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CST6 promoter methylation in circulating cell-free DNA of breast cancer patients

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ABSTRACT

Objectives: We have recently shown that detection of *CST6* promoter methylation in primary breast tumors can provide important prognostic information in patients with operable breast cancer and that *CST6* promoter is also methylated in Circulating Tumor Cells (CTC). In this study we evaluated the presence of *CST6* promoter methylation in cell-free DNA (cfDNA) circulating in plasma of breast cancer patients.

Design and methods: Our study material consisted of: a) a pilot testing group of 27 patients with stage I-III operable breast cancer, 46 patients with verified metastasis and 37 healthy donors and b) an independent cohort of 123 consecutive stage I-III operable breast cancer patients. Methylated and unmethylated *CST6* promoter sequences were detected by using methylation-specific PCR (MSP). CST6 immunohistochemical detection was performed in 20 corresponding primary tumor tissues.

Results: In the pilot testing group, *CST6* promoter was methylated in 8/27 (29.6%) operable breast cancer patients, in 6/46 (13.0%) patients with verified metastasis but none of 37 healthy individuals (0%). In the independent cohort, 49/123 (39.8%) operable breast cancer patients were found positive. During the follow up period, 25/123 (20.3%) patients relapsed and 9/123 (7.3%) died. *CST6* was methylated in cfDNA of 13/25 (52%) patients that relapsed and in 3/9 (33.3%) patients that died.

Conclusions: *CST6* promoter is highly methylated in cfDNA of breast cancer patients, but not in healthy individuals. *CST6* promoter methylation in cfDNA, should be prospectively validated as a novel plasma tumor biomarker for breast cancer in a large cohort of breast cancer patients.

Introduction

In recent years many teams have focused on the development of sensitive assays that allow the specific detection of single tumor cells or small amounts of cell-free tumor DNA in the peripheral blood of cancer patients. These methods allow the detection and characterization of early metastatic spread and will provide unique insights into the biology of metastatic progression of human tumors, including the effects of therapeutic interventions [1]. In the past decade a wealth of information indicating the potential use of circulating nucleic acids for cancer screening, prognosis and monitoring of the efficacy of anticancer therapies has emerged. It is now known that DNA, mRNA and microRNA are released and circulate in the blood of cancer patients. Changes in the levels of circulating nucleic acids have been associated with tumor burden and malignant progression. These findings with a specific focus on the clinical utility of cell-free nucleic acids as blood biomarkers have been recently very nicely reviewed [1,2].

Cell-free DNA (cfDNA) circulating in plasma of breast cancer patients was suggested as a very promising tumor biomarker for early detection and prognosis already since 1999 [3]. Through all these years since then it has also been clearly shown that DNA methylation is one of the most frequently occurring epigenetic events in the mammalian genome and alterations in DNA methylation are very common in cancer cells [4]. In particular, hypermethylation has been reported as an early event in breast cancer [5], frequently leading to gene silencing through methylation of CpG-rich regions near the transcriptional start sites of genes that regulate important cell functions [6]. Several studies have described methylation of tumor suppressor genes in serum or plasma samples and in the corresponding primary breast tumors, while notably DNA methylation was not detected in plasma or serum of healthy donors [7]. DNA methylation of particular genes, especially of *RASSF1A* and *APC*, in pre-treatment sera of breast cancer patients, has been shown to be independently associated with poor outcome, with a relative risk

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for death of 5.7, and a more powerful predictor than standard prognostic parameters [8]. A number of studies have shown that cell free DNA is present in the plasma or serum of cancer patients and its methylation patterns resemble that of the primary tumor DNA [9].

Cystatin M or E/M (encoded by the *CST6* gene) is an endogenous inhibitor of lysosomal cysteine proteases that functions to protect cells against uncontrolled proteolysis [10]. Cystatin M was first identified and cloned by Sotiropoulou et al. by differential RNA display as a transcript that was significantly down-regulated in metastatic breast cancer cells when compared to primary breast cancer cells [11]. Later, the same protein was identified and cloned independently from embryonic lung fibroblasts and was named Cystatin E [12]. Cystatin E/M is a low molecular mass protein sharing 27-32% homology with other cystatins. Cystatin M has been assigned to chromosome region 11q13 [13], which is the site of loss of heterozygosity (LOH) in several cancer types and believed to harbor tumor suppressor genes. Cystatin M was shown to directly inhibit the activity of cathepsins B, V, and L [14,15]. In addition, cystatin M controls the activity of legumain, which is a known oncogene and an indicator of poor prognosis in colorectal and breast cancer but was also found overexpressed in the majority of human solid tumors [16,17]. Thus, imbalance between proteases and their inhibitors cystatins can lead to tumor development, invasion and metastasis [18]. Analysis of the *CST6* gene shows an unusual density of CpGs islands in the promoter and the exon 1 of the gene (~64 CpGs in a 507 bp segment) [19] and it was recently shown that this region is a target for DNA methylation, which concludes to loss of cystatin M expression in breast cancer lines and breast carcinomas [19–21].

