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Oncogenes and Tumor Suppressors

Breast Cancer Metastasis Suppressor-1 Promoter Methylation in Primary Breast Tumors and Corresponding Circulating Tumor Cells

Maria Chimonidou[1], Galatea Kallergi[2], Vassilis Georgoulias[2], Danny R. Welch[3], and Evi S. Lianidou[1]

Abstract

Breast cancer metastasis suppressor-1 (BRMS1) differentially regulates the expression of multiple genes, leading to metastasis suppression without affecting orthotopic tumor growth. For the first time, BRMS1 promoter methylation was evaluated as a prognostic biomarker in primary breast tumors and a subset of corresponding circulating tumor cells (CTC). Formalin-fixed paraffin embedded samples were analyzed for BRMS1 methylation status using methylation-specific PCR in a human specimen cohort consisting of noncancerous tissues, benign fibroadenomas, and primary breast tumors, including some with adjacent noncancerous tissues. Peripheral blood mononuclear cells from a large subset of these patients were fixed in cytospins and analyzed. In addition, BRMS1 expression in cytospins was examined by double-immunofluorescence using anti-BRMS1 and pan-cytokeratin antibodies. BRMS1 promoter methylation was not detected in noncancerous breast tissues or benign fibroadenomas; however, methylation was observed in more than a third of primary breast tumors. Critically, BRMS1 promoter methylation in primary tumors was significantly associated with reduced disease-free survival with a trend toward reduced overall survival. Similarly, a third of cytospin samples were positive for the presence of CTCs, and the total number of detected CTCs was 41. Although a large fraction of CTCs were negative or maintained low expression of BRMS1, promoter methylation was observed in a small fraction of samples, implying that BRMS1 expression in CTCs was either downregulated or heterogeneous. In summary, these data define BRMS1 promoter methylation in primary breast tumors and associated CTCs.

Implications: This study indicates that BRMS1 promoter methylation status has biomarker potential in breast cancer. *Mol Cancer Res; 11(10); 1248–57. ©2013 AACR.*

Introduction

Distant metastasis is the main cause of morbidity and mortality in most patients with cancer and most breast cancer–related deaths occur as a result of treatment failure of metastases (1). Therefore, it is important to better understand the molecular mechanisms related to metastasis and to develop early therapeutic approaches to prevent the dissemination of tumor cells; this will come from better understanding of the metastatic process, including how molecular factors, such as metastasis suppressors, contribute to this process (2). Metastasis suppressors, by definition, inhibit metastasis at any step of the metastatic cascade without blocking primary tumor growth by regulating signaling pathways that inhibit proliferation, cell migration, and growth at the secondary site (3).

The isolation and functional characterization of breast cancer metastasis suppressor-1 (BRMS1), as a novel mediator of metastasis suppression in human breast carcinoma was first described in 2000 (4). This gene encodes for a predominantly nuclear protein that differentially regulates the expression of multiple genes, leading to suppression of metastasis without blocking orthotopic tumor growth. The murine version, Brms1, also suppresses metastasis and exhibits a high level of homology to the human gene (5). By interacting with large chromatin remodeling complexes, BRMS1 regulates chromatin status and therefore modulates the expression of genes functioning in cell apoptosis, cell–cell communication, and cell migration (6–9). In this way, upon

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forced expression in metastatic cells, a nearly complete suppression of metastasis is noted without preventing primary tumor growth (10). In addition, BRMS1 inhibits the activity of NF-κB, a well-known transcription factor that plays significant roles in tumor progression and coordinately regulates the expression of metastasis-associated microRNAs known as metastamirs (11).

In vitro, BRMS1 expression decreased cancer cell survival under stress conditions (hypoxia), increased anoikis, and decreased the ability of cancer cells to adhere (12). Recent results point toward 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 (13). Cook and colleagues have recently shown that cell-type–specific overexpression of Brms1 is important for Brms1-mediated metastasis suppression (14). BRMS1 cellular location is important for its effects as a metastasis suppressor, with nuclear versus cytoplasmic expression associated with invasive and metastatic capacity in a cell-type–specific manner (15, 16).

Circulating tumor cells (CTC) are prognostic in a variety of human cancers and have been proposed as a so-called "liquid biopsy" for follow-up examinations (17). The presence of CTCs in peripheral blood appears to be an early indicator of metastasis and may indicate tumor spread before clinical symptoms or detection by imaging (17). Research on CTCs is gaining attention because they are defined targets for understanding the metastatic process (18). CTC molecular characterization has the potential to provide important information about the cancer cells which could be used to guide individualized targeted treatments (19).

We recently showed that *BRMS1* promoter is methylated in CTCs isolated from peripheral blood from both operable and patients with metastatic breast cancer (20). However, a relationship between the epigenetic silencing of BRMS1 and clinical outcome has not been previously reported. In this study, we aimed to examine the clinical significance of *BRMS1* promoter methylation in early breast cancer, using formalin-fixed, paraffin-embedded (FFPE) and CTCs in patients with long follow-up.

Materials and Methods

The outline of the workflow of our study is shown in Fig. 1.

