

SOX17 Promoter Methylation in Circulating Tumor Cells and Matched Cell-Free DNA Isolated from Plasma of Patients with Breast Cancer

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INTRODUCTION: Detection of circulating tumor cells (CTCs) and cell-free DNA (cfDNA) in the peripheral blood of patients with solid tumors has been widely studied for the early detection of metastatic spread. We evaluated whether there was an association between the origin of cfDNA and CTCs. We investigated whether SRY (sex determining region Y)-box 17 (*SOX17*) promoter methylation in CTCs was associated with the methylation pattern of this gene in matched cfDNA isolated from plasma of patients with breast cancer.

METHODS: We examined *SOX17* methylation in 79 primary breast tumors, in 114 paired samples of DNA isolated from CTCs and cfDNA, and in 60 healthy individuals. Isolated DNA was modified by sodium bisulfite and subjected to methylation specific PCR.

RESULTS: The *SOX17* promoter was methylated in 68 (86.0%) of 79 of primary breast tumors. In CTCs, *SOX17* was methylated in 19 (34.5%) of 55 patients with early breast cancer, 27 (45.8%) of 59 patients with metastatic cancer, and 1 (4.3%) of 23 healthy individuals, whereas in matched cfDNA *SOX17* was methylated in 19 (34.5%) of 55, 24 (40.7%) of 59, and 1 (2.0%) of 49 of these same groups, respectively. There was a significant correlation between *SOX17* methylation in cfDNA and CTCs in patients with early breast cancer ($P = 0.008$), but not in patients with verified metastasis ($P = 0.283$).

CONCLUSIONS: The *SOX17* promoter is highly methylated in primary breast tumors, in CTCs isolated from patients with breast cancer, and in corresponding cfDNA samples. Our findings indicate a direct connection between the presence of CTCs and cfDNA in pa-

tients with operable breast cancer, after surgical removal of the primary tumor.

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In the past decade a wealth of information has emerged indicating the potential use of circulating tumor cells (CTCs)⁴ and circulating nucleic acids such as cell-free DNA (cfDNA) for cancer screening, prognosis determination, and monitoring of the efficacy of anticancer therapies. CTCs, cfDNA, mRNA, and microRNAs circulate in the blood of patients with cancer, and changes in their concentrations have been associated with tumor burden and malignant progression (1–5).

CTCs play a critical role in the metastatic spread of carcinomas. Their detection is associated with prognosis in many cancers and their enumeration has been cleared by the US Food and Drug Administration for follow-up of patients with breast, colon, and prostate cancer who have verified metastasis (1, 4, 5). On the basis of these developments, CTCs are considered a promising new diagnostic tool, especially for patients with advanced-stage cancer, in whom the CTCs can be used as a “liquid biopsy,” allowing physicians to follow cancer changes over time and tailor treatment accordingly (1). However, simple quantification of CTCs is not enough. The molecular characterization of CTCs is absolutely necessary for advancing our understanding of the biology of metastasis and enabling us to identify patients who will benefit from targeted therapy (6).

cfDNA circulates at increased concentrations in the plasma of patients with cancer (7). The identification of this DNA as tumor derived spurred the search for amplifiable tumor DNA markers in patient blood (8). Numerous teams have focused on the development of analytically sensitive assays that allow the spe-

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⁴ Nonstandard abbreviations: CTCs, circulating tumor cells; cfDNA, cell-free DNA; SOX, Sry-related high mobility group box; FFPE, formalin-fixed paraffin-embedded; MSP, methylation-specific PCR; gDNA, genomic DNA; EpCAM, epithelial cell adhesion molecule; SB, sodium bisulfite; LNA, locked nucleic acid; Cq, quantification cycle.

cific detection of a single tumor cell or small amounts of tumor-specific cfDNA in the peripheral blood (1–5). The detection of tumor-specific DNA alterations such as mutations (9) and methylation (10, 11) in cfDNA provides a less invasive, more easily accessible source of DNA for genetic analysis than tumor biopsies. Several studies have described methylation of tumor suppressor genes in serum or plasma samples and in the corresponding primary breast tumors, although DNA methylation was not detected in the plasma or serum of healthy donors (10). In pretreatment sera of patients with breast cancer, DNA methylation of particular genes, especially of Ras association (RalGDS/AF-6) domain family member 1 (*RASSF1*, also known as *RASSF1A*)⁵ and adenomatous polyposis coli (*APC*), is independently associated with poor outcome and a higher relative risk for death and is a more powerful predictor than standard prognostic parameters (11). cfDNA is present in the plasma or serum of patients with cancer and its methylation pattern resembles that of the primary tumor DNA (12).

Therefore, blood may be a reservoir for collecting DNA from different sources, including CTCs, cfDNA, and occult micrometastatic deposits in secondary organs. Combining these DNA analyses with screening for CTCs may provide additional information for molecular staging of tumors and monitoring of tumor progression (2). In 2009 a relationship between the occurrence of CTCs and cfDNA in the blood of patients with prostate cancer was reported (13). In this study, however, this relationship was shown using markers and methods that differ from those we report here and has not yet been studied in prostate cancer patients to see if there is a connection between the presence of cfDNA and CTCs in the same patients, detected with the same marker and the same methods.

