Clinical Chemistry 57:8
1169–1177 (2011)

DNA Methylation of Tumor Suppressor and Metastasis Suppressor Genes in Circulating Tumor Cells

Maria Chimonidou,[1†] Areti Strati,[1†] Alexandra Tzitzira,[1] Georgia Sotiropoulou,[2] Nikos Malamos,[3] Vasilis Georgoulias,[4] and Evi S. Lianidou[1*]

BACKGROUND: Circulating tumor cells (CTCs) are associated with prognosis in a variety of human cancers and have been proposed as a liquid biopsy for follow-up examinations. We show that tumor suppressor and metastasis suppressor genes are epigenetically silenced in CTCs isolated from peripheral blood of breast cancer patients.

METHODS: We obtained peripheral blood from 56 patients with operable breast cancer, 27 patients with verified metastasis, and 23 healthy individuals. We tested DNA extracted from the EpCAM-positive immunomagnetically selected CTC fraction for the presence of methylated and unmethylated *CST6*, *BRMS1*, and *SOX17* promoter sequences by methylation-specific PCR (MSP). All samples were checked for *KRT19* (keratin 19, formerly *CK-19*) expression by reverse-transcription quantitative PCR.

RESULTS: In CTCs of patients with operable breast cancer, promoter methylation of *CST6* was observed in 17.9%, *BRMS1* in 32.1%, and *SOX17* in 53.6% of patients. In CTCs of patients with verified metastasis, promoter methylation of *CST6* was observed in 37.0%, *BRMS1* in 44.4%, and *SOX17* in 74.1%. In healthy individuals, promoter methylation of *CST6* was observed in 4.3%, *BRMS1* in 8.7%, and *SOX17* in 4.3%. DNA methylation of these genes for both operable and metastatic breast cancer was significantly different from that of the control population.

CONCLUSIONS: DNA methylation of tumor suppressor and metastasis suppressor genes is a hallmark of CTCs and confirms their heterogeneity. Our findings add a new dimension to the molecular characterization of CTCs and may underlie the acquisition of malignant properties, including their stem-like phenotype.

Metastasis is a multistage process that selects for circulating tumor cells (CTCs)[5] that have the ability to infiltrate, survive in blood circulation, and colonize distant organs *(1, 2)*. Recent advances support the early dissemination model of metastasis, based on observations that disseminated tumor cells (DTCs) isolated from bone marrow or lymph nodes display disparate changes from primary tumor cells at all levels of genomic resolution *(3)*. Cancer cell dissemination may be followed by a dormancy period before relapse in 1 or more organs *(4)*.

Molecular characterization of CTCs presents a challenge because these cells are well-defined targets for understanding tumor biology and tumor cell dissemination *(2)*. European studies have shown the prognostic impact of DTCs present in the bone marrow of breast cancer patients *(5)*, our group has provided evidence for an association of the detection of CTCs in peripheral blood and prognosis of early breast cancer *(6–8)*, and other groups have shown the same for advanced breast cancer *(9, 10)*. We have shown that detection of CTCs postchemotherapy in breast cancer patients is associated with significantly increased clinical relapses and disease-related deaths *(11)*. Moreover, CTC enumeration and characterization is currently evaluated as a liquid biopsy approach and may play a major role in guiding targeted therapy *(12, 13)*. It was recently discovered that CTCs can reinfiltrate tumors at their primary organs, and possibly also at metastatic sites, and promote tumor growth and progression in a bidirectional process of tumor self-seeding or cross-seeding *(14, 15)*.

[1] Department of Chemistry, University of Athens, Greece; [2] Department of Pharmacy, School of Health Sciences, University of Patras, Greece; [3] Medical Oncology Unit, Elena Venizelou Hospital, Athens, Greece; [4] Laboratory of Tumor Cell Biology, Medical School, University of Crete, Heraklion, Greece.
[†] M. Chimonidou and A. Strati contributed equally to this work.
[*] Address correspondence to this author at: Department of Chemistry, University of Athens, 15771, Greece; Fax +30-210-7274750; e-mail lianidou@chem.uoa.gr.

Received March 22, 2011; accepted June 3, 2011.
Previously published online at DOI: 10.1373/clinchem.2011.165902

[5] Nonstandard abbreviations: CTC, circulating tumor cell; DTC, disseminated TC; miRNA, micro-RNA; CpG, C-phosphate-G; PBMC, peripheral blood mononuclear cell; anti-EpCAM, anti–epithelial cell adhesion molecule; MSP, methylation-specific PCR; SB, sodium bisulfite; gDNA, genomic DNA; TGF-β, transforming growth factor-β.

Whereas methylation of cancer-related genes plays a fundamental role in the development and progression of cancer and is a promising biomarker for early detection and prognosis estimation *(16)*, it has not been studied as yet in CTCs.