We have demonstrated that *CST6* is hypermethylated in breast cancer tissues and that *CST6* promoter methylation provides important prognostic information in patients with operable breast cancer [22]. Moreover we have recently shown that *CST6* is epigenetically silenced in Circulating Tumor Cells (CTC) isolated from peripheral blood of operable and metastatic breast cancer patients [23]. In the present study, we examined for the first time the prevalence of *CST6* promoter methylation in cell-free DNA circulating in plasma of operable and metastatic breast cancer patients as well as in healthy individuals.

Patients and Methods

Patients

Our study material consisted of a total of 233 consecutively collected clinical samples: a) one pilot testing group, consisting of 27 patients with stage I-III operable breast cancer, within 2–4 weeks after the removal of the primary tumor and before the initiation of adjuvant chemotherapy, 46 patients with verified metastasis and 37 healthy female blood donors (all comparable to the cases with respect to other characteristics aside from a diagnosis of breast cancer) and b) one independent cohort consisting of 123 stage I-III operable breast cancer patients whose clinicopathological characteristics and clinical outcome was not known when the analysis was performed, but were later available to us. The median follow up (FU) for these 123 patients was 64 months, with a range of 18–204 months, and the plasma samples were consecutively collected within 2–4 weeks after the removal of the primary tumor and before the initiation of adjuvant chemotherapy. For 20 of these operable breast cancer patients matched formalin fixed paraffin-embedded tissues (FFPEs) samples were also available. All patients were treated in the Medical Oncology Unit, Helena Venizelou Hospital. For every patient enrolled, a complete diagnostic examination to evaluate the presence or absence of distant metastasis was performed consisting of chest x-rays, mammography, ultrasound of the liver and a whole body bone scan. Computed tomography scans and/or magnetic resonance imaging studies were performed if clinically indicated. Peripheral blood in EDTA was centrifuged at 2,000 x g for 10 min at room temperature and 1 mL aliquots of plasma samples were stored at −20 °C. The breast cancer cell line MCF-7 was used as positive control in MSP reactions for the detection of *CST6* promoter methylation. All patients signed an informed consent to participate in the study which was approved by the Ethics and Scientific Committees of our Institution.

Isolation of genomic DNA from paraffin tissues and cell lines

Tissue sections of 5 μm containing >80% of tumor cells were used for DNA extraction and Methylation-Specific PCR (MSP) analysis. Genomic DNA (gDNA) from paraffin tissues and from the breast cancer cell line MCF-7 was isolated with the High Pure PCR Template Preparation kit (Roche, Germany) according to the manufacturer's instructions.

Isolation of cell-free DNA circulating in plasma

Circulating cell-free DNA was isolated from plasma samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany). 200μL of plasma were mixed with 200μL of working solution and 50μL proteinase K (18 mg/mL) and incubated for 10 min at 72 °C. DNA isolation was, then, processed as described in the manufacturer's protocol. DNA concentration was determined in the Nanodrop ND-100 (Nanodrop Technologies, USA).

Sodium bisulfite conversion

Extracted DNA was modified with Sodium Bisulfite (SB), in order to convert all unmethylated, but not methylated-cytosines to uracil. Bisulfite conversion was carried out in 1 μg of denaturated DNA using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer's instructions. The converted DNA was stored at −70 °C until used. In each SB conversion reaction, dH_2O and MCF-7 were included as a negative and positive control, respectively. In order to avoid false negative results, particularly in cell-free SB-converted DNA, we performed MSP for *CST6* using an unmethylated set of primers, as previously described [22].

Methylation Specific PCR (MSP)

The methylation status of *CST6* in circulating cell-free DNA was detected by nested MSP as previously reported [22]. Each MSP reaction was performed in a total volume of 25μL. SB-treated DNA was amplified in two separate MSP reactions, one with primers specific for methylated and one with primers specific for unmethylated *CST6* promoter sequences. Human placental genomic DNA (gDNA; Sigma Aldrich) methylated *in vitro* with SssI methylase (NEB, Ipswich, MA) was used, after SB conversion, as fully methylated (100%) MSP positive control; the same unmethylated placental gDNA, was used, after SB conversion, as a negative MSP control.

