Breast Cancer Metastasis Suppressor-1 promoter methylation in primary breast
tumors and corresponding Circulating Tumor Cells

Running title: BRMS1 promoter methylation in breast cancer

Maria Chimonidou1+, Galatea Kallergi2+, Vasilis Georgoulias2, Danny R. Welch3 and
Evi S. Lianidou1

1) Analysis of Circulating Tumor Cells lab, Lab of Analytical Chemistry, Department
of Chemistry, University of Athens, 15771, Greece.
2) Laboratory of Tumor Cell Biology, Medical School, University of Crete,
Heraklion, Greece.
3) Department of Cancer Biology and the University of Kansas Cancer Center, The
Kansas University Medical Center, Kansas City, KS, 66160, USA
+: these authors contributed equally to this work

Correspondence should be addressed to:
Dr. E.S. Lianidou, Laboratory of Analytical Chemistry,
Department of Chemistry, University of Athens, 15771, Greece
Tel: ++ 30 210 7274319, Fax: ++ 30 210 7274750, E-mail: lianidou@chem.uoa.gr

Keywords: Breast Cancer Metastasis Suppressor gene-1, BRMS1, circulating tumor
cells, CTC, DNA methylation, operable breast cancer

Conflicts of interest statement: There are no conflicts to disclose for all authors
Word count (excluding references): 4079

Total number of figures and tables: 6
ABSTRACT

Breast Cancer Metastasis Suppressor-1 differentially regulates expression of multiple genes, leading to metastasis suppression without affecting orthotopic tumor growth. We evaluated BRMS1 promoter methylation as prognostic biomarker in primary breast tumors and studied BRMS1 promoter methylation in a subset of corresponding Circulating Tumor Cells (CTC) for the first time. We analyzed 118 formalin-fixed paraffin embedded samples: 5 pairs of breast tumors and adjacent non-cancerous tissues, 14 non-cancerous tissues, 10 benign fibroadenomas, and 84 primary breast tumors. Peripheral blood mononuclear cells from 39/84 of these patients were fixed in cytopsins. BRMS1 methylation status was investigated in all FFPE and cytopsin stained CTC using methylation specific PCR. BRMS1 expression in cytopsins was examined by double-immunofluorescence using anti-BRMS1 and pancytokeratin A45-B/B3 antibodies. BRMS1 promoter methylation was not observed in noncancerous breast tissues (0%), and benign fibroadenomas (0%), while it was observed in 36.9% of primary breast tumors. BRMS1 promoter methylation in primary tumors was associated with reduced disease-free interval (P=0.009) while a trend towards a reduced overall survival was also observed (P=0.071). 13/39 cytopsin samples (33.3%) were positive for the presence of CTC and the total number of the detected CTC was 41. Most CTC (80.5%) were negative for BRMS1 or maintained low expression, implying that BRMS1 is down regulated in these cells. BRMS1 promoter methylation was observed in 5/39 (12.8%) samples. BRMS1 promoter methylation in primary breast tumors provides prognostic information for DFS. BRMS1 expression in CTC was highly heterogeneous, between patients and even in the same patient.
INTRODUCTION

Distant metastasis is the main cause of morbidity and mortality in most cancer patients 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 in order 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 remodelling 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 forced expression in metastatic cells, a nearly complete suppression of metastasis is noted without preventing primary tumor growth [10]. Additionally, BRMS1 inhibits the activity of NFκB, 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 et al have recently shown that cell type-specific over-expression of Brms1 is important for Brms1-mediated metastasis suppression [14]. BRMS1 cellular location is important for its effects as a metastasis suppressor, with nuclear vs. 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 CTC in peripheral blood appears to be an early indicator of metastasis and may indicate tumor spread prior to clinical symptoms or detection by imaging [17]. Research on CTC is gaining attention because they are defined targets for understanding the metastatic process [18]. CTC molecular characterization has the potential to provide important information regarding the cancer cells which could be utilized to guide individualized targeted treatments [19].

