

Functional Evidence for a Novel Human Breast Carcinoma Metastasis Suppressor, *BRMS1*, Encoded at Chromosome 11q13¹

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Abstract

We previously showed that introduction of a normal, neomycin-tagged human chromosome 11 reduces the metastatic capacity of MDA-MB-435 (435) human breast carcinoma cells by 70–90% without affecting tumorigenicity, suggesting the presence of one or more metastasis suppressor genes encoded on human chromosome 11. To identify the gene(s) responsible, differential display comparing chromosome 11-containing (neo11/435) and parental, metastatic cells was done. We describe the isolation and functional characterization of a full-length cDNA for one of the novel genes, designated breast-cancer metastasis suppressor 1 (*BRMS1*), which maps to human chromosome 11q13.1-q13.2. Stably transfected MDA-MB-435 and MDA-MB-231 breast carcinoma cells still form progressively growing, locally invasive tumors when injected into mammary fat pads but are significantly less metastatic to lungs and regional lymph nodes. These data provide compelling functional evidence that breast-cancer metastasis suppressor 1 is a novel mediator of metastasis suppression in human breast carcinoma.

INTRODUCTION

When breast carcinoma cells are confined to breast tissue, long-term survival rates are high. But when tumor cells disseminate to and colonize secondary sites, cure rates drop significantly. Likewise, quality of life for patients with stage IV (metastatic) disease is significantly worse than for those with stage I (local) carcinoma. Thus, decreased morbidity and mortality will depend on prevention and/or effective treatment of metastatic disease. To that end, understanding the biological, biochemical, and genetic mechanisms underpinning tumor cell invasion and metastasis will be required.

Metastasis-regulatory genes can be broadly categorized as either metastasis-promoting or metastasis-suppressing. Analogous to the role of oncogenes in tumorigenesis, metastasis promoters drive conversion from nonmetastatic to metastatic. Although similar in other respects, metastasis suppressors are distinguishable from tumor suppressors in that the former block only metastasis when introduced into metastatic tumor cells (*i.e.*, not tumorigenicity). As expected, tumor suppressors suppress both phenotypes because tumorigenicity is a prerequisite to metastasis (1). To date, only six metastasis suppressor genes (*Nm23*, *KISS1*, *Kai1*, *E-cadherin*, *MKK4*, *TIMPs*) have been shown to functionally suppress metastasis using *in vivo* models (reviewed in Ref. 2).

Two general approaches were used to identify these metastasis-controlling genes. The first involved comparison of gene expression in poorly or nonmetastatic cells with matched metastasis-competent cells. The second took advantage of clinical observations that identified nonrandom chromosomal changes that occur during tumor progression. This information localized the gene(s) from which cloning could commence. In this study, we combined aspects of both strategies to identify a novel, functional breast carcinoma metastasis suppressor gene.

A recent cataloging of differential gene/protein gene expression and chromosomal abnormalities occurring as breast carcinoma acquires metastatic potential (2) revealed that some karyotypic changes commonly occur in early-stage breast cancer (8p, 13q, 16q, 17p, 17q, and 19p), whereas others typically occur later in breast cancer progression (1p, 1q, 3p, 6q, 7q, 11p, and 11q). Among the most common changes in both familial or sporadic breast carcinoma are losses of genetic material on chromosome 11q, which occurs in 40–65% of cases. There are several regions spanning the q-arm of chromosome 11 for which associations have been made with breast cancer progression. Among the most common are amplifications and deletions involving regions near band 11q13. Within this region, there is evidence supporting the existence of a number of critical genes, including tumor-promoting, tumor-suppressing, metastasis-promoting and metastasis-suppressing genes. The genes *int-2*, *hst*, *bcl-1*, *glutathione S-transferase*, *CCND1*, and *EMS-1*, which map to 11q13, are amplified in breast cancer at a frequency between 3 and 20%. There exists a high-frequency involvement of rat chromosome 1 (which is syntenic to human chromosome 11) in the development and progression of rat mammary tumors (3). Therefore, based on these observations and high-frequency deletions involving 11q13-q14 in late-stage, metastatic breast carcinomas, we tested the hypothesis that chromosome 11q encodes a metastasis suppressor gene. Upon finding that introduction of a normal human chromosome 11 into metastatic MDA-MB-435 (435) human breast carcinoma suppressed metastasis without affecting tumorigenicity (4), we set out to identify the gene(s) responsible. We report here the isolation and functional characterization of a metastasis suppressor gene from this region.

MATERIALS AND METHODS

Cell Lines and Cell Culture. MDA-MB-435 and MDA-MB-231 are human estrogen receptor- and progesterone receptor-negative cell lines derived from metastatic (pleural effusion), infiltrating ductal breast carcinoma. Both cell lines form progressively growing tumors when injected into the mammary fat pads of immunocompromised mice. MDA-MB-435 cells develop macroscopic metastases in the lungs and regional lymph nodes by 10–12 weeks postinoculation, but rarely metastasize after direct injection into the lateral tail vein. The opposite pattern exists for 231 in athymic mice. MDA-MB-435 cell clones into which a normal, neomycin-tagged human chromosome 11 had been introduced by microcell-mediated transfer (designated neo11/435) are suppressed at least 75% for metastasis from the mammary fat pad (4).

