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Development and validation of a multiplex methylation specific PCR-coupled liquid bead array for liquid biopsy analysis

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ABSTRACT

Background: Liquid biopsy is based on minimally invasive blood tests and has the potential to characterize the evolution of a solid tumor in real time, by extracting molecular information from circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). Epigenetic silencing of tumor and metastasis suppressor genes plays a key role in survival and metastatic potential of cancer cells. Our group was the first to show the presence of epigenetic alterations in CTCs.

Methods: We present the development and analytical validation of a highly specific and sensitive Multiplex Methylation Specific PCR-coupled liquid bead array (MMSPA) for the simultaneous detection of the methylation status of three tumor and metastasis suppressor genes (*CST6*, *SOX17* and *BRMS1*) in liquid biopsy material (CTCs, corresponding ctDNA) and paired primary breast tumors.

Results: In the EpCAM-positive CTCs fraction we observed methylation of: a) *CST6*, in 11/30(37%) and 11/30(37%), b) *BRMS1* in 8/30(27%) and 11/30(37%) c) *SOX17* in 8/30(27%) and 13/30(43%) early breast cancer patients and patients with verified metastasis respectively. In ctDNA we observed methylation of: a) *CST6*, in 5/30(17%) and 10/31(32%), b) *BRMS1* in 8/30 (27%) and 8/31 (26%) c) *SOX17* in 5/30(17%) and 13/31(42%) early breast cancer patients and patients with verified metastasis respectively.

Conclusions: Our results indicate a high cancerous load at the epigenetic level in EpCAM-positive CTCs fractions and corresponding ctDNA in breast cancer. The main principle of the developed methodology has the potential to be extended in a large number of gene-targets and be applied in many types of cancer.

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1. Introduction

Liquid biopsy is based on minimally invasive blood tests and has the potential to characterize the evolution of a solid tumor in real time, by extracting molecular information from circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), in contrast to the classic tissue biopsy approach which is not only an invasive procedure but only captures a single snapshot in the evolution of cancer. Thus, the molecular characterization of CTCs and ctDNA holds considerable promise for the identification of therapeutic targets and resistance mechanisms and for real-time monitoring of the efficacy of systemic therapies [1,2]. The major advantage of CTCs and ctDNA analysis is that they can be serially repeated, thus allowing extracting information from the tumor in real time [3,4].

CTCs are nowadays a well-established target serving as an important weapon in scientific community's arsenal, in order to figure out the underlying mechanisms of tumor development and the metastatic procedure, in a non-invasive way [5]. Cell-free DNA (cfDNA) represents another source of cancer-originated material circulating in elevated concentrations in serum and plasma of cancer patients [6], used as a biomarker to evaluate prognosis, diagnosis and response to treatment [7] and monitor the efficacy of anticancer therapies [8]. ctDNA within total cfDNA is believed to be shed in the bloodstream both by active release of tumor DNA and passive DNA leakage following apoptosis or necrosis of cancer cells [8]. ctDNA and CTCs have complementary roles as ctDNA can be analyzed without the prior need to enrich and isolate a rare population of cells whereas CTCs provide the unique opportunity to study the whole cell giving the potential for functional studies to guide personalized treatment selection [9].

Epigenetic alterations that occur independently of changes in primary DNA sequences, contribute to cancer initiation and progression, constituting a hallmark of all types of cancer. DNA methylation was the first epigenetic modification to be described and is still the most studied in mammals [10,11]. This modification occurs generally in cytosine within CpG dinucleotides, concentrated in large clusters called CpG islands and is generally but not exclusively associated with repression of transcription initiation at CpG-island promoters [12]. Since it takes place early in the process of cancer development, it is considered as a promising

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tumor biomarker for early detection, prognosis and of outmost importance for therapy approaches [13].

Our group was the first to show epigenetic alterations in CTCs and corresponding ctDNA; the promoters of the tumor suppressor genes cystatin E/M (*CST6*) and SRY (sex determining region Y)-box 17 (*SOX17*), and the metastasis suppressor gene breast cancer metastasis suppressor 1 (*BRMS1*) were found methylated in CTCs isolated from the peripheral blood of patients with breast cancer [14] and corresponding ctDNA [15,16]. The molecular characterization of CTCs at the epigenetic level, can give important information on the molecular and biological nature of these cells, as the epigenetic silencing of tumor and metastasis suppressor genes plays a key role in the survival and regulation of their metastatic potential [14,17]. In addition, the methylation status of tumor and metastasis suppressor genes in ctDNA is a very promising approach, since the starting material is accessed non-invasively and low percentages of DNA methylation are detected in plasma of healthy donors [18]. The benefits can be anticipated to improve patient management, reduce unnecessary drug toxicity and accelerate data acquisition from clinical trials [19].

We have recently developed a multiplexed PCR-coupled liquid bead array to detect simultaneously the expression of six genes in CTCs, saving precious sample and reducing the cost and time of analysis [20]. In the present study we present for the first time the development and analytical validation of a novel Multiplex Methylation Specific PCR-coupled liquid bead array assay (MMSPA) for the simultaneous detection of the methylation status of three tumor and metastasis suppressor genes in liquid biopsy material. The main principle of the developed methodology has the potential to be extended in a large number of gene-targets and be applied in many other types of cancer.

2. Patients, materials and methods

2.1. Controls and cell lines

To optimize the assay, we used human placental genomic DNA (gDNA; Sigma-Aldrich, USA) after Sodium Bisulfite (SB)-treatment, as a negative Methylation Specific PCR (MSP) control. The Universal Methylated Human DNA Standard (ZYMO Research, USA), treated with SB, was used as fully methylated (100%) MSP positive control. To evaluate the performance of this assay we used the breast cancer cell lines MCF-7 and SK-BR-3. The cells were dyed with trypan blue to assess their viability and counted in a hemocytometer.

2.2. Clinical samples

The developed assay was applied in SB-treated DNA originated from FFPE tissues, and a number of matched EpCAM-positive immunomagnetically selected CTC fractions and ctDNA samples too. This study was performed in accordance with the 1964 Declaration of Helsinki and was approved by the ethics and scientific committees of the Department of Medical Oncology, University of Crete. All specimens were obtained after written informed consent of all participants included in the study.

