Epigenetic Silencing of the Tumor Suppressor Cystatin M Occurs during Breast Cancer Progression

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Abstract
Cystatin M is a secreted inhibitor of lysosomal cysteine proteases. Several lines of evidence indicate that cystatin M is a tumor suppressor important in breast malignancy; however, the mechanism(s) that lead to inactivation of cystatin M during cancer progression is unknown. Inspection of the human cystatin M locus uncovered a large and dense CpG island within the 5' region of this gene (termed CST6). Analysis of cultured human breast tumor lines indicated that cystatin M expression is either undetectable or in low abundance in several lines; however, enhanced gene expression was measured in cells cultured on the DNA demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC). Increased cystatin M expression does not correlate with a cytotoxic response to 5-aza-dC; rather, various molecular approaches indicated that the CST6 gene was aberrantly methylated in these tumor lines as well as in primary breast tumors. Moreover, 60% (12 of 20) of primary tumors analyzed displayed CST6 hypermethylation, indicating that this aberrant characteristic is common in breast malignancies. Finally, preinvasive and invasive breast tumor cells were microdissected from nine archival breast cancer specimens. Of the five tumors displaying CST6 gene methylation, four tumors displayed methylation in both ductal carcinoma in situ and invasive breast carcinoma lesions and reduced expression of cystatin M in these tumors was confirmed by immunohistochemistry. In summary, this study establishes that the tumor suppressor cystatin M is a novel target for epigenetic silencing during mammary tumorigenesis and that this aberrant event can occur before development of invasive breast cancer.

Introduction
During the process of carcinogenesis, tumor cells arise from normal cells that have acquired abnormal proliferative capability (1). This is a multistep process during which oncogene activity is increased and tumor suppressor activity is constrained. Often concomitant with uncontrolled cellular proliferation is acquisition of enhanced migratory capability, evasion of immune surveillance, and promotion of angiogenesis. Such disease-producing changes in cellular phenotype result from dysregulation of gene expression and/or protein function attributable to genetic and/or epigenetic changes within the genome.

Numerous tumor suppressor or growth-regulatory genes undergo de novo methylation and transcriptional silencing in human cancer cells. Aberrant cytosine methylation leading to gene silencing occurs at clustered 5'-CG-3' dinucleotides (called CpG islands) within the genome (2). In a broader sense, cancer-associated CpG methylation is part of a more complex set of epigenetic events that result in the transformation of chromatin from a transcriptionally active to an inactive state (3). Epigenetic gene silencing is now widely recognized as either a causative or a correlative event in tumor development (4, 5). In breast cancer, a list of well-characterized tumor suppressors, including BRCA1, p16^{INK4a}, 14-3-3e, E-cadherin, and ATM, are targets for epigenetic silencing (6–11). Site-specific DNA methylation is often an early event as shown in tumor suppressor genes with a well-defined pattern of progression, such as colon cancer, and is now widely regarded as one of the "hits" in the Knudsen hypothesis leading to tumor suppressor gene inactivation.

Acquisition of an invasive cellular phenotype in breast cancer is a key event in progression and severity of this disease. The majority of breast cancers are adenocarcinomas arising from the epithelial layers that line the mammary glands. These glands are composed of terminal mammary lobules and lactiferous ducts that connect the lobule units to the nipple. It is now widely recognized that the preponderance of diagnosed cases of breast cancer arise from ductal epithelium at the terminal lobular ductal unit (12). The first recognizable premalignant change in ductal architecture is epithelial hyperplasia (13). Usual ductal hyperplasia can occur normally, regresses spontaneously, and carries a modest risk (~2-fold) of developing into more severe disease (14). However, with continued cell proliferation and acquisition of characteristic morphologic hallmarks, a lesion termed ductal carcinoma in situ (DCIS) develops. Although considered a cancerous lesion and has a higher risk (~10-fold) of developing into a more serious form of the disease (14), the cell growth in DCIS is confined to the mammary duct because the basement membrane and myoepithelial cell layer that surrounds the duct remains intact. Further changes in cells comprising DCIS lesions may lead to the ability to escape the duct structure and invade the surrounding stroma. Such invasive breast carcinoma (IBC) lesions may continue to grow in size, destroying nearby normal breast architecture. Further disease progression can result in far more serious metastatic disease, as malignant cells enter the lymphatics or vasculature and spread to distal sites within the body.

