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Methylation of cystatin M promoter is associated with unfavorable prognosis in operable breast cancer

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The methylation status of cystatin M (*CST6*) gene in breast tumors was investigated and its prognostic significance as a novel breast cancer biomarker was evaluated. Using methylation-specific PCR (MSP), *CST6* promoter methylation was examined in 134 formalin fixed paraffin-embedded tissues (FFPEs): 10 pairs of breast tumors and their surrounding normal tissues, 10 breast fibroadenomas, 11 normal breast tissues and 93 breast tumors. Methylation of *CST6* promoter was observed in 2/21 (9.5%) noncancerous breast tissues, 1/10 (10%) benign breast tumors (fibroadenomas) and 52 (55.9%) operable breast cancer tumor samples. *CST6* was rarely methylated in the normal tissue surrounding the tumor (10%). During the follow-up period, 24 (25.8%) patients relapsed and 19 (20.4%) died. *CST6* methylation was detected in 19 (79.2%) of patients who relapsed and in 15 (78.9%) of patients who died. Disease-free-interval (DFI) and overall survival (OS) were significantly associated with *CST6* promoter methylation ($p = 0.004$ and $p = 0.001$ respectively). Multivariate analysis revealed that *CST6* methylation is an independent prognostic factor for DFI (HR = 3.484; 95% CI: 1.155–10.511; $p = 0.027$). and OS (HR = 9.190; 95% CI: 1.989–42.454; $p = 0.004$). *CST6* promoter methylation status in tumor cells seems to provide important prognostic information in operable breast cancer and merits to be further evaluated and validated in a larger cohort of patients.

Key words: cystatin M; CST6; DNA methylation; operable breast cancer; epigenetic markers

Epigenetics is defined as changes in DNA and histone modifications resulting in heritable but potentially reversible silencing of gene expression that is not due to any alteration in their coding sequence.[1] The best known epigenetic marker is aberrant DNA methylation, which primarily occurs at CpG dinucleotides.[2] Cancer cells, harbor widespread genomic hypomethylation and regional hypermethylation at specific CpG islands.[3] It is well established that methylation of normally unmethylated CpG islands correlates with the loss-of expression of tumor suppressor genes in cancer cell lines and primary tumors, including breast cancer.[4] As methylation of these genes appears to be an early event that plays a fundamental role in the development and progression of cancer,[5] it is considered to be a promising biomarker for early detection and prognosis estimation in cancer patients. Detection of changes in methylation patterns as a consequence of disease or a response to treatment is under evaluation in the clinic and has gained increased interest as a diagnostic or prognostic indicator.

Cystatin M (CST6) is a member of the cystatin family, a group of 14 enzymes that function as endogenous inhibitors of lysosomal cysteine proteases to protect cells against uncontrolled proteolysis.[6] It is involved in regulating the activity of two cysteine proteases, the cathepsins B and L. Impaired regulation of expression and activity of lysosomal cysteine proteases has been implicated in cancer progression, thus, imbalances between these proteases and their inhibitory cystatins can lead to tumor development, invasion and metastasis.[7,8] Cystatin M was first identified and cloned by Sotiropoulou *et al*. by differential display of mRNAs as a transcript that was significantly down-regulated in a metastatic breast cancer cell line when compared to a matched primary tumor cell line.[9] Later, the same inhibitor was independently cloned by Ni *et al*. from embryonic lung fibroblasts and named cystatin E.[10] Cystatin M was mapped to chromosomal locus11q13,[11] which is believed to harbor tumor suppressors because loss-of heterozygosity (LOH) has been frequently observed in several cancer types. This region contains a 507 bp segment that spans the proximal promoter and the first exon and encompasses ~60 CpGs that meet the criteria of a CpG island.[12] Very recently, it has been shown that this region is a target for DNA hypermethylation, which impairs transcription and leads to loss-of cystatin M expression in breast cancer cell lines and breast carcinomas.[12–14]

Despite the fact that epigenetic inactivation of cystatin M has been shown to have a significant impact on the phenotype of breast tumors, the relationship between CpG island hypermethylation of this gene and prognosis in breast cancer has not been studied as yet. Therefore, the aim of our study was to examine the prevalence of *CST6* methylation in primary breast tumors of operable breast cancer patients and evaluate its prognostic significance.

