

DNA methylation-dependent silencing of *CST6* in human breast cancer cell lines

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Cystatin M (*CST6*) is a candidate breast cancer tumor suppressor that is expressed in normal and premalignant breast epithelium, but not in metastatic breast cancer cell lines. *CST6* is subject to epigenetic silencing in MCF-7 breast cancer cells related to methylation of the CpG island that encompasses the *CST6* proximal promoter region and exon 1. In the current study, *CST6* CpG island methylation and expression status was examined in a panel of breast cancer cell lines. Seven of 12 (58%) cell lines lack detectable expression of *CST6* and treatment of these cells with 5-aza-2'-deoxycytidine resulted in a significant increase in *CST6* expression, suggesting that the loss of expression may be related to methylation-dependent epigenetic silencing. Bisulfite sequencing of *CST6* in a subset of breast cancer cell lines revealed CpG island hypermethylation in *CST6*-negative cells, and an absence of CpG island methylation in cells that express *CST6*. The extent of regional methylation was strongly associated with the lack of expression of *CST6* among these cell lines. In particular, hypermethylation of the proximal promoter was significantly associated with *CST6* gene silencing, and methylation of a number of individual CpGs was found to be statistically correlated with extinction of gene expression. These results establish a strong link between *CST6* promoter hypermethylation and loss of *CST6* expression in breast cancer cell lines, and suggest that methylation-dependent epigenetic silencing of *CST6* may represent an important mechanism for loss of *CST6* during breast carcinogenesis *in vivo*.

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Cystatin M (*CST6*) is a member of a family of proteins that function as physiological inhibitors of lysosomal cysteine proteases, and control target proteases by forming high-affinity reversible complexes.^{1,2} Lysosomal cysteine proteases are involved in the degradation of components of connective tissues and basement membranes *in vitro*, and aberrant expression and activity of these proteases accompany cancer invasion and metastasis *in vivo*.^{1,3} Thus, loss of *CST6* expression might contribute to increased proteolysis of tissue architecture, facilitating the spread of cancer cells.^{1,4} The majority of human breast cancer cell lines derived from metastatic breast tumors lack *CST6* expression,

whereas normal and premalignant cells express abundant levels of *CST6*.^{2,5} Consistent with a role in suppression of metastasis, *CST6* has been suggested to function as a breast tumor suppressor gene.² Exogenous expression of *CST6* in MDA-MB-435S breast cancer cells results in the suppression of cell proliferation, migration, matrix invasion, and tumor-endothelial cell adhesion *in vitro*.¹ No deletions or structural rearrangements of *CST6* have been characterized, suggesting that loss of gene expression may be the result of transcriptional silencing in breast cancer cells.^{4,6} *CST6* contains a large CpG island that spans the proximal promoter and exon 1, encompassing the start site for transcription. CpG islands sited in gene promoters represent a major target for DNA hypermethylation, which impairs transcription related to regional or specific methylation events.^{7–9} Promoter CpG island hypermethylation contributes to gene silencing by inhibiting the binding of certain transcription factors to their recognition sequence,^{8,9} attracting methylated DNA-binding proteins,^{10–13} and/or

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through chromatin remodeling.¹⁴ In a previous microarray-based analysis of differential gene expression in MCF-7 breast cancer cells, we identified *CST6* as a methylation-sensitive gene and demonstrated an inverse relationship between *CST6* mRNA expression and methylation of its promoter CpG island.¹⁵ Consistent with this observation, recent studies have shown *CST6* to be silenced by methylation in select breast cancer cell lines and primary breast tumors.¹⁶ Furthermore, *CST6* was identified as a methylation-sensitive gene in glioma cell lines and primary brain tumors.¹⁷ These findings combine to suggest that loss of *CST6* expression in multiple tumor systems may be a direct consequence of methylation-dependent gene silencing.

The aim of the present study was to investigate the direct association between *CST6* CpG island methylation and expression status in multiple breast cancer cell lines in order to definitively determine if aberrant DNA methylation represents an important mechanism for the loss of *CST6* during breast tumorigenesis and/or progression. Gene expression analysis revealed diminished expression of *CST6* in the majority of breast cancer cell lines and treatment with the demethylating agent 5-aza-2'-deoxycytidine (5-aza) produced a significant increase in *CST6* expression, suggesting that *CST6* is subject to methylation-dependent silencing. Consistent with this suggestion, bisulfite sequencing demonstrated extensive CpG island methylation in *CST6*-negative cell lines, while *CST6*-positive cell lines lack CpG island methylation. The extent of methylation within the *CST6* CpG island was inversely correlated with gene expression. In particular, hypermethylation of the proximal promoter was significantly associated with *CST6* silencing, and methylation of several individual CpGs was found to be significantly associated with diminished or absent expression of *CST6*. These results establish a strong link between *CST6* CpG island hypermethylation and transcriptional silencing in breast cancer cell lines, and suggest that methylation-dependent gene repression represents an important mechanism leading to diminished *CST6* expression during breast carcinogenesis and/or cancer progression.