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*) are methylated in CTCs isolated from the peripheral blood of patients with breast cancer (14). *SOX17*, a member of the Sry-related high mobility group box (SOX) family of transcription factors, is conserved in many species and plays critical roles in the regulation of development and stem/precursor cell function, at least partly through repression of the canonical Wnt/ β -catenin signaling pathway (15, 16). Global analysis of CpG island hypermethylation and gene expression in colorectal cancer

cell lines has revealed that *SOX17* gene silencing is associated with DNA hypermethylation (17), and *SOX17* plays a tumor suppressor role through suppression of Wnt signaling (18).

In this study we evaluated whether there was an association between the origins of cfDNA and CTCs. We also investigated whether *SOX17* promoter methylation in CTCs was associated with the methylation pattern of this gene in matched cfDNA isolated from the plasma of patients with breast cancer.

Materials and Methods

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

PATIENTS AND SAMPLES

We evaluated *SOX17* promoter methylation by using real-time methylation-specific PCR (MSP) in primary breast cancer formalin-fixed paraffin-embedded (FFPE) tissues, CTCs isolated from peripheral blood of patients with breast cancer, cfDNA isolated from corresponding plasma of the same patients with breast cancer, and samples from a control population. All study participants signed an informed consent form to participate in the study, which was approved by the ethics and scientific committees of our institutions.

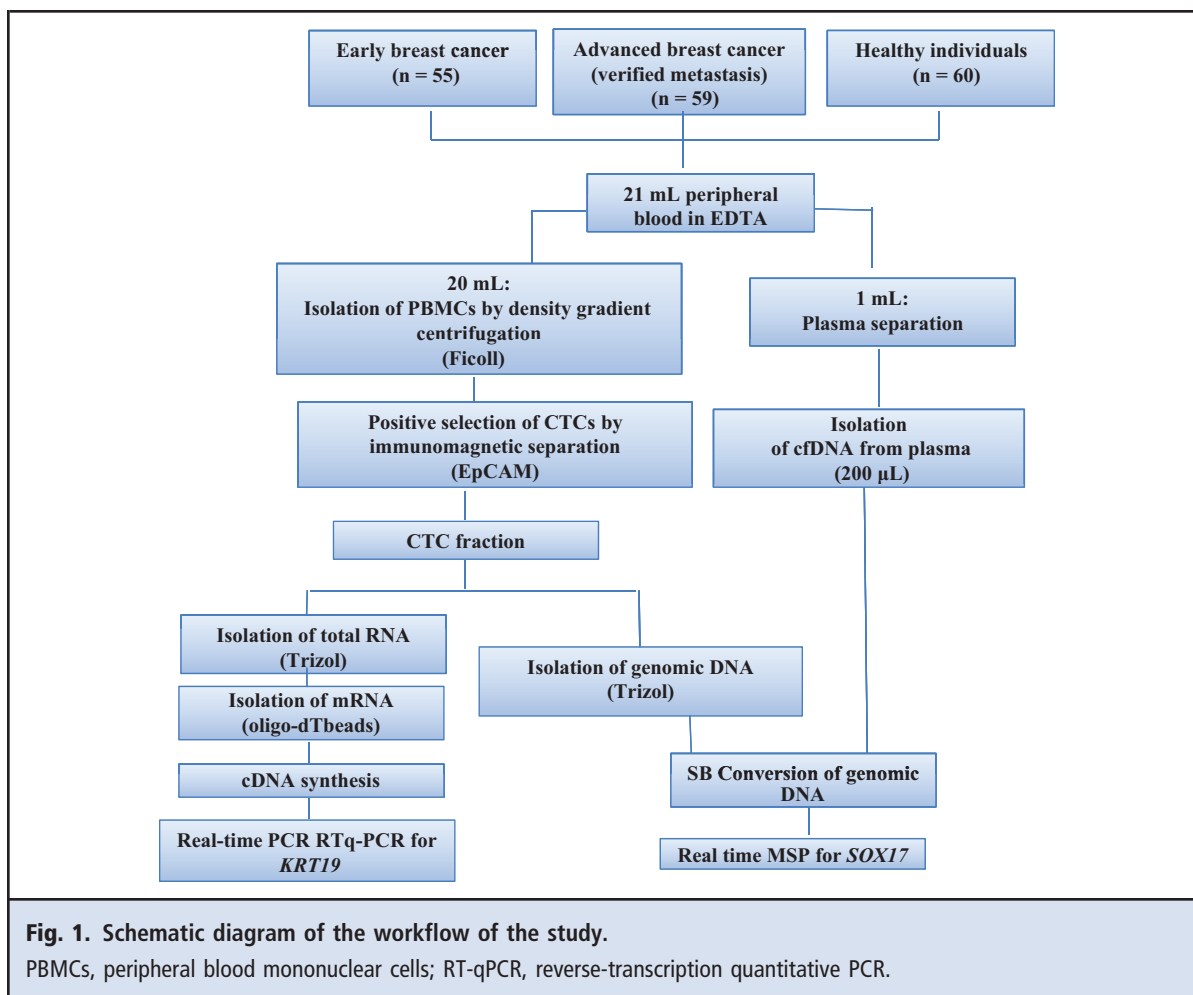
For the primary breast cancer FFPE tissues, we obtained a total of 79 FFPE breast carcinoma samples from patients with operable breast cancer and 15 non-cancerous FFPE breast tissue samples that were used as normal breast tissue controls (8 histologically normal tissues adjacent to tumors and 7 histologically cancer-free specimens from reduction mammoplasty). Additionally, 9 breast fibroadenomas were included as a separate benign tumor group.