Material and methods

Patients and samples

The study material consisted of a total of 134 breast formalin fixed paraffin-embedded tissues (FFPEs): 103 paraffin-embedded breast carcinomas, obtained from patients with operable breast cancer from the Department of Medical Oncology, University Hospital of Heraklion Crete; for 93 of these patients the clinical outcome was available.

Twenty-one noncancerous paraffin-embedded breast tissues were used as normal breast tissue controls: 10 histologically normal tissues adjacent to tumors and 11 histologically cancer-free specimens from reduction mammoplasty. Moreover, 10 breast fibroadenomas. were included as a separate benign tumor group. All samples were collected at diagnosis and all patients gave their informed consent to participate in the study which has been approved by the Ethical and Scientific Committees of our Institution. Patients were enrolled on adjuvant chemotherapy research protocols of the Hellenic Oncology Research Group (HORG) [*i.e*. FEC regimen or sequential docetaxel followed by epirubicin in combination with cyclophosphamide (D/EC regimen) or docetaxel in combination with epirubicin (DE regimen)]; patients with breast conservative surgery also

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received radiation treatment and those with hormone receptor-positive tumors received adjuvant tamoxifen for 5 years. Clinicopathological features for the 93 patients for which the clinical outcome and a long follow-up was available are shown in Table I. Tissue sections of 10 μm containing >80% of tumor cells were used for DNA extraction and methylation-specific PCR (MSP) analysis. The breast cancer cell line MCF-7 was used as positive control in MSP reactions for the detection of *CST6* promoter methylation. Genomic DNA (gDNA) from both paraffin tissues and breast cancer cell line MCF-7 was isolated with the High Pure PCR Template Preparation kit (Roche, Germany). DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). Before proceeding to the sodium bisulfite conversion and MSP reaction steps, the genomic DNA integrity of all our clinical samples was assessed by amplifying BRCA1 exon 20 for mutation analysis by using the same primers as previously described.[15]

Sodium bisulfite conversion

One microgram of 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 using the EZ DNA Methylation Gold Kit (ZYMO Research, Orange, CA), according to the manufacturer's instructions. The converted DNA was stored at –70°C until used. In each sodium bisulfite conversion reaction, dH_2O and MCF-7 were included as a negative and positive control, respectively.

Methylation-specific PCR

CST6 promoter methylation status was detected by nested MSP by using in silico designed specific primer pairs for both the methylated and unmetthylated *CST6* promoter sequences (Table II). Each MSP reaction was performed in a total volume of 25 μL. One microliter (~100 ng) of sodium bisulfite converted DNA was added into a 24 μL reaction mixture that contained 0.1 μL of Taq DNA polymerase (5U/μL, Platinum DNA polymerase; Invitrogen), 2.5 μL of the supplied 10× PCR buffer, 1.0 μL of $MgCl_2$ (50 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, sodium bisulfite treated DNA was amplified with a set of external primers specific for the methylated or unmethylated sequences. Nested MSP was performed using 1 μL of the amplified products and a set of internal primers that were previously described[12] and are specific for the methylated sequences, or newly designed internal primers specific for the unmethylated sequences respectively. Similar thermocycling conditions were used as for the first MSP: 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. MSP products for methylated and unmethylated *CST6* promoter were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH = 8.0) and visualized by ethidium bromide staining. Human placental genomic DNA (gDNA; Sigma-Aldrich) methylated *in vitro* with *SssI* methylase (NEB, Ipswich, MA) was used, after sodium bisulfite conversion, as a fully methylated (100%) MSP positive control; the same unmethylated placental gDNA, was used, after sodium bisulfite conversion, as a negative MSP control. All MSP reactions were performed in a blinded fashion with regards to the patient's clinical outcome.