For all clinical samples studied, 1μL (~100 ng) of SB-converted DNA was added into a 24 μL reaction mixture that contained 0.125 μL of Taq DNA polymerase (5U/μL, Go Taq polymerase; Promega), 2.5 μL of the supplied 5x PCR buffer, 2.0 μL of $MgCl_2$ (25 mmol/L), 0.5 μL of dNTPs (10 mmol/L; Fermentas) and 1 μL of the corresponding forward and reverse primers (10 μmol/L); finally dH_2O was added to a final volume of 25 μL. In the first MSP, SB treated DNA was amplified with a set of external primers specific for methylated or unmethylated sequences. Nested MSP was performed using 1μL of the amplified products and a set of internal primers that are specific for the methylated sequences or internal primers specific for the unmethylated sequences, respectively. All primer sets used were previously described [22]. Similar thermocycling conditions were used for the first and second MSP, when using primers for methylated sequences: 1 cycle at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, with a final extension cycle of 72 °C for 10 min. Thermocycling conditions for the first and the second MSP when using primers for unmethylated sequences were the following: 1 cycle at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min, with a final extension

cycle of 72 °C for 10 min. MSP products for methylated and unmethylated *CST6* promoter were resolved on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH = 8.0) and visualized by ethidium bromide staining.

Immunohistochemical detection of cystatin M

In 20 formalin-fixed, paraffin-embedded tissues that were available from the same breast cancer patients (13 samples from patients with methylated *CST6* promoter and 7 samples from patients with unmethylated *CST6* promoter), an immunohistochemical study was performed, using a rabbit polyclonal antibody specific for cystatin M [11,24] at 1:100 dilution. The streptavidin-biotin-peroxidase method was applied using the Bond II automated immunohistochemistry system (Leica/Menarini).

Statistical Analysis

For the independent cohort (123 consecutive operable breast cancer patients), all clinicopathological data (including disease free survival and overall survival) information were available to us after the *CST6* methylation analysis was completed. Correlations between *CTS6* promoter methylation status and clinicopathological features of the patients were assessed by the Chi-square test. P values <0.05 were considered statistically significant. Statistical analysis was performed by using the SPSS Windows version 17.0 (SPSS Inc., Chicago, IL).

Results

Sensitivity and specificity of nested MSP assay for CST6 promoter methylation

A nested MSP was performed throughout our study, since conventional MSP was not sensitive enough for the detection of *CST6* promoter methylation in cell free DNA. We have previously evaluated in detail both the sensitivity and specificity of this nested MSP [22]. The sensitivity was evaluated by initially preparing synthetic samples of fully (100%) in vitro methylated (through *SssI* methylase) human placental genomic DNA and fully unmethylated (100%) human placental genomic DNA. According to our findings this assay is sensitive enough to detect one methylated sequence of *CST6* in the presence of 20,000 unmethylated sequences (1:20,000) corresponding to 0.005%. The specificity of nested MSP assay for *CST6* promoter methylation, was tested using SB modified human placental gDNA that is not methylated, 11 normal breast tissues obtained from reduction mammoplasty, and 10 breast fibroadenomas used as benign tumor controls.; no amplification of *CST6* promoter was observed in any case [22].

CST6 promoter methylation in circulating cell-free DNA

The methylation status of *CST6* promoter in circulating cell-free DNA was firstly assessed in the pilot testing group. In this group, *CST6* promoter was found methylated in 8/27 (29.6%) patients with operable breast cancer (Fig. 1A), in 6/46 (13.04%) patients with verified metastasis but not in any of 37 healthy individuals (0%) (Fig. 2A). MSP with primers specific for the unmethylated DNA was also performed for all SB-converted samples to exclude failure of PCR reaction when the PCR reaction specific for the methylated DNA sequences was negative (Figs. 1B, 2B). Figs. 1 and 2 are reporting only selected representative samples. These results are supportive for the technical feasibility of the study and clearly suggest the absence of *CST6* promoter methylation in blood from healthy donors.

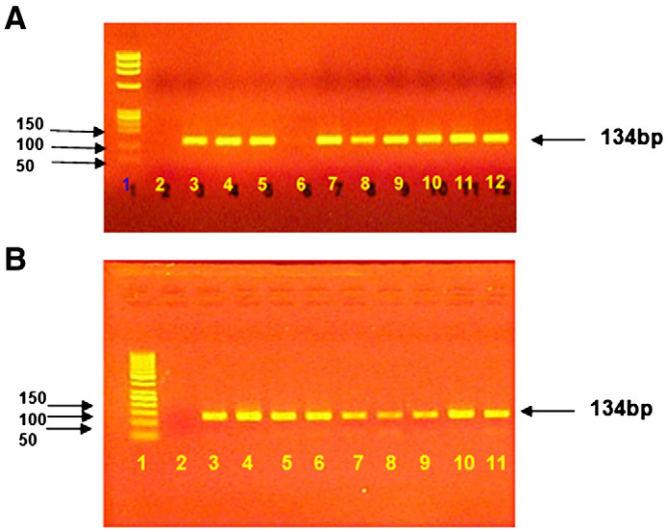

Fig. 1. A: *CST6* promoter methylated sequences in cell-free DNA circulating in plasma of early breast cancer patients: 1) DNA marker 50 bp, 2) Negative control: dH_2O, 3,4,5,7,8-11) plasma samples of early breast cancer patients 6) unmethylated breast cancer plasma sample 12) Positive control: MCF-7. **B:** *CST6* promoter unmethylated sequences cell-free DNA circulating in plasma of early breast cancer patients: 1) DNA marker 50 bp, 2) Negative control: dH_2O, 3–10) plasma samples of early breast cancer patients 11) Positive control: placental DNA.