Materials and methods

Breast Cancer Cell Line Culture

Human breast cancer cell lines were obtained from the Tissue Culture Core Facility of the UNC Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill: BT-20 (ATCC#HTB-19), BT549 (HTB-122), Hs578T (HTB-126), MCF-7 (HTB-22), MDA-MB-231 (HTB-26), MDA-MB-415 (HTB-128), MDA-MB-435S (HTB-129), MDA-MB-436 (HTB-130), MDA-MB-453 (HTB-131), MDA-MB-468 (HTB-132), SK-BR-3

(HTB-30), and ZR-75-1 (CRL-1500). Normal breast epithelial cell lines, MCF12A (CRL-10782) and MCF10-2A (CRL-10781), were obtained from the ATCC (Manassas, VA, USA). All cells were propagated according to recommendations from the ATCC. Cell lines that lack expression of *CST6* (including Hs578T, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MCF-7, and ZR-75-1) were treated with the demethylating agent 5-aza (Sigma Chemical Company, St Louis, MO, USA), as previously described.¹⁵ Briefly, two treatment groups were established from a single founding cell population: (i) control medium and (ii) medium containing 250 nM 5-aza. Cells in the treatment group were exposed to 5-aza for 3 weeks, with weekly subcultivation, followed by a 5-week recovery period in control growth medium with weekly subcultivations during the last 3 weeks. Control cells were subcultivated once per week during the 8-week cell culture period. Cell cultures were fed fresh growth medium three times weekly. The concentration of 5-aza utilized in this study was 4–6-fold lower than traditional methods,^{18–21} eliminating the typically encountered cytotoxic effects^{22,23} and allowing prolonged exposure of breast cancer cells to the demethylating drug.

Semiquantitative RT-PCR

Total RNA was isolated from cultured cells using a modification of the method of Chomczynski and Sacchi²⁴ utilizing TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA (2 μ g) from control cells or cells treated with 5-aza was reverse-transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT) as the primer for 60 min at 42°C, according to standard methodology. *CST6* oligodeoxynucleotide primers were synthesized by the UNC Oligodeoxynucleotide Synthesis Core Facility (Chapel Hill, NC, USA) based on known cDNA sequence (Genbank, www.ncbi.nih.gov): 5'-AAGACCAGGGT CACTGGAGA-3' and 5'-CGGGGACTTATCACATCT GC-3' (163 bp). Verification of equal template concentration was accomplished using primers that amplify a portion of β -actin mRNA (5'-AGA GATGGCCACGGCTGCTT-3' and 5'-ATTTGCGGTG GACGATGGAG-3'). PCR reactions were performed in a 50 μ l total volume of buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.001% gelatin, 200 μ M of each dNTP (EasyStart Micro 50 PCR-mix-in-a-tube, Molecular BioProducts, San Diego, CA, USA), 0.4 μ M of each primer, and 2.5U AmpliTaq enzyme (Perkin Elmer/Cetus, Foster City, CA, USA). Amplifications were carried out in a Perkin-Elmer 9700 Thermocycler using a step-cycle program consisting of 25–30 cycles of 94°C for denaturing (1 min), 58°C for annealing (1 min), and 72°C for extension (2 min).

Quantitative Real-Time PCR

Total RNA samples (20 μ g) from control and treated cells were DNase treated (Promega, Madison, WI, USA), purified using the Qiagen Rneasy mini-kit (Qiagen, Valencia, CA, USA), and reversed transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Real-time primers and probes for *CST6* and β -actin were purchased from Applied Biosystems. Reactions were carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) and the following PCR conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Gene expression levels were normalized using β -actin for each cell line and differences in *CST6* gene expression were determined using the comparative Ct method described in the ABI Prism 7700 User Bulletin #2 (Applied Biosystems).

Sodium Bisulfite Modification of Genomic DNA

Genomic DNA from 2×10^6 cultured cells was isolated using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, PA, USA). Sodium bisulfite modification of genomic DNA was performed by a procedure adapted from Grunau *et al*²⁵ as previously described.¹⁵ Sodium bisulfite-converted DNA was amplified using primers that targeted two regions of the proximal promoter and exon 1 of *CST6*: the first region encompasses nucleotides +242 to -228, which contains 55 CpG dinucleotides (5'-GGTTGGAATGTTGTAGTGGT-3' and 5'-CTACCCATATTATAACTAACC-3'), and the second region encompasses nucleotides +118 to -228, which contains 46 CpG dinucleotides (5'-GGTTTTTTGGGTTTTTTGAATTT-3' and 5'-CTACC CATATTATAACTAACC-3'). PCR amplification was accomplished using a step-cycle program consisting of 40 cycles of 94°C for denaturing (1 min), 52°C for annealing (1.5 min), and 72°C for extension (2 min). PCR products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. A portion of each PCR product (1 to 5 μ l) was cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). Three to five colonies were selected per gene segment and expanded in liquid culture. Plasmid DNA was purified using the Wizard Plus Miniprep DNA purification kit (Promega), before digestion with *NcoI* and *NdeI* (New England Biolabs, Beverly, MA, USA) to confirm the presence and size of the cloned insert. Validated clones were sequenced using the universal M13R3 primer with an Applied Biosystems automated sequencer at the UNC Genome Analysis Facility (Chapel Hill, NC, USA). The bisulfite conversion efficiency was calculated for each sequenced clone based on the ratio of converted Cs (non-CpG) to total number of Cs (non-CpG) in a given gene segment. Only clones

determined to have a conversion efficiency of >95% were included in the present study. The results of methylation analyses were expressed as total methylation index (TMI). This measure of methylation can be applied to single CpG dinucleotides, select groups of CpG dinucleotides, or to continuous groups of CpG dinucleotides in a given gene segment. TMI was calculated for each cell line and clone by dividing the number of methylated CpGs observed by the total CpGs analyzed and expressed as percent methylation. For instance, in an analysis of a gene segment containing 55 CpG dinucleotides and three clones sequenced, TMI would be calculated based upon 165 possible CpG methylation events (3×55).

Statistical Analysis

Values included in the text, table, and Figures represent averages \pm s.e.m. that were calculated using the statistical function of KaleidaGraph Version 3.5 (Synergy Software, Essex Junction, VT, USA). An unpaired *t*-test was performed to determine statistical significance of 5-aza treatment-related differences in gene expression in *CST6*-negative cell lines. Likewise, an unpaired *t*-test was used to examine the association between *CST6* methylation status and gene expression levels among subsets of breast cancer cell lines.