Clinical samples

We evaluated (i) *BRMS1* promoter methylation by methylation-specific PCR (MSP) in a total number of 118 breast tissue samples and (ii) BRMS1 expression and *BRMS1* promoter methylation in CTC from 39 corresponding peripheral blood cytospin samples.

Primary breast cancer tissues (FFPEs). Eighty-four FFPE tissue samples were available from patients with early breast cancer with a known clinical outcome and a median follow-up of 121 months (range, 58–157). FFPE sections were also available from 5 pairs of breast tumors and their surrounding noncancerous tissues and 14 noncancerous breast tissues (histologically cancer-free specimens from reduction mammoplasty) were used as a control set. Ten

benign fibroadenomas were also included as a separate benign tumor group.

CTC (cytospins). Thirty-nine blood samples obtained before the initiation of adjuvant chemotherapy from the same patients with early breast cancer were analyzed. Peripheral blood (10 mL in EDTA) was drawn from the middle of vein puncture after the first 5 mL of blood were discarded. This precaution was undertaken to avoid contamination of the sample with epithelial cells from the skin during sample collection. Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Hypaque density gradient ($d = 1,077$g/mol) centrifugation at $660 \times g$ for 30 minutes. PBMCs were washed 3 times with PBS and centrifuged at $470 \times g$ for 10 minutes. Aliquots of 250,000 cells were centrifuged at $400 \times g$ for 2 minutes on glass slides (Superfrost Plus). Cytospins were dried up and stored at $-80°C$. Four slides were analyzed from the same blood sample. For all these cytospins, DNA was isolated and *BRMS1* promoter methylation was evaluated by MSP.

All patients signed an informed consent to participate in the study that was approved by the Ethics and Scientific Committees of our Institutions.

DNA isolation from FFPEs

Tissue sections of 10 μm containing more than 80% of tumor cells were used for DNA extraction and MSP (21). The breast cancer cell line MCF-7 was used as positive control in MSP reactions for the detection of *BRMS1* promoter methylation as previously described (20). gDNA from both FFPEs and MCF-7 was isolated with the High Pure PCR Template Preparation Kit (Roche) as previously described (20). DNA concentration was determined in the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies).

Double staining experiments for BRMS1 and pan-cytokeratin A45/B-B3 in CTCs

Control cytospins were first analyzed with confocal laser scanning microscope module (Leica Lasertechnik) and with ARIOL system (Genetix) for the evaluation of immunofluorescence. Consequently, patients samples were analyzed for the expression status of CK and BRMS1 in CTCs using the ARIOL Analysis System (Genetix) as previously described (22–24).

For the evaluation of BRMS1 expression in CTCs, we first carried out control experiments in cytospins prepared with MCF-7 cells spiked in normal PBMCs. We used spiked experiments with normal PBMCs as control because PBMCs would be the internal positive control (baseline expression) in each slide and allowed the quantification of BRMS1 expression in cancer cells with the Ariol System. Consequently, control experiments were carried out in blood samples of 14 patients with CK-positive metastatic breast cancer to identify BRMS1 expression status in patients with high number of CTCs. PBMC cytospins were fixed with 3% paraformaldehyde (PFA) for 30 minutes. Cell membrane permeabilization was conducted with 0.5% Triton for 10 minutes followed by overnight incubation with blocking
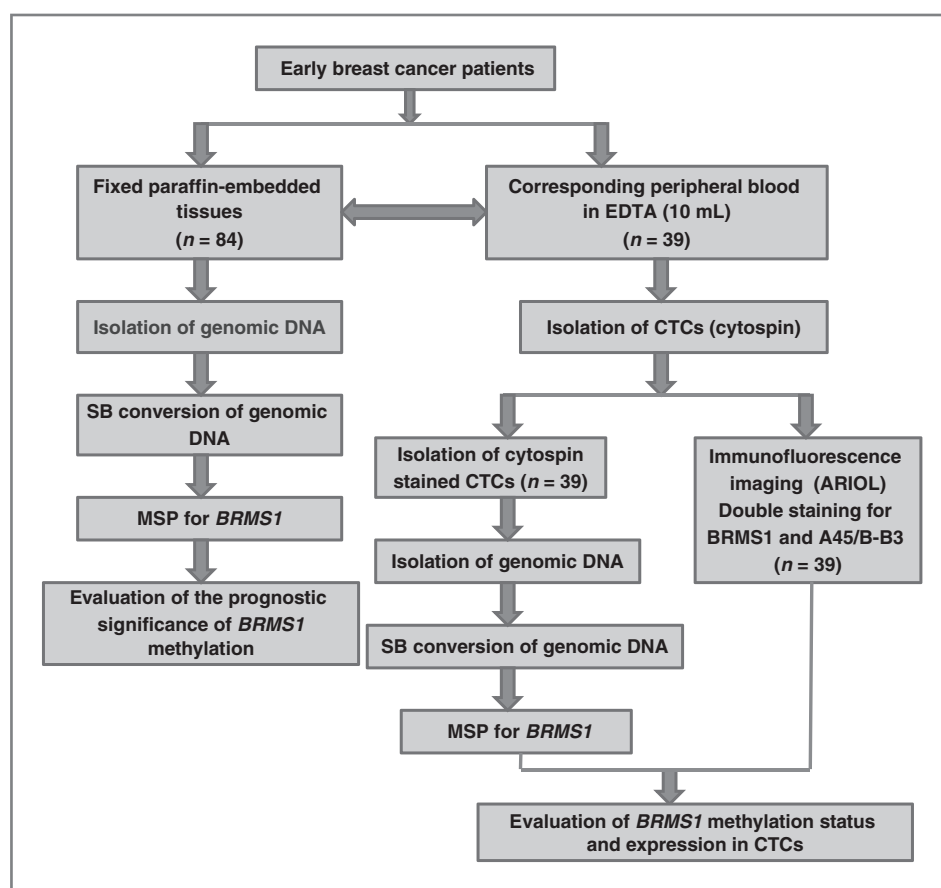
Figure 1. Schematic diagram of the workflow of the study.

