant (p=0.03) inverse correlation between BRMS1 mRNA expression and expression of the mRNA corresponding to the large variant of the X-chromosome RBM10 gene was found. The degree of direct correlation with another member of the X-chromosome RBM gene family, RBMX, almost attained statistical significance (p=0.06). These results point towards a possible link between BRMS1 expression and apoptosis in human breast cancer through a relationship with the expression of genes belonging to the X-chromosome RBM family.*

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The breast cancer metastasis suppressor-1 gene (*BRMS1*) was identified in 2000 in MDA-MB-435 breast cancer cells and was found to be located on the long arm of chromosome 11 (1). Shortly thereafter, it was shown by the same group to suppress metastasis in experimental melanoma models after transfection and, therefore, postulated to be a potent antimetastatic gene (2). However, in 2005, Kelly *et al*. (3) and our own group (4) independently reported that down-regulation of *BRMS1* expression did not correlate with metastasis to axillary lymph nodes in clinical breast cancer specimens. Furthermore, both studies were also coincident in that *BRMS1* expression was not related to any other clinical or pathological feature (size, tumor grade, hormone receptor status, *etc*.) usually studied in breast cancer. Three further studies, correlating *BRMS1* expression with survival of breast cancer patients showed completely opposite results: in the first two, Hicks *et al*. (5) reported that loss of BRMS1 protein expression was significantly associated with reduced disease-free survival in subsets of patients stratified for loss of hormone receptors or HER-2-expression and, correspondingly, Zhang *et al*. (6) found that higher levels of *BRMS1* mRNA expression were associated with longer disease-free survival. In contrast with this, Lombardi *et al*. (7), almost at the same time, reported that elevated *BRMS1*-mRNA expression was significantly associated with shorter disease-free and overall survival. Pending a plausible explanation for these contradictory results, Liu *et al*. suggested that *BRMS1* may exert its physiological role through the modulation of apoptosis (8). In order to test this hypothesis in a series of human breast carcinoma samples, we studied the expression of *BRMS1* and a number of key genes of the mitochondrial apoptotic pathway, together with the X-chromosome RNA binding motif (*RBM*) genes, which our group for the first time related in human breast cancer to the expression of the *BAX* gene, which plays a central role in apoptosis (9).

Materials and Methods

For the present investigation, 94 primary breast cancer tumor samples from our tumor bank, already used for our previous *BRMS1* study (4), were analyzed. No patient had received any kind of treatment prior to surgery and all had given their written informed consent for the use of the tumor specimens for research purposes. The histology of the tumors was as follows: ductal infiltrating carcinoma, 76; lobular infiltrating carcinoma, 15; pure tubular carcinoma, 3. The postsurgical (pTNM) stage distribution, in its turn, was as follows: T1, 63; T2, 26; T3, 2. Three of the tumors, finally, were unclassifiable diffuse carcinomas, encompassing the whole breast with tiny invasive foci.

By means of differential RT-PCR, the expression of mRNA corresponding to the following genes was studied: *BRMS1, BCL 2, BAX, CASP3* and the X-chromosome RBM genes (*RBMX, RBM3, RBM10* small and large variant). Because of the correlation previously reported by us between the expression of the X-chromosome *RBM* genes and the angiogenesis-associated vascular endothelial growth factor (*VEGF* gene) (9), and between the expression of *RBMX* and the angiogenesis-associated *CD105* (endoglin) gene (10), using the same method we also studied the expression of both these genes.

The technique used (primers, RT-PCR conditions, *etc*.) has been described extensively in previous papers by our own group devoted to each of the studied genes, and the reader is referred to them for details (4,9-12). Briefly, RNA was extracted from the tumor specimens using the RNeasy™ commercial kit (AMBION Inc., Austin, TX, 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 the RNA was then frozen at –80˚C until further use. The RT-PCR reaction was carried out in a thermal cycler using a commercial one-step RT-PCR kit (AMBION Inc.). To evaluate the expression of the studied genes, a semiquantitative differential RT-PCR method was used which has been described extensively elsewhere (8-12). It involves the inverse transcription of both the mRNA corresponding to the target gene and to a constitutive one (in our case β-actin) 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. The amplification products were then resuspended in loading buffer with 10% bromophenol blue and run at 100 V in a 1.5-2% agarose gel prepared in 1XTAE 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, 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. The different levels of expression obtained constitute a continuous variable for each gene. Spearman's rank correlation test was used to compare the expression levels of *BRMS1* with those corresponding to all the other genes studied. The statistical analysis was performed using the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, USA). Values were considered significant when the *p*-value was <0.05.