TABLE I – ASSOCIATION OF *CST6* METHYLATION IN BREAST CANCER PATIENTS WITH CLINICOPATHOLOGICAL FEATURES

Clinic opathological features	*n* (*n* = 93)	*CST6* methylation (%)	*p*[1]
Menopausal status			
Pre	38	23 (60.5)	0.456
Post	55	29 (52.7)	
Tumor size (cm)			
0–2.0	26	12 (46.2)	0.187
2.1–5.0	56	31 (55.4)	
>5.0	10	8 (80.0)	
Unknown	1		
Axillary lymph node			
0	27	8 (29.6)	0.007
1–3	30	19 (63.3)	
≥4	34	23 (67.6)	
Unknown	2		
Tumor grade			
I, II	42	17 (40.5)	0.010
III	42	27 (64.3)	
Lobular	5	5 (100.0)	
Unknown	4		
Tumor stage			
I	23	7 (30.4)	0.015
II	36	22 (61.1)	
III	34	23 (67.6)	
Estrogen receptor			
Positive	56	29 (51.8)	0.324
Negative	37	23 (62.2)	
Progesterone receptor			
Positive	30	20 (66.7)	0.150
Negative	63	32 (50.8)	
HER2 score			
Negative (0–2+)	72	40 (55.6)	0.950
Positive (3+)	11	6 (54.5)	
Unknown	10		

[1]Chi-square test.

Statistical analysis

The prognostic significance of *CTS6* promoter methylation was assessed according to our results of the FFPEs samples from the 93 breast cancer patients for which the clinical outcome was available. Correlation between *CTS6* promoter methylation status and clinicopathological features of these patients was assessed by using the Chi-square test. Disease-free interval (DFI) and overall survival (OS) curves were calculated by using the Kaplan-Meier method and comparisons were performed using the log rank test. A Cox-regression analysis was performed in order to determine the relative contribution of various variables to the assessment of DFI and OS. *p*-values ≤0.05 were considered statistically significant. Statistical analysis was performed using the SPSS Windows version 11.0 (SPSS, Chicago, IL).

Results

Sensitivity of CST6 promoter MSP assay

The sensitivity of the developed nested MSP assay for *CST6* was evaluated by initially preparing 1 μg of fully (100%) in vitro methylated (through *SssI* methylase) human placental genomic DNA and 1 μg of the same fully unmethylated (100%) human placental genomic DNA through SB conversion reaction in a total volume of 10 μL. Serial dilutions of this fully methylated DNA sample in quantities ranging from 1 μg (undiluted) to 10 pg in a final volume of 10 μL were performed and then 5 μL of these dilutions were mixed with the same constant amount of 5 μL (500ng) of the unmethylated DNA sample (final volume 10 μL). One microliter of these samples, corresponding to a methylated DNA quantity ranging from 100 ng down to 1pg was used in the nested MSP reaction in the presence of a constant amount of 50ng unmethylated DNA. The nested MSP assay for *CST6* was performed in triplicate and the sensitivity was 1:20,000 corresponding to 0.005% (5 pg) *CST6* methylated gDNA (Fig. 1*a*). A nested MSP was performed throughout our study, since conventional MSP was not sensitive enough for the detection of *CST6* promoter methylation in many of our clinical samples (Supporting Information Fig. 1).

TABLE II – SEQUENCES OF *CST6* PRIMERS USED IN THIS STUDY

CST6	Use	Name	Oligonucleotide sequence (5′-3′)
Methylated	Outer primer set	CST6 M F1	5′-GGA TTT CGG TAA TTT CGA GTT TC-3′
		CST6 M R1	5′-TTC GAA CGC GCC ATA ACC G-3′
	Inner primer set	CST6 M F2	5′-TCG AGT TTC GTT TTA GTT TTA GGT C-3′
		CST6 M R2	5′-CAT AAC CGT CAA TAC CGT CG-3′
Unmethylated	Outer primer set	CST6 U F1	5′-GGA TTT TGG TAA TTT TGA GTT TT-3′
		CST6 U R1	5′-TTC AAA CAC ACC ATA ACC A-3′
	Inner primer set	CST6 U F2	5′-TTG AGT TTT GTT TTA GTT TTA GGT T-3′
		CST6 U R2	5′-CAT AAC CAT CAA TAC CAT CA-3′