buffer [PBS/1% bovine serum albumin (BSA)]. Subsequently, slides were stained with pan-cytokeratin A45-B/B3 (detecting CK8, CK18, and CK19; Micromet) antibody conjugated with Zenon secondary antibody (Invitrogen) and with BRMS1 antibody (25). Zenon antibodies were prepared within 30 minutes before use. Cells were then incubated with the corresponding anti-mouse Alexa555 secondary antibody (Invitrogen) for 45 minutes. Finally, slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) conjugated with antifade (Invitrogen). In each experiment, positive and negative controls (without incubation with the corresponding primary antibody but only with the IgG fluorescence isotype) were prepared.

DNA isolation from cytospin-stained CTC

Initially, to evaluate the efficacy of DNA isolation from cytospin-stained CTCs, we first carried out control experiments using MCF-7 cells immobilized on cytospins as a positive control for the whole process including the first step of isolation of CTCs from glass slides up to the final step of MSP.

To diffuse CTCs from the cytospins, 500 μL of cold PBS was added on the surface of glass slides containing the immobilized CTCs and incubated for 3 to 4 minutes at room temperature. Then, CTCs were removed from the glass slides by scrapping with a plastic tip. The isolated cell pellet in PBS was centrifuged at 530 × g for 10 minutes,

before DNA extraction. CTCs were resuspended in 200 μL PBS and then were used for DNA extraction. gDNA from both CTCs and MCF-7 cells immobilized on cytospins was isolated with the High Pure PCR Template Preparation Kit (Roche) using the protocol for isolation of nucleic acids from mammalian whole blood or cultured cells as described (20). DNA concentration was determined in the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies).

Sodium bisulfite conversion

Samples containing 500 pg to 2 μg of DNA extracted from FFPE and cytospins were modified with sodium bisulfite (SB), to convert all unmethylated but not methylated cytosines to uracil. SB conversion was carried out using the EZ DNA Methylation Gold Kit (Zymo Research Co.), according to the manufacturer's instructions following the short program (20). The converted DNA was stored at −70°C until used. In each SB conversion reaction, dH$_2$O and MCF-7 were included as a negative and positive controls, respectively.

Methylation-specific PCR

BRMS1 promoter methylation was detected by nested MSP by using specific primer pairs for both the methylated and nonmethylated BRMS1 promoter sequences. The primer sets for BRMS1 used in this study (Supplementary Table S1) were first in silico designed using the PrimerPremier 5

software (Premier Biosoft International) and synthesized by FORTHNET (FORTHNET). For MSP, 2 pairs of primers were designed: one specific for SB modified and methylated DNA (M pair) and the other for SB modified and non-methylated DNA (U pair). For maximal discrimination between methylated and nonmethylated alleles, both primers contained several CpGs. In addition, both primer sets contained T bases derived from modified nonmethylated C regions so as to discriminate and amplify the converted from unconverted DNA (26).

Each MSP reaction was conducted in a total volume of 25 µL. One microliter of sodium bisulfite–converted DNA was added into a 24 µL reaction mixture that contained 0.1 µL of Taq DNA polymerase (5 U/L, DNA polymerase; Promega), 2.5 µL of the supplied PCR buffer, 1.0 µL of MgCl$_2$ (50 mmol/L), 0.5 µL of dNTP (10 mmol/L; Fermentas), and 1 µL of the corresponding forward and reverse primers (10 µmol/L); finally, dH$_2$O was added to a final volume of 25 µL. In the first MSP, SB-treated DNA was amplified with a set of external primers specific for the methylated or unmethylated sequences. Nested MSP was conducted using 1 µL of the amplified products and a set of internal primers that are specific for the methylated sequences.

For the MSP reaction using the primer set for the methylated *BRMS1* sequence, thermocycling conditions were: 1 cycle at 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a final extension cycle of 72°C for 10 minutes. Thermocycling conditions were exactly the same for both MSP reactions for the methylated sequence (both outer and inner primer set).