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*BRMS1*⁵ transfectants were derived after transfection of full-length *BRMS1* cDNA (see below) cloned into the constitutive mammalian expression vector, pcDNA3 (Invitrogen, San Diego, CA). All cell lines were cultured in a 1:1 (v/v) mixture of DMEM and Ham's F-12 medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% nonessential amino acids, and 1.0 mM sodium pyruvate, but no antibiotics or antimycotics. Transfected cells and neo11/435 hybrids also received 500 µg/ml Geneticin (G-418; Life Technologies, Inc., Gaithersburg, MD). *BRMS1*-transfected 435 cells acquired an unexplained acute sensitivity to trypsin; therefore, cultures were thereafter passaged using 2 mM EDTA solution in calcium- and magnesium-free Dulbecco's PBS. Hybrid clones and transfectants were used before passage 11 in all cases to minimize the impacts of clonal diversification and phenotypic instability. For all functional and biological assays, cells between 70 and 90% confluence were used with viability >95%. All lines were routinely checked and found to be negative for *Mycoplasma* spp. contamination using the GenProbe method (Fisher Scientific, Pittsburgh, PA).

Cell line nomenclature was developed to identify the origin and nature of each cell line as unambiguously as possible. Single-cell clones are identified by the parental cell line name preceding a “.” followed by a clonal designation. Uncolonized populations are identified by a “-” after the parental cell line name. Microcell hybrids are identified by the tagged chromosome number followed by a “/” (e.g., neo11/435.A3 is single cell clone A3 derived after microcell-mediated transfer of chromosome 11 into MDA-MB-435). Where appropriate, numbers in parentheses following the cell line designation indicate the number of subcultures following cloning or establishment of the cell line. Numbers preceded by a “TE” indicate that the cells were passaged in a mixture of 0.125% trypsin-2 mM EDTA in calcium- and magnesium-free Dulbecco's PBS. Numbers preceded by a “P” indicate the cells were passaged using EDTA alone.

Transfections. *BRMS1* was cloned into the constitutive mammalian expression vector, pcDNA3 (Invitrogen). To detect *BRMS1* protein expression, a chimeric molecule was also constructed with an NH₂-terminal epitope tag (SV40T epitope 901; Ref. 5). Epitope-tagged and native *BRMS1* plasmids as well as pcDNA3 vector only were transfected into 435 and 231 cells by electroporation. After selection in G-418, single-cell clones were isolated by limiting dilution. Stable transfectants were assessed for their expression by RNA blot and, as appropriate, by immunoblot.

DD-RT-PCR. To identify differences in mRNA expression between metastatic and nonmetastatic neo11/435 hybrid cells, a DD-RT-PCR approach was undertaken. The methods used involved modifications of the method described by Liang and Pardee (6) as available in the Delta Differential Display kit (Clontech Laboratories, Inc., Palo Alto, CA). To minimize the impact of clonal heterogeneity, a mixture of equal parts mRNA from three neo11/435 hybrid clones [neo11/435.A3 (TE5), neo11/435.B1 (TE4), and neo11/435.D1 (TE10)] were used. To reduce the chances of proceeding with irrelevant cDNAs, we validated findings at several intermediate steps during the experiment. In short, every step was replicated using independent samples. Once PCR products were validated, they were used as probes to examine differential expression in Northern blots using progressively more extensive series of cell-line mRNAs. As full-length cDNA clones were obtained, replicate Northern blots were done with those as well to verify prior results.

Differentially expressed cDNAs and the primers used for the initial DD-RT-PCR reactions were as follows: *BRMS1* (P9/P9); *F5A3* (P9/T5), *8A3* (P3/T4); adenine phosphoribosyltransferase (P1/T9); *N*-acetyl-galactosamine-6-sulfate sulfatase (P6/P6); hexokinase II (P10/T8):

P1: 5'-ATTAACCCTCACTAAATGCTGGGGG-3'
 P3: 5'-ATTAACCCTCACTAAATGCTGGTGG-3'
 P6: 5'-ATTAACCCTCACTAAATGCTGGGTG-3'
 P9: 5'-ATTAACCCTCACTAAATGTGGCAGG-3'
 P10: 5'-ATTAACCCTCACTAAAGCACCGTCC-3'
 T4: 5'-CATTATGCTGAGTGATATCTTTTTTTTCA-3'
 T5: 5'-CATTATGCTGAGTGATATCTTTTTTTTCC-3'
 T8: 5'-CATTATGCTGAGTGATATCTTTTTTTTGC-3'
 T9: 5'-CATTATGCTGAGTGATATCTTTTTTTTGG-3'

⁵ The abbreviations used are: *BRMS1*, breast cancer metastasis suppressor-1; DD-RT-PCR, differential display; DAPI, 4',6-diamidino-2-phenylindole; RT-PCR, reverse transcription-PCR; EST, expressed sequence tags.

F5A3 and *8A3* were provisional nomenclature used during the initial part of these studies.