2.2.1. Primary breast cancer formalin-fixed paraffin-embedded (FFPE) tissues

FFPEs from 20 patients with operable breast cancer and 15 breast cancer patients with verified metastasis were obtained. We also used 11 samples obtained from reduction mammoplasties as a control group.

2.2.2. Positive immunomagnetic enrichment of CTCs

Sixty EpCAM-positive CTCs fractions were analyzed, 30 from patients with operable breast cancer and 30 from breast cancer patients with verified metastasis, while 35 of them matched the above mentioned FFPEs. EpCAM-positive CTCs fractions were isolated from 20 mL peripheral blood in EDTA as previously described [14] using immunomagnetic anti–EpCAM-coated capture beads (Dynabeads® Epithelial Enrich, Invitrogen) to enrich for epithelial cells. Peripheral blood was also collected from 33 healthy individuals, analyzed in exactly the same way and used as a control group.

2.2.3. ctDNA

ctDNA was isolated from plasma samples obtained from peripheral blood in EDTA of 30 patients with operable breast cancer and 31 breast cancer patients with verified metastasis, while 28 samples were obtained from the same patients as above. ctDNA samples from 28 healthy blood donors were used as the ctDNA control group.

2.3. Samples preparation

2.3.1. Isolation of gDNA from FFPEs

FFPE tissue sections of 10 mm containing >80% of tumor cells were used for DNA extraction. gDNA was isolated with the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol. All DNA preparation and handling steps took place in a laminar-flow hood under DNase-free conditions. The DNA concentration was measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific, USA). The isolated gDNA was stored at −70 °C until further use.

2.3.2. gDNA extraction from CTCs and cell lines

gDNA was extracted from the EpCAM-positive CTCs fractions and cell lines using the Trizol reagent as previously described [14]. Isolated gDNA was dissolved in 50 μL of 8 mmol/L NaOH. DNA concentration was measured with the Nanodrop-1000 spectrophotometer and the samples were kept at −70 °C until further use.

2.3.3. ctDNA isolation from plasma samples

For each sample, 1 mL of peripheral blood in EDTA was centrifuged at 1600*g* for 10 min, the plasma was carefully transferred into 2 mL tubes and stored at −70 °C until ctDNA isolation. The High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Switzerland) was used to extract ctDNA from 200 μL of plasma according to the manufacturer's protocol.

2.4. SB-treatment

Before proceeding to the SB-treatment and MSP steps, we assessed the gDNA integrity of all our clinical samples by amplifying the *PIK3CA* exon 20 as previously described [21]. Only samples that were positive for amplification were further processed to SB-treatment. gDNA extracted from cell lines, FFPEs and EpCAM-positive CTCs fractions, as well as ctDNA samples, was modified with SB, to convert all non-methylated cytosines to uracil, while methylated cytosines were not converted, using the EZ DNA Methylation Gold Kit (ZYMO Research, USA) according to the manufacturer's instructions, starting from approximately 0.5 μg of DNA. Converted DNA was stored at −70 °C until use. In each SB reaction, dH_2O and 100% methylated DNA were included as negative and positive control respectively. Converted DNA from each cancer cell line was used for the assay evaluation, prior to the analysis of clinical samples. The quality of SB-treated DNA was checked by a real-time PCR (qPCR) assay for *β-actin* (*ACTB*).

2.5. MMSPA primers and capture probes design

We designed *in silico* novel primer pairs for the MMSPA using Primer Premier 5.00 software (Premier Biosoft, USA) avoiding the formation of stable hairpin structures, primer dimers, cross dimers and false priming sites. Upstream primers consist of the T7 common extension sequence and about 20–25 nucleotides (nt) of gene-specific sequence. Downstream primers consist of about 20–25 nt of gene-specific sequence and the T3 common extension at the 5′ end respectively. These common extensions and the common biotinylated T7 (b-T7) primer as well were the same as previously described [20].

Capture probes were also designed to match in length a target-specific sequence of about 30 nt, complementary to the biotinylated strand of the MMSP products and were modified with a reactive amino group and a 12-carbon spacer separating the reactive group from the 5′ end of the oligonucleotide for optimum hybridization. The specificity of all primers and capture probes sequences was first tested *in silico* using the FastPCR software (version 6.0.157, PrimerDigital, Finland) in order to avoid possible cross-hybridizations.

For maximal discrimination between methylated and unmethylated alleles, both primers and probes for *CST6, SOX17* and *BRMS1* contained several CGs. In addition, both primers and probes for these genes contained T bases derived from modified unmethylated C bases to allow discrimination and amplification of the converted from the unconverted DNA.

All primers and capture probes were synthesized by Integrated DNA Technologies (IDT, USA). Primers and capture probes sequences are given upon request.

2.6. MMSP

MMSP was carried out with 2.0 μL SB-treated DNA in a final volume of 25 μL while a PCR negative control containing no target was included in each assay run. After extensive optimization of all experimental conditions for all primer pairs the reaction consisted of 12.5 μL Master Mix, 2.5 μL Q-Solution (Multiplex PCR Kit, Qiagen, Germany) and 0.2 μM of each primer for the 3 gene targets. MMSP was performed in a Mastercycler® epgradient (Eppendorf, Germany) with the following final conditions: denaturation at 95 °C for 15 min and 45 cycles of denaturation at 95 °C for 30 s, annealing at 67 °C for 45 s and extension at 72 °C for 30s. Samples were then held for final extension at 72 °C for 10 min and kept at 4 °C until use.

2.7. Biotinylation of MMSP products

A b-T7 primer common for the 3 targets was used for biotinylation of the MMSP products in a final volume of 20 μL. 1.0 μL of MMSP products was placed in a 19 μL reaction volume containing 0.1 μM of the upstream b-T7 primer, 0.2 μM of each dNTP, 2.5 μM $MgCl_2$, 0.05 U/μL GoTaq® Hot Start Polymerase (Promega, USA) and 1× PCR buffer. The PCR reaction conditions were as follows: 95 °C for 5 min, 10 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final step at 72 °C for 10 min. Samples were kept at 4 °C until use.