CST6 promoter methylation in circulating cell-free DNA in an independent cohort of operable breast cancer patients

The methylation status of *CST6* promoter in circulating cell-free DNA was further evaluated in an independent cohort that consisted of 123 operable breast cancer patients with a median follow up of 64 months, (range : 18–204 months). In this independent cohort, 49/123 (39.8%) patients were found positive for *CST6* methylation in circulating cell-free DNA. These patients' characteristics in relation to the methylation status of *CST6* are presented in Table 1. Chi-square analysis revealed no statistically significant correlation between *CST6* promoter methylation and the major clinicopathological features of the patients.

During the follow up period, 25/123 (20.3%) patients relapsed. *CST6* was methylated in circulating cell-free DNA of 13/25 (52%) patients that relapsed. During the follow up period, 9/123 (7.3%) patients died as a consequence of disease progression. *CST6* was methylated in circulating

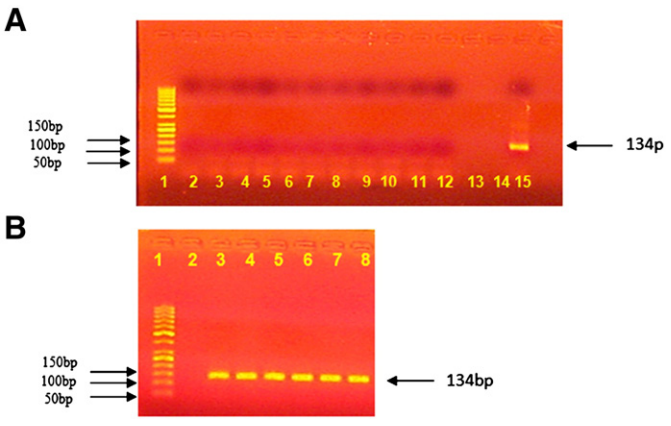

Fig. 2. A: *CST6* promoter methylated sequences in cell-free DNA circulating in plasma of healthy individuals 1) DNA marker 50 bp, 2) Negative control: dH_2O, 3–12) plasma samples of healthy individuals, 13–14) no sample 15) Positive control: MCF-7. **B:** *CST6* promoter unmethylated sequences in plasma of healthy individuals 1) DNA marker 50 bp, 2) Negative control: dH_2O, 3–7) plasma samples of healthy individuals, 8) Positive control: placental DNA.

Table 1
Association of *CST6* methylation in cell-free circulating DNA with clinicopathological features of operable breast cancer patients (n = 123). p-values are derived from a Chi-square test.

Patients enrolled	*CST6 Methylation*			
	All	Positive	Negative	P
	n (%)	n (%)	n (%)	
	123 (100)	49 (39.8)	74 (60.2)	
Age, years				0.071
≥ 51	65 (52.8)	21 (32.3)	44 (67.7)	
<51	58 (47.2)	28 (48.3)	30 (51.7)	
Menopausal status				0.266
Premenopausal	58 (47.2)	26 (44.8)	32 (69.0)	
Postmenopausal	63 (51.2)	22 (34.9)	41 (65.1)	
Unknown	2 (1.6)			
Tumor Size, cm				0.118
T1 (≤2)	45 (36.6)	22 (48.9)	23 (51.1)	
T2/T3 (>2)	78 (63.4)	27 (34.6)	51 (65.4)	
Grade				0.760
1 or 2	47 (38.2)	20 (42.6)	27 (57.4)	
3	68 (55.3)	27 (39.7)	41 (60.3)	
Unknown	8 (6.5)			
Lymph nodes				0.661
None	41 (33.3)	15 (36.6)	26 (63.4)	
1-3	50 (40.7)	22 (44.0)	28 (56.0)	
>4	26 (21.1)	9 (34.6)	17 (65.4)	
Unknown	6 (4.9)			
ER				0.969
Positive	65 (52.8)	26 (40.0)	39 (60.0)	
Negative	58 (47.2)	23 (39.7)	35 (60.3)	
PR				0.696
Positive	74 (60.2)	30 (40.5)	44 (59.5)	
Negative	46 (37.4)	17 (37.0)	29 (63.0)	
Unknown	3 (2.4)			
HER2				0.606
Positive	49 (39.8)	18 (36.7)	31 (63.3)	
Negative	70 (56.9)	29 (41.4)	41 (58.6)	
Unknown	4 (3.3)			

cell-free DNA in 3/9 (33.3%) of patients that died. As can be seen in Table 2 however, the incidence of relapses was not significantly higher in patients with methylated 13/49 (26.5%) than in patients with unmethylated *CST6* promoter 12/74 (16.2%). The same was found in respect to the incidence of deaths: it was not significantly higher in patients with methylated 3/49 (6.1%) than in patients with unmethylated *CST6* promoter 6/74 (8.1%).