Results

CST6 is Differentially Expressed among Breast Cancer Cell Lines

CST6 gene expression was examined by semi-quantitative RT-PCR in MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, BT-549, BT-20, Hs578T, MCF-7, SK-BR-3, and ZR-75-1 breast cancer cell lines and two normal mammary epithelial cell lines, MCF10-2A and MCF12A. Five of the 12 breast cancer cell lines (42%) express detectable levels of *CST6* mRNA: MDA-MB-468 and SK-BR-3 cells express low levels of *CST6*, MDA-MB-231 and MDA-MB-415 cells express moderate levels of *CST6*, and BT-20 cells strongly express *CST6*. However, no *CST6* mRNA was found in the remaining seven cell lines (58%) (Figure 1a). Both MCF10-2A and MCF12A cells transcribe *CST6* at low levels, and β -actin mRNA was expressed evenly across all cell lines examined (Figure 1a). These results are consistent with the recently published studies on MCF-7, SK-BR-3, and MDA-MB-231 cells.¹⁶ This analysis identified subsets of breast cancer cell lines that differentially express *CST6* mRNA, providing the cellular reagents for examination of methylation-dependent epigenetic regulation of *CST6* in breast cancer cells.

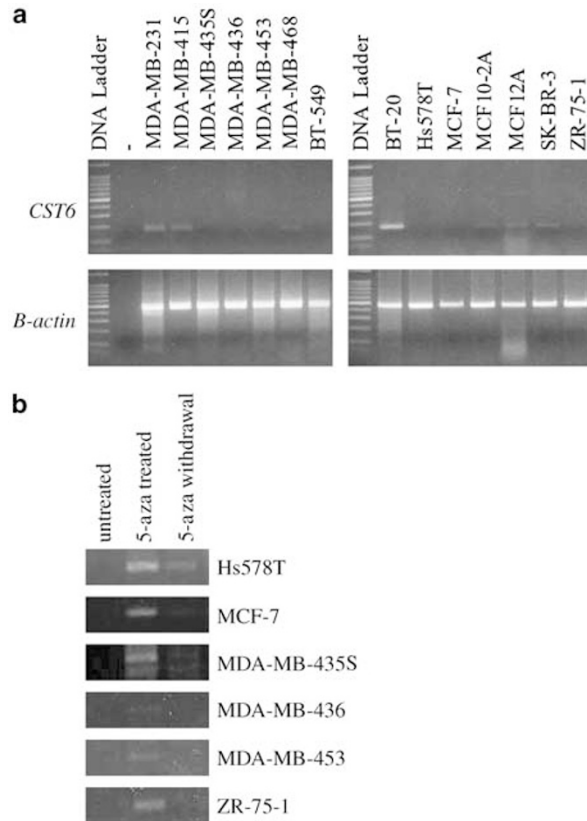


Figure 1 *CST6* expression in human breast cancer cell lines and normal mammary epithelial cells. Representative agarose gels of RT-PCR products are shown. (a) Differential expression of *CST6* among breast cancer cell lines and two normal mammary epithelial cell lines (MCF10-2A and MCF12A). The *CST6* RT-PCR product is 163 bp in size. β -Actin was utilized as a sample control. (b) Lane 1 corresponds to cDNA from the indicated untreated breast cancer cell lines; lanes 2 and 3 correspond to cells after 5-aza treatment or treatment withdrawal, respectively. β -Actin RNA was evenly expressed in all samples (data not shown).

5-Aza Treatment Induces *CST6* Expression in Breast Cancer Cell Lines

CST6-negative cell lines Hs578T, MCF-7, MDA-MB-435S, MDA-MB-436, MDA-MB-453, and ZR-75-1 were treated with 5-aza as described previously,¹⁵ to determine if gene silencing was the likely result of DNA methylation. Four of six (67%) of these cell lines expressed significantly increased levels of *CST6* mRNA in response to 5-aza treatment (Figure 1b). The significant induction of *CST6* mRNA in response to 5-aza treatment in MCF-7 cells observed in this study is consistent with similar published studies.^{15,16} In contrast, 5-aza exposure of MDA-MB-453 and MDA-MB-436 cells resulted in a modest, but detectable increase in *CST6* mRNA (Figure 1b). The 5-aza-induced increase in *CST6* expression was completely reversible. Following a period of recovery after treatment withdrawal, *CST6* mRNA diminished to control levels (corresponding to untreated cells) in all cell lines examined (Figure 1b). The

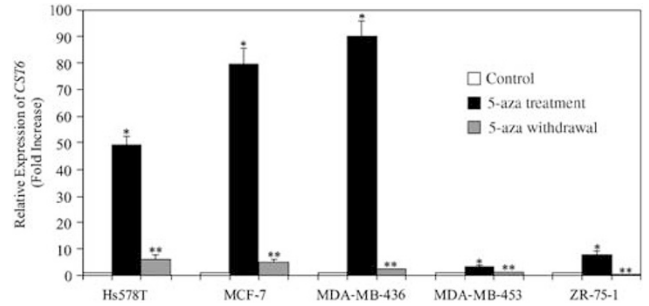


Figure 2 Quantitative real-time PCR analysis of *CST6* in breast cancer cell lines. β -Actin was used to normalize gene expression levels for each cell line and differences in *CST6* expression were determined using the comparative Ct method. *CST6* gene expression for cell lines treated with 5-aza (black bars) and after treatment withdrawal (gray bars) are expressed as relative fold-change compared to control values (set at 1.0). Error bars reflect s.e.m. Values for gene expression that do not show error bars reflect data where the s.e.m. could not be depicted graphically. *Denotes statistical significance at $P < 0.05$ compared to control values. **Denotes statistical significance at $P < 0.04$ compared to 5-aza treatment values.