For the MSP reaction using the primer set for the unmethylated *BRMS1* sequence, thermocycling conditions were as follows: 1 cycle at 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute, with a final extension cycle of 72°C for 10 minutes. MSP products were fractionated on 2% agarose gels containing 40 mmol/L Tris-acetate/1.0 mmol/L EDTA (pH 8.0) and visualized by ethidium bromide staining. Human placental gDNA (Sigma-Aldrich) methylated *in vitro* with *Sss*I methylase (NEB) was used, after SB conversion, as a fully methylated (100%) MSP positive control; the same unmethylated placental gDNA was used, after SB conversion, as a negative MSP control.

Statistical analysis

Correlations between *BRMS1* promoter methylation status and the clinicopathologic features were assessed by using the χ^2 test. Disease-free interval (DFI) and overall survival (OS) curves were calculated by using the Kaplan–Meier method and comparisons were conducted using the log rank test. $P < 0.05$ was considered statistically significant. Statistical analysis was conducted using the SPSS Windows version 19.0 (SPSS).

Results

Analytical sensitivity and specificity of *BRMS1* promoter MSP assay

The analytic sensitivity of the developed nested MSP assay for *BRMS1* promoter was evaluated by initially subjecting 1 µg of fully methylated DNA (100%) and 1 µg of fully

Figure 2. A, analytic sensitivity of nested MSP for *BRMS1* using methylated primer set and synthetic mixtures, containing: (1) DNA marker 50 bp, (2) negative control: dH$_2$O, (3) 0%, (4) 0.1%, (5) 1%, (6) 10%, and 7) 50% SB-converted positive control (100% methylated DNA). B, analytical specificity of nested MSP for *BRMS1* using methylated primer set and control samples: (1) DNA marker 50 bp, (2) negative control: dH$_2$O, (3) unconverted DNA, (4) SB-converted placental DNA (0% methylated), (5) SB-converted DNA from the MCF-7 cell line, and (6) 100% methylated DNA. C, nested MSP for *BRMS1* promoter for methylated sequences: (1) DNA marker 50 bp, (2) negative control: dH$_2$O, (3–6) noncancerous breast tissues, (7–15) breast tumors, and (16) MSP-positive control (100% methylated DNA).
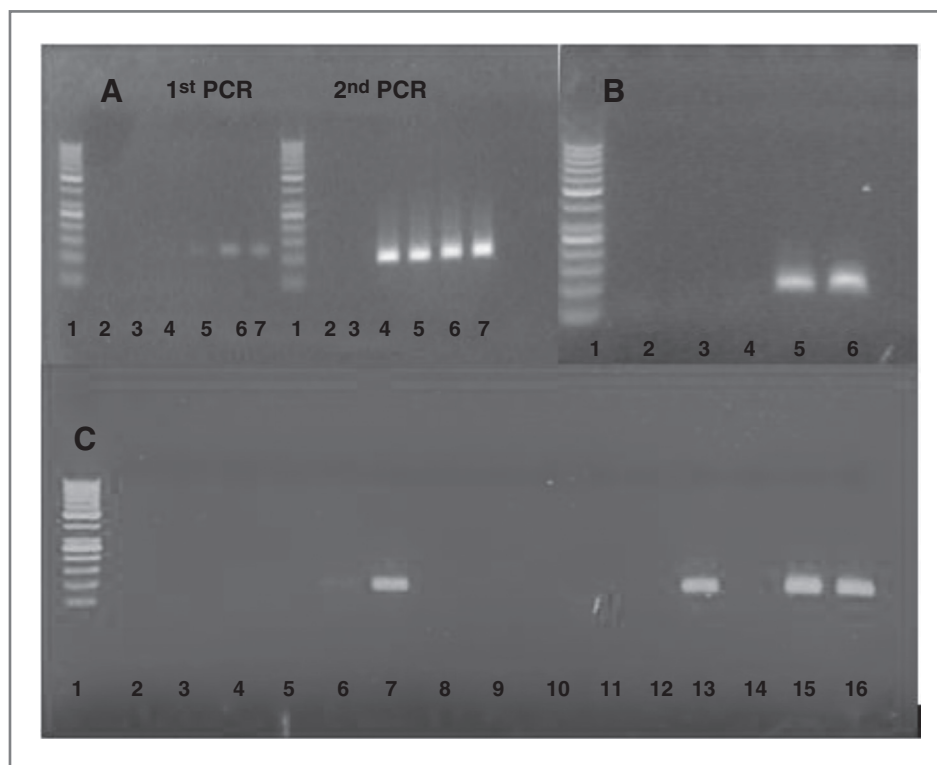
unmethylated (100%) human placental gDNA to SB conversion. Synthetic mixtures based on serial dilutions of these SB-converted DNA samples were prepared containing various percentages of methylation (0.1%, 1%, 10%, and 50%), and 1 μL of these samples was used in the MSP reaction. As can be seen in Fig. 2A, by using nested MSP, we could detect methylated *BRMS1* promoter sequences with a sensitivity of 0.1%, in the presence of 99.9% unmethylated *BRMS1* promoter sequences.