Increased extracellular proteolytic activity can lead to destruction of the extracellular glycoprotein scaffolds that maintain tissue architecture, thus facilitating invasion of normal tissue and...
promoting metastasis of cancer cells (15). In addition, extracellular proteolytic activity can result in the activation of latent growth-promoting molecules or inactivation of growth-inhibiting molecules (16). For example, enhanced activity of several matrix metalloproteinases (MMP) was observed in cultured breast tumor cell lines (17), induced expression of MMP3 in mammary epithelium promotes mammary tumors in mice (18), and MMP1 has recently been identified as a possible early biomarker in breast tumors (19). In addition to MMPs, lysosomal cysteine proteases are known to degrade extracellular matrix components in vitro (20–22), and the cysteine proteinases cathepsins B and L have been implicated in tumor cell invasion and metastasis (23–25).

As an alternative to increased expression or export of proteases, decreased expression or activity of their normal endogenous inhibitors will predictably result in a similar outcome. Indeed, an imbalance between proteases and their inhibitors is viewed as important in driving tumorigenesis (26). Cystatins are a class of protease inhibitors originally shown to inhibit the activity of cathepsins and other cysteine proteases (27). At present, cystatins are recognized as a superfamily of proteins and are classified into five families: stefins, cystatins, latexins, fetuins, and kininogens (28). Cystatin M (also known as cystatin E) was originally identified by two groups. One group identified this protein by using differential display to compare gene expression in primary and metastatic breast tumor lines (29) and the other group used inspection of expressed sequence tag sequences obtained from cDNAs clones from a variety of tissue sources (30). Characterization of this protein determined that cystatin M is produced and secreted in both an unglycosylated (14 kDa) form and a glycosylated (17 kDa) form. Since the identification of cystatin M and the proposal that this gene could function as a tumor suppressor (29), several subsequent studies point toward a critical role for this protease inhibitor in cancer progression. For example, the breast tumor line MDA-MB-435S is highly tumorigenic, invasive, and metastatic in nude mice (31). Shridhar et al. (32) observed that this line expresses undetectable levels of cystatin M but that expression of recombinant cystatin M reduced cell proliferation, migration, and Matrigel invasion. Further, although cystatin M expression was detectable in normal human mammary epithelium, reduced or subdetectable levels of cystatin M were observed in IBC lesions (33). These workers also found that expression of cystatin M in MDA-MB-435S cells delayed primary tumor growth and reduced the burden of lung metastases in scid mice orthotopically implanted with this human breast cancer line. Thus, ample evidence supports the view that cystatin M expression is down-regulated in breast cancer and that this event is likely to contribute to development and/or progression of this tumor type; however, the mechanism underlying reduced cystatin M expression in breast malignancy remains unknown.

Based on the gene structure, Kepler (28) first proposed that cystatin M may be subject to epigenetic silencing through aberrant gene methylation. More recently, Rivenbark et al. (34) uncovered that cystatin M expression increased in cultured MCF-7 breast tumor cells on treatment with the global DNA demethylating agent 5-aza-2’-deoxycytidine (5-aza-dC) and that this cell line displayed CpG methylation within a region directly upstream of the CST6 gene. Here, we show that the CST6 gene is commonly subject to aberrant hypermethylation not only in cultured breast tumor cells but also in primary breast tumors. This study firmly establishes cystatin M as a target for epigenetic silencing in breast cancer.