5-aza treatment-related increases in *CST6* expression, combined with the loss of *CST6* expression following treatment withdrawal, suggests that *CST6* may be subject to methylation-dependent silencing in these breast cancer cell lines.

Expression of *CST6* was analyzed using real-time PCR in *CST6*-negative cell lines Hs578T, MCF-7, MDA-MB-436, MDA-MB-453, and ZR-75-1 to quantitate the 5-aza treatment-related increases in gene expression. Consistent with the RT-PCR results, untreated cell lines express extremely low levels of *CST6* mRNA, and exposure to 5-aza resulted in significant increases in *CST6* mRNA (Figure 2). Exposure of Hs578T cells to 5-aza led to a 49-fold increase in *CST6* expression ($P = 0.0036$ compared to control) and withdrawal of 5-aza resulted in a significant reduction ($P = 0.0021$ compared to 5-aza treated) of gene expression to a level that approaches that of control (untreated) cells (Figure 2). Likewise, 5-aza treatment of MCF-7 and MDA-MB-436 cells produced 80-fold ($P = 0.0058$) and 90-fold ($P = 0.0042$) increases in *CST6* expression, respectively, and withdrawal of the 5-aza treatment lead to significant reductions in *CST6* expression in both cell lines ($P = 0.0062$ and $P = 0.0043$, respectively) (Figure 2). Of note, the induction of *CST6* mRNA in MDA-MB-436 cells in response to 5-aza treatment was consistently demonstrable with all methods, but the magnitude of increased gene expression detected by real-time RT-PCR was greater than that detected using RT-PCR, possibly due to the increased sensitivity of the real-time method. Exposure of MDA-MB-453 and ZR-75-1 cells to 5-aza produced modest increases in *CST6* mRNA (three- and eight-fold, respectively) that were statistically significant ($P = 0.0017$ and $P = 0.0479$), and withdrawal of treatment resulted in significant decreases

in *CST6* expression to levels that were comparable to untreated cells ($P=0.0070$ and $P=0.0393$, respectively) (Figure 2). These data show that 5-aza treatment of *CST6*-negative cell lines results in statistically significant increases in *CST6* mRNA, and suggests strongly that *CST6* is subject to methylation-dependent silencing in a variety of breast cancer cell lines.

Methylation-Dependent Silencing of *CST6* in Select Breast Cancer Cell Lines

To facilitate a correlative analysis of *CST6* gene expression and *CST6* CpG island methylation status, we analyzed 55 CpG dinucleotides from a segment of the proximal promoter region and exon 1 (+242 to -228) in normal breast epithelial cells (MCF12A), two breast cancer cell lines (BT-20 and SK-BR-3) that express *CST6*, and five breast cancer cell lines (Hs578T, MCF-7, MDA-MB-435S, MDA-MB-436, and MDA-MB-453) that lack expression of *CST6*. Multiple clones ($n=3-5$) corresponding to the *CST6* promoter and exon 1 from each cell line were analyzed by sodium bisulfite sequencing and individual CpGs were scored for methylation status. In MCF12A cells, 35/55 (64%) CpGs were not methylated, 18/55 (33%) CpGs were methylated at an intermediate level, and 2/55 (3%) CpGs were 100% methylated, producing a TMI for the promoter/exon 1 of 16% (Figure 3). BT-20 cells exhibit sparse methylation of *CST6*: 53/55 (96%) CpGs were unmethylated in all clones analyzed, resulting in a TMI of 1%. The *CST6* gene in SK-BR-3 cells was significantly more methylated than MCF12A or BT-20 cells (especially within exon 1): 39/55 (71%) CpGs show some level of methylation, producing a TMI of 45% (Figure 3). Overall, the *CST6*-positive cell lines (MCF12A, BT-20, and SK-BR-3) exhibit low levels of methylation within the proximal promoter/exon 1 of *CST6* (mean TMI = $21 \pm 13\%$)

(Table 1). In contrast, breast cancer cell lines that do not express *CST6* exhibit hypermethylation of the *CST6* promoter/exon 1 region, with TMI values ranging from 72 to 98% (Figure 3). MDA-MB-435S and MDA-MB-453 cells were 100% methylated at 52/55 (95%) and 51/55 (93%) CpGs, respectively (Figure 3). In MCF-7 cells, 42/55 (76%) CpGs were 100% methylated, 12/55 (22%) CpGs were methylated at an intermediate level, and 1/55 (2%) CpGs was unmethylated (Figure 3). Hs578T and MDA-MB-436 cells were 100% methylated at 19/55 (35%) and 13/55 (24%) CpGs, respectively, with the remaining CpGs methylated at an intermediate level. The average TMI for the *CST6*-negative cell lines was $87 \pm 5\%$ ($n=5$), reflecting *CST6* promoter/exon 1 hypermethylation among these cells (Table 1). When the methylation status of *CST6* promoter/exon 1 was compared between groups of cell lines that differentially express *CST6*, a significant association ($P=0.0227$) between *CST6* promoter/exon 1 region methylation (CpGs 1-55) and *CST6* gene expression was found (Table 1). However, hypermethylation of the proximal promoter of *CST6* (CpGs 1-23 and 1-31, $P<0.001$) was more strongly associated with loss of *CST6* expression status than methylation involving exon 1 (CpGs 24-55, NS) (Table 1). These observations suggest that hypermethylation of the *CST6* CpG island contributes to the silencing of *CST6* expression in breast cancer cell lines, and that hypermethylation of the proximal promoter is most important for downregulation of *CST6* gene expression.