For the CTCs isolated from the peripheral blood of patients with breast cancer, we obtained a total of 114 samples of peripheral blood from 55 patients with operable breast cancer and 59 patients with verified metastasis. For the isolation of CTCs, peripheral blood (20 mL collected in EDTA) was obtained at the midpoint of sample collection by venipuncture as previously described (14).

For the cfDNA isolated from corresponding plasma of the same patients with breast cancer, a total of 114 matched samples of plasma in EDTA were obtained from the same patients as above. For the isolation of cfDNA, plasma from blood collected in EDTA (200 μ L) was used.

Lastly, for the control population, peripheral blood was collected from 60 healthy individuals. For 12 of these individuals both the CTC fraction and plasma samples were available.

⁵ Human genes: *RASSF1A*, Ras association (RalGDS/AF-6) domain family member 1; *APC*, adenomatous polyposis coli; *CST6*, cystatin E/M; *SOX17*, SRY (sex determining region Y)-box 17; *BRMS1*, breast cancer metastasis suppressor 1; *KRT19*, keratin 19.



DNA ISOLATION FROM PRIMARY BREAST CANCER FFPE

Tissue sections of 10 μm containing $>80\%$ tumor cells were used for DNA extraction and MSP. The breast cancer cell line MCF-7 was used as a positive control in MSP reactions for the detection of *SOX17* promoter methylation. Genomic DNA (gDNA) from both FFPEs and MCF-7 cells was isolated with the High Pure PCR template preparation kit (Roche) as previously described (19). DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies).

POSITIVE IMMUNOMAGNETIC SELECTION OF CTCs

CTCs were isolated from 20 mL peripheral blood as previously described (14, 20). More specifically, after dilution of peripheral blood with 20 mL PBS (pH 7.3), peripheral blood mononuclear cells were obtained by gradient density centrifugation using Ficoll-Paque TM PLUS (GE Healthcare, Bio-Sciences AB) at 670g for 30 min at room temperature. The interface cells were removed, washed twice with 40 mL of sterile PBS (pH 7.3,

4 $^{\circ}\text{C}$) at 530g for 10 min, and resuspended in 10 mL of PBS. Cells were dyed with trypan blue and counted in a hemocytometer. Immunomagnetic Ber-EP4 [anti-epithelial cell adhesion molecule (EpCAM)]-coated capture beads (Dynabeads[®] Epithelial Enrich, Invitrogen) were used to enrich for epithelial cells.

RNA EXTRACTION FROM CTCs

Total RNA isolation from the CTC fraction was performed using Trizol (Invitrogen) as previously described (16). All RNA preparation and handling steps took place in a laminar flow hood, under RNase-free conditions. The isolated RNA from each fraction was dissolved in 20 μL of RNA storage buffer (Ambion) and stored at -70°C until used. The RNA concentration was determined by absorbance readings at 260 nm performed with the Nanodrop-1000 spectrophotometer (NanoDrop Technologies). mRNA was isolated from the total RNA by use of the Dynabeads mRNA purification kit (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed

Table 1. Oligonucleotide Sequences of SOX17 primers and LNA probe used in this study.

Oligonucleotide	Sequence 5'-to-3' direction	PCR product, bp
Primers and probe for SOX17 real-time MSP		
Methylated forward primer 5'-3'	-GTTGCGTTAGTCGTTTGCGTTC-	76
Methylated reverse primer	-AACGAATCCCGTATCCGACG-	76
Methylated specific probe (LNA modified)	-F-AGTTTATATTATGAAAG ^b C+GTTTAT+C+G+GT-Q	76
Primers for SOX17 (both methylated and unmethylated) designed for SB converted DNA		
Forward primer 5'-3'	-CGTTTTATGGTGTGGGTTAAGGA-	99
Reverse primer 5'-3'	-AGCTTGAGTGGGGTTGTAGAATGAT-	99

^a SOX17 accession number: NT_008183.
^b Position of LNA-modified nucleotide.

using the High-Capacity RNA-to-cDNA kit (Applied Biosystems) and was used for keratin 19 (*KRT19*, also known as *CK-19*) expression studies in CTCs as previously described (21).