Materials and Methods

Cell culture and drug treatment. The breast tumor cell lines BT-549, MCF-7, MDA-MB-231, MDA-MB-468, SK-BR-3, and T-47D were obtained from American Type Culture Collection (ATCC, Manassas, VA). The nontumor breast epithelium-derived lines MCF-10A and 184B5 were also obtained from ATCC. Cell lines were maintained in medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitro, Carlsbad, CA), 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO2 humidified atmosphere. MCF-7, MDA-MB-231, MDA-MB-468, and SK-BR-3 were grown in Eagle’s MEM with 2 mmol/L l-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium pyruvate. BT-549 and T-47D were cultured in RPMI 1640 with 2 mmol/L l-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate. For MCF-7 and T-47D cells, the medium was further supplemented with 0.01 mg/mL bovine insulin (Sigma, St. Louis, MO). Both MCF-10A and 184B5 cells were cultured on MEGM (Clonetics, San Diego, CA) supplemented with cholera toxin (Biomol, Plymouth Meeting, PA).

Unless otherwise indicated, 5-aza-dC treatment was conducted as described previously (35). Following a 24-h incubation with 5 mmol/L 5-aza-dC, the cells were rinsed with PBS, fresh culture medium was added, and the cells returned to the incubator for 24 hours. At the end of this incubation, 5 mmol/L 5-aza-dC was again added, and the cells were incubated with drug for another 24 hours until harvest. 5-Aza-dC (Sigma) was freshly prepared as 5 mL/L stock solution in PBS before use.

Reverse transcription-PCR. Total RNA was extracted from cultured cells and tissues using TRIzol reagent (Invitrogen). This RNA was then used in first-strand cDNA synthesis reactions using Stratascript first-strand synthesis system (Stratagene, La Jolla, CA) using supplied random hexamer primers. Cystatin M expression was subsequently analyzed by PCR. CST6-specific primers were used forward 5′-GACTGCGCGAAGACC-3′ and reverse 5′-GAAGTGCCCTTCACAACA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control for RNA integrity and was amplified using primers forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCAC- CACCCGTGTTGCTGA-3′. PCR thermocycling conditions used were 1 cycle at 95°C for 5 minutes; 38 cycles at 94°C for 45 seconds, 56°C for 1 minute, and 72°C for 90 seconds; and final extension at 72°C for 10 minutes. Thermocycling conditions for GAPDH were the same as given for CST6, except that the annealing temperature of 58°C was used and PCR was conducted for 25 cycles. Following thermocycling, reactions were analyzed by electrophoresis on 10% polyacrylamide gels followed by staining with ethidium bromide.

Quantitative PCR. Total RNA was digested with RNase-free DNase (Promega, Madison, WI) following the manufacturer’s protocol and DNA-free total RNA (1.0 μg) was used in a 20 μl cDNA synthesis. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for quantitative PCR (qPCR) and 2 μL cDNA was added to final 20 μl PCR solution using above-outlined reverse transcription-PCR (RT-PCR) primer sets from cystatin M or GAPDH. Thermocycling conditions for qPCR are 1 cycle at 95°C for 10 minutes and total 40 cycles at 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. Thermocycling was conducted using a Bio-Rad MiniOpticon System (Bio-Rad Laboratories, Hercules, CA) and C1 was calculated using supplied software. Cystatin M transcript abundance relative to GAPDH transcript abundance (ΔC1) was calculated and subsequently used to calculate changes (2-ΔΔCt) in cystatin M expression. For analysis of gene expression following 5-aza-dC treatment, cystatin M transcript abundance in untreated cells was set at 1 and relative transcript abundance in treated cells was subsequently calculated using this reference value. For analysis of matched breast tumor and normal samples, cystatin M transcript abundance in normal tissues was defined as 100% and relative transcript abundance in tumors was calculated accordingly.

SDS-PAGE and Immunoblotting. Culture medium from MDA-MB-231 and MDA-MB-468 cells with or without 5-aza-dC treatment was collected. The medium was subsequently concentrated using iCON concentrators (Pierce, Rockford, IL) and protein concentration was assayed by BCA.
(Pierce). SDS-PAGE and immunoblotting was done using established protocols (36). Biotinylated goat anti-human cystatin E/M antibody was purchased from R&D Systems (Minneapolis, MN). Streptavidin-conjugated horseradish peroxidase (HRP; Kirkegaard and Perry Labs, Gaithersburg, MD) was used for antibody detection, and the blot was developed using SuperSignal West Pico chemiluminescent substrate (Pierce) followed by exposure on X-ray film. Alternatively, SDS-PAGE gels were stained with Coomassie brilliant blue R250 (0.1%, w/v) dissolved in 10% methanol/10% acetic acid.