2.8. Coupling of gene-specific capture probes to the spectrally distinct microspheres

The capture probes were designed *in silico* to be highly gene-specific using selected sequences of the corresponding biotinylated MMSP products. Each target-specific capture probe was coupled to a spectrally distinct xMAP® carboxylated microspheres set (Luminex Corporation, USA) by a modification of the carbodiimide coupling method, as previously described [22]. We stored each capture probe-microsphere conjugate separately in the dark at 4 °C to 8 °C and prepared a fresh bead solution containing all conjugates for each samples batch. The following microsphere sets were used in this assay: LC10080-*BRMS1*, LC10020-*CST6* and LC10070-*SOX17*.

2.9. Bead array hybridization

For each sample we prepared a bead solution consisting of 3000 beads of each gene–target conjugate in 1.5× TMAC hybridization buffer (4.5 M tetramethyl ammonium chloride, 0.15% *N*-Lauroylsarcosine sodium salt solution, 75 mM Tris–HCl, pH 8.0 and 6.0 mM EDTA, pH 8.0) to a final volume of 51 μL. We added 4.0 μL of the biotinylated MMSP products to the bead mix, denatured the sample at 95 °C for 10 min and allowed the biotinylated strands of MMSP products to hybridize with the capture probes on the beads at 65 °C for 15 min. The coupled microspheres were pelleted by microcentrifugation at 11,340*g* for 4 min, the supernatant was removed, followed by two washes with the specific wash buffer (10 mM Tris, 200 mM sodium acetate, 5 mM EDTA, and 0.05% Tween 20, pH 7.7) at 65 °C and microcentrifugation at 11,340*g* for 4 min. After the final microcentrifugation, the coupled microspheres were resuspended in 75 μL of detection reagent solution (10 μg/mL streptavidin-phycoerythrin in 1× TMAC hybridization buffer) and incubated at room temperature for 15 min.

2.10. Bead analysis

Resuspended microspheres were placed in 96-well microtiter plates and analyzed with a Luminex® 200 instrument (Luminex Corporation, USA). The sample volume was set at 50 μL, and the flow rate at 60 μL/min. A minimum of 100 events was recorded for each bead set, mean fluorescence intensities (MFIs) were computed and analysis was completed in <60 s for each sample. A sample is considered positive when the ratio $MFI_{sample}/MFI_{negative\ control}$ (signal-to-noise, S/N) is >2.

2.11. Comparison with real time MSP

All samples used in this study were also analyzed by our previously described real time MSP assays for *CST6* [15], *SOX17* [16] and *BRMS1* [23].

2.12. Statistical analysis

The assessment of agreement between the developed assay and real time MSP for *CST6, SOX17* and *BRMS1* methylation status was performed using chi-square test and Cohen's kappa coefficient [24]. The assessment of possible correlations for each gene promoter methylation status between the different categories of clinical samples was performed using Fisher's exact test. $P \leq 0.05$ in all these tests was considered statistically significant. Statistical analysis was conducted using the SPSS Statistics version 23.0 for Windows (IBM, USA).

3. Results

An outline of the present study is presented in Fig. 1 and a schematic representation of the whole experimental procedure is shown in Fig. 2.

3.1. Development of the MMSPA assay

3.1.1. Optimization of experimental conditions

To optimize the assay, we used a 100% methylated DNA sample after SB treatment. Experimental conditions in every step of the assay were optimized according to the best S/N ratio. The conditions of MMSP were optimized for the number of PCR cycles, the annealing temperature and time. The biotinylation protocol was optimized in terms of the quantity of MMSP product added. The hybridization protocol was optimized according to the number of fluorescent microspheres used for each target, the temperature and time of the hybridization step and the volume of biotinylated MMSP products used for each sample (data not shown).

3.1.2. Specificity

To verify that we could specifically detect only the targeted methylated sequences in the presence of all others, we used 3 control samples: gDNA not submitted to SB-treatment (unconverted DNA), placental DNA submitted to SB-treatment (0% methylated) and 100% methylated DNA sample after SB-treatment. The developed assay is highly specific for the SB-treated targeted methylated sequences (Fig. 3a). We further checked the analytical specificity of the assay both in the presence and in the absence of each target by using 100% methylated SB-treated DNA. First, we assessed analytical specificity when a single amplified