Cystatin M or E/M [encoded by cystatin E/M (*CST6*)[6]] is an endogenous inhibitor of cathepsins B and L *(17)* that was first identified by differential display of mRNAs as being markedly downregulated in metastatic breast cancer *(18)* and mapped to chromosomal locus 11q13 *(19)*. DNA hypermethylation in this region impairs transcription and leads to loss of cystatin M expression in cancer *(20–23)*. Cystatin M has generated much recent interest and is postulated to be a tumor suppressor in breast cancer *(21)*. Indeed, exogenous cystatin M expression in a breast cancer cell line resulted in significant reduction of cell proliferation, migration, Matrigel invasion, and adhesion to endothelial cells *(20)*. *CST6* promoter methylation provides important prognostic information in patients with operable breast cancer *(24)*.

Breast cancer metastasis suppressor 1 (*BRMS1*), a known suppressor of metastasis, is a predominantly nuclear protein that differentially regulates expression of multiple genes, leading to suppression of metastasis without blocking orthotopic tumor growth *(25)*. *BRMS1* is significantly downregulated in some breast tumors, especially in metastatic disease, by epigenetic silencing *(26)*. *BRMS1* coordinately regulates expression of multiple metastasis–associated micro-RNAs (miRNAs) *(27)*, whereas its expression in metastatic human breast cancer cells leads to selective repression of epidermal growth factor receptor and downstream Akt signaling *(28)*.

SRY (sex-determining region Y)-box 17 (*SOX17*) plays a critical role in the regulation of development and stem/precursor cell function, at least partly through repression of the canonical Wnt/β-catenin signaling pathway. *SOX17* gene silencing is associated with DNA hypermethylation of a C-phosphate-G (CpG) island located in the promoter region *(29)*. It is well established that SOX17 plays a tumor suppressor role through suppression of Wnt signaling *(30)*.

The aim of our study was to investigate, for the first time, the methylation status of tumor suppressor and metastasis suppressor genes in CTCs. The *CST6*, *BRMS1*, and *SOX17* tumor suppressors were selected because their epigenetic inactivation has been causally associated with cancer.

[6] Human genes: *CST6*, cystatin E/M; *SOX17*, SRY (sex-determining region Y)-box 17; *BRMS1*, breast cancer metastasis suppressor 1; *KRT19*, keratin 19, formerly *CK-19*. To come later.

Materials and Methods

PATIENTS

We recruited 83 breast cancer patients: (*a*) 56 patients with operable breast cancer at least 2 weeks after removal of the primary tumor and before the initiation of adjuvant chemotherapy and (*b*) 27 patients with metastatic breast cancer. We enrolled 23 healthy female blood donors as a control group. Peripheral blood (20 mL in EDTA) was obtained at the middle of vein puncture after the first 5 mL of blood was discarded. All blood samples were processed within 4 h of collection as described *(31, 32)*. All individuals signed an informed consent to participate in the study, which was approved by the ethics and scientific committees of our institutions. A diagram outlining the procedure is shown in Fig. 1.

IMMUNOMAGNETIC ISOLATION OF CTCs

After dilution of peripheral blood with 20 mL PBS (pH 7.3), we obtained peripheral blood mononuclear cells (PBMCs) by gradient density centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Bio-Sciences AB) at 670*g* for 30 min at room temperature. The interface cells were removed, washed twice with 40 mL sterile PBS (pH 7.3, 4 °C), centrifuged at 530*g* for 10 min, and resuspended in 1 mL PBS. Cells were dyed with Trypan blue and counted in a hemocytometer. We used immunomagnetic Ber-EP4 [anti–epithelial cell adhesion molecule (anti-EpCAM)]–coated capture beads (Dynabeads® Epithelial Enrich, Invitrogen) to enrich epithelial cells according to the manufacturer's protocol.

RNA EXTRACTION FROM CTCs

We performed total RNA isolation with Trizol (Invitrogen) and measured RNA concentrations using the Nanodrop-1000 (NanoDrop Technologies). mRNA was isolated from total RNA using the Dynabeads mRNA Purification kit (Invitrogen) as described *(32)*. cDNA synthesis was performed using the SuperScript™ First-Strand Synthesis System (Invitrogen) and the cDNA was used for *KRT19* expression studies in CTCs as described *(31)*.

DNA EXTRACTION FROM CTCs

After removal of the aqueous phase of Trizol, DNA was precipitated from the interphase by adding 150 μL of 100% ethanol. Samples were mixed by inversion and kept at room temperature for 2–3 min, and the DNA was sedimented by centrifugation (2000*g*, 5 min, 4 °C) and washed twice in a solution containing 0.1 mol/L sodium citrate in 10% ethanol (500 μL). In each wash, the DNA pellet was stored in the washing solution for 30 min at room temperature with periodic mixing and