DNA EXTRACTION FROM CTCs

gDNA was extracted from CTCs as previously described (14). After removal of the aqueous phase of Trizol, DNA was precipitated (from the interphase) by adding 150 μ L of 100% ethanol. Samples were mixed by inversion and kept at room temperature for 2–3 min, and then DNA was sedimented by centrifugation (2000g, 5 min, 4 °C) and washed twice in a solution containing 0.1 mol/L sodium citrate in 10% ethanol (500 μ L). After each wash, the DNA pellet was stored in the washing solution for 30 min at room temperature with periodic mixing and centrifuged (2000g, 5 min, 4 °C). Following 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 (2000g, 5 min, 4 °C). Isolated gDNA was then air dried for 15 min and dissolved in 50 μ L of 8 mmol/L NaOH. The DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer.

ISOLATION OF cfDNA FROM PLASMA

One milliliter of peripheral blood was collected and processed immediately. All samples were centrifuged at 1600g (10 min), and the plasma was carefully transferred into 2-mL tubes and stored at –20 °C until DNA isolation. The High Pure Viral nucleic acid kit (Roche Diagnostics) was used to extract cfDNA from plasma (200 μ L) according to the manufacturer's protocol.

SODIUM BISULFITE CONVERSION

Before proceeding to the sodium bisulfite (SB) conversion and MSP reaction steps, we assessed the gDNA integrity of all of our clinical samples by amplifying the *BRCA1*

exon 20 as previously described (22). We modified the gDNA extracted from both isolated CTC fractions and plasma with SB using the EZ DNA methylation–Gold kit (Zymo Research). Briefly, approximately 1 μ g of gDNA was treated with the conversion reagent and incubated at 98 °C for 10 min and 64 °C for 2.5 h. Samples were applied to columns, washed, desulfonated, washed again, and then eluted with 10 μ L of elution buffer. SB-converted DNA was stored at –70 °C until use. In each SB reaction, deionized water and DNA 100% methylated (DNA methylation standard, Zymo Research) were included as a negative and positive controls, respectively.

PRIMER AND PROBE DESIGNS FOR REAL-TIME MSP

For MSP, 1 primer pair specific for SB-modified and methylated DNA (M pair) and 1 pair specific for SB-modified and unmethylated DNA (U pair) are needed (23). In this project, we designed a specific primer set and a hydrolysis-locked nucleic acid (LNA) probe for methylated DNA to distinguish the methylated sequence of the *SOX17* promoter. The primer sets and the LNA probe (Table 1) were designed in silico using the Primer Premier 5 software (Premier Biosoft International) and synthesized by Forthnet and TIB Molbiol, respectively. For maximal discrimination between methylated and unmethylated alleles, both primers and probe contained several CpGs. Additionally, both primers and probes contained T bases derived from modified unmethylated C regions to allow discrimination and amplification of the converted from the unconverted DNA. To verify that we could specifically detect only the methylated sequence, we used 4 controls: gDNA not submitted to SB conversion (unconverted DNA), placental DNA submitted to SB conversion (placental converted DNA, 0% methylated), 1% methylated control (synthetic mix of 0% methylated and fully methylated standard), and 100% methylated

standard. To certify the quality of DNA in the same genomic region in our SB-converted samples that were found to be negative by real-time MSP, we specifically designed a primer set that equally amplifies both methylated and unmethylated *SOX17* SB converted sequences, approximately in the same region (see Fig. 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol59/issue1>).

REAL-TIME MSP

Each reaction was performed in a total volume of 10 μL . One microliter of SB-converted DNA was added to a 9- μL reaction mixture containing 0.1 μL of *Taq*DNA polymerase (5 U/ μL DNA polymerase; Promega), 2 μL of the supplied PCR buffer (5 \times), 1.0 μL of MgCl_2 (25 mmol/L), 0.2 μL of dNTPs (10 mmol/L; Fermentas), 0.3 μL of the forward and reverse primers (10 $\mu\text{mol/L}$), 0.15 μL BSA (10 $\mu\text{g}/\mu\text{L}$), and 1 μL hydrolysis LNA probe (3 $\mu\text{mol/L}$). Finally, deionized water was added to a final volume of 10 μL . Similar thermocycling conditions were used: 1 cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 10s and 63°C for 1 min. SB-converted DNA from the DNA methylation standard (100%) was included in every run as a positive control.

Results

ANALYTICAL SENSITIVITY AND SPECIFICITY OF REAL-TIME MSP

The analytical sensitivity of the developed real-time MSP for *SOX17* was evaluated by using synthetic mixtures based on serial dilutions of SB-converted DNA control samples (0% and 100% methylated) at various percentages of methylation (0.1%, 1%, 10%, 25%, and 50%). One microliter of these synthetic samples were used in the real-time MSP reaction. The developed real-time MSP assay for *SOX17* methylation could specifically and reliably detect the presence of the 1% methylated *SOX17* sequence in the presence of 99% unmethylated *SOX17* sequence (Fig. 2A). Moreover, we obtained a semiquantitative estimation of the methylation status of these samples through evaluation of the quantification cycle (Cq) parameters; Cq values of synthetic mixtures containing different concentrations of *SOX17* methylated sequences were differentiated so that samples with a high level of methylation had much lower Cq values than samples with a low level of methylation (Fig. 2A). However, the method used was not fully quantitative. To validate the analytical specificity of the *SOX17* real-time MSP, we initially tested the primers *in silico* and then in PCR, using SB-modified human placental gDNA that was not methylated; no amplification of the *SOX17* promoter was observed (data not shown).