Cystatin M expression in primary breast tumors

In 20 FFPE tissues that were available from the same breast cancer patients the expression of *CST6* was also studied using an antibody specific for cystatin M [11]. All tissues of the 20 patients (100%) showed a diffuse strong cytoplasmic staining for cystatin M indicating that the expression of the *CST6* gene is not lost in primary breast tumors, irrespectively of the receptor methylation detected in cell free DNA circulating in plasma. Cystatin M expression was also observed in foci of carcinoma *in situ* (CIS) as well as in benign breast luminal epithelium. Myoepithelial cells were negative for cystatin M expression both in CIS and in the benign structures (Figs. 3A, B and 4). No apparent difference in the levels of cystatin M immunohistochemical expression was observed between breast carcinomas from patients with methylated *CST6* and patients with unmethylated *CST6* promoter.

Discussion

Concentrations of circulating cell-free tumor DNA are high in cancer patients compared to healthy individuals [1,2]. Early in tumor development, apoptotic and necrotic cells of the primary tumor release DNA into the bloodstream. Inactivation of tumor suppressor genes by promoter hypermethylation that plays a crucial role in tumorigenesis has been detected in cell free DNA [1,2]. In this work we report for the first time that *CST6* promoter methylation can be detected early on in circulating cell-free DNA of breast cancer patients, while it is not present in healthy individuals.

Cystatin M was originally described as a putative tumor suppressor, whose expression is often diminished or completely lost in metastatic breast cancers [11] however its cellular functions are virtually unknown. Loss of expression is likely associated with the progression of a primary tumor to a metastatic phenotype, suggesting a role of *CST6* as a tumor suppressor gene [24]. When exogenously expressed in human MDA-MB 435 breast cancer cells, Cystatin M expression significantly alters the neoplastic phenotype *in vitro*, resulting in diminished cell proliferation, loss of migration and reduced cell adhesion [24]. More recently, it was shown that *CST6* is epigenetically regulated by strong hypermethylation of the *CST6* gene promoter in breast cancer cell lines [19], in breast cancer and metastatic lesions in the lymph nodes [25], in malignant gliomas [26], in cervical [27] and prostate cancer [28]. Because promoter hypermethylation does not account for the loss of *CST6* expression in all tumors alternative modes of *CST6* repression are likely. Re-expression of *CST6* was observed in cervical cancer cell lines following Trichostatin A (TSA) treatment, suggesting that histone deacetylation and repressive chromatin structure may be involved [27], since silencing of *CST6* has been associated with repressive trimethyl-H3K27 and dimethyl-H3K9 histone marks [29].

Recently, a novel high-throughput mass spectrometry on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) silicochip was used to determine semi-quantitative methylation changes of 22 candidate genes (a total of 42,528 CpG dinucleotides) in 48 breast tumor and 48 paired normal paraffin-embedded tissues, and methylation profiles were classified by a two-way hierarchical cluster analysis. *CST6* was identified among the 10 hypermethylated genes (*APC, BIN1, BMP6, BRCA1, CST6, ESRb, GSTP1, P16, P21, TIMP3*) that distinguish between cancerous and normal tissues according to the extent of methylation [30]. Moreover, a whole-genome approach using a human gene promoter tiling microarray platform to identify genome-wide and gene-specific epigenetic signatures of breast cancer metastasis to lymph nodes led to functional associations between the methylation status and expression of genes *CDH1, CST6, EGFR, SNAI2 and ZEB2* associated with epithelial-mesenchymal transition [31]. In addition, a recent functional epigenetic study of renal cell carcinoma (RCC) cell lines and primary tumors by

Table 2
Incidence of disease-relapse and disease-related death according to the methylation status of *CST6* in circulating cell-free DNA (n = 123).

CST6 Methylation status	Clinical outcome						Total
	Relapse	No relapse	P[a]	Death	Alive	P[b]	
	n (%)	n (%)		n (%)	n (%)		n (%)
Methylated	13 (26.5)	36 (73.5)	0.164	3 (6.1)	46 (93.9)	1	49 (39.8)
Unmethylated	12 (16.2)	62 (83.8)		6 (8.1)	68 (91.9)		74 (60.2)
Total	25 (20.3)	98 (79.7)		9 (7.3)	114 (92.7)		123 (100)

a: Chi square test.
b: Fisher's Exact Test.