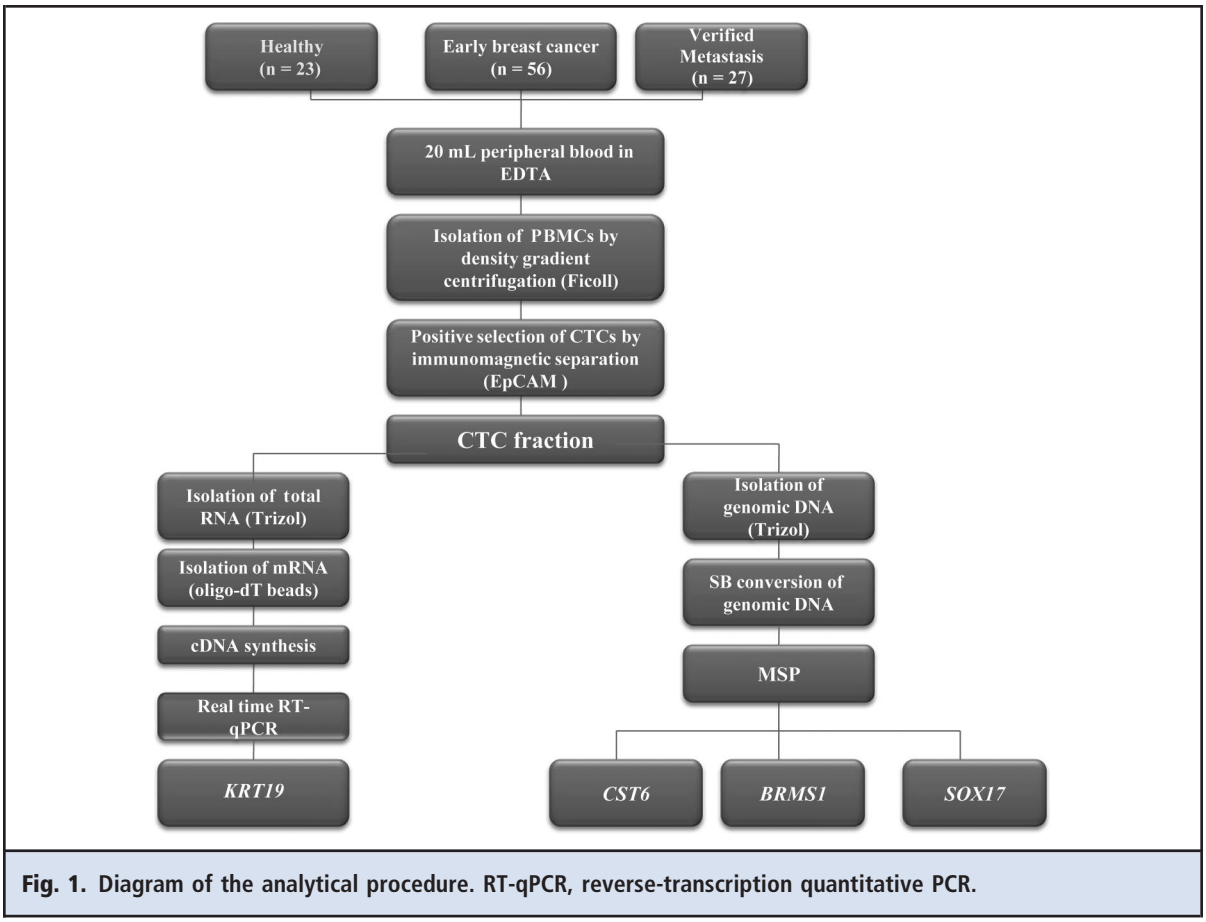

Fig. 1. Diagram of the analytical procedure. RT-qPCR, reverse-transcription quantitative PCR.

centrifuged (2000*g*, 5 min, 4 °C). After these 2 washes, the DNA pellet was suspended in 1 mL of 75% ethanol, kept for 10–20 min at room temperature with periodic mixing, and centrifuged (2000*g*, 5 min, 4 °C). Isolated DNA was then air-dried for 15 min and dissolved in 50 μL of 8 mmol/L NaOH. We determined the DNA concentration in the Nanodrop-1000.

SODIUM BISULFITE CONVERSION

We modified the DNA extracted from isolated CTCs by a sodium bisulfite (SB) reaction carried out in denatured DNA using the EZ DNA Methylation Gold Kit (Zymo) following the short program (reaction time 160 min). The converted DNA was stored at −70 °C until use. In each SB reaction, distilled H_2O (PCR grade) and MCF-7 cells (or MDA-MB-231 cells) were included as negative and positive controls, respectively.

METHYLATION-SPECIFIC PCR

All methylation-specific PCR (MSP) reactions were performed in an Eppendorf Mastercycler. MSP products were fractionated on 2% agarose gels containing 40 mmol/L Tris-acetate/1.0 mmol/L EDTA (pH 8.0). To avoid false-negative results, we amplified SB-treated DNA in 2 separate MSP reactions, 1 with a set of primers specific for methylated and 1 for unmethylated *CST6* promoter sequences *(24)*. We used human placental genomic DNA (gDNA; Sigma-Aldrich) methylated in vitro with *SssI* methylase (NEB), after SB conversion, as a fully methylated (100%) MSP positive control; we used the same unmethylated placental gDNA, after SB conversion, as a negative MSP control. MSP primers are shown in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue8, and MSP reaction conditions for each gene are shown in detail in online Supplemental Table 2.

STATISTICAL ANALYSIS

We performed statistical evaluation of data using SPSS (SPSS Statistics 17.0). We used the χ^2 test of independence (SPSS, version 17.0) for data analysis and for the evaluation of the significance of differences between groups, and Fisher exact test for the evaluation of the correlation between methylation of each gene in re-

Table 1. Correlation of DNA methylation in CTCs and early breast cancer patients' characteristics.