In order to identify CpG dinucleotides that are critical in *CST6* silencing, 5 *CST6*-negative cell lines (Hs578T, MCF-7, MDA-MB-435S, MDA-MB-436, and MDA-MB-453) were treated with 5-aza, and 46 CpG dinucleotides from the proximal promoter region and exon 1 (+118 to -228, CpGs 10-55) were evaluated by sodium bisulfite sequencing (Figure 4). The *CST6* promoter/exon 1 became significantly demethylated in response to 5-aza

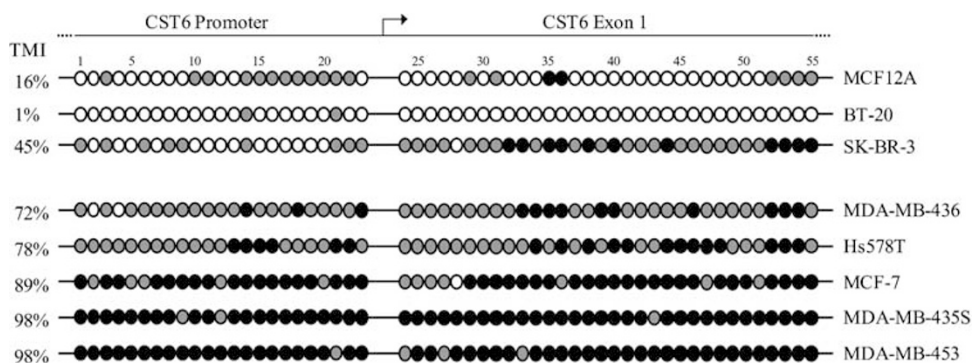


Figure 3 Methylation analysis of the *CST6* proximal promoter and exon 1 in breast cancer cell lines and normal mammary epithelial cells that differentially express *CST6*. A summary of the methylation analysis of the *CST6* promoter (23 CpGs) and exon 1 (32 CpGs) is shown. The black arrow indicates the start of transcription between CpGs 23 and 24. Black circles correspond to fully (100%) methylated CpGs, gray circles correspond to CpGs with intermediate methylation, and open circles correspond to unmethylated CpGs. TMI values for the entire promoter/exon 1 region (55 CpGs) are given for each breast cancer cell line. MCF12A, SK-BR-3, and BT-20 cells express *CST6*, while the remaining cell lines lack *CST6* expression.

Table 1 Segmental CpG methylation analysis of the *CST6* promoter and proximal exon 1 among *CST6*-positive and *CST6*-negative breast cancer cell lines

Cell line	CpGs 1–55 ^a control	CpGs 10–55 ^b control	CpGs 10–55 ^b , 5-aza treatment	CpGs 10–55 ^b , 5-aza withdrawal	CpGs 1–23 ^c control	CpGs 24–55 ^d control	CpGs 1–31 ^e control
<i>CST6</i> -positive							
BT-20	1%	1%	—	—	3%	0%	2%
MCF12A	16%	18%	—	—	19%	14%	15%
SK-BR-3	45%	50%	—	—	16%	67%	19%
<i>CST6</i> -negative							
MDA-MB-435S	98%	99%	42%	40%	96%	99%	97%
MDA-MB-453	98%	97%	64%	41%	99%	97%	97%
MCF-7	89%	90%	33%	64%	90%	89%	83%
Hs578T	78%	86%	1%	73%	72%	80%	72%
MDA-MB-436	72%	76%	26%	75%	65%	77%	66%
<i>CST6</i> -positive	21 ± 13	23 ± 14	—	—	13 ± 5	27 ± 20	12 ± 5
<i>CST6</i> -negative	87 ± 5	90 ± 4	33 ± 10	59 ± 8	84 ± 7	88 ± 4	83 ± 6
<i>t</i> -test	$P = 0.0227^f$	$P = 0.0349$	$P = 0.0033^g$	NS ^h	$P < 0.0001^f$	NS ⁱ	$P < 0.0001^f$

^aCpG dinucleotides from a segment of the promoter region and exon 1 (+242 to –228).

^bCpG dinucleotides from a segment of the proximal promoter region and exon 1 (+118 to –228). The values indicated under 5-aza treatment are from cell lines at the conclusion of a 3-week treatment period. The values indicated under 5-aza withdrawal are from cell lines that had recovered from 5-aza treatment for 5 weeks.

^cCpG dinucleotides from the proximal promoter region (0 to –228).

^dCpG dinucleotides from exon 1 (0 to +242).

^eCpG dinucleotides from the proximal promoter region and 8 CpGs from exon 1 (+50 to –228).

^f*P*-values provided reflect a statistical comparison of average TMI values corresponding to *CST6*-positive ($n = 3$) and *CST6*-negative ($n = 5$) cell lines for the designated gene segments.

^g*P*-value for the comparison of average TMI values corresponding to control and 5-aza-treated *CST6*-negative cell lines for the designated gene segment.

^h*P*-value for the comparison of average TMI values corresponding to 5-aza-treated *CST6*-negative cell lines and the same cell lines after 5-aza withdrawal.