Chromosomal Localization of *BRMS1*. *BRMS1* cDNA was used to screen bacterial artificial chromosome and P1 artificial chromosome libraries at Genome Systems, Inc. (St. Louis, MO). Bacterial artificial chromosome clones 412(n24) and 536(h18) harbored *BRMS1* as confirmed by direct sequencing.⁶ The genomic sequence was determined using the 412(n24) clone. DNA was isolated and labeled with digoxigenin dUTP by nick translation and combined with sheared human DNA before hybridization to metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood leukocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by exposing the hybrid cell lines to antidigoxigenin antibodies followed by counterstaining with DAPI. Specific labeling was seen along the proximal long arm of a group C chromosome, which was subsequently confirmed to be chromosome 11 based on cohybridization with genomic probes known to map to 11p15 and 11cen. Measurements of 71 of 80 specifically labeled chromosomes 11 in metaphase spreads demonstrated that *BRMS1* is located at a position that is 19% of the distance from the centromere to the telomere of chromosome 11q. This corresponds to band 11q13.1-q13.2 (data not shown).

Metastasis Assays. Immediately prior to injection, cells (7–11 passages after transfection) at 80–90% confluence were detached with a 2 mM EDTA solution. Cells were washed, counted on a hemacytometer, and resuspended in ice-cold HBSS to a final concentration of 2.5 × 10⁶ cells/ml for 231 cells and 1 × 10⁷ cells/ml for 435 cells. MDA-MB-231 cells and derivatives (0.5 × 10⁶ in 0.2 ml) were injected i.v. into the lateral tail vein of 3- to 4-week-old female athymic mice (Harlan Sprague-Dawley, Indianapolis, IN). Mice were killed 4 weeks post-injection and examined for the presence of metastases. Lungs were removed, rinsed in water, and fixed in Bouin's solution before quantification of surface metastases as described previously (7).

Similar procedures were used for the spontaneous metastasis assay using MDA-MB-435 cells, except that 1 × 10⁶ cells (0.1 ml) were injected into exposed axillary mammary fat pads of anesthetized 5- to 6-week-old female athymic mice. When the mean tumor diameter reached 1.5–2.0 cm, tumors were surgically removed under ketamine: xylazine (80–85 mg/kg; 14–16 mg/kg) anesthesia, and the wounds were closed with sterile stainless steel clips. Four weeks later, mice were sacrificed, and visible metastases were counted. Lung tissues were handled as above. Metastases were also observed in the ipsilateral and contralateral axillary lymph nodes of control mice. Occasional recurrences developed at the site of tumor removal, but the presence of hematogenous metastases did not necessarily correlate with presence of recurrent tumor.

Animals were maintained under the guidelines of the NIH and the Pennsylvania State University College of Medicine. All protocols were approved by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*.

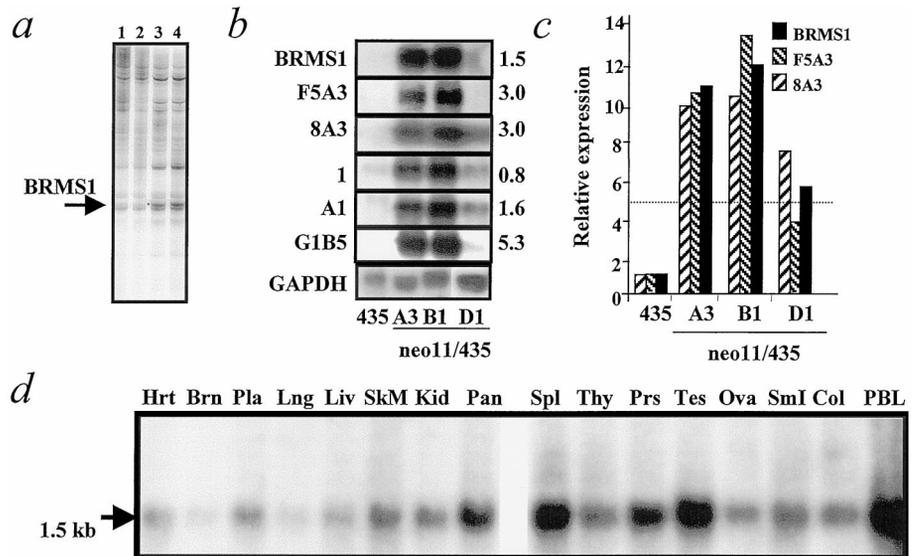
Statistical Analyses. The number of lung metastases was compared for *BRMS1* transfectants and corresponding parental and vector-only-transfected MDA-MB-435 and MDA-MB-231 cells. For experimental metastasis assays, one-way ANOVA followed by Tukey's Honestly Significant Difference *post hoc* test was used. For spontaneous metastasis assays, a Kruskal-Wallis ANOVA of ranks procedure was used with Dunn's *post hoc* test. Statistical differences in adhesion and motility assays were done using Student's *t* test comparing *BRMS1*-transfected to vector-only-transfected cells. Calculations were performed using SigmaStat statistical analysis software (Jandel Scientific, San Rafael, CA). Statistical significance was designed as *P* ≤ 0.05 using two-tailed tests.