		CST6			*BRMS1*			*SOX17*		
	All	Methylated	Unmethylated	*P*[a]	Methylated	Unmethylated	*P*	Methylated	Unmethylated	*P*
n (%)	56 (100)	10 (17.9)	46 (82.1)		18 (32.1)	38 (67.9)		30 (53.6)	26 (46.4)	
Age, years				0.486			0.558			0.588
≥49	31 (55.4)	7 (22.6)	24 (77.4)		11 (35.5)	20 (64.5)		15 (48.4)	16 (51.6)	
<49	24 (42.9)	3 (12.5)	21 (87.5)		6 (25.0)	18 (75.0)		14 (58.3)	10 (41.7)	
Unknown	1 (1.80)	0	1 (100)		1 (100)	0		1 (100)	0	
Menopausal status				0.032			0.753			0.776
Premenopausal	28 (50.0)	2 (7.10)	26 (92.9)		7 (25.0)	21 (75.0)		15 (53.6)	13 (46.4)	
Postmenopausal	22 (39.3)	7 (31.8)	15 (68.2)		7 (31.8)	15 (68.2)		10 (45.5)	12 (54.5)	
Unknown	6 (10.7)	1 (16.7)	5 (83.3)		4 (66.7)	2 (33.3)		5 (83.3)	1 (16.7)	
Tumor size, cm				0.049			0.051			0.552
T1 (δ2.0)	16 (28.6)	6 (37.5)	10 (62.5)		8 (50.0)	8 (50.0)		10 (62.5)	6 (37.5)	
T2/T3 (>2.0)	38 (67.9)	4 (10.5)	34 (89.5)		8 (21.1)	30 (78.9)		19 (50.0)	19 (50.0)	
Unknown	2 (3.60)	0	2 (100)		2 (100)	0		1 (50.0)	1 (50.0)	
Histology grade				0.684			0.311			0.534
1 or 2	14 (25.0)	3 (21.4)	11 (78.6)		6 (42.9)	8 (57.1)		6 (42.9)	8 (57.1)	
3	39 (69.6)	6 (15.4)	33 (84.6)		10 (25.6)	29 (74.4)		22 (56.4)	17 (43.6)	
Unknown	3 (5.40)	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)		2 (66.7)	1 (33.3)	
Lymph node status				1			1			0.404
N0	25 (44.6)	4 (16.0)	21 (84.0)		7 (28.0)	18 (72.0)		12 (48.0)	13 (52.0)	
N1–N3	26 (46.4)	5 (19.2)	21 (80.8)		8 (30.8)	18 (69.2)		16 (61.5)	10 (38.5)	
Unknown	5 (8.90)	1 (20.0)	4 (80.0)		3 (60.0)	2 (40.0)		2 (40.0)	3 (60.0)	
Estrogen receptor status				0.720			1			1
Positive	31 (55.4)	6 (19.4)	25 (80.6)		9 (29.0)	22 (71.0)		17 (54.8)	14 (45.2)	
Negative	22 (39.3)	3 (1.60)	19 (86.4)		7 (31.8)	15 (68.2)		12 (54.5)	10 (45.5)	
Unknown	3 (5.40)	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)		1 (33.3)	2 (66.7)	
Progesterone receptor status				0.726			0.541			1
Positive	26 (46.4)	4 (15.4)	22 (84.6)		9 (34.6)	17 (65.4)		14 (53.8)	12 (46.2)	
Negative	25 (44.6)	5 (20.0)	20 (80.0)		6 (24.0)	19 (76.0)		14 (56.0)	11 (44.0)	
Unknown	5 (8.90)	1 (20.0)	4 (80.0)		3 (60.0)	2 (40.0)		2 (40.0)	3 (60.0)	
HER-2/neu status				0.277			0.768			1
Positive (3+)	22 (39.3)	2 (9.10)	20 (90.9)		6 (27.3)	16 (72.7)		12 (54.5)	10 (45.5)	
Negative (0/1+/2+)	31 (55.4)	7 (22.6)	24 (77.4)		10 (32.3)	21 (67.7)		17 (54.8)	14 (45.2)	
Unknown	3 (5.40)	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)		1 (33.3)	2 (66.7)	

[a] Fisher exact test, n = 56.

spect to the patient's characteristics and DNA methylation of each gene in respect to *KRT19* gene expression.

Results

ANALYTICAL SENSITIVITY AND SPECIFICITY OF MSP ASSAYS

We extensively evaluated the analytical sensitivity and specificity of the MSP assay for *CST6* promoter methylation in our previous study *(24)*. The analytical sensitivity and specificity of the MSP assays for *BRMS1* and *SOX17* promoter methylation have been evaluated *(26, 29, 33)*. MSP for *CST6* with primers specific for the unmethylated DNA was performed for all SB-converted samples to exclude failure of PCR reaction when the PCR reaction specific for the methylated DNA sequences was negative.