**Human breast tumor and normal tissue genomic DNA extraction.**

Twenty fresh-frozen archival breast tumors were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank. All specimens and pertinent patient information were treated in accordance with policies of the institutional review board of the University of Florida Health Sciences Center. Tumors analyzed in this study were examined by a surgical pathologist and identified as invasive breast adenocarcinoma (stage II or III). Where indicated, matched normal breast tissue was obtained from disease-free surgical margin. Genomic DNA (gDNA) was isolated from these samples, as well as cultured cell lines, using a Qiagen Blood and Cell Culture DNA kit (Qiagen, Inc., Valencia, CA) and stored at −20°C before use.

**Sodium bisulfite DNA modification.** DNA was modified with EZ DNA Methylation kit (ZYMO Research Co., Orange, CA) according to the manufacturer's instructions. Briefly, ~1.5 μg denatured gDNA was treated with sodium bisulfite at 30°C for ~16 hours in the dark. Following this, samples were applied to supplied columns, columns were washed, deamination was conducted, samples were washed again, and DNA was subsequently eluted with 20 μL elution buffer. Generally, 2 μL modified DNA was used in subsequent PCRs.

**Methylation-specific PCR.** Qiagen HotStarTaq DNA polymerase was used for PCR. Reactions contained supplied 1X PCR buffer supplemented with 0.1 mMol/L deoxynucleotide triphosphate, 2.5 mMol/L MgCl₂, and 0.5 μMol/L of each forward and reverse primers. The methylation-specific PCR (MSP) primers specific for methylated CST6 gene are the forward primer 5’-TCAGATTTCGTGATTAGTTTAGTGC-3’ and reverse primer 5’-CATACCGTCAACACGTG-3’. The MSP primers specific for unmethylated CST6 gene are the forward primer 5’-TGAGTTTTGTTTTAGTTT-3’ and reverse primer 5’-CCATAACCATCAATACCATCAA-3’. Thermocycling conditions used are 1 cycle at 95°C for 15 minutes; 38 cycles at 94°C for 30 seconds, 60°C (for M primer set) or 55°C (for U primer set) for 1 minute, and 72°C for 30 seconds; and final extension at 72°C for 10 minutes. PCR products were loaded 10% polyacrylamide gels and the gel was stained with ethidium bromide and directly visualized under UV illumination. A positively methylated DNA control was run with each reaction, and the resulting bands were compared to determine the degree of methylation.

**Bisulfite genomic sequencing.** Bisulfite genomic sequencing (BGS) was conducted as outlined previously (11). A 362-bp segment (nucleotides −117 to +242; transcriptional start site, +1) was amplified from bisulfite-modified gDNA. The primer sequences used for BGS are 5’-GTTTTTTTGGTGGGTTTA-3’ and 5’-TTACTACCATATTTACTAACACAC-3’. The following thermocycling conditions were used: 1 cycle at 95°C for 15 minutes; 38 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute; and final extension at 72°C for 10 minutes. Resultant amplicons were subcloned into the plasmid pCR-TOPO (Invitrogen), and recombinants were identified by EcoRI digestion and subsequently sequenced at the University of Florida ICBR DNA sequencing facility using a vector-encoded primer (M13-Forw).

**Laser capture microdissection and DNA extraction from paraffin-embedded tumor samples.** Archival blocks of formalin-fixed, paraffin-embedded breast tumors (invasive ductal carcinomas) were obtained from the Department of Pathology of the University of Florida Shands Hospital in accordance with institutional review board policy. All tumors were confirmed by histologic examination to contain both IBC and DCIS lesions. For each tumor, 10-μm-thick sections were mounted on PEN membrane slides (MicroDissect GmbH Co., Mittenaer, Germany). H&E staining of the sections was conducted according to a Leica protocol. After staining, 10 to 15 individual IBC or DCIS lesions (~10,000 cells) were microdissected from the sections using a Leica AS LMD Laser Capture Microdissection (LCM) System (Leica, Wetzlar, Germany). Following microdissection, 45 μL lysis buffer [10 mmol/L Tris-HCl (pH 8.4), 0.1% Tween 20 containing 5 μL proteinase K (100 mg/mL)] was added and the cells were incubated at 55°C for 12 hours. Subsequently, proteinase K was inactivated by incubation at 99°C for 10 minutes, and these samples were used without further processing in sodium bisulfite modification reactions.