SOX17 METHYLATION IN DNA ISOLATED FROM PRIMARY BREAST TUMORS

When *SOX17* promoter methylation was evaluated in FFPE breast carcinomas from 79 patients diagnosed with operable breast cancer, methylation was found in 68 (86.0%) of 79 samples (see online Supplemental Fig. 1). *SOX17* promoter methylation was further evaluated in 7 normal breast tissue samples obtained from reduction mammoplasty, 8 histologically tested noncancerous samples of breast tissue surrounding breast tumors, and 9 breast fibroadenomas used as benign tumor controls. The *SOX17* promoter was found to be methylated in 2 (25%) of 8 noncancerous breast tissues adjacent to tumors, as well as 2 (22.2%) of 9 breast fibroadenomas and 1 (14.3%) of 7 reduction mammoplasty tissues. In total, 3 (20%) of 15 noncancerous FFPE breast tissues and 2 (22.2%) of 9 benign breast tumors were positive for *SOX17* methylation.

SOX17 METHYLATION IN DNA ISOLATED FROM CTCs

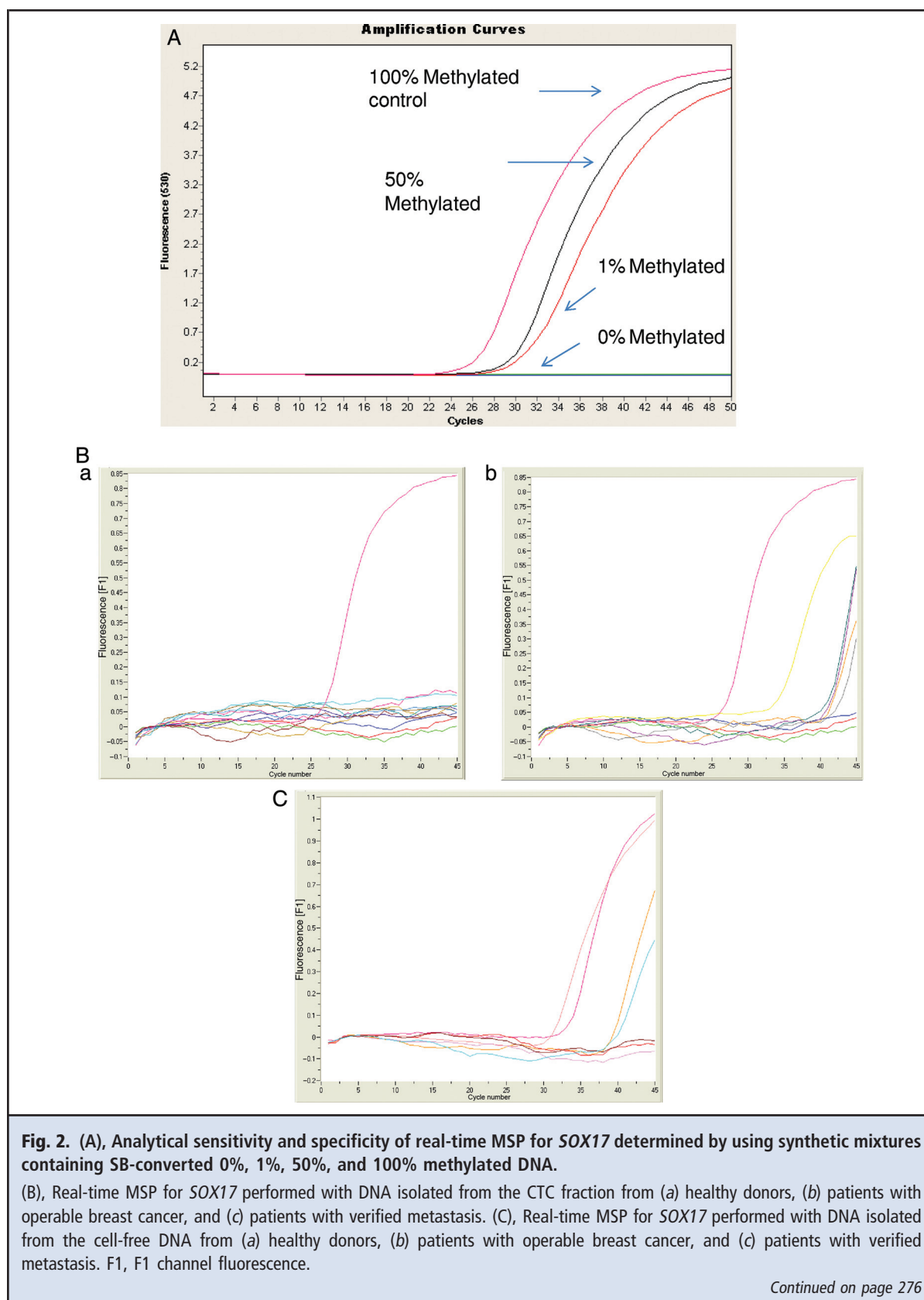
SOX17 promoter methylation was evaluated in DNA isolated from the EpCAM-positive CTC fraction from 55 patients with operable breast cancer, 59 patients with verified metastasis, and 23 healthy individuals. *SOX17* was observed in 19 (34.5%) of 55 patients with operable breast cancer, 27 (45.8%) of 59 of patients with verified metastasis, and 1 (4.3%) of 23 healthy individuals. Real-time MSP amplification of *SOX17* in DNA isolated from the CTC fraction of healthy donors, patients with operable breast cancer, and patients with verified metastasis is shown in Fig. 2B.