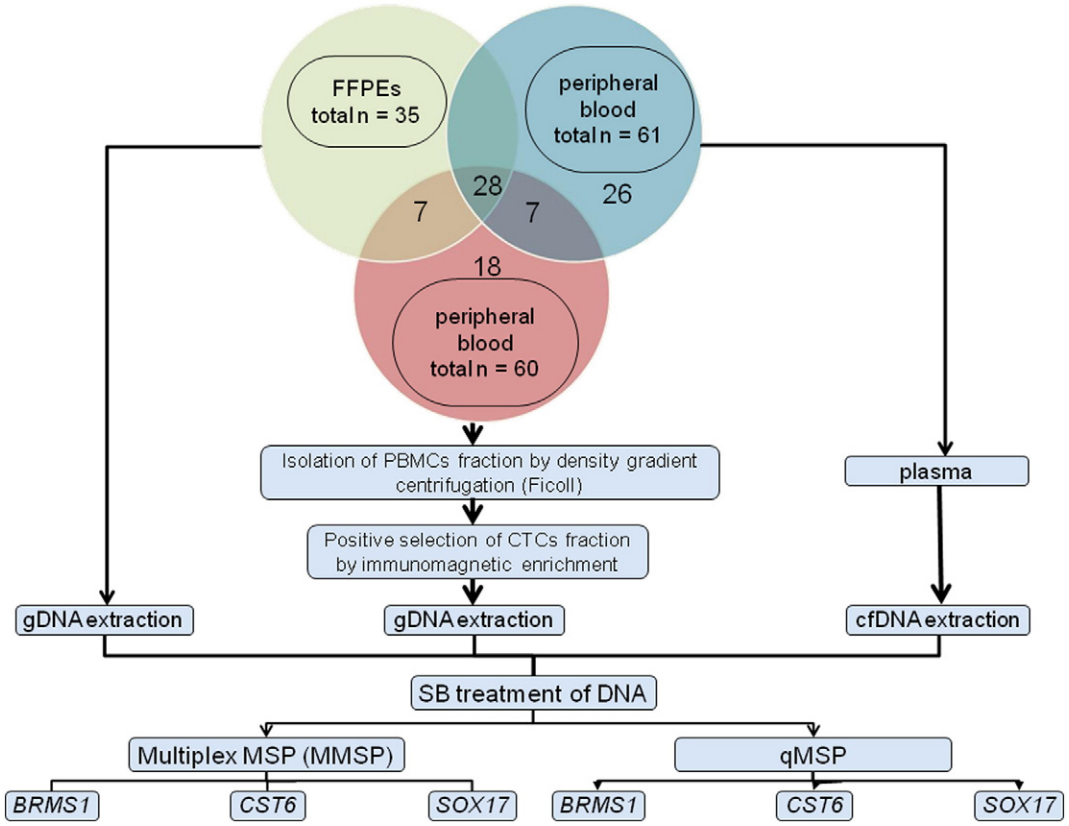

Fig. 1. Flow diagram: The number of matched samples used in this study is shown as a Venn diagram. Extracted genomic DNA and isolated ctDNA undergo SB treatment followed by the MMSPA assay and real time MSP for *BRMS1*, *CST6* and *SOX17* promoters.

target per sample is hybridized in the presence of all the conjugated microspheres. For this reason, a single PCR for each gene was performed as described in methods section, using the corresponding specific primers at a final concentration of 0.2 μM. Then each single biotinylated amplicon was hybridized in the presence of all 3 microspheres sets. The assay was highly specific since we detected the expression of each individual gene target while we did not observe any of the 6 nonspecific interactions that theoretically could have occurred between the MMSP products and the specific oligonucleotides attached on the microspheres (Fig. 3a).

Moreover, we assessed the analytical specificity of the MMSPA assay in the absence of each single gene target but in the presence of all other targets and the 3 microsphere sets. In the bead sets mix of 51 μL, 3.0 μL of each biotinylated MMSP product of the 3 single gene targets were added thus one different target was missing in each sample. The assay was highly specific in this case too, since only the amplicons that were present in each sample were detected (Fig. 3b).

3.1.3. Analytical sensitivity

The analytical sensitivity of the developed MMSPA assay was evaluated by using synthetic mixtures based on serial dilutions of SB-treated DNA control samples (0% and 100% methylated) at various percentages of methylation (0.01%, 0.1%, 0.2%, 1.0% and 10%). 2.0 μL of these synthetic samples were used following the entire analytical procedure as outlined in Fig. 2. The developed assay detected specifically and reliably methylated DNA sequences of *BRMS1, CST6* and *SOX17* at 0.01% (CV = 12.9%), 0.1% (CV = 14.4%) and 0.01% (CV = 10.4%) in the presence of 99.09%, 99.9% and 99.09% of unmethylated DNA respectively (Fig. 3c).

3.1.4. Precision

We evaluated intra-assay (within-run) precision by analyzing in triplicate: a) a 100% methylated and b) a 10% methylated converted DNA sample following the entire analytical procedure as outlined in Fig. 2. We evaluated inter-assay (between-run) precision by analyzing the same two samples in 3 separate assays in 3 different days. Intra-assay CVs of the recorded MFI units ranged from 1.7% to 5.2% and inter-assay CVs ranged from 6.5% to 11.6% (Table 1).

3.2. Evaluation of the performance of the MMSPA assay in cancer cell lines

We evaluated the performance of this assay by analyzing SB-treated DNA samples kept in aliquots at −70 °C from breast cancer cell lines MCF-7 and SK-BR-3. MCF-7 was found highly methylated for *CST6* and *SOX17* while no methylation was detected for *BRMS1*. SK-BR-3 was found methylated for *BRMS1* and highly methylated for *CST6* and *SOX17*. These data are consistent with our previous findings using MSP [14].

3.3. DNA methylation status in primary breast cancer FFPEs

We further applied the developed MMSPA assay to evaluate the DNA methylation status of *BRMS1, CST6* and *SOX17* in all available FFPE samples. *BRMS1* promoter was found highly methylated in FFPE samples; methylation was detected in 10/20 (50%) samples from early breast cancer patients, in 8/15 (53%) of patients with verified metastasis and in 4/11 (36%) of FFPE samples from mammoplasties. *CST6* promoter was found methylated in 9/20 (45%) early breast cancer patients, 3/15 (20%) patients with verified metastasis and in only 1/11 (9%) mammoplasties. *SOX17* promoter was also highly methylated in FFPE samples as it was found methylated in 11/20 (55%) of early breast cancer patients, in 12/15 (80%) of metastatic patients and in 5/11 (45%) mammoplasties.