RESULTS

DD-RT-PCR was used to compare gene expression in metastasis-competent (435) *versus* metastasis-suppressed (neo11/435) variants. Initially, 64 cDNA fragments were detected as up-regulated in the neo11/435 hybrids. A representative result is shown in Fig. 1a. Eighteen bands amplified reproducibly in replicate RT-PCR reactions (data not shown). Of those, six fragments exhibited ≥5-fold higher mRNA expression in

⁶ R. S. Samant, M. T. Debies, and D. R. Welch, unpublished observations.

Fig. 1. Identification of differentially expressed genes in metastasis-suppressed neo11/435 hybrid cell clones using differential display. *a*, DD-RT-PCR result in which *BRMS1* was identified. Replicate reactions were done in parallel to compare parental, metastatic MDA-MB-435 (Lanes 1 and 2) with mixtures (1:1:1) of metastasis-suppressed neo11/435 clones A3, B1, and D1 (Lanes 3 and 4). Lanes 2 and 4 contain twice as much starting material as Lanes 1 and 3. An equal mixture of neo11/435 hybrid cell clones was used to minimize the impact of tumor heterogeneity on the differential display reaction. *b*, Northern blot analyses using candidate differential display products as probes. Only candidates displaying ≥ 5 -fold higher expression in neo11/435 hybrid cell clones are presented. Poly(A)⁺ mRNA (2 μ g) was electrophoresed on denatured agarose gels, transferred to a nylon membrane, fixed, and probed with random prime-radiolabeled PCR products from the differential display reaction. Equal loading was verified by probing with *GAPDH* cDNA (data not shown). Approximate transcript sizes are depicted to the right of each gel. *c*, differential expression of the novel genes was quantified using phosphorimage analysis. Relative expression was compared with parental MDA-MB-435, and only genes showing ≥ 5 -fold higher (reference line shown as dotted line) expression in the neo11/435 hybrid cell clones were chosen for further study. *d*, multi-tissue Northern blots (Clontech) showing mRNA expression of *BRMS1*.



neo11/435 hybrid cell clones as detected by Northern blotting using the PCR product as a probe and quantified by phosphor image analysis (Fig. 1, *b* and *c*). None of the PCR products detected mRNAs expressed exclusively in the metastasis-suppressed cells. The differentially expressed cDNA inserts were sequenced, and homology to known genes and ESTs was assessed by comparing with the GenBank/European Molecular Biology Laboratory/DDBJ/PDB combined database. Three of the cDNAs were homologous to known human genes (*N*-acetylgalactosamine-6-sulphatase, adenine phosphoribosyltransferase, and hexokinase II). The remaining three cDNA fragments were novel and became priorities for further study. Here we report on the isolation and functional characterization of one of these novel cDNAs, *BRMS1*.

Full-length clones were obtained from a human kidney cDNA λ TriplEx library after this tissue was found to express high levels of all three novel cDNAs. Tissue-specific splice variants were not expected because only a single band of ~ 1.5 kb was detected. This pattern was replicated using full-length *BRMS1* cDNA as a probe. *BRMS1* is widely expressed to varying levels in every normal human tissue examined (Fig. 1*d*).

Nucleotide sequence analysis of *BRMS1* cDNA initially revealed no significant homologies to any known genes, ESTs, or proteins deposited in the databases. Regions of *BRMS1* cDNA showed numerous homologies to short ESTs isolated from fetal liver and spleen as well as the human tumor cell line HeLa, consistent with the wide tissue expression observed in the multitissue Northern blots. *BRMS1* has been detected in Southern blots in multiple species at the DNA level, including yeast, human, mouse, rat, rabbit, and cow (data not shown). A murine cDNA recently has been isolated and is 89.8% homologous at the nucleotide level,⁷ suggesting that the gene is relatively well conserved. The *BRMS1* human cDNA sequence was submitted to GenBank as a novel human gene with an Accession Number of AF159141.

After the *BRMS1* sequence was submitted, a deposited partial sequence (Accession Number AL050008; designated "hypothetical human protein sequenced by AGOWA within the cDNA sequencing consortium of the German Genome Project") had 91% homology at the predicted protein level for the region bounded by amino acids 60–244. However, no information regarding function for AL050008 is available. This may indicate that there is a *BRMS1* gene family.

Computerized analysis (DNAsis; Hitachi Software) shows that the

BRMS1 cDNA length is 1485 bp with the largest open reading frame of 741 bp (from nucleotides 122 to 862; Fig. 2). *BRMS1* encodes a novel protein of 246 amino acids ($M_r \approx 28,500$), a result confirmed using *in vitro* transcription and translation (data not shown). The genomic structure of *BRMS1* is organized as 10 exons spanning ~ 10 kb.⁸ Exon 1 is untranslated. Fluorescence *in situ* hybridization mapping places the location of *BRMS1* gene at human chromosome 11q13.1-q13.2 (Fig. 3*a*).