DNA METHYLATION OF TUMOR SUPPRESSOR GENES IN CTCs

CST6 promoter methylation was observed in 10 of 56 (17.9%) patients with operable breast cancer and 10 of 27 (37.0%) patients with metastasis; *CST6* promoter methylation was observed in 1 (4.3%) of 23 blood samples from healthy individuals using exactly the same procedure.

Interestingly, previous studies have reported differences in the methylation status of *BRMS1* in primary tumors *(26)*. In this study, we found that the *BRMS1* promoter was highly methylated in CTCs of breast cancer patients. *BRMS1* promoter methylation was observed in 18 of 56 (32.1%) patients with operable breast cancer, 12 of 27 (44.4%) patients with metastasis, and 2 (8.7%) of 23 peripheral blood samples from healthy individuals.

The importance of epigenetic silencing of *SOX17* gene was shown very recently *(29, 33)*. We observed that *SOX17* promoter is highly methylated in CTCs. *SOX17* promoter methylation was observed in 30 of 56 (53.6%) patients with operable breast cancer and 20 of 27 (74.1%) patients with distant metastasis. Conversely, *SOX17* promoter methylation was observed in only 1 (4.3%) of 23 peripheral blood samples obtained from healthy individuals.

When we correlated the patient's characteristics with our results on DNA methylation, we found no association of DNA methylation of these genes, except for a correlation between *CST6* methylation and menopausal status ($P = 0.03$) and tumor size ($P = 0.05$), and between *BRMS1* methylation and tumor size ($P = 0.05$) (Table 1). Table 2 shows the localization of metastases with respect to the methylation profile of these genes in CTCs for each individual patient.

Using χ^2 test analysis, we found that methylation of the promoter of each gene was different between the 3 groups: *CST6*, Pearson χ^2 8640, df 2, $P = 0.013$; *BRMS1*, Pearson χ^2 7746, df 2, $P = 0.021$; *SOX17*, Pearson χ^2 25604, df 2, $P = 0.000$.

Table 2. DNA methylation in CTCs and metastasis site for individual breast cancer patients with verified metastasis (n = 27).

		Methylation of gene promoter in CTCs		
Sample	Metastasis site	*CST6*	*SOX17*	*BRMS1*
1	Brain	−	+	+
2	Brain/lung	−	+	−
3	Lung	+	+	+
4	Lung	+	−	+
5	Lung	−	−	+
6	Lung	−	+	−
7	Lung	−	−	−
8	Lung	+	+	+
9	Lung	+	+	+
10	Lung	+	+	+
11	Lung/liver	+	+	−
12	Liver	−	−	−
13	Liver	−	+	−
14	Liver	+	+	−
15	Liver	−	+	+
16	Skin	+	+	+
17	Skin	−	−	−
18	Skin	−	−	−
19	Lymph node enlargements	+	+	+
20	Bone	−	+	−
21	Stomach	−	+	−
22	Breast	+	+	−
23	Generalized disease	−	+	+
24	Generalized disease	−	−	−
25	Generalized disease	−	+	−
26	Generalized disease	−	+	−
27	Unknown	−	−	+

CST6, BRMS1, AND *SOX17* PROMOTER METHYLATION AND *KRT19* EXPRESSION IN CTCs

KRT19 has been extensively used as an epithelial marker, characteristic for the presence of CTCs *(6–8, 11)*. For this reason, we also studied in all these clinical samples the expression of *KRT19* in the EpCAM-positive, immunomagnetically isolated CTC fraction. We found 24 samples positive for *KRT19* expression of 56 early breast cancer patients studied (42.8%) and 14 of 27 (51.8%) patients with verified metastasis, whereas none of the 23 healthy individual samples was positive for *KRT19* expression. Table 3 shows the results for *CST6, BRMS1*, and *SOX17* promoter methylation in comparison to the expression of *KRT19* expression in CTCs.

Table 3. *CST6, BRMS1,* and *SOX17* promoter methylation and *KRT19* expression in CTCs of breast cancer patients (n = 83).

Gene	*KRT19*-positive	*KRT19*-negative	*P*[a]	Concordance, %
Early breast cancer patients (n = 56)				
CST6				
Methylated	4	6	NS	53.6
Unmethylated	20	26		
BRMS1				
Methylated	6	12	NS	46.4
Unmethylated	18	20		
SOX17				
Methylated	8	22	0.014	32.1
Unmethylated	16	10		
Verified metastasis (n = 27)				
CST6				
Methylated	4	6	NS	40.7
Unmethylated	10	7		
BRMS1				
Methylated	6	6	NS	48.1
Unmethylated	8	7		
SOX17				
Methylated	11	9	NS	55.5
Unmethylated	3	4		

[a] Fisher exact test. NS, nonsignificant.

In early breast cancer, the concordance between *CST6* promoter methylation and *KRT19* gene expression was 53.6% (30/56), whereas in patients with metastasis, the concordance was 40.7% (11/27) (Table 3). The concordance between *BRMS1* promoter methylation and *KRT19* gene expression in early breast cancer was 46.4% (26/56), whereas in patients with overt metastasis, the concordance was 48.1% (13/27). In early breast cancer, the concordance between *SOX17* promoter methylation and *KRT19* gene expression was 32.1% (18/56). We found an inverse correlation between *SOX17* promoter methylation and *KRT19* expression. In patients with verified metastasis, the concordance between *SOX17* promoter methylation and *KRT19* gene expression was 55.5% (15/27).