We recently showed that BRMS1 promoter is methylated in CTC isolated from peripheral blood from both operable and metastatic breast cancer patients [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 FFPE and CTC in patients with long follow-up.

MATERIALS AND METHODS

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

Clinical samples

We evaluated: a) BRMS1 promoter methylation by methylation specific PCR in a total number of 118 breast tissue samples b) BRMS1 expression and BRMS1 promoter methylation in CTC from 39 corresponding peripheral blood cytospin samples. More specifically:

a) primary breast cancer tissues (FFPEs): 84 formalin fixed paraffin-embedded tissue samples (FFPEs) 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 non-cancerous tissues and 14 non-cancerous breast tissues (histologically cancer-free specimens from reduction mammoplasty) were used as a control set. 10 benign fibroadenomas, were also included as a separate benign tumor group.

b) CTC (cytospins): 39 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 in order to avoid contamination of the sample with epithelial cells from the skin during sample collection. PBMC were isolated with Ficoll-Hypaque density gradient (d=1.077g/mol) centrifugation at 660g for 30min. PBMC were washed three times with PBS and centrifuged at 470g for 10min. Aliquots of 250,000 cells were centrifuged at 400g for 2 min 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 methylation specific PCR.

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 >80% of tumor cells were used for DNA extraction and methylation-specific PCR (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]. Genomic DNA (gDNA) from both FFPEs and MCF-7 was isolated with the High Pure PCR Template Preparation kit (Roche, Germany) as previously described [20]. DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

Double staining experiments for BRMS1 and pan-cytokeratin A45/B-B3 in CTC
Control cytospins were first analysed with confocal laser scanning microscope module (Leica Lasertechnik, Heidelberg, Germany) and with ARIOL system (Genetix, New Milton, UK) for the evaluation of immunofluorescence. Consequently patients samples were analysed for the expression status of CK and BRMS1 in CTC using the ARIOL analysis system (Genetix, New Milton, UK) as previously described [22-24].

For the evaluation of BRMS1 expression in CTC we first performed control experiments in cytospins prepared with MCF-7 cells spiked in normal PBMC. 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 performed in blood samples of 14 CK-positive metastatic breast cancer patients in order to identify BRMS1 expression status in patients with high number of CTC. PBMC cytospins were fixed with 3% paraformaldehyde (PFA) for 30 min. Cell membrane permeabilization was performed with 0.5% Triton for 10 min followed by overnight incubation with blocking buffer (PBS/1%BSA). Subsequently, slides were stained with pancytokeratin A45-B/B3 (detecting CK8, CK18 and CK19) (Micromet Munich, Germany) antibody conjugated with Zenon secondary antibody (Invitrogen, Carlsbad, CA, USA) and with BRMS1 antibody [25]. Zenon antibodies were prepared within 30 min before use. Cells were then incubated with the corresponding anti-mouse Alexa555 secondary antibody (Invitrogen, Carlsbad, CA, USA) for 45 min. Finally, slides were stained with DAPI conjugated with antifade (Invitrogen, Carlsbad, CA, USA). 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, in order to evaluate the efficacy of DNA isolation from cytospin stained CTC we first performed control experiments using MCF-7 cells immobilized on cytospins as a positive control for the whole process including the first step of isolation of CTC from glass slides up to the final step of methylation specific PCR.

In order to diffuse CTC from the cytospins, 500 μL of cold PBS were added on the surface of glass slides containing the immobilized CTC and incubated for 3-4 min at room temperature. Then, CTC were removed from the glass slides by scrapping with a plastic tip. The isolated cell pellet in PBS was centrifuged at 530g for 10 min, prior to DNA extraction. CTC were resuspended in 200 μL PBS and then were used for DNA extraction. Genomic DNA (gDNA) from both CTC and MCF-7 cells immobilized on cytospins was isolated with the High Pure PCR Template Preparation kit (Roche, Germany) 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, USA).