The predicted amino acid sequence of *BRMS1* was analyzed for structural and sequence homologies to obtain clues regarding mechanism of action. The identified structural domains of the *BRMS1* protein are shown schematically in Fig. 3*b*. Regions of homology were identified using the algorithms listed below. Using PROSITE⁹ several putative phosphorylation sites for cAMP/cGMP ([R/K]₂-x-[S/T]; amino acids 55–58 and 240–243), protein kinase C ([S/T]-[R/K]; amino acids 111–113, 147–149, 190–192, and 200–202), and casein kinase II ([S/T]-x₂-[D/E]; amino acids 19–22, 30–33, 37–40, 39–42, 41–44, and 46–49) were detected. PSORT II¹⁰ identified two putative nuclear localization sequences (amino acids 198–205 and 239–245), which were shown to be functional according to subcellular fractionation and immunofluorescence studies using *BRMS1*-transfected 231 cells (data not shown). For both studies, antibodies to epitope-tagged *BRMS1* (SV40T901 on the NH₂ terminus) were used. Additionally, *BRMS1* contains two coiled-coil (amino acids 51–81 and 147–180) motifs and several imperfect leucine zipper (L-x₆-L-x₆-L-x₆-L) motifs at amino acids 67–88, 131–152, 138–159, 153–174, and 160–181. No signal peptide motifs were identified, but there was a potential endoplasmic reticulum retention sequence at amino acids 243–246. These predictions were corroborated using the ExPASy search engine.¹¹ The four cysteine residues within the *BRMS1* protein are apparently not utilized for intra- or interprotein disulfide linkages because mobility in SDS-PAGE is unaffected by reducing agents (data not shown).

To assess the effect of *BRMS1* on breast carcinoma biological behavior, *BRMS1* was transfected into two independently derived metastatic human breast carcinoma cell lines, MDA-MB-435 (435) and MDA-MB-231 (231). The morphologies of 435 and 231 *BRMS1*-transfected cells

⁸ R. S. Samant, M. T. Debies, M. J. Seraj, and D. R. Welch, manuscript in preparation.

⁹ <http://www.genebio.com/prosite.html>.

¹⁰ <http://www.psорт.nibb.ac.jp:8800>.

¹¹ <http://www.expasy.ch>.

⁷ R. S. Samant, M. T. Debies, and D. R. Welch, manuscript in preparation.

agaaaagggagccgagcagcctacgggagtcggcgagcagccggtaccggcaaccacgggagctctcaggaa
tctcgtgtaggcccagaggctccagtcctccgagtcag

ATG CCT GTC CAG CCT CCA AGC AAA GAC ACA GAA GAG ATG GAA GCA GAG GGT GAT TCT GCT
1- **M P V Q P P S K D T E E M E A E G D S A** -20
GCT GAG ATG AAT GGG GAG GAG GAA GAG AGT GAG GAG GAG CGG AGC GGC AGC CAG ACA GAG
21- **A E M N G E E E E S E E E R S G S Q T E** -40
TCA GAA GAG GAG AGC TCC GAG ATG GAT GAT GAG GAC TAT GAG CGA CGC CGC AGC GAG TGT
41- **S E E E S S E M D D E D Y E R R S E C** -60
GTC AGT GAG ATG CTG GAC CTA GAG AAG CAG TTC TCG GAG CTA AAG GAG AAG TTG TTC AGG
61- **V S E M L D L E K Q F S E L K E K L F R** -80
GAA CGA CTG AGT CAG CTG CGG TTG CGG CTG GAG GAA GTG GGG GCT GAG AGA GCC CCT GAA
81- **E R L S Q L R L R L E E V G A E R A P E** -100
TAC ACG GAG CCC CTT GGG GGG CTG GAG CGG AGC CTC AAG ATT CGC ATT CAG GTG GCA GGG
101- **Y T E P L G G L Q R S L K I R I Q V A G** -120
ATC TAC AAG GGC TTC TGT CTG GAT GTG ATC AGG AAT AAG TAC GAA TGT GAG CTG CAG GGA
121- **I Y K G F C L D V I R N K Y E C E L Q G** -140
GCC AAA CAG CAC CTG GAG AGT GAG AAG CTG CTG CTC TAT GAC ACG CTG CAG GGG GAG CTG
141- **A K Q H L E S E K L L Y D T L Q G E L** -160
CAG GAG CGG ATC CAG AGG CTG GAG GAG GAC CGC CAG AGC CTG GAC CTC AGC TCT GAA TGG
161- **Q E R I Q R L E E D R Q S L D L S S E W** -180
TGG GAC GAC AAA CTG CAC GCC AGA GGC AGC TCC AGG TCT TGG GAC TCC CTG CCG CCP AGC
181- **W D D K L H A R G S S R S W D S L P P S** -200
AAG AGG AAG AAG GCA CCT CTG GTT TCT GGC CCA TAC ATC GTG TAC ATG CTT CAA GAG ATC
201- **K R K K A P L V S G P Y I V Y M L Q E I** -220
GAC ATC CTG GAG GAC TGG ACA GCC ATC AAA AAG GCT AGG GCA GCT GTG TCC CCT CAG AAG
221- **D I L E D W T A I K K A R A A V S P Q K** -240
AGA AAA TCG GAT GGA CCT TGA cctgctgttcacagccagggggaccctcagagcagctggcactgacacca
241- **R K S D G P ***
ggattctcgtcttctcctcctgcagacagggcggaccacagggccctcagggtctgcccagccaggctcctgtggtgctgct
ggccctcccactccatctggcactggcctggactcctcctctgcccctcctcagggcctgcacagctgtggcctggagc
tgacctgaccaggaaggctgctgtctccatccctgagcgcctgccacctcccactcctgaagatccatctcttggggc
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tggaggcaagatgccagggcccccaaggttctcagggcaagttcttgggtgtctgcttctcagattccaaggactgga
atataaaccttctcctgggaa
aaaaaaaaaaaaa