To validate the analytic specificity of *BRMS1*-nested MSP, methylated primers were initially tested *in silico* and then in PCR, using SB-modified human placental gDNA that was not methylated (negative control), unconverted DNA, DNA extracted from the MCF-7 cell line, and our positive control (100% methylated DNA). As can be seen in Fig. 2B, no amplification of *BRMS1* promoter could be observed in the first 2 controls, while both MCF-7 cells and our positive control gave the expected bands. The specificity of *BRMS1* promoter methylation was further confirmed by conducting nested MSP in FFPEs obtained from 5 pairs of breast tumors and their surrounding noncancerous tissues, 14 non-cancerous breast tissues (histologically cancer-free specimens from reduction mammoplasty), and 10 benign breast tumors (fibroadenomas), that were also included as a separate benign tumor group.

MSP with primers specific for the unmethylated DNA was also conducted for all SB-converted samples to exclude false-negative cases, for example, negative MSP reactions (specific for the methylated DNA sequences) that could be due to bad quality of DNA. By using this quality control approach, *BRMS1* promoter was found to be nonmethylated in all these noncancerous tissues.

BRMS1 methylation in DNA isolated from primary breast tumors

Using the above described highly specific and sensitive nested MSP assay, we examined *BRMS1* promoter methylation in 84 operable breast cancer FFPEs. Methylation of *BRMS1* promoter was observed in 0 of 19 (0%) noncancerous breast tissues, in 0 of 10 (0%) fibroadenomas, and in 31 of 84 (36.9%) breast tumors. *BRMS1* methylation status in the primary tumors in respect to the clinical characteristics of the patients are shown in Table 1. As can be seen in Table 1, there was no correlation between *BRMS1* methylation and tumor size, number of lymph nodes, tumor grade, tumor stage, the presence of progesterone (PR) and estrogen receptors (ER), HER2 status, and age.

### *BRMS1* promoter methylation and BRMS1 expression in CTC

Cytospins were available for 39 of 84 patients for which the primary tumor was analyzed for *BRMS1* promoter methylation. Thirteen samples (33.3%) were positive for the presence of CTCs and the total number of the detected CTC was 41. In these cytospins, double immunofluorescence staining experiments for BRMS1 and pan-cytokeratin A45/B-B3 were conducted.

Table 1. Clinical characteristics of patients with early breast cancer in respect to *BRMS1* methylation status in FFPEs ($n = 84$)

Clinicopathologic features	Number of patients	*BRMS1* methylation (%)	*P* (χ-test)
Tumor size, cm			
0–2.0	13	3	0.364
>2	69	26	
Unknown	2		
Number of lymph nodes			
0	24	10	
1–3	24	7	0.711
4–9	21	9	
>9	10	3	
Unknown	5		
Histologic grade			
I–II	38	15	
III	36	11	0.472
Unknown	10		
Tumor stage			
I	21	10	
II	55	20	0.436
Unknown	8		
PR			
Positive	26	11	0.455
Negative	53	17	
Unknown	5		
ER			
Positive	46	18	
Negative	32	9	0.344
Unknown	6		
HER2 Status			
Positive	7	3	0.492
Negative	57	20	
Unknown	20		
Age, y			
<54	43	17	
>54	40	13	0.331
Unknown	1		

All detected CTCs were pan-cytokeratin A45/B-B3–positive and BRMS1 expression levels was differentiated as: (i) high when BRMS1 expression was higher or equal to the average expression in normal PBMCs, (ii) low when BRMS1 expression was lower than the average expression in normal PBMCs, and (iii) negative (no expression; Fig. 3A–C). As shown in Table 2, CTCs with high BRMS1 expression were found in 7 of 13 (53.8%) patients, whereas 3 of 13 (23.0%) patients were found with exclusively high BRMS1 expression in CTCs. CTCs with low BRMS1 expression were found in 3 of 13 (23.0%) patients, whereas CTCs negative for BRMS1 were found in 8 of 13 (61.5%) patients. Exclusively CTCs with low or negative BRMS1 expression were identified in 6 of 13