**Immunohistochemistry.** For cystatin M immunostaining, formalin-fixed, paraffin-embedded human breast tumors were sectioned (5 μm thick) and mounted on glass microscope slides. Sections were subsequently deparaffinized, rehydrated, and subsequently processed for antigen retrieval by steaming in citrate buffer [10 mmol/L sodium citrate, 0.05% Tween 20 (pH 6.0)] for 20 minutes. After incubation in 0.3% H₂O₂ and blocking with 5% bovine serum albumin in PBS, tissues were incubated in affinity-purified, biotinylated goat anti-human cystatin M antibody (R&D Systems; working concentration, 10 μg/mL) at room temperature for 2 hours. Following three washes with 0.2% Tween 20 in PBS, 1 to 2 drops of streptavidin-conjugated HRP solution were layered on the tissues for 30 minutes. Subsequently, the sections were washed again and 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA) was layered on the sections according to the manufacturer's directions. After this, sections were counterstained with hematoxylin. Control staining was carried out using the same procedure, except that cystatin M antibody was replaced with biotinylated goat anti-rabbit IgG (KPL, Gaithersburg, MD) used at the same concentration. Stained sections were observed using a Zeiss Axioplan 2 microscope and photomicrographs were taken using a CCD camera.

## Results

**Architecture of the human CST6 gene.** Reduced expression of the cystatin M gene (CST6) is a commonly encountered event in both cultured human breast cancer lines and primary breast malignancies (29, 33). As first proposed by Keppler (28) and consistent with a recent study using a genome-wide analysis approach (34), we hypothesized that reduced expression of this gene may stem from aberrant methylation of CpG dinucleotides within the proximal gene region of the CST6 gene in breast cancer. To initially investigate this possibility, we examined the architecture of the human CST6 gene using public genome sequence databases. Using the University of California at Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) to search the May 2004 assembly (National Center for Biotechnology Information Build 35) of repositioned human genome sequences, we observed that the CST6 gene maps to chromosome 11q13.1 (Fig. 1A). This is consistent with the location of this gene as determined previously by fluorescence in situ hybridization (FISH; ref. 37). Analysis of the 35-kbp region of genome in which the CST6 gene resides shows this locus to contain a moderate number of genes (Fig. 1B). Telomerically flanking the CST6 gene is the CATSPER1 gene that encodes a voltage-gated calcium channel located in the plasma membrane of the sperm tail and is necessary for sperm motility and fertility in mice (38). Centromerically flanking CST6 is the barrier to autointegration factor-1 (BANF1) gene, known to function as a retroviral integration cofactor (39), and MGCI11102, a theoretical protein with sequence homology to a putative translation initiation factor in plants that shares limited homology with translation initiation factor 1A.

Analysis of the CST6 gene shows it to be a rather compact gene contained within a 1,513-bp genomic interval (Fig. 1C). The gene is composed of three exons: exon 1 (294 bp), exon 2 (126 bp), and exon 3 (188 bp). Exon 1 encodes the 5’ untranslated region (UTR) of the cystatin M transcript as well as the translational initiation...
To examine cystatin M protein expression, the MDA-MB-468 cell line was cultured on 5-aza-dC, and because the cystatin M protein is a secreted protein, tissue culture medium was collected and concentrated. Subsequently, concentrated medium from untreated MDA-MB-468 cells as well as cells treated with 5-aza-dC for 1 or 2 days was immunoblotted with anti-cystatin M. For these experiments, an affinity-purified rabbit anti-cystatin M antiserum found to be specific for the unglycosylated (14 kDa) and glycosylated (17 kDa) forms of cystatin M by immunoblot analysis of tissue culture medium and whole-cell extracts (see Supplementary Fig. S1A) was used. The results (Fig. 2C) clearly show that no cystatin M was detected in the medium of untreated cells or cells cultured on 5-aza-dC for 1 day. However, MDA-MB-468 cells cultured on 5-aza-dC for 2 days showed strong expression of both unglycosylated and glycosylated forms of cystatin M. Analysis of medium from untreated MDA-MB-231 cells displayed detectable levels of cystatin M expression (Fig. 2C) and expression of this protein in this line did not change on treatment with 5-aza-dC.