Fig. 2. Nucleotide (*upper case letters*) and predicted amino acid sequence (in *bold*) of *BRMS1* cDNA. *BRMS1* cDNA GenBank Accession Number is AF159141. *, termination codon.

were not noticeably or uniformly different from parental cells. Nor were *in vitro* growth rates or saturation densities different. In the experiment shown, *BRMS1*-transfected cells exhibited a slight delay following seeding; however, this did not impact routine culture. *BRMS1* transfectants also tended to aggregate more readily after detachment. However, these properties were not evident in every clone isolated. Surprisingly, 435 cells, but not 231 cells, transfected with *BRMS1* acquired an acute sensitivity to trypsin (data not shown). Whereas parental cells previously were routinely passaged using a mixture of trypsin and EDTA, the *BRMS1* transfectants died when exposed to even low concentrations of trypsin. Therefore, subsequent cultures were handled using EDTA to detach the cells from the substrata.

Clones representing low, medium, and high *BRMS1* mRNA and protein expression (the latter were evaluated using epitope-tagged *BRMS1*) were chosen for *in vivo* functional studies. The transfected cells were then tested for tumorigenicity and metastasis in athymic mice. *BRMS1*-transfected 231 cells were injected i.v. and assessed for their ability to form macroscopic metastases in lung. Compared with vector-only transfectants, *BRMS1* transfectants exhibited a dose-dependent, significant ($P < 0.001$) decrease in metastatic potential (Fig. 3c). Similar conclusions were drawn using mRNA to assess expression (data not shown). This implies that the epitope tag does not deleteriously influence *BRMS1* functionality. As expected, the ability of 231 cells to form progressively growing tumors in the mammary fat pad was not suppressed. Because the parental 231 cell line does not metastasize from an orthotopic site, metastatic suppression using the spontaneous metastasis assay could not be assessed.

Parental and *BRMS1*-transfected MDA-MB-435 cells grew progres-

sively after injection into an orthotopic (*i.e.*, mammary fat pad) site. Growth of *BRMS1*-transfected 435 tumors was somewhat delayed compared with the parental and/or vector controls. In general, once the tumors began to grow, their sizes were ~1 week behind the parental, metastatic populations, suggesting that rate of tumor growth, once established, is the same. Failure to suppress tumorigenicity indicates that *BRMS1* is not a tumor suppressor gene. The histologic appearance of parental 435 and *BRMS1* transfectants were similar, except that the latter exhibited fewer fibrous bands in the stromal compartment of the tumors (data not shown). Whereas tumorigenicity was unaltered, the incidence and number of metastases to lung and regional lymph nodes were significantly ($P = 0.004$) suppressed in the MDA-MB-435 *BRMS1* transfectants (Table 1). Parental and vector-only transfectant cells formed axillary lymph node and lung metastases in 100% of the mice injected, whereas in the *BRMS1* transfectants, the incidence dropped by 50–90%. Of the metastases that formed, all were noticeably smaller than the parental lesions at a comparable time following injection. Even if the metastases were given more time to grow, most did not develop into grossly visible lesions. *BRMS1* expression was still detectable in the *BRMS1*-transfected 435 locally growing tumors. Because parental 435 cells do not form lung metastases following i.v. injection, i.v. inoculation studies like those with 231 were not done.

DISCUSSION

DD-RT-PCR was used to discover genes more highly expressed in metastasis-suppressed neo11/435 than in their metastatic counterparts. *BRMS1*, which maps to a region frequently involved in breast carci-