SOX17 METHYLATION IN cfDNA

The methylation status of the *SOX17* promoter was evaluated in cfDNA isolated from matched plasma from the same patients as above and 49 healthy individuals. Promoter methylation of *SOX17* was observed in 19 (34.5%) of 55 patients with operable breast cancer, 24 (40.7%) of 59 patients with verified metastasis, and 1 (2.0%) of 49 healthy individuals. Real-time MSP amplification of *SOX17* in DNA isolated from cfDNA from healthy donors, patients with operable breast cancer, and patients with verified metastasis is shown in Fig. 2C.

COMPARISON AND ASSOCIATION OF *SOX17* METHYLATION IN MATCHED CTCs AND cfDNA SAMPLES WITH CLINICAL AND PATHOLOGICAL CHARACTERISTICS

In tissue samples of early breast cancer there was a concordance (Table 2) between *SOX17* methylation in the cfDNA and CTC fraction for 39 (70.9%) of 55 patients ($P = 0.008$, Pearson's χ^2 test, 2 sided). However, there was no statistically significant concordance in the group of patients with verified metastasis, for which



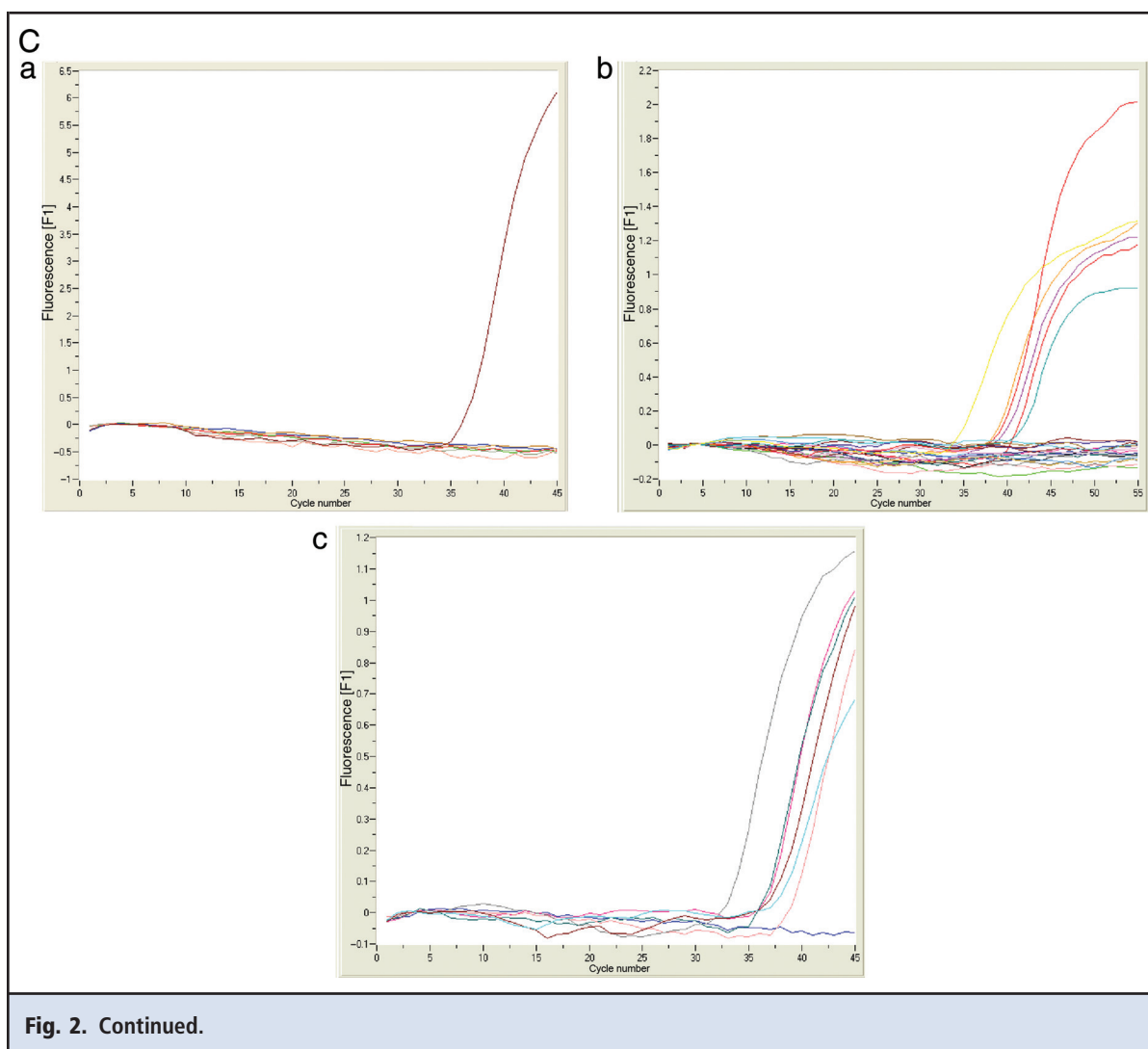


Fig. 2. Continued.

there was agreement for 34 (57.6%) of 59 patients ($P = 0.283$, Pearson's χ^2 test, 2 sided). The presence of *SOX17* methylation both in CTCs and cfDNA was not associated with any of the clinical and pathological characteristics of patients with early breast cancer. Only the absence of *SOX17* methylation in cfDNA correlated with the absence of positive lymph nodes (see online Supplemental Table 1).

KRT19 EXPRESSION AND *SOX17* METHYLATION IN CTCs AND cfDNA

We evaluated *KRT19* mRNA expression in all matched EpCAM-positive immune-magnetically isolated CTC fractions, because this epithelial marker has been extensively used to verify the presence of CTCs (1, 4, 5, 20, 21). In early breast cancer we found 20 (36.4%) of 55 samples to be positive for *KRT19* expression, whereas in the group of patients with verified me-

tastasis 24 (40.7%) of 59 were positive. Using the same procedure, none of 28 healthy individual samples was found positive for *KRT19* expression (20). The *SOX17* methylation status in CTCs did not correlate with *KRT19* expression (Table 3). In early breast cancer, there was a concordance for 34 (61.8%) of 55 samples, whereas in the group of patients with