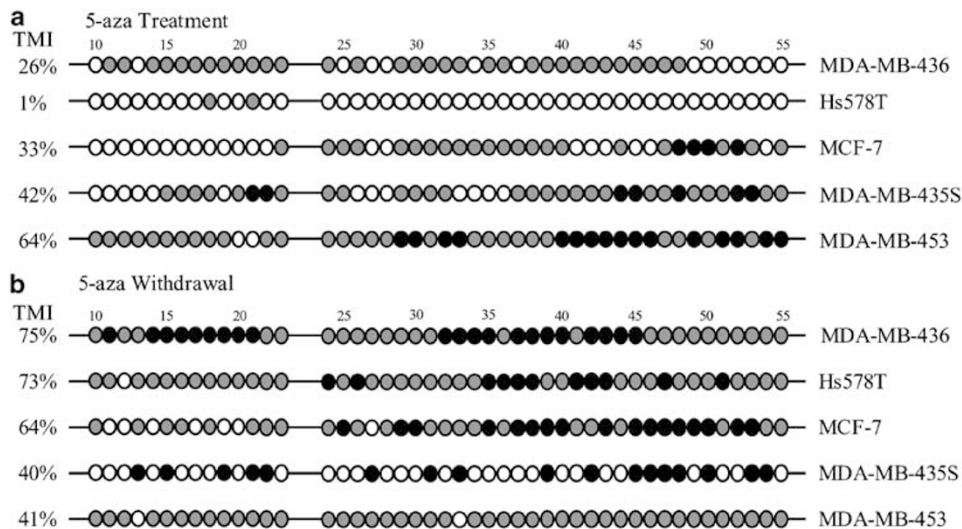


Figure 4 Methylation analysis of the *CST6* proximal promoter and exon 1 in *CST6*-negative breast cancer cell lines that have been exposed to demethylating treatment. A summary of the methylation analysis of the *CST6* promoter (14 CpGs) and exon 1 (32 CpGs) is shown. Black circles correspond to fully (100%) methylated CpGs, gray circles correspond to CpGs with intermediate methylation, and open circles correspond to unmethylated CpGs. TMI values for the promoter/exon 1 region (46 CpGs) are given for each breast cancer cell line. (a) CpG methylation analysis of the *CST6* promoter/exon 1 region after treatment with 5-aza. (b) CpG methylation analysis of the *CST6* promoter/exon 1 region after withdrawal of 5-aza treatment.

treatment in each cell line examined ($P < 0.0001$), resulting in lower values for TMI ($33 \pm 10\%$, range 1–64%) (Figure 4a). Following withdrawal of 5-aza

treatment, remethylation of the *CST6* promoter/exon 1 occurred in MDA-MB-436, Hs578T, and MCF-7 cells (Figure 4b). The extent of methylation after

5-aza withdrawal was indistinguishable from that of controls for MDA-MB-436 and Hs578T cells, reflecting a complete remethylation of the promoter/exon 1 ($P < 0.0001$) (Figures 3 and 4b). Likewise, significant remethylation of *CST6* occurred in MCF-7 cells after treatment withdrawal, with methylation levels approaching that of control (TMI of 64 vs 89%) (Figures 3 and 4b). In contrast, there was no apparent change in *CST6* methylation in MDA-MB-435S cells after treatment withdrawal based upon calculated TMIs (42 vs 40%), but there was a significant qualitative change in the methylation pattern (Figure 4b). MDA-MB-435S cells exhibit a loss of *CST6* expression when the demethylating treatment was withdrawn, suggesting that some or all of the 12 CpGs that were differentially remethylated (CpGs 13, 15, 19, 27, 31, 33, 39, 42, 46, 47, 50, and 54, Figure 4) may be critical for *CST6* silencing. *CST6* expression after 5-aza withdrawal was diminished in MDA-MB-453 cells in the absence of a dramatic increase in *CST6* methylation, although loss of expression was accompanied by a redistribution of methylation across the *CST6* promoter and exon 1 (Figure 4). There is a significant association

between *CST6* methylation status and expression among *CST6*-negative cell lines treated with 5-aza for both the promoter/exon 1 region (CpGs 10–55, control vs 5-aza, $P = 0.0033$), as well as exon 1 alone (CpGs 24–55, control vs 5-aza, $P = 0.0127$) (Table 1).

A comparative analysis of CpG dinucleotides in the promoter and exon 1 regions of *CST6*-positive and -negative cell lines was performed to identify methylation events involving individual CpGs or regions of CpGs that are important for the silencing of *CST6*. Average TMI values for individual CpG dinucleotides were calculated for *CST6*-positive (BT-20, MCF12A, and SK-BR-3) and *CST6*-negative cell lines (Hs578T, MCF-7, MDA-MB-435S, MDA-MB-436, and MDA-MB-453) (Figure 5). The *CST6*-positive cell lines exhibit a low level of methylation (TMI $\leq 33\%$) for the 15 CpG dinucleotides contained in segment 1 (corresponding to -50 to -200). Of note, six CpGs (CpGs 2, 4, 5, 7, 12, and 13) were not methylated in these cell lines, suggesting a possible role for these CpGs and/or the sequences containing these CpGs in the positive regulation of *CST6* expression (Figure 5b). In contrast, a high level of methylation for CpGs in segment 1 (TMI