Figure 3. BRMS1 expression in cytospin-stained CTCs of patients with early breast cancer. Cells were stained with A45-B/B3 antibody (green), BRMS1 antibody (red), and DAPI (blue). Quantification of the samples was conducted with the ARIOL system and scored as (A) BRMS1 high expression, (B) BRMS1 low expression, (C) negative for BRMS1. D, heatmap for BRMS1 expression and matched DNA samples for *BRMS1* promoter methylation in cytospin-stained CTCs (*n* = 39): black: no CTC cells, red: no BRMS1 expression at top row, *BRMS1* methylation at bottom row, green: normal BRMS1 expression at top row, *BRMS1* nonmethylated at bottom row, brown: low BRMS1 expression.
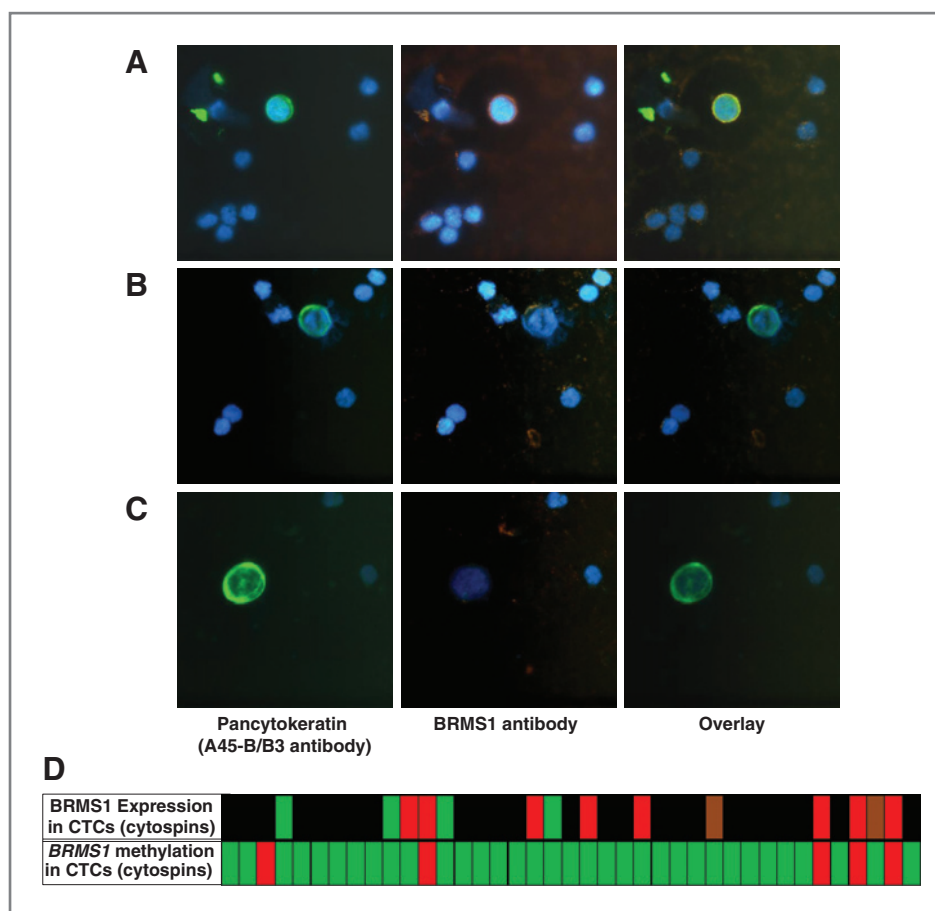
(46.1%) patients. Four of 13 (30.8%) patients had both high and low or negative BRMS1 expression in their CTC. Only 8 of 41 (19.5%) of the total analyzed CTC had high BRMS1 expression in patients with early breast cancer, whereas the majority of the observed CTC (33 of 41; 80.5%) had low or negative expression for BRMS1.

Using nested MSP, we examined *BRMS1* promoter methylation in DNA isolated from these identical cytospin CTC samples which were also tested for nonmethylated *BRMS1* to check their DNA quality. As shown in Table 2, *BRMS1* promoter methylation was observed in 5 of 39 (12.8%) samples, whereas in the remaining samples, *BRMS1* nonmethylated sequences were observed. This was expected, as cytospins also included PBMCs. By immunofluorescence, BRMS1 protein was not expressed in 8 samples, whereas 4 of 5 samples that were found positive in MSP for *BRMS1* promoter methylation did not express BRMS1. In 2 samples where BRMS1 was expressed at a very low level, *BRMS1* promoter methylation was not observed, whereas 4 CTC samples had no BRMS1 expression or promoter methylation. In Fig. 3D, a heat map showing the expression of BRMS1 and *BRMS1* promoter methylation in CTCs isolated from the same cytospins is presented.

Disease relapse and disease-free survival

After a median follow-up of 121 months (range, 58–157), 27 of 84 (32.1%) patients relapsed and *BRMS1* methylation was detected in 15 of 27 (55.6%) of these patients. The incidence of relapses was significantly higher in patients with methylated (15 of 31; 48.4%) than in patients with nonmethylated *BRMS1* promoter (12 of 53; 22.6%). Even using these limited cases, the diagnostic sensitivity of *BRMS1* methylation for prediction of relapses was estimated as 55.6% (15/27) and the diagnostic specificity was 71.9% (41/57), respectively. It is interesting to note that the Kaplan–Meier estimates of the cumulative DFI for patients with methylated and nonmethylated *BRMS1* promoter were significantly different in favor of patients with nonmethylated *BRMS1* promoter ($P = 0.009$; Fig. 4A).