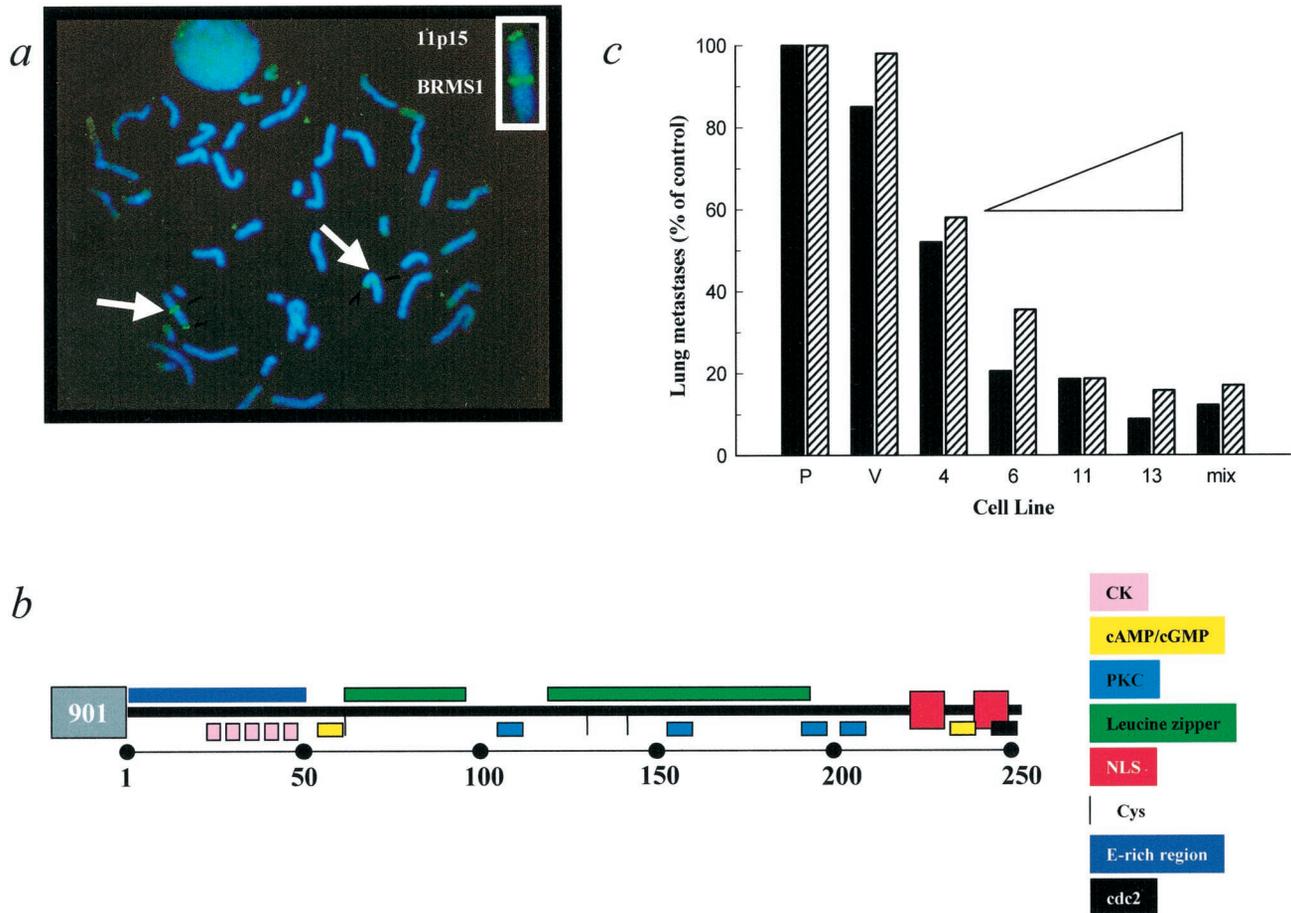


Fig. 3. *a*, The *BRMS1* gene maps to 11q13.1 by fluorescence *in situ* hybridization. Arrows show two chromosome 11 with *BRMS1* staining. An 11p15 probe and *BRMS1* probe are shown on DAPI-counterstained chromosome spread from normal human fibroblasts. *Inset* shows magnified chromosome 11 probed with two markers. *b*, schematic representation of the predicted domains of the *BRMS1* protein. Phosphorylation domains for casein kinase (CK), *cAMP/cGMP*, and protein kinase C (PKC) are shown. Several imperfect *leucine zipper* motifs are located within the regions highlighted. A bipartite nuclear localization signal (NLS) is present at the COOH terminus, and a glutamate-rich region is located near the NH₂ terminus. *c*, *BRMS1* suppresses metastasis of MDA-MB-231 cells. Parental line (P) and pcDNA3 vector-only transfectant (V) are controls for two independent experiments (filled and hatched columns). An uncloned population of *BRMS1*-transfected MDA-MB-231 (mix) is also included in these experiments. Metastasis suppression generally correlated with the level of *BRMS1* mRNA and protein expression (triangle) as determined in independent experiments.

Table 1 *BRMS1* suppresses metastasis of MDA-MB-435

Data are pooled from two independent experiments involving seven to eight mice per group. Data for the native *BRMS1* (*i.e.*, not epitope-tagged) was collected from a single study.

Cell line	Mean tumor diameter ^a (mm)	Incidence of metastases ^b			
		Lung		Extrapulmonary	
		No. mice with metastases/No. mice injected	<i>P</i> < 0.05	No. mice with metastases/No. mice injected	<i>P</i> < 0.05
MDA-MB-435	7.8 ± 0.6	9/15		15/15	
MDA-MB-435/pcDNA3	8.9 ± 1.1	9/13		13/13	
BRMS1.3 ⁹⁰¹ -epitope	6.3 ± 0.3	2/15	***	3/15	***
BRMS1.4 ⁹⁰¹ -epitope	5.6 ± 0.4	5/14		5/14	
BRMS1.6 ⁹⁰¹ -epitope	5.8 ± 0.4	2/15	***	0/15	***
BRMS1.1	4.7 ± 0.3	2/8	***	0/8	***
BRMS1.3	4.7 ± 0.4	1/8	***	3/8	***
BRMS1.5	6.7 ± 0.4	3/7		5/8	

^a Cells (1×10^6) were injected into the axillary mammary pads of 5–6-week-old female athymic mice. To compare relative tumor growth, mean tumor diameter of tumors 42 days after injection is shown. To compensate for delayed tumor growth, tumors were removed when the mean diameter (square root of the product of orthogonal diameters) reached 1.3–1.5 cm. Four weeks later, mice were killed, and the presence of metastases determined.