A heat map for *CST6, BRMS1*, and *SOX17* promoter methylation and *KRT19* expression in the CTC fraction of each patient in all 3 groups studied is shown in Fig. 2. Each patient had a different profile concerning DNA methylation of these genes in CTCs. In 5 of 56 (8.9%) patients with early breast cancer and 6 of 27 (22.2%) patients with verified metastasis, all 3 genes were methylated.

Discussion

Our study provides the first evidence that DNA methylation of tumor suppressor and metastasis suppressor genes exists in CTCs. We found that the promoters of the tumor suppressor genes *CST6* and *SOX17* and metastasis suppressor *BRMS1* are methylated in CTC isolated from peripheral blood of breast cancer patients, in both groups of early disease and verified metastasis. In operable breast cancer, the percentage of DNA methylation in all 3 of these genes was lower but not significantly different from that found in the group of patients with overt metastasis, whereas for both operable and metastatic breast cancer this was significantly different from the control population.

Whole genome studies identified *CST6* as among the 10 hypermethylated genes that distinguish between cancerous and normal tissues *(34)*, and its methylation was associated with epithelial-to-mesenchymal transition *(35)*. A recent functional epigenetic study identified *CST6* as 1 of 8 genes that showed frequent (>30%) tumor-specific promoter region hypermethylation associated with transcriptional silencing and that its re-

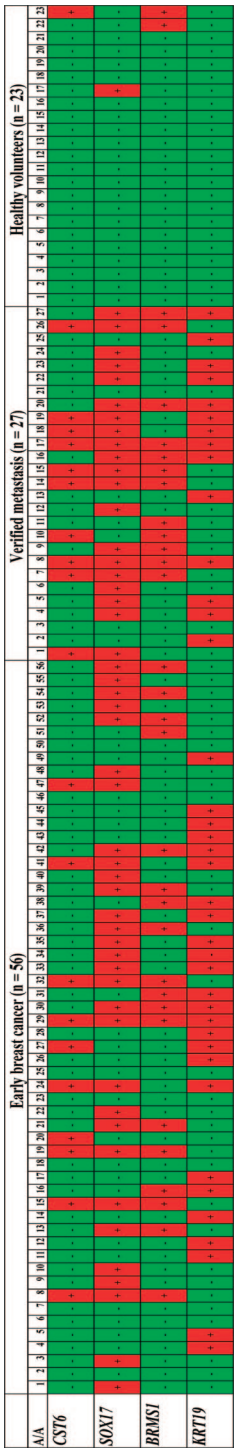

Fig. 2. Heat map of *CST6*, *BRMS1*, and *SOX17* promoter methylation in the CTC fraction of patients.

Operable breast cancer (n = 56); verified metastasis (n = 27); healthy individuals (n = 23). Red, positive; green, negative. A/A, sample number.

expression suppressed the growth of renal cell carcinoma cell lines *(36)*. Moreover, according to our recent findings, *CST6* methylation is a promising biomarker for prediction of relapses and deaths in operable breast cancer *(24)*. Here we provide evidence that the *CST6* promoter is methylated in CTCs.

BRMS1 suppresses breast cancer metastasis in multiple experimental models by reducing solitary cell survival and inhibiting growth initiation *(37)*. Loss of BRMS1 expression has been shown to predict reduced disease-free survival in subsets of breast cancer patients *(38)*. We found that *BRMS1* is methylated in CTC, a finding that is consistent with other studies that showed significant downregulation of *BRMS1* in some breast tumors, especially in metastatic disease, because of epigenetic silencing *(26)*. Recent observations demonstrated that methylation of the promoter-associated CpG island in *BRMS1* results in its transcriptional repression and highlighted the potential clinical relevance of this event with respect to non–small cell lung cancer tumor histology and pathological stage *(39)*.

Similarly, *SOX17* plays a critical role in the regulation of development and stem/precursor cell function, at least partly through repression of Wnt pathway activity *(30)*. *SOX17* has been shown to alter adult lung progenitor cell fate, decrease the expression of transforming growth factor-β (TGF-β)–responsive cell cycle inhibitors, and inhibit TGF-β1–mediated transcriptional responses in vitro, while blocking Smad3 DNA binding and transcriptional activity *(40)*. Silencing of *SOX17* due to promoter hypermethylation is a frequent event and may contribute to aberrant activation of Wnt signaling in breast cancer *(33)*. Our data demonstrate that *SOX17* promoter is methylated in the CTC fraction in the majority of operable (53.6%) and metastatic (74.1%) breast cancer patients.

Further molecular characterization of CTCs is important not only to confirm their malignant origin but also to identify diagnostically and therapeutically relevant targets to help stratify cancer patients for individual therapies. Molecular characterization of CTCs can expand our knowledge of basic molecular pathways of invasion, migration, and immune surveillance. The big question is how soon that knowledge will be translated into new clinical concepts for diagnosis and therapy. Toward this, our group has very recently developed a multiplexed PCR-coupled liquid bead array to detect the expression of multiple genes in CTCs *(32)*.