Specificity of CST6 promoter MSP assay

To validate the specificity of *CST6* promoter MSP, the primers were initially tested in silico and then in PCR reactions, using bisulfite modified human placental gDNA that is not methylated; no amplification of *CST6* promoter could be observed. The specificity of *CST6* promoter methylation was further confirmed by performing nested MSP in 11 normal breast tissues obtained from reduction mammoplasty, 10 histologically tested noncancerous breast tissues surrounding the corresponding tumors and 10 breast fibroadenomas used as benign tumor controls. *CST6* promoter was found methylated in 1 out of 10 (10%) noncancerous breast tissues adjacent to tumors, as well as in 1 out of 10 (10%) breast fibroadenomas and in 1 out of 11 (9.1%) reduction mammoplasty tissues. In total 2/21 (9.5%) noncancerous paraffin-embedded breast tissues and 1/10 (10%) benign breast tumors were positive for *CST6* methylation (Fig. 1*b*). 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 (Fig. 1*c*).

CST6 promoter methylation in breast tumors

Clinicopathological features. The methylation status of *CST6* promoter was evaluated in paraffin-embedded breast carcinomas from 103 patients diagnosed with operable breast cancer in double-blinded experiments. Patients' clinicopathological characteristics and clinical outcome data became available for 93 patients upon completion of analysis and were compared to the methylation status of *CST6* (Table I). *CST6* promoter was found methylated in 52 (55.9%) out of 93 breast tumor samples. Chi-square analysis revealed a statistically significant correlation between *CST6* promoter methylation and the number of axillary lymph nodes involved ($p = 0.007$), as well as the tumor stage ($p = 0.015$) and the tumor grade ($p = 0.010$) (Table I). According to our data, there was no difference ($p = 0.456$) in the methylation status of *CST6* between groups of premenopausal ($n = 38$) and postmenopausal women ($n = 55$) (Table I).

Disease relapse and disease-free survival

After a median follow-up period of 76 months (range 3–116 months), 24 (25.8%) patients had relapsed and *CST6* methylation was detected in 19 (79.2%) of these patients. The incidence of relapses was significantly higher in patients with methylated (36.5%) than in patients with nonmethylated *CST6* promoter (12.2%; $p = 0.008$, Table III). By using these limited (concerning the number of cases) data, the diagnostic sensitivity of *CST6* methylation for prediction of relapses was estimated as 79.2% (19/24) and the diagnostic specificity as 52.2% (36/69) respectively. Furthermore, the Kaplan–Meier estimates of the cumulative DFI for patients with methylated and nonmethylated *CST6* promoter were significantly different in favor of patients with nonmethylated *CST6* promoter ($p = 0.004$; Fig. 2*a*).

Overall survival

During the follow-up period, 19 (20.4%) patients died as a consequence of disease progression and *CST6* methylation was detected in 15 (78.9%) of these patients. The incidence of deaths was higher in patients with methylated (28.8%) than in patients with nonmethylated *CST6* promoter (9.8%; $p = 0.023$, Table III). By using these limited (concerning the number of cases) data, the diagnostic sensitivity of *CST6* methylation for prediction of deaths was estimated as 78.9% (15/19) and the diagnostic specificity as 50% (37/74) respectively. Furthermore, the Kaplan–Meier estimates of the cumulative OS for patients with methylated and nonmethylated *CST6* promoter were significantly different in favor of patients with nonmethylated *CST6* promoter ($p = 0.001$; Fig. 2*b*).