Overall survival

During the follow-up period, 19 of 84 (22.6%) patients died as a consequence of disease progression and *BRMS1* methylation was detected in 10 of 19 (52.6%) of these patients. The incidence of deaths was higher in patients with methylated *BRMS1* promoter (10 of 31; 31.3%) than in patients with nonmethylated *BRMS1* promoter (9 of 53; 17.0%). Diagnostic sensitivity of *BRMS1*

Table 2. BRMS1 expression and *BRMS1* methylation status in CTCs as evaluated in cytospins (*n* = 39) corresponding to FFPEs, in relation to the clinical outcome of patients with early breast cancer

| Patients | Patient ID | Numbers of CTCs in cytospins | | | | CTC (cytospin) | Primary tumor |
		(CK$^+$, CD45$^-$, DAPI$^+$)	BRMS1-negative	BRMS1+++	BRMS1 low+	*BRMS1* methylated	*BRMS1* methylated
1	175	0	0	0	0	−	+
2	106	0	0	0	0	−	−
3	92	0	0	0	0	+	−
4	501	1	0	1	0	−	−
5	365	0	0	0	0	−	−
6	587	0	0	0	0	−	+
7	439	0	0	0	0	−	+
8	309	0	0	0	0	−	−
9	567	0	0	0	0	−	−
10	341	1	0	1	0	−	−
11	56	12	11	0	1	−	−
12	308	2	1	1	0	+	−
13	394	1	0	1	0	−	+
14	443	0	0	0	0	−	−
15	358	0	0	0	0	−	−
16	472	0	0	0	0	−	−
17	479	0	0	0	0	−	−
18	356	1	1	0	0	−	−
19	452	0	0	0	0	−	+
20	450	0	0	0	0	−	+
21	432	2	1	1	0	−	+
22	340	0	0	0	0	−	−
23	55	0	0	0	0	−	+
24	284	4	4	0	0	−	−
25	198	0	0	0	0	−	+
26	369	0	0	0	0	−	+
27	473	0	0	0	0	−	−
28	23	10	0	0	10	−	−
29	520	0	0	0	0	−	−
30	477	0	0	0	0	−	+
31	232	0	0	0	0	−	−
32	363	0	0	0	0	−	−
33	376	0	0	0	0	−	−
34	429	1	1	0	0	+	+
35	255	0	0	0	0	−	−
36	357	2	1	1	0	+	−
37	543	2	0	1	1	−	−
38	368	1	1	0	0	+	−
39	345	0	0	0	0	−	−

methylation for prediction of deaths was estimated as 52.6% (10 of 19) and the diagnostic specificity as 66.7% (44 of 66), respectively. The Kaplan–Meier estimates of the cumulative OS for patients with methylated and nonmethylated *BRMS1* promoter were not significantly different in favor of patients with nonmethylated *BRMS1* promoter, however, there was a trend (*P* = 0.071; Fig. 4B).

Discussion

We examined for the first time the relationship between epigenetic silencing of *BRMS1* and clinical outcome in operable breast cancers and evaluated the expression of BRMS1 protein and *BRMS1* methylation status in CTCs using a highly sensitive and specific MSP assay for *BRMS1* promoter methylation (20). *BRMS1* promoter methylation was detected only in primary breast tumors but never

Figure 4. A, Kaplan–Meier estimates of disease-free interval (DFI) for patients with early breast cancer with (green) or without (blue) *BRMS1* promoter methylation (*P* = 0.009). B, Kaplan–Meier estimates of overall survival (OS) for patients with early breast cancer with (green) or without (blue) *BRMS1* promoter methylation (*P* = 0.071).
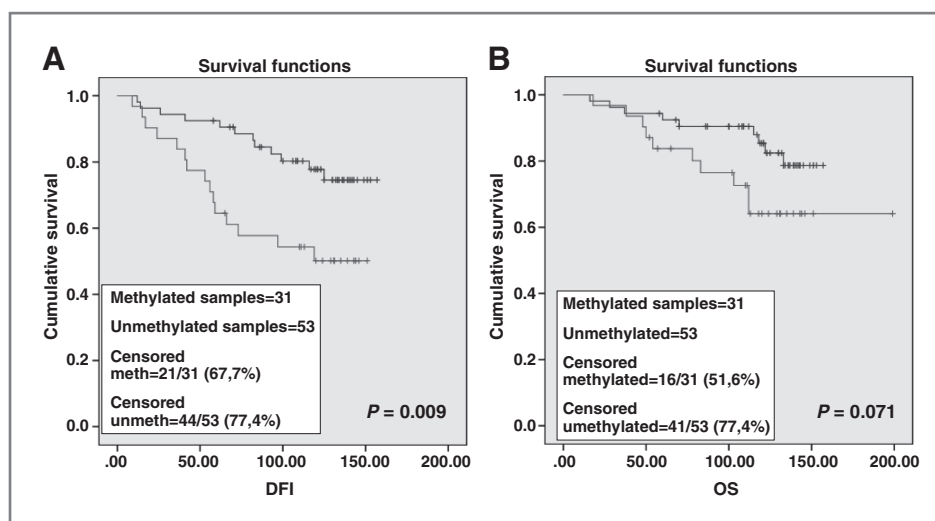
in normal breast or benign breast disease. *BRMS1* promoter methylation in the primary tumor predicted poorer disease-free survival. We could not analyze these primary tumors both for BRMS1 protein and *BRMS1* promoter methylation, as the amount of available sample was very limited. For this reason, we preferred to conduct *BRMS1* methylation analysis in these samples, as the prognostic significance of *BRMS1* promoter methylation has not been shown till now.