**Sodium bisulfite conversion**
Samples containing 500 pg-2 μg of DNA extracted from FFPE and cytospins were modified with sodium bisulfite (SB), in order 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., Orange, CA), 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, dH2O and MCF-7 were included as a negative and positive control, respectively.

**Methylation-specific PCR**

*BRMS1* promoter methylation was detected by nested MSP by using specific primer pairs for both the methylated and non-methylated *BRMS1* promoter sequences. The primer sets for *BRMS1* used in this study (Supplementary Table 1) were first *in silico* designed, using the PrimerPremier 5 software (Premier Biosoft International, USA) and synthesized by FORTHNET (FORTHNET, Heraklion, Greece). For MSP, two 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 non-methylated alleles, both primers contained several CpGs. Additionally, both primer sets contained T bases derived from modified non-methylated C regions so as to discriminate and amplify the converted from unconverted DNA [26].

Each MSP reaction was performed 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 (5U/L, DNA polymerase; Promega), 2.5μL
of the supplied PCR buffer, 1.0 μL of MgCl₂ (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 dH₂O 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 performed 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 min, followed by 30 cycles of 94°C for 30s, 60°C for 1min and 72°C for 1min, with a final extension cycle of 72°C for 10min. 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 min, followed by 30 cycles of 94°C for 30s, 55°C for 1min and 72°C for 1 min, with a final extension cycle of 72°C for 10 min. MSP products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. Human placental genomic DNA (gDNA; Sigma-Aldrich) methylated in vitro with SsSI methylase (NEB, Ipswich, MA) 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 clinicopathological features were assessed by using the Chi-square test. Disease-free interval (DFI) and overall survival (OS) curves were calculated by using the Kaplan-Meier method and comparisons were performed using the log rank test. P-values <0.05 were considered statistically significant. Statistical analysis was performed using the SPSS Windows version 19.0 (SPSS, Chicago, IL).

RESULTS

Analytical sensitivity and specificity of $BRMS1$ promoter MSP assay

The analytical sensitivity of the developed nested MSP assay for $BRMS1$ promoter was evaluated by initially subjecting 1 $\mu$g of fully methylated DNA (100%) and 1 $\mu$g of fully unmethylated (100%) human placental genomic DNA 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 $\mu$L of these samples were used in the MSP reaction. As can be seen in Figure 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 analytical 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 Figure 2B no amplification of BRMS1 promoter could be observed in the first two controls, while both MCF-7 cells and our positive control gave the expected bands; The specificity of BRMS1 promoter methylation was further confirmed by performing nested MSP in FFPEs obtained from 5 pairs of breast tumors and their surrounding non-cancerous 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 performed for all SB converted samples to exclude false negative cases, e.g., 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 non-methylated in all these non-cancerous 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 0/19 (0%) noncancerous breast tissues, in 0/10 (0%) fibroadenomas and in 31/84 (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 and estrogen receptors, 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 CTC and the total number of the detected CTC was 41. In these cytospins double immunofluorescence staining experiments for BRMS1 and Pancytokeratin A45/B-B3 were performed.

All detected CTC were Pancytokeratin A45/B-B3 positive and BRMS1 expression levels was differentiated as: a) high when BRMS1 expression was higher or equal to the average expression in normal PBMC, b) low when BRMS1 expression was lower than the average expression in normal PBMC and c) negative (no expression) (Figure 3A, 3B, 3C). As shown in Table 2, CTC with high BRMS1 expression were found in 7/13 (53.8%) patients while 3/13 (23.0%) patients were found with exclusively high BRMS1 expression in CTC. CTC with low BRMS1 expression were found in 3/13 (23.0%) patients, while CTC negative for BRMS1 were found in 8/13 (61.5%) patients. Exclusively CTC with low or negative BRMS1-expression were identified in 6/13 (46.1%) patients. Four of 13 (30.8%) patients had both high and low or negative BRMS1 expression in their CTC. Only 8/41 (19.5 %) of the total analysed CTC had high BRMS1 expression in early breast cancer patients, while the majority of the observed CTC 33/41 (80.5%) had low or negative expression for BRMS1.