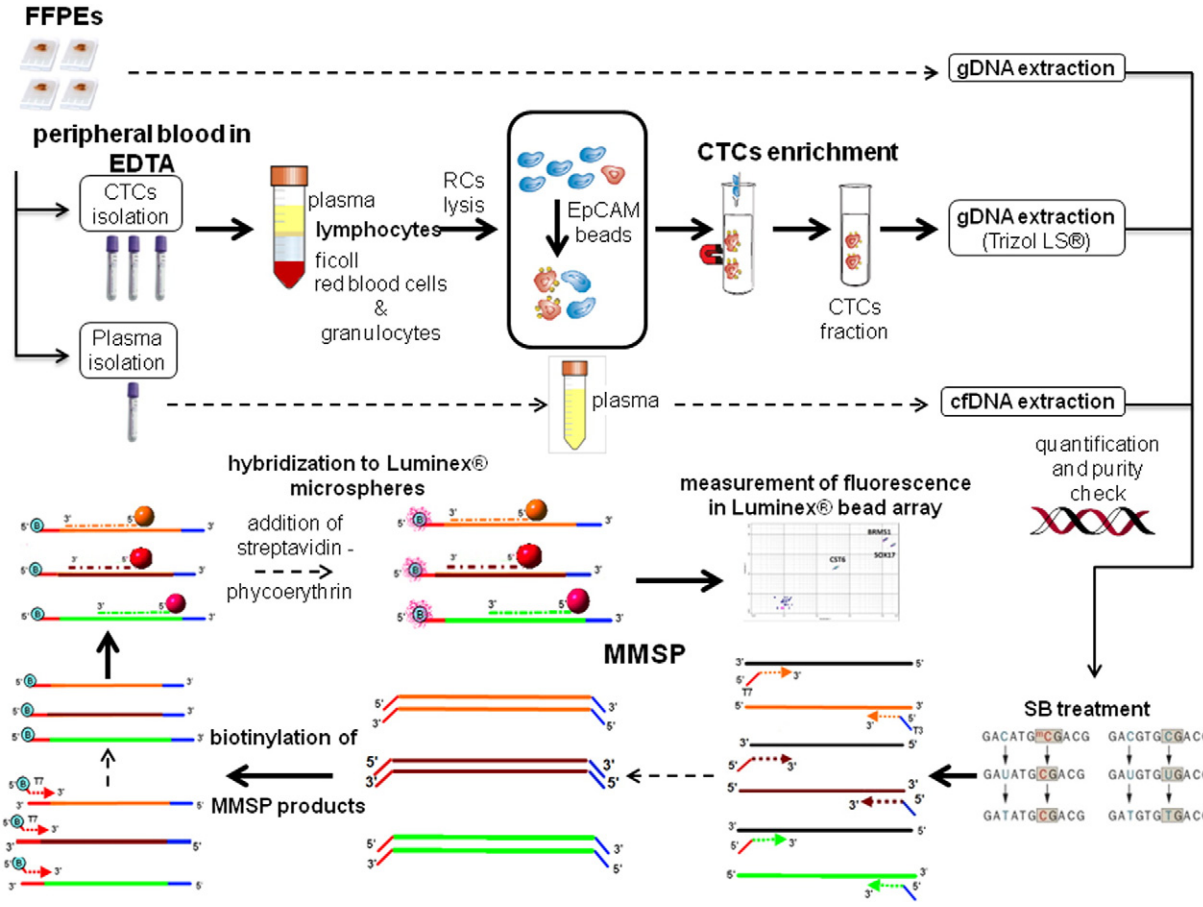

Fig. 2. Schematic representation of the experimental procedure of the MMSP-coupled liquid bead array assay for the study of the methylation status of tumor suppressor and metastasis suppressor genes. Genomic DNA and ctDNA are extracted directly from FFPEs and plasma samples respectively. Genomic DNA is also extracted from immunomagnetically enriched CTCs. Extracted DNA is quantified and then submitted to SB treatment. Multiplex MSP is performed followed by biotinylation of MPCR products. Biotinylated MPCR products are hybridized with the specific capture probes on the coupled microspheres, the reporter reagent is added and MFI values are computed. (RCs: red blood cells).

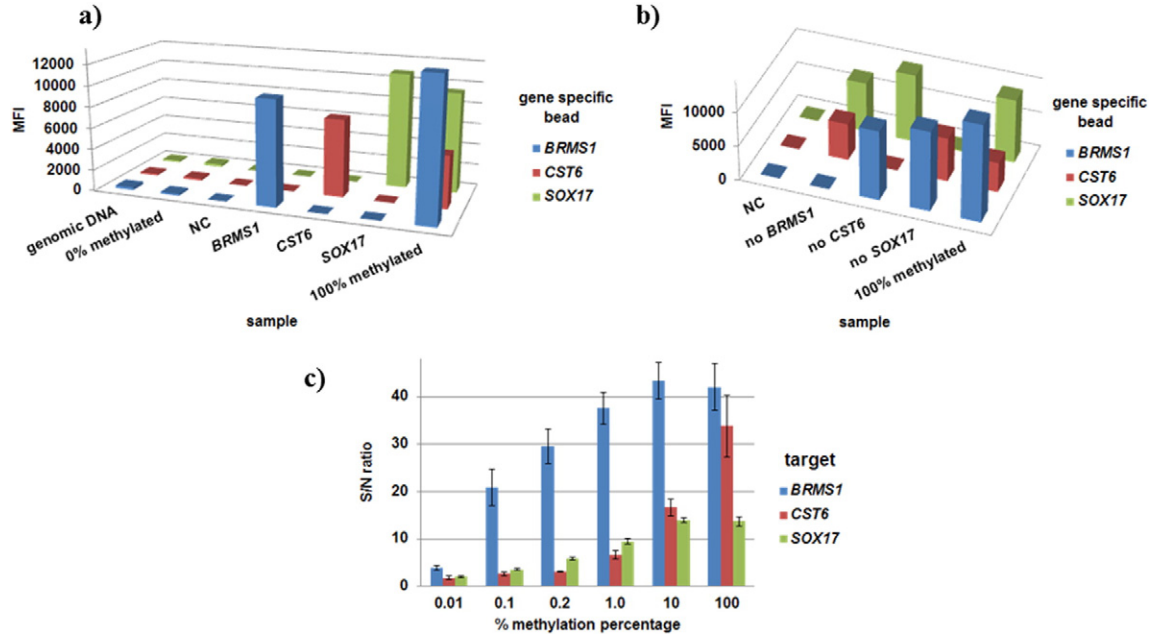

Fig. 3. Specificity and sensitivity of the MMSP-coupled liquid bead array assay. a) The developed assay detects only the methylated sequences as untreated genomic DNA and unmethylated converted DNA are negative. Each individual biotinylated MMSP product is hybridized with all 3 microspheres sets providing only the specific MFI values. b) All 3 microspheres sets are hybridized with 2 out of 3 biotinylated MMSP products. The missing MPCR product in each case is not detected. c) Sensitivity of the assay.

Table 1
Precision of the MMSP-coupled liquid bead array assay.