About half of the patients from which FFPE samples were used had corresponding available peripheral blood samples that were used to isolate pan-cytokeratin–positive CTCs, which were fixed in cytospins. We have assessed both BRMS1 expression and *BRMS1* methylation status in these identical cytospins, so that there would be no bias in CTC isolation. More specifically, we first evaluated BRMS1 protein expression status by immunofluorescence in the Ariol System, by using BRMS1-specific Ab, and then we used the same cytospins to detach all cells and extract their DNA. It was this DNA sample that was further used in MSP reactions to evaluate for *BRMS1* promoter methylation. BRMS1 expression in CTCs was highly heterogeneous between patients and even in the same patient. This was expected as heterogeneity of CTCs has already been reported for many other markers in many studies up to now mainly at the gene expression level (27–31).

Besides this observation, it is interesting that the majority of the analyzed CTC (80.5%) was negative for BRMS1 or maintain low expression, as quantified with the Ariol system, implying that BRMS1 is downregulated in these cells. This assumption was confirmed by the fact that 4 of 5 patients, who had methylated *BRMS1* promoter, were negative for BRMS1 expression in their CTCs. Furthermore, the number of patients who displayed CTCs with exclusively high expression of BRMS1 (comparable to PBMCs level) was rather low (28.6%). This high BRMS1 expression in CTCs could be related to a good prognosis group of patients, nevertheless, due to the small number of available samples, this remains to be explored in the future.

Zhang and colleagues provided evidence to support the notion that *BRMS1* is a breast carcinoma metastasis suppressor gene, suggesting that BRMS1 expression will help to identify those patients with breast cancer with worse disease-free survival (32). More specifically, they reported that patients with breast cancer with high levels of expression of *BRMS1* mRNA have a better prognosis than those with low expression and that *BRMS1* mRNA is an independent prognostic factor for disease-free survival in breast cancer (32). Hicks and colleagues showed for the first time a significant correlation between loss of BRMS1 protein expression and reduced disease-free survival when patients with breast cancer were stratified by either loss of ER or PR or HER2 overexpression suggesting a mechanistic relationship between BRMS1 expression, hormone receptor status, and HER2 growth factor (33). Our results confirm these findings if we take into account that the *BRMS1* promoter methylation is indicating a lower expression of *BRMS1* mRNA.

The clinical significance of BRMS1 has been very recently evaluated in several other malignancies as well, mainly at the protein level. Low expression of the metastasis suppressor BRMS1 was recently shown to be an independent prognostic factor for poor prognosis both for metastasis-free survival and overall survival in patients with nasopharyngeal carcinoma (34). In a recent study, Wu and colleagues found that the expression level of BRMS1 was significantly downregulated in hepatocellular carcinoma (HCC) tissues and that BRMS1 sensitizes HCC cells to apoptosis (35).

Nagji and colleagues experimentally verified that methylation contributes to BRMS1 transcriptional repression (36). They cloned the *BRMS1* promoter region, including the promoter-associated CGI, into a luciferase reporter gene and found that *BRMS1* promoter activity was dramatically inhibited under methylated conditions. They showed that nuclear BRMS1 expression is reduced in lung cancer specimens compared with normal bronchial epithelium and found that pathological tumor stage was associated with increased *BRMS1* methylation in squamous cell cancers (36). Moreover, Yang and colleagues analyzed associations

between the methylation status of *BRMS1* in patients with non–small cell lung cancer (NSCLC) separately and available epidemiologic and clinical information including smoking status, gender, age, and histologic type, or the stage of the tumor. Their results provide clinical evidence to support the notion that *BRMS1* is an NSCLC metastasis suppressor gene. Measuring methylation status of *BRMS1* promoter is a useful marker for identifying patients with NSCLCs with worse disease-free survival (37). Very recently, detailed quantitative analysis of the metastatic process in lung showed that BRMS1 expression significantly reduced the numbers of solitary single cells that survive after initial arrest within the lung microvasculature and also inhibited the initiation of growth subsequent to arrest (38).

BRMS1 may play a critical role in promoting migration, invasion, and angiogenesis of ovarian cancer cells as well. Sheng and colleagues recently investigated the mechanisms of BRMS1 involvement in ovarian cancer metastasis and they suggest that BRMS1 may regulate the metastatic potential, at least in part, through upregulation of CXCR4 via NF-κB activation (39).

In conclusion, our data show that *BRMS1* promoter methylation results in the transcriptional repression of this gene and highlight the potential clinical relevance of this methylation event in operable breast cancer. We show that *BRMS1* promoter is methylated in primary tumors of patients with early breast cancer and in corresponding CTC samples but not in noncancerous breast tissues. *BRMS1* promoter methylation and BRMS1 protein expression was evaluated in identical CTCs from the same patients for the first time. According to our results, CTCs were highly heterogeneous in respect to BRMS1 expression even in the same patient. Furthermore, CTC-expressing epithelial markers but no BRMS1 are identified in patients with breast cancer and probably seem to identify patients with worse prognosis. According to our results, *BRMS1* promoter methylation provides important prognostic information for disease-free survival in early breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Chimonidou, V. Georgoulias, D.R. Welch, E.S. Lianidou
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Chimonidou, G. Kallergi, D.R. Welch, E.S. Lianidou
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