Decreased cystatin M expression in cultured breast cancer cells correlates with cystatin M gene methylation. To further explore the possibility that the \( \text{CST6} \) gene is a target for epigenetic silencing in breast cancer, we analyzed a panel of cultured human breast cancer cell lines for cystatin M expression. Cells were cultured in the presence or absence of the DNA demethylating drug 5-aza-dC and total RNA was harvested. Following this, cDNA was synthesized from total RNA and used in RT-PCR assays to examine expression of the cystatin M transcript. Our results indicated that the line MDA-MB-231 displayed expression of the cystatin M transcript and that the relative abundance of this transcript was unaltered by culture on 5-aza-dC (Fig. 2A). In contrast, cystatin M transcript abundance in five other breast tumor lines (MCF-7, T-47D, SK-BR-3, MDA-MB-468, and BT-549) was either very low or subdetectable but was markedly increased following culture on 5-aza-dC.

To independently confirm these results and quantify changes in gene expression, qPCR was done. We measured that cultivating MCF-7 cells with 5-aza-dC resulted in a 13.1-fold (SD, 2.1; \( n = 4 \)) increase in cystatin M transcript abundance compared with untreated cultures of MCF-7 cells (Fig. 2B). Similarly, significant increases in cystatin M transcripts were measured in T-47D (27.5-fold; SD, 3.1; \( n = 4 \)), BT-549 (57.3-fold; SD, 7.4; \( n = 4 \)), SK-BR-3 (35.3-fold; SD, 4.3; \( n = 4 \)), and MDA-MB-468 (49.9-fold; SD, 6.8; \( n = 4 \)) cells. Consistent with our RT-PCR results, we measured an insignificant (1.1-fold; SD, 0.2; \( n = 4 \)) increase in cystatin M mRNA in 5-aza-dC treated MDA-MB-231 cells by qPCR.
(data not shown). Additional studies indicate that increased cystatin M transcript abundance in response to 5-aza-dC does not correlate with a cytotoxic response to this drug in MCF-7 cells and that staurosporine, a potent inducer of apoptosis (43), does not promote increased cystatin M expression (Supplemental Fig. S2). Taken together, these experiments indicate that the majority of breast cancer cell lines analyzed in this study display reduced cystatin M expression and that this defect can be complemented by culture on the global DNA demethylating drug 5-aza-dC. Moreover, these findings clearly suggest that cystatin M expression is controlled, either directly or indirectly, by DNA methylation.

We next sought to directly assess CpG methylation in human breast cancer cell lines. To accomplish this, we developed a set of oligonucleotide primers useful in analysis of the methylation status of the CST6 gene by MSP (44) and BGS (ref. 45; Fig. 3A). For MSP, oligonucleotide primers were designed to amplify a segment of the CST6 gene existing in either a methylated or an unmethylated state based on the modification status of CpG dinucleotides following sodium bisulfite modification. The primers designed to amplify unmethylated CST6 gene sequence yield a 137-bp amplicon spanning from nucleotides −78 to +58, and the methylated primer set yield a 134-bp amplicon spanning from nucleotides −76 to +57 (numbering system based on location of the cystatin M transcriptional start site). Amplification of the CST6 gene from bisulfite-modified human placental gDNA was observed using the unmethylated specific primer set but not with the methylated specific primer set and this amplification was restricted to bisulfite-modified DNA (Fig. 3B). Conversely, when this gDNA was methylated in vitro using SssI methylase and subsequently subjected to bisulfite modification before PCR, we observed amplification of the methylated specific amplicon but not the unmethylated specific product. Sequence analysis of both unmethylated and methylated amplicons indicated that these primer sets specifically amplify the targeted portion of the CST6 gene (data not shown). These validation experiments indicate that our MSP primers amplify gDNA in a manner that clearly ascertains the methylation status of the CST6 gene.