Using nested MSP, we examined **BRMS1** promoter methylation in DNA isolated from these identical cyto spin CTC samples which were also tested for non-methylated **BRMS1** in order to check their DNA quality. As shown in Table 2, **BRMS1** promoter
methylation was observed in 5/39 (12.8%) samples, while in the remaining samples
BRMSI non-methylated sequences were observed. This was expected, since cytospins
also included PBMC. By immunofluorescence, BRMS1 protein was not expressed in
eight samples, while 4/5 samples that were found positive in MSP for BRMS1
promoter methylation did not express BRMS1. In two samples where BRMS1 was
expressed at a very low level, BRMSI promoter methylation was not observed, while
four CTC samples had no BRMS1 expression or promoter methylation. In Figure 3D
a heat map showing the expression of BRMS1 and BRMSI promoter methylation in
CTC isolated from the same cytospins is presented.

Disease relapse and disease-free survival

After a median follow-up of 121 months (range 58-157), 27/84 (32.1%) patients
relapsed and BRMSI methylation was detected in 15/27 (55.6%) of these patients. The
incidence of relapses was significantly higher in patients with methylated
(15/31=48.4%) than in patients with non-methylated BRMSI promoter
(12/53=22.6%). Even using these limited cases, the diagnostic sensitivity of BRMSI
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 non-
methylated BRMSI promoter were significantly different in favor of patients with
non-methylated BRMSI promoter (p=0.009) (Figure 4A).

Overall survival
During the follow-up period, 19/84 (22.6%) patients died as a consequence of disease progression and BRMS1 methylation was detected in 10/19 (52.6%) of these patients. The incidence of deaths was higher in patients with methylated BRMS1 promoter (10/31=31.3%) than in patients with non-methylated BRMS1 promoter (9/53=17.0%). Diagnostic sensitivity of BRMS1 methylation for prediction of deaths was estimated as 52.6% (10/19) and the diagnostic specificity as 66.7% (44/66) respectively. The Kaplan–Meier estimates of the cumulative OS for patients with methylated and non-methylated BRMS1 promoter were not significantly different in favor of patients with non-methylated BRMS1 promoter however there was a trend (p= 0.071; Figure 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 CTC 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 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, since the amount of available sample was very limited. For this reason, we preferred to perform BRMS1 methylation analysis in these samples, since 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 pancytokeratin positive-CTC, which were fixed in cytopsins. We have assessed both BRMS1 expression and \textit{BRMS1} methylation status in these identical cytopsins, 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 cytopsins to detach all cells and extract their DNA. It was this DNA sample that was further used in MSP reactions to evaluate for \textit{BRMS1} promoter methylation. BRMS1 expression in CTC 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 analysed CTC (80.5\%) were negative for BRMS1 or maintain low expression, as quantified with the Ariol system, implying that BRMS1 is down regulated in these cells. This assumption was confirmed by the fact that four out of five patients, who had methylated \textit{BRMS1} promoter, were negative for BRMS1 expression in their CTC. Furthermore, the number of patients who displayed CTC with exclusively high expression of BRMS1 (comparable to PBMCs level) was rather low (28.6\%). This high BRMS1 expression in CTC 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 et al provided evidence to support the notion that \textit{BRMS1} is a breast carcinoma metastasis suppressor gene, suggesting that BRMS1 expression will help to identify
those breast cancer patients with worse disease-free survival [32]. More specifically, they reported that breast cancer patients with high levels of expression of \textit{BRMS1} mRNA have a better prognosis than those with low expression and that \textit{BRMS1} mRNA is an independent prognostic factor for disease-free survival in breast cancer [32]. Hicks et al. showed, for the first time, a significant correlation between loss of BRMS1 protein expression and reduced disease-free survival when breast cancer patients 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 \textit{BRMS1} promoter methylation is indicating a lower expression of \textit{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 nasopharyngeal carcinoma patients [34]. In a recent study, Wu et al found that the expression level of BRMS1 was significantly down-regulated in hepatocellular carcinoma (HCC) tissues and that BRMS1 sensitizes HCC cells to apoptosis [35].