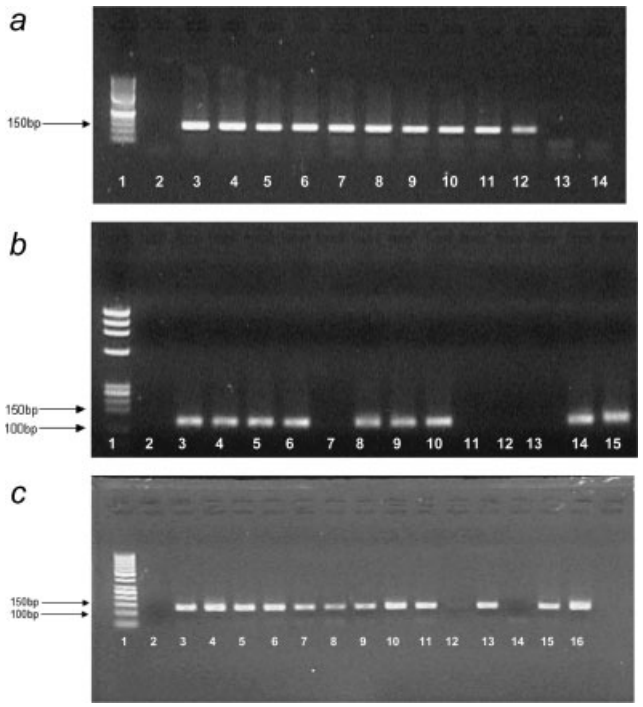

FIGURE 1 – (*a*) Sensitivity of Methylation-Specific PCR for *CST6*: (1) DNA marker 50 bp, (2) Negative control (dH_2O), (3) 100% methylated DNA, (4) 50% methylated DNA, (5,6) 5% methylated DNA, (7,8) 0.5% methylated DNA, (9,10) 0.05% methylated DNA (1:2,000), (11,12). 0.005% methylated DNA (1:20,000), (13,14) 2×10^{-3}% methylated DNA (1:50,000) (*b*) Nested methylation-specific PCR for *CST6* promoter methylated sequences: (1) DNA marker 50 bp, (2) Negative control: dH_2O, 3–6,8,9,14, Breast tumors: 3–6, 8, 9, 14, (7) Breast tumor: 7, (10,11) Pair of breast tumor and adjacent normal tissue: 1, (12) Breast fibroadenoma: 1, (13) Normal breast tissue: 1, (15) Positive control: MCF-7. (*c*) Nested methylation-specific PCR for *CST6* promoter unmethylated sequences: (1) DNA marker 50bp, (2) Negative control: dH_2O, (3–6,8,9,14) Breast tumors: 3–6, 8, 9, 14, (7) Breast tumor: 7, (10,11) Pair of breast tumor and adjacent normal tissue: 1, (12) negative control,:dH_2O, (13) Breast fibroadenoma: 1, (15) Normal breast tissue: 1, (16) Positive control: placental gDNA.

TABLE III – INCIDENCE OF DISEASE-RELAPSE AND DISEASE-RELATED DEATH ACCORDING TO THE METHYLATION STATUS OF *CST6* PROMOTER

Gene	Methylation status	Relapses (%)	p^1	Median DFI (range)	Deaths (%)	p^1	Median OS (range)
CST6	M[2] ($n = 52$)	19 (36.5)	0.008	82 (73–91)	15 (28.8)	0.023	97 (88–105)
	U[3] ($n = 41$)	5 (12.2)		109 (101–117)	4 (9.8)		116 (110–123)

DFI, disease-free interval; OS, overall survival.
[1]Chi-square test.–[2]methylated.–[3]Nonmethylated.