^b The number and incidence of lung metastases were compared with vector-only transfectant MDA-MB-435 using the Kruskal-Wallis ANOVA followed by Dunn's post-test. Incidence of extrapulmonary metastases (usually ipsilateral axillary lymph nodes, but occasionally ribcage, diaphragm, and chest wall) was similarly examined.

noma progression (*i.e.*, 11q13.1-q13.2) was identified and was expressed 5–10-fold more in the metastasis-suppressed neo11/435 clones than in their metastatic parents. When transfected into MDA-MB-435 and MDA-MB-231 cells, both the incidence of metastasis and the number of lung metastases per mouse were significantly

inhibited compared with controls. Although tumor development in *BRMS1*-transfected 435 cells was slightly delayed compared with controls, tumors still formed and grew at a similar rate. Even when tumor-bearing animals were allowed more time for metastases to grow (*i.e.*, to compensate for the slower growth of the locally growing

tumor), metastasis was suppressed. *BRMS1* mRNA was still detectable within the primary tumors (data not shown). Taken together, these data fulfill the functional definition that *BRMS1* is a metastasis-suppressor gene, *i.e.*, metastasis is suppressed, whereas tumorigenicity is not.

The mechanism by which BRMS1 suppresses metastasis is still not fully determined. *In vitro* assays assessing individual steps in the metastatic cascade predict a complex role for this molecule. The step(s) at which BRMS1 functions are downstream of local invasion: invasive cords are observed at the edge of locally growing tumors. This finding is consistent with the lack of gross changes in the ability of the cells to produce and activate matrix metalloproteinase-2 and -9 as detected by zymography.⁶ Efficient invasion occurs despite a modest but reproducible decrease in motility as measured using *in vitro* wound migration assays. Adhesion to fibronectin, laminin, and type IV collagen are likewise unaffected by BRMS1 expression in 231 and 435 cells.¹²

Analysis of the predicted BRMS1 amino acid sequence hints that BRMS1 interacts with other proteins (*i.e.*, phosphorylation sites, coiled-coil periodicity, and leucine zipper). Because these motifs are often found in components of transcriptional machinery, we hypothesized that BRMS1 might suppress metastasis by regulating expression of other metastasis-suppressor genes; however, there appears to be no correlation between the expression of Kai1, Nm23, KiSS1 or E-cadherin with BRMS1.⁶ Although this hypothesis is not formally disproved, these data argue that it is not the case.

BRMS1 transcript (1.5 kb) was detected in every human tissue examined, albeit at different levels. The uniform size of *BRMS1* transcript argues that it is not alternatively spliced in various tissues. Homology to *BRMS1* DNA was detected using Southern blotting. This suggests that *BRMS1* is relatively well conserved; however, the genomic organization is apparently different (data not shown).

Compared with the many normal tissues examined, *BRMS1* mRNA expression was very low in the 435 and 231 cells by both RT-PCR and poly(A)⁺-enriched mRNA Northern blots. Analysis of protein levels awaits the development of BRMS1-specific antibodies (in progress). An RNA blot composed of a panel of human breast carcinoma cell lines with different malignant properties—MCF10A, MCF7, T47D^{CO}, MDA-MB-435, MDA-MB-231, LCC15, SUM185, SUM1315, and MKL-4 (8–12) was probed with full-length *BRMS1* cDNA to assess expression levels. These cell lines were chosen because all have characteristics that labeled them as “aggressive.” In our hands, however, only 435 and 231 are reproducibly metastatic in athymic mouse models. *BRMS1* mRNA expression was high in LCC15 and MKL-4, but expression was also observed in MCF10A, T47D, SUM185, and SUM1315 cell lines.⁶ Sequencing is underway to determine whether BRMS1 is wild-type or mutant in these cell lines.

In summary, we found a new human breast carcinoma metastasis suppressor gene by DD-RT-PCR comparison of metastatic 435 cells

and metastasis-suppressed neo11/435 cells. The *BRMS1* gene maps to a “hot spot” in breast cancer progression, human chromosome 11q13, further supporting the likelihood that BRMS1 is important in human breast cancer progression toward metastasis. In general, low expression of BRMS1 correlates with the metastatic potential in human breast carcinoma cell lines in nude mice. It will be necessary to further analyze the *BRMS1* gene in other breast carcinomas during various stages of progression. Presently, it is not possible to state whether defects in human breast carcinoma are due to down-regulation of BRMS1, mutation, or both. Such experiments will require collection of matched samples from primary tumors and metastases. Because the most lethal attribute of breast cancer cells is their ability to spread and colonize distant sites, understanding BRMS1 function may help prevent metastasis and improve breast cancer survival.

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