Table I. *Correlation of BRMS1 expression with the expression of genes involved in apoptosis and angiogenesis in human breast cancer. N=94. Spearman's rank correlation test*

Gene	Correlation coefficient (r)	*p*-value
Bcl-2	–0.004	0.97
Bax	0.13	0.23
Caspase-3	–0.08	0.51
RBMX	0.20	0.06
RBM3	0.02	0.86
RBM10 small	–0.05	0.64
RBM10 large	–0.22	0.03
VEGF	–0.03	0.77
CD105 (endoglin)	0.01	0.94

Results

The possible correlation between the expression of the putative antimetastatic gene *BRMS1* and a series of genes involved in apoptosis was studied in 94 human breast tumors.

A significant inverse correlation between *BRMS1* mRNA expression and expression of the mRNA corresponding to the large variant of the X-chromosome *RBM10* gene was found. The degree of direct correlation with another member of the X-chromosome *RBM* gene family, *RBMX*, almost attained statistical significance ($p=0.06$). All results are summarized in Table I.

Discussion

To our knowledge, this is the first study on *BRMS1* expression and the expression of genes involved in apoptosis in human breast cancer. No correlation was found between the expression of *BRMS1* and the expression of genes known to be major players in apoptosis (*BCL2, BAX* and *CASP3*). However, a significant inverse correlation was found with the expression of a gene playing a still undefined role in apoptosis (*RBM10* large), and a direct correlation with the expression of another member of the same family, *RBMX*, almost attained statistical significance. All members of the X-chromosome RBM family were shown by our group for the first time to be significantly coexpressed with the proapoptotic *BAX* gene in human breast cancer (9). That the *RBM* genes are most probably involved in apoptosis had been ventured previously by Sutherland *et al*. (11). In a further study, our group reported that the caspase-3 gene is significantly coexpressed with the small variant of *RBM10*. The effector caspases, of which caspase-3 is a prominent member, are the final step upon which both apoptotic pathways, the extrinsic and the mitochondrial one, converge before the triggering of apoptosis. Taking all these results

together, it seems that *BRMS1* might indeed be linked to apoptosis in breast cancer through its possible relationship with some of the X-chromosome *RBM* genes. The exact biological role of *BRMS1*, on the other hand, is still undefined. Although it was initially postulated as a potent antimetastasis gene based upon experimental studies in melanoma cell lines (12), all clinical studies carried out until present have failed to establish a link between the expression of *BRMS1* and metastasis to the regional axillary nodes in breast cancer, which is believed to be one of the earliest manifestations of the metastatic spread of this tumor. On the other hand, at least two different studies (5, 6), as was mentioned earlier, have established a significant link between down-regulation of *BRMS1* and a shorter disease-free survival, but interestingly not overall survival. However, breast cancer kills through distant metastasis, not local recurrence. Therefore, if *BRMS1* indeed plays a role in metastatic spread, this should be reflected in overall survival, or at least in the appearance of distant metastases, not local recurrence, which is an entirely different process. Unfortunately, the patients were not stratified by local or distant (*i.e.* metastatic) recurrence in the two mentioned studies. Therefore, it cannot be concluded from them that *BRMS1* functions as an antimetastasis gene in human breast cancer, as the authors do, but only that *BRMS1* expression is significantly linked to earlier recurrence (local and/or distant). The findings of Hicks *et al*. and Zhang *et al*., furthermore, are challenged by those of Lombardi *et al*. (7), who arrived at quite the opposite conclusion from the former, after a particularly thorough study.

In conclusion, our results point towards a possible link between *BRMS1* expression and apoptosis in human breast cancer through a relationship with the expression of genes belonging to the X-chromosome *RBM* family. How this might in its turn be related to the presumed antimetastatic role of *BRMS1* deserves further study.

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