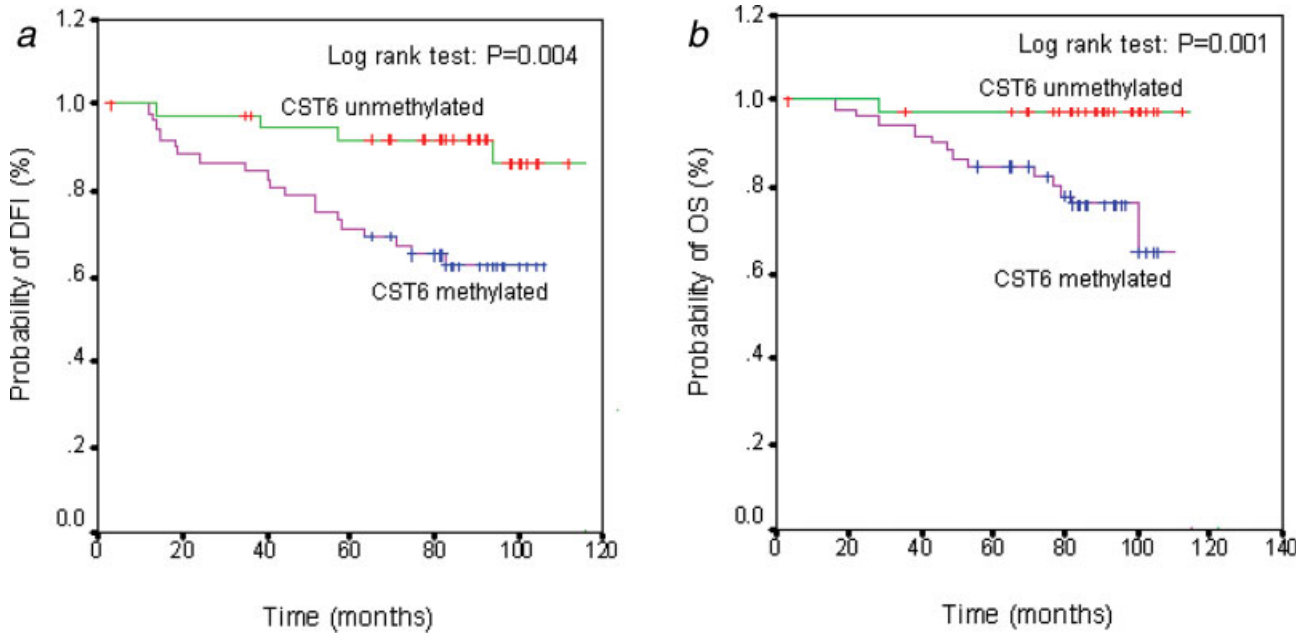

FIGURE 2 – (*a*) Kaplan-Meier estimates of disease-free interval (DFI) for early breast cancer patients with or without *CST6* promoter methylation ($p = 0.004$) (*b*) Kaplan-Meier estimates of overall survival (OS) for early breast cancer patients with or without *CST6* promoter methylation ($p = 0.012$), (HR = 9.311, 95% CI: 1.975 – 43.90; $p = 0.005$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Univariate and multivariate analysis

CST6 promoter methylation, menopausal and axillary lymph node status, tumor size, hormone receptor (estrogen and progesterone) and HER2 status were tested in univariate analysis for association with DFI (Table IV). Axillary lymph node status (positive vs negative), HER2 status and *CST6* promoter methylation were significantly associated with decreased DFI ($p = 0.031$, $p = 0.048$, $p = 0.009$ respectively). The same clinical and epigenetic variables were also tested in univariate analysis for association with OS. Detection of *CST6* promoter methylation was significantly associated with decreased OS ($p = 0.006$). In addition, HR status (ER/PR negative vs ER or PR positive), was significantly associated with decreased OS ($p = 0.043$).

All factors which were derived from the univariate analysis as significant and independent were tested in multivariate analysis for association with DFI and OS, in a standardized way using a Cox proportional hazard regression model. All results with *p*-values and HRs and 95% CI both for OS and disease-free interval (DFI) are shown in Table IV. After the process of backward elimination, multivariate analysis demonstrated that methylation of *CST6* promoter (HR = 3.484; 95% CI: 1.155–10.511; $p = 0.027$) and HER2 status (HR = 2.889; 95% CI: 1.026–8.133; $p = 0.044$) were independently associated with decreased DFI. In the same way, multivariate analysis demonstrated that methylation of *CST6* promoter (HR = 9.190; 95% CI: 1.989–42.454; $p = 0.004$), and HR status (ER/PR negative vs ER or PR positive), (HR = 3.270; 95% CI: 1.136–9.409; $p = 0.028$) were independently associated with a shorter OS.