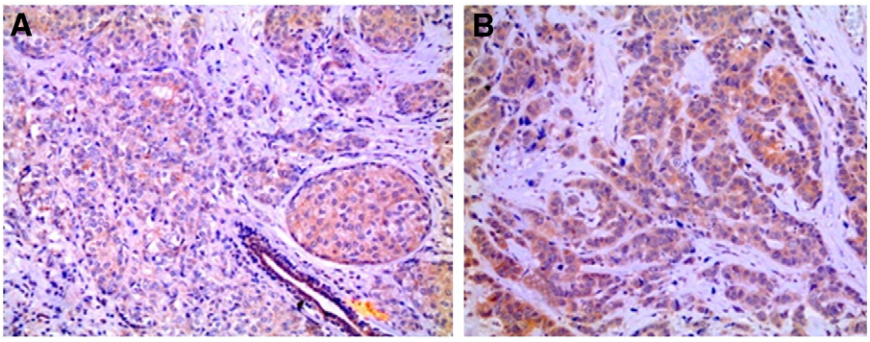

Fig. 3. A: CST6 cytoplasmic immunohistochemical expression in benign luminal epithelium, carcinoma in situ (CIS) and infiltrating breast carcinoma in patients with methylated *CST6* promoter. Myoepithelial cells are negative, **B**: CST6 cytoplasmic immunohistochemical expression in infiltrating breast carcinoma in patients with methylated *CST6* promoter. (all magnifications: x200).

high-density gene expression microarrays identified *CST6* as one of eight genes that showed frequent (>30%) tumor-specific promoter region hypermethylation associated with transcriptional silencing (epigenetically inactivated candidate RCC TSGs). According to this study, re-expression of *BNC1, CST6, RPRM and SFRP1* suppressed the growth of RCC cell lines [32]. All these recent studies are in support of the importance of *CST6* promoter methylation in metastasis.

In breast cancer, *CST6* methylation is an early event since methylation of the *CST6* promoter was reported in 7 out of 28 corresponding normal tumor-adjacent breast tissues samples [21]. Our group has shown the prognostic significance of *CST6* promoter methylation in patients with operable breast cancer [22]. According to our findings, the diagnostic sensitivity and specificity of *CST6* methylation as a biomarker for prediction of relapses and deaths in operable breast cancer seems to be quite promising [22]. Moreover, we have recently shown that *CST6* promoter was methylated in Circulating Tumor Cells (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 *CST6* promoter methylation was lower but not significantly different in comparison to that found in the group of patients with overt metastasis, while for both operable and metastatic breast cancer this was significantly different in respect to the control population [23].

In the present study we tested the expression of cystatin M by immunohistochemistry in a limited number of samples where we had both the primary tumor (FFPE) and circulating cell free DNA. Interestingly, we found that cystatin M tumor suppressor is not expressed in myoepithelial cells in CIS and in benign structures in the tumor microenvironment, which may also contribute to breast cancer development. It should be noted that, although breast cancer arises mainly from the luminal epithelium, myoepithelial cells express proteins that have been shown to suppress transformation of luminal epithelial cells *in vivo*, thus allowing myoepithelial cells to act as tumor suppressors in the breast [33]. A recent study has shown that cystatin M loss may be associated with the losses of ER, PR, and HER4 in invasive breast cancer [34].

In our study, we found that cystatin M expression could be detected in the primary tumor of all breast cancer patients tested, regardless of *CST6* promoter methylation status in their circulating cell free DNA. Therefore, it is likely that loss of *CST6* expression primarily occurs in a small subpopulation of cells in the primary tumor which could not be detectable by the classical immunohistochemical observation. Epigenetic silencing of *CST6* may be associated to the switch of these cells to an invasive phenotype. This subpopulation of more aggressive cells is likely the source of methylated cell-free DNA circulating in plasma. This hypothesis is supported by our recent findings that *CST6* promoter is highly methylated in DNA isolated from circulating tumor cells from operable and metastatic breast cancer patients [23]. According to our data, the present assay could be clinically useful to monitor early relapse in operable breast cancer patients, however this has to be shown in a larger number of patients and a longer follow up.

Cell-free DNA circulating in plasma is an optimal source for finding new tumor biomarkers for early detection, prognosis and probably monitoring of therapeutic response. During recent years numerous studies have reported methylated cell-free DNA in serum or plasma of breast cancer patients at diagnosis [4–6,35] for prognosis [8], as a marker for monitoring the efficacy of chemotherapy [36] and the absence of methylated DNA in normal controls [1,37]. Circulating DNA in plasma/serum is a new promising minimally invasive diagnostic and prognostic tool requiring only a blood sample, and overcomes all the difficulties associated with the use of invasive procedures during screening for breast cancer [4–8,34,38].