verified metastasis there was a concordance for 30 (50.8%) of 59. We further compared *KRT19* mRNA expression in all EpCAM-positive immune-magnetically isolated CTC fractions and *SOX17* methylation in corresponding cfDNA. *SOX17* methylation did not correlate with *KRT19* expression in cfDNA (Table 3). More specifically, in early breast cancer, there was a concordance for 34 (61.8%) of 55 samples, whereas in the group of patients with verified metastasis there was a concordance for 37 (62.7%) of 59. *SOX17* methylation and *KRT19* expression in the CTC fraction, and *SOX17*

Table 2. SOX17 methylation status in cfDNA and CTC fractions.

Sample characteristics		cfDNA		
Operable breast cancer (n = 55)				
CTC fraction	Methylated SOX17	Unmethylated SOX17	Total	
Methylated SOX17	11	8	19	
Unmethylated SOX17	8	28	36	
Total	19	36	55	
Agreement 39/55 (70.9%), <i>P</i> = 0.008 ^a				
Verified metastasis (n = 59)		cfDNA		
CTC fraction	Methylated SOX17	Unmethylated SOX17	Total	
Methylated SOX17	13	14	27	
Unmethylated SOX17	11	21	32	
Total	24	35	59	
Agreement 34/59 (57.6%), <i>P</i> = 0.283				

^a Pearson χ^2 , 2 sided.

methylation in the corresponding cfDNA samples for each individual patient are shown in Fig. 3. In 6 (10.9%) of 55 cases of early breast cancer all markers were found to be positive in the same patient, whereas in verified metastasis 8 (13.6%) of 59 cases were found to be positive for all markers in the same patient.

Discussion

Both CTCs and cfDNA are being intensely explored as promising sources of novel tumor biomarkers. In this study we investigated whether a direct connection between the presence of CTCs and cfDNA occurs in patients with operable breast cancer in which the primary tumor has already been resected.

Our group previously showed the prognostic importance of the detection of CTCs in the peripheral blood of patients with early breast cancer after the surgical removal of the primary breast tumor, before and after chemotherapy, through the epithelial molecular marker *KRT19* (24–28). We also showed that the detection of CTCs after chemotherapy in patients with breast cancer is associated with involvement of more than 3 auxiliary lymph nodes, with significantly increased clinical relapses and disease-related deaths (29).