Discussion

Epigenetic silencing due to DNA hypermethylation often leads to inactivation of the wild-type allele at sites of LOH that introduces one hit in the well-known Knudson's model for tumorigenesis that accounts for loss-of–function of tumor suppressor genes.[16] The potential of DNA methylation as a novel area of new biomarkers discovery is very promising. There is a number of interesting tumor suppressor genes, whose silencing through DNA methylation has been evaluated in many types of cancer and especially in breast cancer as novel prognostic and predictive biomarkers.[17–20]

Cystatin M is involved in regulating the activity of cathepsins B and L which have been clearly implicated in cancer progression.[8] In this way, imbalances between the expression of cathepsins B and L and their inhibitor cystatin M can lead to tumor development. According to recent studies, methylation-dependent epigenetic silencing of *CST6* represents an important mechanism for loss-of cystatin M during breast tumorigenesis and progression to metastasis.[21] Cystatin M (*CST6*) was also identified as a frequent target of epigenetic silencing in gliomas.[22] Moreover, it was shown that epigenetic silencing of *CST6* is frequent in adult and pediatric brain tumors and occurs in glioma tumor initiating cells (TICs), which are thought to give rise to the tumor. *CST6* methylation may therefore represent a novel prognostic marker and therapeutic target specifically altered in TICs.[23] In cervical cancer, cystatin M was found inactivated by somatic mutations and promoter hypermethylation.[24]

TABLE IV – UNIVARIATE AND MULTIVARIATE ANALYSIS FOR DFI (DISEASE-FREE INTERVAL) AND OS (OVERALL SURVIVAL) OF PATIENTS WITH EARLY-STAGE BREAST CANCER

	Univariate analysis [1]			Multivariate analysis [1]		
	Hazard ratio	95% CI	*p*	Hazard ratio	95% CI	*p*
Disease-free interval						
Menopausal Status ("post" *vs.* "pre")	1.398	0.592–3.301	0.445			
HR status (ER/PR"−" *vs.* ER or PR "+")	1.928	0.844–4.401	0.119			
HER-2 ("+" *vs.* "−")	2.818	1.011–7.854	0.048	2.889	1.026–8.133	0.044
Methylation of *CST6* ("M" *vs.* "U")	4.233	1.438–12.463	0.009	3.484	1.155–10.511	0.027
Lymph Nodes"positive" *vs.* "negative"	4.937	1.153–21.134	0.031	2.634	0.586–11.835	0.207
Tumor size">2 *vs.* 0–2"	2.871	0.849–9.707	0.090			
Overall survival						
Menopausal status ("post" *vs.* "pre")	1.545	0.534–4.468	0.422			
HR status (ER/PR"−" *vs.* ER or PR "+")	2.816	1.034–7.671	0.043	3.270	1.136–9.409	0.028
HER-2 ("+" *vs.* "−")	2.494	0.777–8.002	0.124			
Methylation of *CST6* ("M" *vs.* "U")	8.174	1.832–36.465	0.006	9.190	1.989–42.454	0.004
Lymph Nodes "positive" *vs.* "negative"	2.753	0.614–12.344	0.186			
Tumor size">2 *vs.* 0–2"	2.969	0.669–13.172	0.152			

[1]Cox-regression analysis.
M, methylated; U, unmethylated.

A strong correlation between *CST6* promoter methylation and loss-of *CST6* mRNA expression in a panel of breast cancer cell lines and in a small number of primary breast tumors was clearly shown, suggesting an important role of DNA methylation in transcriptional silencing of this gene in breast cancer.[12–14] Despite the fact that epigenetic inactivation of cystatin M was shown to severely impact on the phenotype of breast tumors, the relationship between promoter methylation of this gene and prognosis in breast cancer has not been studied.