Nagji et al experimentally verified that methylation contributes to BRMS1 transcriptional repression [36]. They cloned the \textit{BRMS1} promoter region, including the promoter-associated CGI, into a luciferase reporter gene and found that \textit{BRMS1} promoter activity was dramatically inhibited under methylated conditions. They demonstrated that nuclear BRMS1 expression is reduced in lung cancer specimens
compared to normal bronchial epithelium and found that pathological tumour stage was associated with increased \textit{BRMS1} methylation in squamous cell cancers [36]. Moreover, Yang et al analysed associations between the methylation status of \textit{BRMS1} in NSCLC patients separately and available epidemiologic and clinical information including smoking status, gender, age, and histological type, or the stage of the tumor. Their results provide clinical evidence to support the notion that \textit{BRMS1} is a NSCLC metastasis suppressor gene. Measuring methylation status of \textit{BRMS1} promoter is a useful marker for identifying NSCLC patients with worse disease-free survival [37]. Very recently, detailed quantitative analysis of the metastatic process in lung showed that \textit{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].

\textit{BRMS1} may play a critical role in promoting migration, invasion and angiogenesis of ovarian cancer cells as well. Sheng et al recently investigated the mechanisms of \textit{BRMS1} involvement in ovarian cancer metastasis and they suggest that \textit{BRMS1} may regulate the metastatic potential at least in part through upregulation of CXCR4 via NF-\kappa B activation [39].

In conclusion, our data demonstrate that \textit{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 \textit{BRMS1} promoter is methylated in primary tumors of early breast cancer patients and in corresponding CTC samples, but not in non-cancerous breast tissues. \textit{BRMS1} promoter methylation and \textit{BRMS1} protein expression was evaluated in identical CTC from the same
patients for the first time. According to our results, CTC 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.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. M. Papadaki for technical assistance and Dr. D. Hurst, University of Alabama at Birmingham for advice.

GRANT SUPPORT

The present work was partly funded by SYNERGASIA 2009 PROGRAMME. This Program is co-funded by the European Regional Development Fund and National Resources (General Secretariat of Research and Technology in Greece), Project code: Onco-Seed diagnostics. Partial funding for this research was also by Susan G. Komen for the Cure (SAC110037), the National Foundation for Cancer Research and the U.S. National Cancer Institute (CA87728 and P30-CA168524.

REFERENCES


FIGURE LEGENDS

**Figure 1.** Schematic diagram of the workflow of the study

**Figure 2.** A) Analytical sensitivity of nested MSP for *BRMS1* using methylated primer set and synthetic mixtures, containing: 1) DNA marker 50bp, 2) Negative control: dH2O, 3) 0%, 4) 0.1%, 5) 1%, 6) 10%, 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 50bp, 2) Negative control: dH2O, 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 50bp, 2) Negative control: dH2O, 3–6) non-cancerous breast tissues, 7-15) breast tumors, 16) MSP positive control (100% methylated DNA).