Gene/sample		Intra – assay precision				Inter – assay precision			
		10% methylated control (n = 3)		100% methylated control (n = 3)		10% methylated control (n = 3)		100% methylated control (n = 3)	
		MFI × 10^3	S/N	MFI × 10^3	S/N	MFI × 10^3	S/N	MFI × 10^3	S/N
BRMS1	Mean ± SD	15.1 ± 0.3	75.0 ± 1.5	14.2 ± 0.3	71.3 ± 1.5	13.9 ± 0.9	43.4 ± 3.8	13.5 ± 1.2	42.1 ± 4.9
	% CV	2.0		2.1		6.5	8.7	8.9	11.6
CST6	Mean ± SD	3.9 ± 0.1	15.3 ± 0.4	10.4 ± 0.3	34.8 ± 1.0	5.7 ± 0.6	16.6 ± 1.8	11.4 ± 1.2	33.8 ± 6.5
	% CV	2.6		2.9		11.0	10.8	10.5	19.2
SOX17	Mean ± SD	9.6 ± 0.5	33.0 ± 1.7	12.0 ± 0.2	40.3 ± 0.7	10.7 ± 1.0	5.4 ± 0.5	12.9 ± 1.5	6.8 ± 1.0
	% CV	5.2		1.7		9.3	9.3	11.6	14.7

3.4. DNA methylation status in EpCAM-positive CTCs fractions

We further applied the developed MMSPA assay to evaluate the DNA methylation status of *BRMS1, CST6* and *SOX17* in all EpCAM-positive CTCs fractions. *BRMS1* promoter methylation was detected in 8/30 (27%) early breast cancer patients, in 11/30 (37%) metastatic patients and in 2/33 (6%) healthy donors. *CST6* promoter was found methylated in 11/30 (37%) early breast cancer patients and 11/30 (37%) patients with verified metastasis. None of the 33 samples from healthy individuals was found positive for *CST6* promoter methylation. *SOX17* promoter was highly methylated in CTCs samples and methylation was detected in 8/30 (27%) early breast cancer patients, in 13/30 (43%) metastatic patients and in 4/33 (12%) healthy donors' blood samples.

3.5. DNA methylation status in ctDNA

We further applied the developed MMSPA assay to evaluate the DNA methylation status of *BRMS1, CST6* and *SOX17* in all ctDNA samples. *BRMS1* promoter was methylated in 8/30 (27%) ctDNA samples from early breast cancer patients, in 8/31 (26%) of metastatic patients and in 3/28 (11%) ctDNA samples from healthy donors. *CST6* promoter was found methylated in 5/30 (17%) ctDNA samples from early breast cancer patients, 10/31 (32%) of patients with verified metastasis and in 3/28 (11%) of ctDNA samples from healthy individuals. *SOX17* promoter methylation was detected in 5/30 (17%) of early breast cancer patients ctDNA samples, in 13/31 (42%) of metastatic patients and in 5/28 (18%) of healthy donors' blood samples.

3.6. Comparison of DNA methylation status in matched FFPEs, CTCs and ctDNA clinical samples

The concordances found between the matched FFPEs, CTCs and ctDNA samples, as well as the results of Fisher's exact test are shown in Table 2. The results for the methylation status for each individual patient are shown as a heatmap in Fig. 4.

There was a slightly non-significant concordance ($p = 0.057$) between methylation for *BRMS1* in primary tumors and the corresponding EpCAM-positive CTCs fraction for 5/20 (25%) early breast cancer patients. There was no statistically significant concordance for the same matched samples for *CST6*; agreement of 12/20 (60%) patients ($p = 0.642$). There was no statistically significant concordance for the same matched samples neither for *SOX17*; agreement for 9/20 (45%) patients ($p = 1.000$). In the group of patients with verified metastasis, there was agreement for 7/15 (47%), 7/15 (47%) and 6/15 (40%) patients comparing the methylation status of FPPEs and matched EpCAM-positive CTCs fraction samples for *BRMS1* ($p = 1.000$), *CST6* ($p = 0.505$) and *SOX17* ($p = 0.229$) respectively.

Comparing the primary tumors and corresponding ctDNA, there was an agreement for 7/14 (50%) of early breast cancer patients for *BRMS1* methylation status ($p = 1.000$), for 5/14 (36%) patients for *CST6* ($p = 0.462$) and 7/14 (50%) patients for *SOX17* ($p = 1.000$). As far as the comparison between the methylation status of FFPEs and matched ctDNA samples in metastatic patients is concerned, there was an agreement for 7/14 (50%) patients for *BRMS1* methylation status ($p = 1.000$), for 8/14 (57%) patients for *CST6* ($p = 1.000$) and 4/14 (29%) patients for *SOX17* ($p = 0.505$).

Concerning the comparison between the EpCAM-positve CTCs fraction samples and matched ctDNA samples of early breast cancer patients, there was a nevertheless statistically non-significant concordance between methylation for *BRMS1* for 13/16 (81%) patients ($p = 0.063$). However, there was no statistically significant concordance for the same matched samples for *CST6* with an agreement of 9/16 (56%) patients ($p = 1.000$) neither for *SOX17* for which there was an agreement for 12/16 (75%) patients ($p = 0.313$). Finally, comparing the CTCs samples and matched ctDNA samples of patients with verified metastasis, there was an agreement for 9/19 (47%) patients for *BRMS1* methylation status ($p = 1.000$), for 9/19 (47%) patients for *CST6* ($p = 0.603$) and 13/19 (68%) patients for *SOX17* ($p = 0.141$).

3.7. Comparison between MMSP-coupled liquid bead array assay and real-time MSP

We further compared the developed MMSPA assay with real time MSP for each gene separately, for all 228 samples available (Table 3).

For *BRMS1* methylation, 144 samples were found negative and 12 samples were found positive by both assays, while 22 samples were positive for real time MSP and negative for MMSPA and 50 samples were positive by MMSPA and negative by real time MSP. There was no statistically significant agreement between the two methods (chi-square test, $p = 0.250$), therefore the kappa value indicated a slight agreement (kappa = 0.071).

For *CST6* methylation, 148 samples were found negative and 20 samples were found positive by both assays, while 27 samples were positive for real time MSP and negative for MMSPA and 33 samples were positive by MMSPA and negative by real time MSP. There was a statistically significant agreement (chi-square test, $p = 0.001$), considered fair based on the Cohen's kappa coefficient (kappa = 0.232).