In this study, we aimed to molecularly characterize CTCs at the epigenetic level. Our data show that tumor suppression is severely disarmed in CTC via progressive DNA hypermethylation that leads to epigenetic silencing of key tumor suppressors and metastasis suppressors known to affect hallmark properties of tumor cells, including growth and proliferation, invasiveness,

epithelial phenotype, and stemness. We have chosen to first study *CST6, BRMS1*, and *SOX17* gene methylation in CTCs, after a very careful search in the literature, since the available material for CTC analysis is extremely low. These 3 genes, as already established through other studies in cell lines and primary tumors, each play an important role in preventing metastasis *(18, 19, 25, 27, 28, 30)*. Methylation of their promoters is highly correlated with their epigenetic silencing, development of metastasis, and poor prognosis *(21, 23, 24, 26, 29)*.

Our results also confirm the heterogeneity of CTCs, since the methylation profile was different in individual patients. Evaluation of these different profiles in respect to the clinical outcome, metastasis site, and patient characteristics will be possible only when a larger number of clinical samples with a known clinical outcome are analyzed. Interestingly, we also found highly methylated gene promoter sequences in the CTC fraction of EpCAM-positive/*KRT19*-negative patients. This confirms the observation that multiparametric evaluation of CTCs is of critical importance *(32)*.

Epigenetic silencing of tumor and metastasis suppressor genes in CTCs by increased DNA methylation with advanced disease provides new insights into the dynamics of metastasis. Combinatorial patterns of aberrant DNA methylation associated with the sequential silencing of key tumor suppressors is consistent with the wide heterogeneity of CTCs and may play a critical role in the evolutionary selection of increasingly aggressive CTC variants with acquired capacity for self-seeding at the primary tumor site, but also at distinct metastatic sites, thus accelerating tumor progression. Elucidating the interplay between epigenetic gene silencing and other tumorigenic processes in CTCs will be crucial to our understanding of tumor evolution and metastasis.

Our findings add a new dimension to the malignant nature and metastatic potential of CTCs. Whether the presence or absence of DNA methylation in CTCs is of prognostic, predictive, or therapeutic importance has yet to be determined. We believe that our findings should be explored in a prospective study, in respect to the clinical outcome of breast cancer patients with *CST6, BRMS1*, and *SOX17* promoter methylation in CTCs.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Research grant of the Greek General Secretariat of Research and Technology (PENED 2003) and Research Grant on Oncology from the Greek Ministry of Health.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Dr. A. Kastania, Bioinformatics and Medical Informatics team, Biomedical and Research Foundation of the Academy of Athens, Greece, for her advice on the statistical evaluation of our data.

References

1. Chiang AC, Massagué J. Molecular basis of metastasis. N Engl J Med 2008;359:2814–23.
2. Pantel K, Alix-Panabières C, Riethdorf S. Cancer micrometastases. Nat Rev Clin Oncol 2009;6:339–51.
3. Klein CA. Parallel progression of primary tumours and metastases. Nat Rev Cancer 2009;9:302–12.
4. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer 2007;7:834–46.
5. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. N Engl J Med 2005;353:793–802.
6. Stathopoulou A, Vlachonikolis I, Mavroudis D, Perraki M, Kouroussis Ch, Apostolaki S, et al. Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. J Clin Oncol 2002;20:3404–12.
7. Xenidis N, Perraki M, Kafousi M, Apostolaki S, Bolonaki I, Stathopoulou A, et al. Predictive and prognostic value of peripheral blood cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in node-negative breast cancer patients. J Clin Oncol 2006;24:3756–62.
8. Ignatiadis M, Kallergi G, Ntoulia M, Perraki M, Apostolaki S, Kafousi M, et al. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. Clin Cancer Res 2008;14:2593–600.
9. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005;23:1420–30.
10. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
11. Xenidis N, Ignatiadis M, Apostolaki S, Perraki M, Kalbakis K, Agelaki S, et al. Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol 2009;27:2177–84.
12. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008;14:6302–9.
13. Scher HI, Jia X, de Bono JS, Fleisher M, Pienta KJ, Raghavan D, Heller G. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. Lancet Oncol 2009;10:233–9.
14. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. Cell 2009;139:1315–26.
15. Leung CT, Brugge JS. Tumor self-seeding: bidirectional flow of tumor cells. Cell 2009;139:1226–8.
16. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nature Rev Genet