While our study was still ongoing, Radpour et al. have shown that aberrant DNA methylation patterns might be used as a biomarker for diagnosis and management of cancer patients. Using eight genes, among

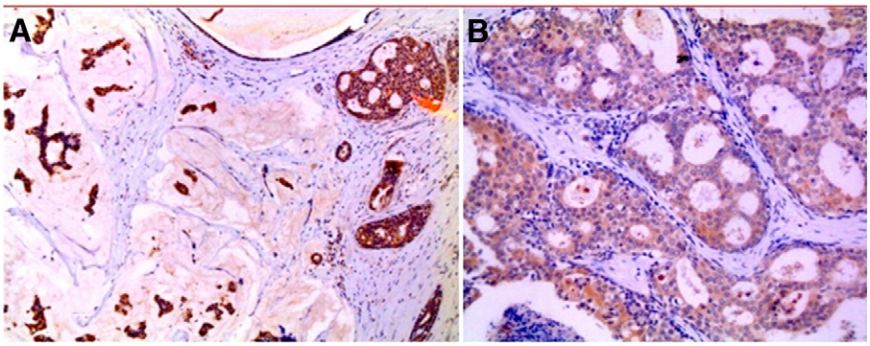

Fig. 4. A: Immunohistochemical detection of cystatin M in CIS and infiltrating breast carcinoma in patients with unmethylated *CST6* promoter. Diffuse cytoplasmic staining is observed. Myoepithelial cells are negative. **B:** Immunohistochemical detection of cystatin M in CIS in patients with unmethylated *CST6* promoter. Myoepithelial cells are negative, (all magnifications: x200, except Fig. 4A: x100).

them *CST6*, as a panel to develop a blood-based test for breast cancer, they achieved a sensitivity and specificity of more than 90% in distinguishing between tumor and normal samples [39]. Moreover, in a very recent study, Avraham et al. suggest that tumor derived DNA methylation of *RASSF1A* in serum may reflect changes in tumor burden and allow early recognition of responders versus nonresponders to adjuvant chemotherapy [40].

In this study, we provide evidence for the specific presence of methylated *CST6* promoter sequences in cell-free DNA circulating in plasma of early breast cancer patients. According to our findings *CST6* promoter methylation in cell-free plasma DNA is a highly specific tumor biomarker since it was not detected in plasma of healthy individuals. Even if we found that the incidence of relapses and deaths was not significantly higher in patients with methylated than in patients with unmethylated *CST6* promoter, we believe that the prognostic significance of these findings could be better evaluated in a larger number of clinical samples with a known clinical outcome and a long follow up period.

Conclusions

CST6 promoter methylation in cell-free plasma DNA is a highly specific tumor biomarker since it was not detected in plasma of healthy individuals. It merits to be further investigated in prospective cohort studies as a potential epigenetic based predictive and prognostic biomarker for breast cancer, since serum DNA methylation has be shown to be useful for monitoring response to neoadjuvant chemotherapy in breast cancer patients.

