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

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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 cytopins. *BRMS1* methylation status was investigated in all FFPE and cytospin stained CTC using methylation specific PCR. *BRMS1* expression in cytopins 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 cytospin 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.

Keywords

Breast Cancer Metastasis Suppressor gene-1; *BRMS1*; circulating tumor cells; CTC; DNA methylation; operable breast cancer

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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 NF κ B, 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 cytopsin 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 noncancerous 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 (cytopsins):** 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 ($d=1,077\text{g/mol}$) 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). Cytopsins were dried up and stored at -80°C . Four slides were analyzed from the same blood sample. For all these cytopsins 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 cytopsins 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 cytopsins 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 CKpositive metastatic breast cancer patients in order to identify BRMS1 expression status in patients with high number of CTC. PBMC cytopspins 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 45min. 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 cytopsin stained CTC

Initially, in order to evaluate the efficacy of DNA isolation from cytopsin stained CTC we first performed control experiments using MCF-7 cells immobilized on cytopspins 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 cytopspins, 500 μ L 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 μ L PBS and then were used for DNA extraction. Genomic DNA (gDNA) from both CTC and MCF-7 cells immobilized on cytopspins 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 μ g of DNA extracted from FFPE and cytopspins 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, dH_2O 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 μ L. One microliter of sodium bisulfite converted DNA was added into a 24 μ L reaction mixture that contained 0.1 μ L of Taq DNA polymerase (5U/L, DNA polymerase; Promega), 2.5 μ L of the supplied PCR buffer, 1.0 μ L of $MgCl_2$ (50 mmol/L), 0.5 μ L of dNTP (10 mmol/L; Fermentas) and 1 μ L of the corresponding forward and reverse primers (10 μ mol/L); finally dH_2O was added to a final volume of 25 μ L. 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 μ L 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 μ g of fully methylated DNA (100%) and 1 μ 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 μ 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 nonmethylated 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 cytopins 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 cytospin 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 *BRMS1* non-methylated sequences were observed. This was expected, since cytopins 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, *BRMS1* 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 *BRMS1* promoter methylation in CTC isolated from the same cytopins 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 *BRMS1* 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 *BRMS1* promoter (12/53=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 non-methylated *BRMS1* 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 nonmethylated *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 cytopins. We have assessed both BRMS1 expression and *BRMS1* methylation status in these identical cytopins, 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 cytopins 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 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 *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 *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 *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 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 *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 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 *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 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 *BRMS1* methylation in squamous cell cancers [36]. Moreover, Yang et al analysed associations between the methylation status of *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 *BRMS1* is a NSCLC metastasis suppressor gene. Measuring methylation status of *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 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 et al 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- κ B activation [39].

In conclusion, our data demonstrate 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 early breast cancer patients and in corresponding CTC samples, but not in non-cancerous breast tissues. *BRMS1* promoter methylation and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 cytopsin 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).

Clinicopathological features	number of patients	BRS1 Methylation (%)	P (Chi square test)
Tumor size (cm)			
0–2.0	13	3	0.364
>2	69	26	
	*unknown 2		
Number of lymph nodes			
0			0.711
1–3	24	10	
4–9	24	7	
>9	21	9	
	10	3	
	*unknown 5		
Histological grade			
I–II	38	15	0.472
III	36	11	
	*unknown 10		
Tumor stage			
I	21	10	0.436
II	55	20	
	*unknown 8		
Progesterone receptor			
Positive		11	0.455
Negative	26	17	
	53		
	*unknown 5		
Estrogen receptor			
Positive	46	18	0.344
Negative	32	9	
	*unknown 6		
HER-2 status			
Positive	7	3	0.492
negative	57	20	
	*unknown 20		
Age			
<54	43	17	0.331
>54	40	13	

Clinicopathological features	number of patients	BRS1 Methylation (%)	P (Chi square test)
	*unknown 1		

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

Table 2

Patients	Patient ID	Numbers of Circulating Tumor Cells (CTC) in cytopins				CTC (cytopin)		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	-	-	+	

Patients	Patient ID	Numbers of Circulating Tumor Cells (CTC) in cytospins				CTC (cytospin)	primary tumor
		(CK+, CD45-, DAPI+)	BRMSI negative	BRMSI +++	BRMSI low+		
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	-	-