The release of nucleic acids into the blood is thought to be related to the apoptosis and necrosis of cancer cells in the tumor microenvironment. Secretion has also been suggested as a potential source of cfDNA (3). In cases in which the primary tumor is already resected, such as in operable breast cancer, tumor cells that circulate in the blood and micrometastatic deposits that are present at distant sites, such as the bone marrow and liver, can also contribute to the release of

cfDNA. Previous studies based on different markers and different methodologies have shown a connection between the presence of CTCs and cfDNA in prostate cancer (13). However, it has recently been shown that patients with breast cancer can have different gene expression profiles in CTCs (20), and individual CTCs present in the blood of the same patient can be very heterogeneous (30).

For this reason, to address the question of whether there is a direct connection between the presence of CTCs and cfDNA in patients with operable breast cancer for which the primary tumor is already resected, we chose to use the same marker and the same methodology in matched clinical samples. We chose as a marker *SOX17*, one of the tumor suppressor genes shown to be epigenetically silenced in CTCs of patients with breast cancer (14). For this study we report our findings in a qualitative matter as positive or negative for the presence of *SOX17* methylated sequences. According to our analytical specificity study, only *SOX17* methylated sequences are recognized by the primers and probe used. On the basis of this finding we did not use a cutoff, and a sample with even 1% methylation was considered as positive because this result indicated that the sample contained a few cells in which *SOX17* was epigenetically inactivated. Although the data presented here indicate which patients were arbitrarily considered to be positive or negative, we have no indication of the percentage of promoter methylation that existed in each individual patient and in different patient groups compared to the percentage of methylation in healthy patients. Presenting the data in this manner facilitates visualization of the differences in levels of methylation between healthy individuals and patients with malignant conditions.

Table 3. SOX17 methylation status in cfDNA and CTCs in respect to KRT19 mRNA expression.

Sample characteristics	KRT19		
Operable breast cancer (n = 55)			
SOX17 methylation status in CTC	KRT19 positive	KRT19 negative	Total
Methylated SOX17	9	10	19
Unmethylated SOX17	11	25	36
Total	20	35	55
Agreement: 34/55 (61.8%), $P^a = 0.218$			
SOX17 methylation status in cfDNA	KRT19 positive	KRT19 negative	Total
Methylated SOX17	9	10	19
Unmethylated SOX17	11	25	36
Total	20	35	55
Agreement: 34/55 (61.8%), $P = 0.218$			
Verified metastasis (n = 59)			
SOX17 methylation status in CTC	KRT19 positive	KRT19 negative	Total
Methylated SOX17	11	16	27
Unmethylated SOX17	13	19	32
Total	24	35	59
Agreement: 30/59 (50.8%), $P = 0.993$			
SOX17 methylation status in cfDNA	KRT19 positive	KRT19 negative	Total
Methylated SOX17	13	11	24
Unmethylated SOX17	11	24	35
Total	24	35	59
Agreement: 37/59 (62.7%), $P = 0.081$			

^a P, Pearson χ^2 , 2 sided.

We have shown that the *SOX17* promoter is highly methylated in primary breast tumors, in CTCs isolated both from patients with early breast cancer and those with metastasis-verified breast cancer, and in corresponding cfDNA samples. A key finding is that *SOX17* promoter methylation in CTCs and in matched cfDNA is highly correlated. This finding points toward a direct connection between the presence of CTCs and cfDNA in patients with operable breast cancer, after surgical removal of the primary tumor. The importance of this finding has to be evaluated later, when the clinical outcome of these pa-

tients with early breast cancer is known. In the group of patients with verified metastasis no such connection was observed, even if in many cases there was a concordance between *SOX17* methylation in CTCs and cfDNA. This result could be due to the fact that in these cases the metastasis was already present and cfDNA can also be released from apoptotic cells escaping from the metastatic site.

In conclusion, our findings indicate for the first time a direct connection between the presence of CTCs and cfDNA in patients with operable breast cancer, after surgical removal of the primary tumor.

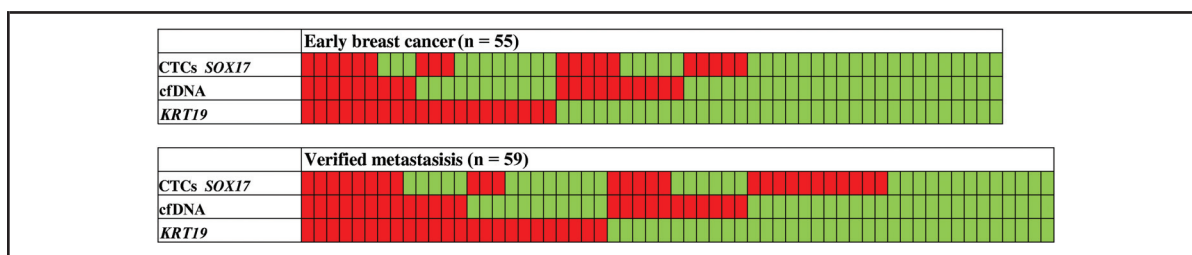


Fig. 3. Heat map of SOX17 promoter methylation and KRT19 expression in the CTC fraction and cfDNA in matched samples of patients with (a) operable breast cancer (n = 55), and (b) verified metastasis (n = 59).

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.

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