For *SOX17* methylation, 111 samples were found negative and 42 samples were found positive by both assays, while 41 samples were positive for real time MSP and negative for MMSPA and 34 samples were positive by MMSPA and negative by real time MSP. In this case too, there was a statistically significant agreement (chi-square test, $p = 0.001$), considered fair based on the Cohen's kappa value (kappa = 0.277).

The lack of agreement between these two different methods could be explained by the fact that the methylation sites that were checked were different in all cases, since the primers were designed at different positions.

4. Discussion

During the last years DNA methylation is gaining ground as a potential biomarker for diagnosis, staging, prognosis, and monitoring of

Table 2
Methylation status in matched FFPEs, CTCs and ctDNA.

Sample/gene	Early breast cancer patients								Patients with verified metastasis							
	BRMS1															
	CTCs				ctDNA				CTCs				ctDNA			
		−	+	Total		−	+	Total		−	+	Total		−	+	Total
FFPEs	−	4	6	10	−	5	2	7	−	3	4	7	−	6	1	7
	+	9	1	10	+	5	2	7	+	4	4	8	+	6	1	7
	Total	13	7	20	Total	10	4	14	Total	7	8	15	Total	12	2	14
Agreement	5/20 (25%), $p = 0.057$*				7/14 (50%), $p = 1.000$*				7/15 (47%), $p = 1.000$*				7/14 (50%), p = 1.000*			
CTCs						−	+	Total						−	+	Total
					−	10	1	11					−	7	3	10
					+	2	3	5					+	7	2	9
					Total	10	4	16					Total	14	5	19
Agreement					13/16 (81%), $p = 0.063$*								9/19 (47%), $p = 1.000$*			
Sample/gene	*CST6*															
	CTCs				ctDNA				CTCs				ctDNA			
		−	+	Total		−	+	Total		−	+	Total		−	+	Total
FFPEs	−	8	3	11	−	5	2	7	−	7	5	12	−	8	3	11
	+	5	4	9	+	7	0	7	+	3	0	3	+	3	0	3
	Total	13	7	20	Total	12	2	14	Total	10	5	15	Total	11	3	14
Agreement	12/20 (60%), $p = 0.642$*				5/14 (36%), $p = 0.462$*				7/15 (47%), $p = 0.505$				8/14 (57%), $p = 1.000$*			
CTCs						−	+	Total						−	+	Total
					−	9	2	11					−	8	4	12
					+	5	0	5					+	6	1	7
					Total	14	2	16					Total	14	5	19
Agreement					9/16 (56%), $p = 1.000$*								9/19 (47%), $p = 0.603$*			
Sample/gene	*SOX17*															
	CTCs				ctDNA				CTCs				ctDNA			
		−	+	Total		−	+	Total		−	+	Total		−	+	Total
FFPEs	−	6	3	9	−	6	0	6	−	0	3	3	−	1	2	3
	+	8	3	11	+	7	1	8	+	6	6	12	+	8	3	11
	Total	14	6	20	Total	13	1	14	Total	6	9	15	Total	9	5	14
Agreement	9/20 (45%), $p = 1.000$*				7/14 (50%), $p = 1.000$*				6/15 (40%), 0.229*				4/14 (29%), $p = 0.505$*			
CTCs						−	+	Total						−	+	Total
					−	11	0	11					−	8	1	9
					+	4	1	5					+	5	5	10
					Total	15	1	16					Total	13	6	19
Agreement					12/16 (75%), $p = 0.313$*								13/19 (68%), $p = 0.141$*			

* Fisher's exact test.

response to therapy. The field of DNA methylation based markers for prognosis and diagnosis is still emerging and its widespread use in clinical practice needs to be implemented [11]. There is also an urgent need for blood-based, minimally invasive molecular tests to assist in the detection, diagnosis and prognosis of cancers in a non-invasive and cost-effective manner. Tumor-associated methylation can actually be detected in cell-fractions enriched for CTCs and ctDNA, rendering the liquid biopsy as a very promising biomarker toward this direction. Very recently, the Epi proColon® (Epigenomics AG, Germany) blood-based colorectal cancer screening test was cleared by the FDA as an *in vitro* diagnostic PCR test for the qualitative methylation detection of *SEPT9* in EDTA plasma derived from patient whole blood specimens [25,26].

CST6 is identified in whole genome studies as one of the genes allowing the discrimination between cancerous and normal tissues according to the extent of their methylation [27] and serving efficiently for cancer prediction in a genes panel comprising *SOX17* as well [28]. Hypermethylation of *CST6* is also associated with the epithelial-to-mesenchymal transition (EMT) in a breast cancer metastasis model [29]. In the present study significant percentage of primary tumors from patients with breast cancer and corresponding EpCAM-positive CTCs

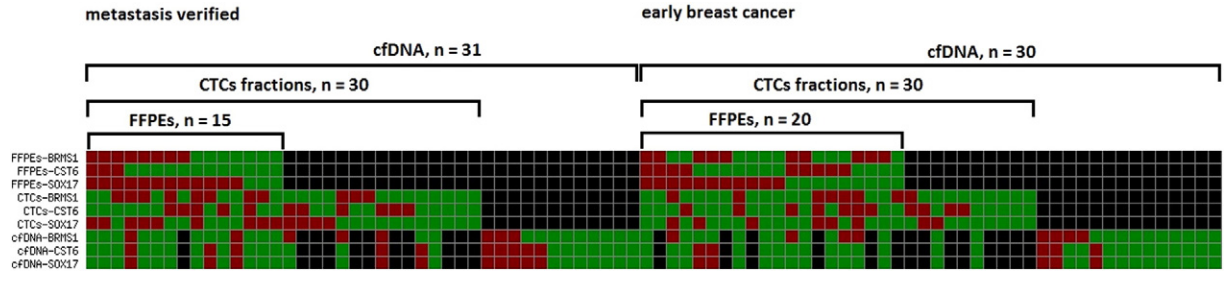

Fig. 4. Results of the methylation status for each patient in the form of a heatmap [42] (red: positive, green: negative, black: data not available).

Table 3
Contingency table for the comparison of the MMSPA and individual real time MSP.