In the current study, we have evaluated the methylation status of *CST6* promoter methylation in a group of 21 noncancerous breast tissues (10 histologically normal tissues adjacent to tumors and 11 histologically cancer-free specimens from reduction mammoplasty), 10 benign breast tumors (fibroadenomas) and 103 breast tumors. Moreover, our study addresses whether tumor-associated *CST6* promoter methylation should be used as a prognostic biomarker in early breast cancer by using nested MSP. Conventional MSP, has already been established as highly specific and sensitive for assessing DNA methylation status of CpG islands.[25] However, since for many of our clinical samples convetional MSP was found not to be enough sensitive for the detection of *CST6* promoter methylation, nested MSP was performed throughout our study.

Although *CST6* methylation may be very common in carcinomas, our study suggests that this gene may also be methylated at a low percentage in adjacent normal breast tissues. This has also been previously reported but at higher percentages (25%) by Schagdarsurengin *et al*.[14] This finding may be attributed to a general hypothesis suggesting that DNA methylation occurs in normal samples as part of the aging process,[26] or to contamination of the normal samples by tumor cells. However, according to our data, there was no difference in the methylation status of *CST6* between groups of premenopausal and postmenoposal women. The results presented in the current study indicate that *CST6* is frequently epigenetically inactivated during breast carcinogenesis since its promoter was found methylated in 52 out of 93 (55.9%) breast carcinomas.

Our data are in accordance with previous studies of Ai *et al*.[12] and Schagdarsurengin *et al*.[14] who, in a limited number of clinical samples ($n = 20$ and $n = 40$, respectively), have shown that 60% of primary breast tumors displayed *CST6* hypermethylation. Association of Cystatin M inactivation with progression of a primary tumor to a metastatic phenotype has already been suggested at the time of its cloning and first characterization.[9] Subsequently, Shridhar *et al*.[27] demonstrated that exogenous expression of cystatin M in human MDA-MB-435S cell line reduces *in vivo* cell proliferation, migration, endothelial cell adhesion and inhibits matrigel invasion. Zhang *et al*.[28] by *in vivo* experiments with SCID mice proposed that cystatin M may function as a candidate tumor suppressor gene for breast carcinogenesis. Expression of cystatin M resulted in significantly delayed growth of primary tumors and lower metastatic burden in the lungs and liver. However, the prognostic value of *CST6* methylation in early breast cancer has not been reported so far.

Our results demonstrate that *CST6* promoter methylation provides important prognostic information in patients with operable breast cancer. According to our data, patients with *CST6* promoter methylation had worse DFI and OS than those without. Moreover, the detection of *CST6* promoter methylation emerged as an independent risk factor affecting both DFI and OS in a multivariate Cox proportional hazard model. It is probable that *CST6* gene silencing due to promoter methylation deactivates its tumor suppressor role and can thus possibly contribute to a shorter survival in breast cancer patients. Furthermore, statistical analysis showed that *CST6* promoter methylation correlated with conventional prognostic factors, such as the number of axillary lymph nodes involved, tumor stage and grade, suggesting that tumors with *CST6* promoter methylation show a biologically aggressive phenotype.

Moreover, the detection of *CST6* promoter methylation emerged in the multivariate analysis to be an independent prognostic factor for disease relapse and disease-related death. *CST6* gene silencing due to its promoter methylation leads to loss-of protein function and can, thus, possibly contribute to a shorter survival in breast cancer patients. Whereas additional studies are mandated to define the biological implications of *CST6* promoter methylation, our results support the notion that *CST6* promoter methylation may be useful as a novel biomarker in the study of breast cancer progression.

In conclusion, our data demonstrate for the first time that cystatin M promoter methylation provides important prognostic information in patients with operable breast cancer and that this methylation plays an important role in the clinical behavior of breast tumors. 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. Nevertheless, the methylation status of this gene should be prospectively evaluated

as a promising prognostic biomarker in a larger cohort of patients with operable breast cancer. Moreover, by using quantitative MSP and cutoff points for normal levels of *CST6* methylation in breast tissues, a better and more accurate assessment of the diagnostic sensitivity and sensitivity of *CST6* methylation as a test of prognosis could be derived.

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