**Figure 3.** BRMS1 expression in cytospin stained CTC of early breast cancer patients. Cells were stained with A45-B/B3 antibody (green), BRMS1 antibody (red) and Dapi (blue). Quantification of the samples was performed with the ARIOL system and scored as A): BRMS1 high expression, (B): BRMS1 low expression, C) negative for BRMS1. D): Heat map for BRMS1 expression and matched DNA samples for *BRMS1* promoter methylation in cytospin stained CTC (n=39): black: no CTC cells, red: no BRMS1 expression at upper row, *BRMS1* methylation at lower row, green: normal BRMS1 expression at upper row, *BRMS1* non-methylated at lower row, brown: low BRMS1 expression.
**Figure 4.** A) Kaplan-Meier estimates of disease-free interval (DFI) for early breast cancer patients with (green) or without (blue) BRMS1 promoter methylation (p=0.009), B) Kaplan-Meier estimates of overall survival (OS) for early breast cancer patients with (green) or without (blue) BRMS1 promoter methylation (p= 0.071)
Table 1: Clinical characteristics of early breast cancer patients in respect to *BRMS1* methylation status in FFPEs (n=84).

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>number of patients</th>
<th>BRMS1 Methylation (%)</th>
<th>P (Chi square test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor size (cm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–2.0</td>
<td>13</td>
<td>3</td>
<td>0.364</td>
</tr>
<tr>
<td>&gt;2</td>
<td>69</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of lymph nodes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24</td>
<td>10</td>
<td>0.711</td>
</tr>
<tr>
<td>1–3</td>
<td>24</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>4–9</td>
<td>21</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>&gt;9</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Histological grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>38</td>
<td>15</td>
<td>0.472</td>
</tr>
<tr>
<td>III</td>
<td>36</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>10</td>
<td>0.436</td>
</tr>
<tr>
<td>II</td>
<td>55</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Progesterone receptor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>11</td>
<td>0.455</td>
</tr>
<tr>
<td>Negative</td>
<td>53</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Estrogen receptor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>46</td>
<td>18</td>
<td>0.344</td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HER-2 status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>3</td>
<td>0.492</td>
</tr>
<tr>
<td>negative</td>
<td>57</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;54</td>
<td>43</td>
<td>17</td>
<td>0.331</td>
</tr>
<tr>
<td>&gt;54</td>
<td>40</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><em>unknown</em></td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: BRMS1 expression and BRMS1 methylation status in CTC as evaluated in cytospins (n=39) corresponding to FFPEs, in relation to the clinical outcome of early breast cancer patients.

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

Early breast cancer patients

- Fixed paraffin-embedded tissues (n=84)
  - Isolation of genomic DNA
  - SB conversion of genomic DNA
  - MSP for BRMS1
  - Evaluation of the prognostic significance of BRMS1 methylation

- Corresponding peripheral blood in EDTA (10 mL) (n=39)
  - Isolation of CTCs (cytospin)
  - Isolation of cytospin stained CTCs (N=39)
  - Immunofluorescence imaging (ARIOL)
  - Double staining for BRMS1 and A45/B-B3 (N=39)

- SB conversion of genomic DNA
  - MSP for BRMS1
  - Evaluation of BRMS1 methylation status and expression in CTCs
Figure 3

A

B

C

Pancytokeratin (A45-B/B3 antibody)  BRMS1 antibody  Overlay
Figure 3D

BRMS1 expression in CTCs (cytospins)

BRMS1 methylation in CTCs (cytospins)

© 2013 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A: Methylated samples = 31
Unmethylated samples = 53
Censored
Methylated = 21/31 (67.7%)
Unmethylated = 44/53 (77.4%)
P = 0.009

B: Methylated samples = 31
Unmethylated = 53
Censored
Methylated = 16/31 (51.6%)
Unmethylated = 41/53 (77.4%)
P = 0.071
Molecular Cancer Research

Breast Cancer Metastasis Suppressor-1 promoter methylation in primary breast tumors and corresponding Circulating Tumor Cells

Maria Chimonidou, Galatea Kallergi, Vasilis Georgoulias, et al.

Mol Cancer Res Published OnlineFirst June 6, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-13-0096

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2013/06/06/1541-7786.MCR-13-0096.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.