2002;3:415–28.
17. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. Nat Rev Cancer 2006;6:764–75.
18. Sotiropoulou G, Anisowicz A, Sager R. Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, downregulated in breast cancer. J Biol Chem 1997;272: 903–10.
19. Stenman G, Astrom AK, Roijer E, Sotiropoulou G, Zhang M, Sager R. Assignment of a novel cysteine proteinase inhibitor (*CST6*) to 11q13 by fluorescence in situ hybridization. Cytogenet Cell Genet 1997;76:45–6.
20. Shridhar R, Zhang J, Song J, Booth BA, Kevil CG, Sotiropoulou G, et al. Cystatin M suppresses the malignant phenotype of human MDA-MB-435S cells. Oncogene 2004;23:2206–15.
21. Ai L, Kim WJ, Kim TY, Fields CR, Massoll NA, Robertson KD, Brown KD. Epigenetic silencing of the tumor suppressor cystatin M occurs during breast cancer progression. Cancer Res 2006;66: 7899–909.
22. Pulukuri SM, Gorantla B, Knost JA, Rao JS. Frequent loss of cystatin E/M expression implicated in the progression of prostate cancer. Oncogene 2009;28:2829–38.
23. Rivenbark AG, Jones WD, Coleman WB. DNA methylation-dependent silencing of *CST6* in human breast cancer cell lines. Lab Invest 2006;86: 1233–42.
24. Kioulafa M, Balkouranidou I, Sotiropoulou G, Kaklamanis L, Mavroudis D, Georgoulias V, Lianidou E. Methylation of cystatin M promoter is associated with unfavorable prognosis in operable breast cancer. Int J Cancer 2009;125: 2887–92.
25. Phadke PA, Vaidya KS, Nash KT, Hurst DR, Welch DR. BRMS1 suppresses breast cancer experimental metastasis to multiple organs by inhibiting several steps of the metastatic process. Am J Pathol 2008;172:809–17.
26. Metge BJ, Frost AR, King JA, Dyess DL, Welch DR, Samant RS, Shevde LA. Epigenetic silencing contributes to the loss of BRMS1 expression in breast cancer. Clin Exp Metastasis 2008;25:753–63.
27. Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, Welch DR. Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. Cancer Res 2009;69:1279–83.
28. Vaidya KS, Harihar S, Phadke PA, Stafford LJ, Hurst DR, Hicks DG, et al. Breast cancer metastasis suppressor-1 differentially modulates growth factor signaling. J Biol Chem 2008;283: 28354–60.
29. Zhang W, Glöckner SC, Guo M, Machida EO, Wang DH, Easwaran H, et al. Epigenetic inactivation of the canonical Wnt antagonist SRY-box containing gene 17 in colorectal cancer. Cancer Res 2008;68:2764–72.
30. Du YC, Oshima H, Oguma K, Kitamura T, Itadani H, Fujimura T, et al. Induction and downregulation of Sox17 and its possible roles during the course of gastrointestinal tumorigenesis. Gastroenterology 2009;137:1346–57.
31. Stathopoulou A, Ntoulia M, Perraki M, Apostolaki S, Mavroudis D, Malamos N, et al. A highly specific real-time RT-PCR method for the quantitative determination of CK-19 mRNA positive cells in peripheral blood of patients with operable breast cancer. Int J Cancer 2006;119:1654–9.
32. Markou A, Strati A, Malamos N, Georgoulias V, Lianidou ES. Molecular characterization of circulating tumor cells in breast cancer by a liquid bead array hybridization assay. Clin Chem 2011; 57:421–30.
33. Fu DY, Wang ZM, Li-Chen, Wang BL, Shen ZZ, Huang W, Shao ZM. Sox17, the canonical Wnt antagonist, is epigenetically inactivated by promoter methylation in human breast cancer. Breast Cancer Res Treat 2010;119:601–12.
34. Radpour R, Kohler C, Haghighi MM, Fan AX, Holzgreve W, Zhong XY. Methylation profiles of 22 candidate genes in breast cancer using high-throughput MALDI-TOF mass array. Oncogene 2009;28:2969–78.
35. Rodenhiser DI, Andrews J, Kennette W, Sadikovic B, Mendlowitz A, Tuck AB, Chambers AF. Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. Breast Cancer Res 2008;10:R62.
36. Morris MR, Ricketts C, Gentle D, Abdulrahman M, Clarke N, Brown M, et al. Identification of candidate tumour suppressor genes frequently methylated in renal cell carcinoma. Oncogene. 2010 29:2104-17.
37. Hedley BD, Vaidya KS, Phadke P, MacKenzie L, Dales DW, Postenka CO, et al. BRMS1 suppresses breast cancer metastasis in multiple experimental models of metastasis by reducing solitary cell survival and inhibiting growth initiation. Clin Exp Metastasis 2008;25:727–40.
38. Hicks DG, Yoder BJ, Short S, Tarr S, Prescott N, Crowe JP, et al. Loss of breast cancer metastasis suppressor 1 protein expression predicts reduced disease-free survival in subsets of breast cancer patients. Clin Cancer Res 2006;12:6702–8.
39. Nagji AS, Liu Y, Stelow EB, Stukenborg GJ, Jones DR. BRMS1 transcriptional repression correlates with CpG island methylation and advanced pathological stage in non-small cell lung cancer. J Pathol 2010;221:229–37.
40. Lange AW, Keiser AR, Wells JM, Zorn AM, Whitsett JA. Sox17 promotes cell cycle progression and inhibits TGF-beta/Smad3 signaling to initiate progenitor cell behavior in the respiratory epithelium. PLoS One 2009;4:e5711.