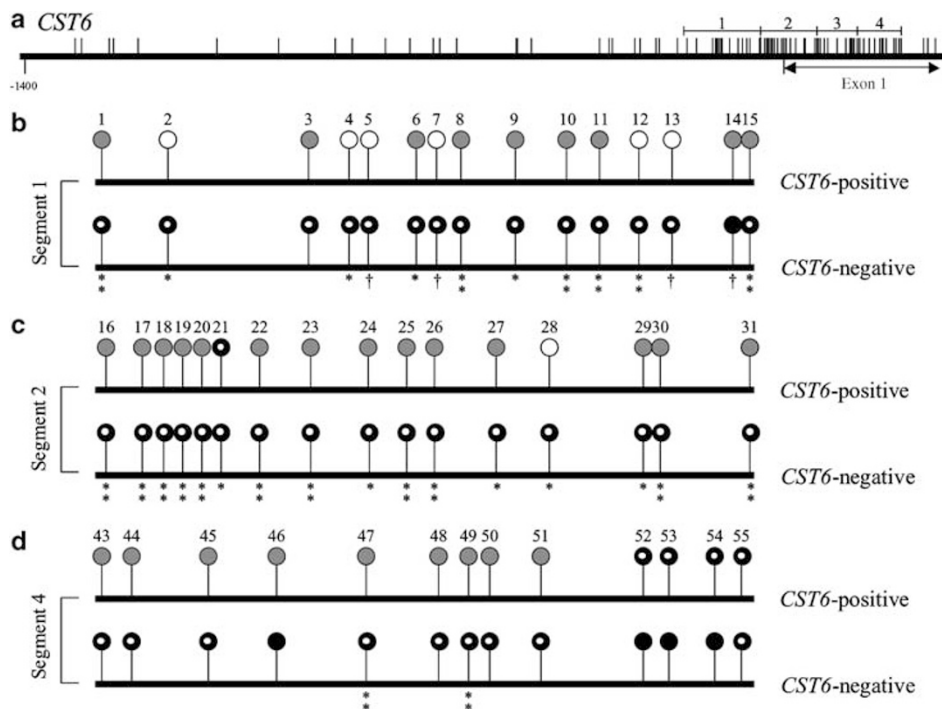


Figure 5 Methylation analysis for individual CpG dinucleotides in *CST6*-positive and *CST6*-negative breast cancer cell lines. (a) Distribution of CpG dinucleotides proximal to the transcriptional start site in the promoter (0 to -1400 nucleotides) and exon 1 (0 to +294 nucleotides) of *CST6* are depicted schematically. Vertical lines represent the relative position of individual CpG dinucleotides and the segmented horizontal lines (designated 1–4) indicate the location of individual CpG dinucleotides depicted in (b) (segment 1), (c) (segment 2), and (d) (segment 4). The results for CpGs in segment 3 are not shown. Representative CpG dinucleotides are indicated by lollipop corresponding to various segments of the promoter or exon 1 (+242 to -228). TMI values represent averages of the three *CST6*-positive and five *CST6*-negative breast cell lines: black circles correspond to 100% methylated CpGs, partially-filled circles correspond to >40% methylated CpGs, gray circles correspond to <40% methylated CpGs, and white circles correspond to unmethylated CpGs. †Denotes statistical significance at $P < 0.001$; **Denotes statistical significance at $P < 0.01$; and *Denotes statistical significance at $P < 0.03$, when comparing individual CpG methylation to *CST6* gene expression status. (b) CpGs 1–15 from segment 1 (-50 to -200) within the promoter region. (c) CpGs 16–31 from segment 2 (+50 to -50) spanning the transcriptional start site. (d) CpGs 43–55 from segment 4 (+242 to +138) within exon 1.

≥73%) was found in *CST6*-negative cell lines, including CpG 14, which was fully (100%) methylated in all cell lines (Figure 5b). The extent of methylation of individual CpG dinucleotides correlated with *CST6* gene expression for 14/15 CpGs in segment 1 ($P=0.0197$ to $P<0.0001$). Segment 2 spans the transcriptional start site of *CST6* (+50 to -50), and consists of CpG dinucleotides 16–31 (Figure 5c). *CST6*-positive cell lines have a relatively low level of methylation at individual CpG dinucleotides (TMI range: 0–44%), whereas *CST6*-negative cell lines contain high levels of methylation in this region of the *CST6* promoter/exon 1 region (TMI range: 66–93%) (Figure 5c). The extent of methylation of individual CpG dinucleotides was significantly associated with *CST6* gene expression for all CpGs in segment 2 ($P=0.0343$ to $P=0.0046$). Segments 3 and 4 encompass CpG dinucleotides 32–55 of *CST6* exon 1 (+242 to +50). CpG dinucleotides in this region were moderately methylated in *CST6*-positive cell lines with TMI values ranging from 11 to 67% (Figure 5d). Among *CST6*-negative cell lines, the calculated TMI values ranged from 80 to 100%, reflecting exon 1 hypermethylation in both segments. With few exceptions, there was no significant correlation between *CST6* gene expression and methylation status of individual CpG dinucleotides in segments 3 and 4.

Discussion

CST6 (which encodes cystatin M) was originally identified as a gene whose expression is lost in metastatic breast cancer, suggesting a possible role for this gene in suppression of the invasive/metastatic phenotype.⁵ Consistent with this suggestion, normal human breast epithelial cells express high levels of cystatin M, while invasive ductal carcinoma cells do not express or express very low levels of this protein.² When exogenously expressed in human MDA-MB-435S breast cancer cells, *CST6* significantly alters the neoplastic phenotype *in vitro*, resulting in diminished cell proliferation, loss of cell migration, inhibition of Matrigel invasion, and reduced endothelial cell adhesion.¹ Furthermore, expression of *CST6* in MDA-MB-435S cells delays tumorigenesis by transplanted cells and suppresses spontaneous formation of liver and lung metastases.² Cystatin M is a member of a family of proteins that function as inhibitors of lysosomal cysteine proteases, which include the cathepsin proteases.^{4,26} Increased levels of cathepsin protease activity have been noted in breast cancer,²⁷ and predict poor prognosis among these patients.²⁸ Thus, breast cancer invasiveness may be a direct consequence of inappropriate cathepsin protease activity in the absence of their inhibitory molecules, which include cystatin M.²⁹ While *CST6* expression is known to be lost in many primary breast tumors and cancer cell lines, the mechanism that accounts

for loss of *CST6* expression has only recently been investigated. Given a role for *CST6* in tumor suppression and/or metastasis suppression, possible mechanisms for loss of expression include genetic alterations (mutation or deletion) and epigenetic silencing. Southern blotting in breast cancer cell lines failed to identify gross structural rearrangements of the *CST6* gene or deletion of the gene locus,⁵ leading to the more recent suggestion that *CST6* expression may be lost due to gene silencing, either in response to a transcriptional repressor protein or as a consequence of promoter hypermethylation.⁴