	MMSPA											
	BRMS1	–	+	Total	*CST6*	–	+	Total	*SOX17*	–	+	Total
MSP	–	144	50	194	–	148	33	181	–	111	34	145
	+	22	12	34	+	27	20	47	+	41	42	83
	Total	166	62	228	Total	175	53	228	Total	152	76	228
Agreement	156/228 (68.4%), $p = 0.250$[a]				168/228 (73.7%), $p = 0.001$[a,b]				153/228 (67.1%), $p = 0.001$[a,b]			
Kappa value	k = 0.071				k = 0.232				k = 0.277			

[a] Chi-square test.
[b] Statistically significant.

were found hypermethylated for *CST6*. These results are in agreement with our previously reported results using nested MSP [14].

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]. Silencing of *SOX17* due to promoter hypermethylation is a frequent event and may contribute to aberrant activation of Wnt signaling in breast cancer [31,32]. In the present study, *SOX17* methylation was detected in a significant percentage of EpCAM-positive CTCs samples from patients with verified metastasis; this finding is in accordance with the previously reported high frequency of *SOX17* methylation in CTCs from metastatic patients [16].

Although the mechanism through which BRMS1 acts as a metastasis suppressor is not yet clearly understood, the loss of *BRMS1* expression has been shown to predict reduced disease-free survival in subsets of breast cancer patients [33]. It has also been shown that significant downregulation of *BRMS1* occurs in some breast tumors, especially in metastatic disease, because of epigenetic silencing [34]. Using nested MSP our group has shown that *BRMS1* is methylated in a significant percentage in the EpCAM-positive CTCs fractions isolated from samples of patients with both operable and metastatic breast cancer [14]. We confirm these results in the present study, where *BRMS1* methylation was found in almost the same percentages.

In the present study we observed no statistically significant correlations between matched FFPEs, EpCAM-positive CTCs fractions and corresponding ctDNA in the clinical samples tested. This result is in partial disagreement with our previous findings [16,35] and it could be attributed to the lower number of paired samples available and used in the present study in comparison to our previous studies. Moreover, CTCs constitute a heterogeneous population of cells not only in the gene expression level but epigenetically as well. More specifically, cases where methylation is found in ctDNA sample and is not present in CTCs are consistent with the passive DNA leakage following apoptosis of cancer cells [8].

The Luminex® platform has already been used for the determination of methylation status in limited number of cases so far, such as for the loss of imprint methylation in sperm from subfertile men [36] and for the detection of aberrant imprint methylation in the ovarian cancer by the same group [37]. In another study using methylation independent PCRs (MIPs) and multiplex hybridization in Luminex® system the methylation status of the E2 binding sites of HPV16 in cervical lesions has been studied [38]. The developed MMSPA assay is a locus-specific DNA methylation approach with a relatively low cost and easily interpretable data rendering this method cost-effective and useful for the clinical practice. In order to achieve a standardized use in the clinical laboratory routine further automatization of this assay is required to minimize the hands-on experience and the total time needed to obtain the result. Moreover, in all steps of the developed assay commercially available kits can be used: a) isolation of ctDNA from plasma, b) SB conversion reaction. After DNA isolation, the assay does not require specifically trained personnel to perform the analysis. Moreover, Luminex® liquid bead array technology has already been successfully used in a variety of assays in the clinical lab setting so far, since all preparation steps are based on a very familiar ELISA microwell plate format, and instrumentation that is available in most clinical labs.

The developed MMSP-coupled liquid bead array assay for the study of the methylation status of tumor suppressor and metastasis suppressor genes combines the advantages of multiplex PCR and the liquid bead microarray technology. Its main advantage over our previously reported real-time MSP methods is that it enables the reliable methylation analysis for three genes in parallel using a very limited amount of sample. The assay is specific for each included target in complex multiplexed formats thereby saving precious sample and reducing the costs and time of analysis. The assay produces results comparable to those of real time MSP for each individual promoter studied. The agreement between the two methods is just fair probably because of the different sets of primers used in MMSP. Although the initial design of primer pairs was based on the same primer sets used in our real time MSP assays, the *in silico* study provided prohibitive results, forcing the modification of selected primers. Discrepancies between the two methods are also observed as the MMSPA assay represents an endpoint approach. For instance, a low amplified sample in real time MSP is considered negative but the same amplification in MMSPA assay provides a MFI value clearly indicating a positive sample. The developed MMSPA has the potential to be further expanded, testing the methylation status of more genes of interest such as *RASSF1A* [39], *KISS1* [40] *etc.*

The developed assay was applied in clinical samples from breast cancer patients but could be used in other types of cancer as well. We have recently shown that the detection of *BRMS1* promoter methylation in ctDNA provides important prognostic information for non-small cell lung cancer (NSCLC) patients [23] and that hypermethylation of *SOX17* promoter in ctDNA of patients with operable gastric cancer, is associated with a poorer outcome [41]. The present methodology was developed independently from the way CTCs are isolated. A variety of systems for CTCs isolation that are based on different technologies, can be used upstream to this assay; such as the one presented here, based on EpCAM, the CellSearch® that is also based on EpCAM, or filter based and size based technologies. The main aim of this study is to present a methodology that has the potential to analyze methylated DNA sequences isolated from ctDNA from plasma or CTCs, independently from the way CTCs were isolated.

To the extent of our knowledge this is the first time that a direct multiplex MSP is applied for methylation analysis based on a classic protocol, modified properly for this type of analysis [20]. Furthermore, to the best of our knowledge, this is the first time that the Luminex® system is used for the study of the methylation status of tumor suppressor and metastasis suppressor genes in liquid biopsy samples and ctDNA originated from cancer patients.

5. Conclusion

The developed Multiplex Methylation Specific PCR-coupled liquid bead array assay is highly specific, sensitive and reproducible. The assay presents a satisfactory agreement when compared with our previously developed real-time MSPs for *BRMS1*, *CST6* and *SOX17*. The MMSPA has been successfully applied for the simultaneous study of the methylation status of tumor suppressor and metastasis suppressor genes in primary tumors (FFPEs), CTCs and corresponding ctDNA originating from patients with operable and metastasis verified breast

cancer. The main principle of the developed methodology has the potential to be extended in a large number of gene-targets and be applied in many other types of cancer.

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