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References

[1] Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11(6):426-37.
[2] Alix-Panabières C, Schwarzenbach H, Pantel K. Circulating tumor cells and circulating tumor DNA. Annu Rev Med 2012;63:199-215.
[3] Silva JM, Dominguez G, Garcia JM, Gonzalez R, Villanueva MJ, Navarro F, et al. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. Cancer Res 1999;59:3251-6.
[4] Laird PW. The power and promise of DNA methylation markers. Nat Rev Cancer 2003;3:253-66.
[5] Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, et al. High frequency of hypermethylation at the 14.3.3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A 2000;97:6049-54.
[6] Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415-28.
[7] Skvortsova TE, Rykova EY, Tamkovich SN, Bryzgunova OE, Starikov AV, Kuznetsova NP, et al. Cell-free and cell-bound circulating DNA in breast tumours: DNA quantification and analysis of tumour-related gene methylation. Br J Cancer 2006;94:1492-5.
[8] Müller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, et al. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res 2003;63:7641-5.
[9] Jahr S. DNA fragments in the blood plasma of cancer patients: quantification and evidence for their origin from apoptotic-necrotic cells. Cancer Res 2001;611: 659–1665.
[10] Turk V, Bode W. The cystatins: protein inhibitors of cystein proteininases. FEBS Lett 1991;285:213-9.
[11] Sotiropoulou G, Anisowicz A, Sager R. Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer. J Biol Chem 1997;272:903-10.
[12] Ni J, Abrahamson M, Zhang M, Fernandez MA, Grubb A, Su J, et al. Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins. J Biol Chem 1997;272:10853-8.
[13] 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.
[14] Zeeuwen PL, Cheng T, Schalkwijk J. The biology of cystatin M/E and its cognate target proteases. J Invest Dermatol 2009;129:1327-38.
[15] Cheng T, Hitomi K, van Vlijmen-Willems IM, de Jongh GJ, Yamamoto K, Nishi K, et al. Cystatin M/E is a high affinity inhibitor of cathepsin V and cathepsin L by a reactive site that is distinct from the legumain-binding site. A novel clue for the role of cystatin M/E in epidermal cornification. J Biol Chem 2006;281:15893-9.
[16] Gawenda J, Traub F, Lück HJ, Kreipe H, von Wasielewski R. Legumain expression as a prognostic factor in breast cancer patients. Breast Cancer Res Treat 2007;102:1-6.
[17] Murthy RV, Arbman G, Gao J, Roodman GD, Sun XF. Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer. Clin Cancer Res 2005;11:2293-9.
[18] Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. Nat Rev Cancer 2006;6:764-75.
[19] Ai L, Kim W, Kim TY, Fields CR, Massoll NA, Robertson KD, et al. Epigenetic silencing of the tumor suppressor cystatin M occurs during breast cancer progression. Cancer Res 2006;66:7899-909.
[20] Rivenbark AG, Jones WD, Coleman WB. DNA methylation-dependent silencing of CST6 in human breast cancer cell lines. Lab Invest 2006;86:1233-42.
[21] Schagdarsurengin U, Pfeifer GP, Dammann R. Frequent epigenetic inactivation of cystatin M in breast carcinoma. Oncogene 2007;26:3089-94.
[22] Kioulafa M, Balkouranidou I, Sotiropoulou G, Kaklamanis L, Mavroudis D, Georgoulias V, et al. Methylation of cystatin M promoter is associated with unfavorable prognosis in operable breast cancer. Int J Cancer 2009;125:2887-92.
[23] Chimonidou M, Strati A, Tzitzira A, Sotiropoulou G, Malamos N, Georgoulias V, et al. DNA methylation of tumor suppressor and metastasis suppressor genes in circulating tumor cells. Clin Chem 2011;57(8):1169-77.
[24] 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.
[25] Rivenbark AG, Livasy CA, Boyd CE, Keppler D, Coleman WB. Methylation-dependent silencing of CST6 in primary human breast tumors and metastatic lesions. Exp Mol Pathol 2007;83:188-97.
[26] Qiu J, Ai L, Ramachandran C, Yao B, Gopalakrishnan S, Fields CR, et al. Invasion suppressor cystatin E/M (CST6): high-level cell type-specific expression in normal brain and epigenetic silencing in gliomas. Lab Invest 2008;88:910-25.
[27] Veena MS, Lee G, Keppler D, Mendonca MS, Redpath JL, Stanbridge EJ, et al. Inactivation of the cystatin E/M tumor suppressor gene in cervical cancer. Genes Chromosomes Cancer 2008;47:740-54.
[28] 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.
[29] Lin HJ, et al. Breast cancer-associated fibroblasts confer AKT1-mediated epigenetic silencing of Cystatin M in epithelial cells. Cancer Res 2008;68:10257-66.
[30] 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.
[31] Rodenhiser DI, Andrews J, Kennette W, Sadikovic B, Mendlowitz A, Tuck AB, et al. Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. Breast Cancer Res 2008;10:R62.
[32] 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(14):2104-17.
[33] Bisell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer 2001;1:46-54.
[34] Ko E, Park SE, Cho EY, Kim Y, Hwang JA, Lee YS, et al. Cystatin M loss is associated with the losses of estrogen receptor, progesterone receptor, and HER4 in invasive breast cancer. Breast Cancer Res 2010;12(6):R100.
[35] Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, et al. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. J Clin Oncol 2006;24(26):4262-9.
[36] Fiegl H, Millinger S, Mueller-Holzner E, Marth C, Ensinger C, Berger A, et al. Circulating tumor-specific DNA: a marker for monitoring efficacy of adjuvant therapy in cancer patients. Cancer Res 2005;65:1141-5.
[37] Dulaimi E, Hillinck J, Ibanez de Caceres I, Al-Saleem T, Cairns P. Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. Clin Cancer Res 2004;10:6189-93.
[38] Anker P, Mulcahy H, Stroun M. Circulating nucleic acids in plasma and serum as an non invasive investigation for cancer: time for large scale clinical studies? Int J Cancer 2003;103:149-52.
[39] Radpour R, Barekati Z, Kohler C, Lv Q, Bürki N, Diesch C, et al. Hypermethylation of tumor suppressor genes involved in critical regulatory pathways for developing a blood-based test in breast cancer. PLoS One 2011;6(1):e16080.
[40] Avraham A, Uhlmann R, Shperber A, Birnbaum M, Sandbank J, Sella A, et al. Serum DNA methylation for monitoring response to neoadjuvant chemotherapy in breast cancer patients. Int J Cancer Mar 12 2012, doi:10.1002/ijc.27526 [Epub ahead of print].