The *CST6* gene contains a large CpG island that encompasses the proximal promoter and exon 1, consisting of 55 CpG dinucleotides distributed over a 470 bp region (CpGPlot, <http://www.ebi.ac.uk/emboss/cpgplot/>). In a previous microarray-based gene expression study, we identified *CST6* as a target for methylation-dependent gene silencing in MCF-7 breast cancer cells, and showed that loss of *CST6* expression in these cells is related to hypermethylation of its CpG island.¹⁵ More recently, Ai *et al*¹⁶ has shown aberrant methylation of *CST6* in select breast cancer cell lines and primary breast tumors. Additionally, Kim *et al*¹⁷ reported *CST6* to be frequently methylated in glioma cell lines and primary brain tumors. In the current study, we established a direct association between *CST6* promoter hypermethylation and gene silencing in a panel of human breast cancer cell lines that differentially express *CST6*. The majority of *CST6*-negative cell lines examined were originally isolated from invasive/metastatic breast neoplasms,^{30–32} whereas the *CST6*-positive BT-20 cells (which express very high levels of *CST6*) were derived from a primary breast carcinoma.³³ These results suggest strongly that (i) CpG island hypermethylation contributes to *CST6* silencing in breast cancer cell lines and (ii) the loss of *CST6* expression is associated with the invasive/metastatic phenotype of the breast cancer cell line.

Epigenetic silencing of gene expression is a consequence of DNA hypermethylation and/or chromatin remodeling related to direct interference with the binding of transcription factors to their recognition sequences,^{8,9} indirect mechanisms associated with recruitment and binding of methylated DNA-binding proteins,^{10–13} and/or modification of histone proteins and alteration of chromatin structure.³⁴ In the current study, we generated evidence for CpG island hypermethylation in the epigenetic silencing of *CST6* in human breast cancer cell lines. However, the precise mechanism and the temporal order of events related to *CST6* gene silencing have not been determined. Nonetheless, we were able to gain significant insight into the process through comparison of the natural methylation pattern of *CST6*-positive and -negative cell lines. *CST6*-negative cell lines are characterized by extensive CpG island methylation, suggesting the possibility that

regional methylation across the *CST6* proximal promoter and exon 1 may be required for gene silencing. However, a subset of *CST6*-positive cell lines contain a significant level of methylation in exon 1, indicating that methylation in this portion of the CpG island does not negatively impact on *CST6* transcription. In contrast, methylation of CpG dinucleotides in the proximal promoter is strongly associated with loss of *CST6* expression, suggesting that regional methylation or specific methylation events affecting this portion of the promoter contribute to gene silencing. There is evidence that discrete methylation events within a larger methylation target (CpG island) can negatively affect gene expression. The AP-2 α tumor suppressor gene is an example of a gene that is silenced in response to CpG methylation of a discrete region that is contained within a larger CpG island.⁷ Discrete methylation events may negatively impact on promoter function by direct interference with transcription factor binding or through indirect interference related to binding by methylated DNA-binding proteins. Among the 55 CpGs that comprise the *CST6* CpG island, seven CpGs in the proximal promoter were found to be unmethylated in all cell lines that express the gene, and these CpGs were frequently methylated in *CST6*-negative cell lines. Specific methylation events affecting these CpG dinucleotides may be required for *CST6* gene silencing, but it is not clear if these specific methylation events occur in isolation or if they always take place in conjunction with more extensive regional methylation. Methylation of these CpGs may directly impact on the binding of specific transcription factors to their recognition sequence. Analysis of the *CST6* promoter using ProSpector (<http://prospector.nci.nih.gov>) identified 16 CpGs within the proximal promoter that directly impinge upon transcription factor binding sites and are associated with loss of *CST6* expression when methylated, including sequences for AP-2, AP-4, Egr-1, MEIS1, NF- κ B, Sp1, and YY1. Methylated CpGs in the binding sites for AP-2 and Sp1 transcription factors have been shown to directly downregulate gene expression.^{35,36} Of note, CpG dinucleotide 13, which impinges on three transcription factor binding sites, is never methylated in *CST6*-positive cell lines. The transcription factor requirements for expression of the *CST6* promoter have not been determined. Nonetheless, CpG methylation of the proximal promoter of *CST6* is likely to inhibit or impair gene transcription by either direct or indirect interference with the transcription machinery.

The differential CpG island methylation profile among *CST6*-positive and -negative breast cancer cell lines indicates that certain methylation events and/or specific promoter regions are strongly associated with gene silencing. Nevertheless, *CST6*-negative cell lines tend to be heavily methylated across the entire promoter/exon 1 CpG island. We posit that

there is a succession of methylation events that progressively leads to *CST6* gene silencing in metastatic breast cancer cell lines: (i) individual CpG dinucleotides within the promoter region are preferentially methylated resulting in transient silencing of *CST6*, (ii) methylation spreads throughout the promoter/exon 1 CpG island, (iii) chromatin remodeling occurs resulting in stable silencing of *CST6*. Alternatively, chromatin remodeling might occur before the completion of regional methylation affecting the entire CpG island. Additional studies will be required to establish the temporal order of events and the nature of chromatin alterations that accompany *CST6* silencing in breast cancer cell lines. Likewise, additional studies will be required to determine if methylation-dependent gene silencing accounts for loss of *CST6* expression in primary breast tumors and their metastatic lesions.

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