MSP analysis was subsequently conducted on two nontumor, mammary epithelium-derived cell lines. MSP analysis of 184B5 and MCF-10A cells determined that the CST6 gene is unmethylated in both of these cell lines (Fig. 3C). RT-PCR analysis of 184B5 and MCF-10A cells indicated that each of these lines express detectable levels of cystatin M transcript (Fig. 3C).

gDNA was harvested from our panel of breast cancer cell lines either cultured in the absence or presence of 5-aza-dC and was subsequently bisulfite modified and used in MSP assays. We observed amplification of the unmethylated specific amplicon in modified DNA from MDA-MB-231, indicating that the CST6 gene is unmethylated in this cell line (Fig. 3D). Analysis of DNA from T-47D and SK-BR-3 cells not cultured on 5-aza-dC showed amplification with both unmethylated and methylated specific amplicons. Although amplification of both unmethylated and methylated specific MSP products is a common finding in MSP analysis (44) potentially attributable to heterogeneous gene methylation within the tumor cell population, this result indicates the presence of methylated CpG within the CST6 gene in at least a subset of cells of each line. In nontreated cultures of MCF-7, MDA-MB-468, and BT-549, we observed exclusive amplification with the methylated specific primer set, indicating CST6 gene methylation in the lines. Further, MSP analysis conducted on these lines after treatment with 5-aza-dC indicated a clear increase in the abundance of the unmethylated specific amplicon, consistent with demethylation of the CST6 gene and increased expression of the cystatin M transcript.

To independently assess CST6 gene methylation, bisulfite-modified gDNA from several breast cancer cell lines was analyzed by BGS. This entailed sequencing an amplicon obtained using primers designed to amplify a segment of the CST6 gene in a methylation-independent manner (see Fig. 3A). Specifically, PCR primers were designed to regions of the CST6 gene devoid of CpG dinucleotides; thus, amplification proceeds in a manner unbiased by gene methylation status. The 362-bp BGS amplicon, which

![Figure 2](image-url)
contains 46 CpG dinucleotides, was subsequently subcloned into a vector, recombinants were identified, and multiple clones were subjected to automated DNA sequencing. These sequences were compared with nonmodified CST6 gene sequence and the methylation status of CpG dinucleotides was determined by characteristic chemical changes associated with cytosines existing in either a methylated or an unmethylated state before bisulfite treatment (45). Results from BGS analysis of modified gDNA from the cell lines MDA-MB-231, BT-549, and MDA-MB-468 (with and without culture on 5-aza-dC) are shown (Fig. 3). MDA-MB-231 cells, which contain an unmethylated CST6 gene as judged by MSP, showed very limited CpG methylation by BGS. Alternatively, the majority of clones from analysis of cell lines BT-549 and MDA-MB-468 displayed dense methylation of the portion of the CST6 gene analyzed. This finding is consistent with the observation that both of these lines are positive for CpG methylation as judged by MSP. Further, only sparse methylation of the CST6 gene was observed when gDNA was harvested and analyzed from MDA-MB-468 cells cultured in the presence of 5-aza-dC. Thus, based on both MSP and BGS analyses, we conclude that the CST6 gene is a target for aberrant methylation in numerous breast cancer cell lines and that reduced expression of cystatin M correlates with this epigenetic event.

Aberrant methylation of the CST6 gene is common in human breast tumors. We next analyzed CST6 methylation in a panel of four patient-matched breast tumor/normal mammary tissue samples. Fresh-frozen IBC tumors (stage II or III) and normal breast tissue from the same patient were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank. gDNA was extracted from these samples (designated BrCA-11-BrCA-14), bisulfite modified, and used in MSP (Fig. 4A). In all normal breast tissue samples, we observed exclusive amplification of the unmethylated specific amplicon, indicating that the CST6 gene is not methylated in normal mammary tissue. In the tumor sample of BrCA-13, we also observed only amplification of the unmethylated specific amplicon, indicating that the CST6 gene is not methylated in this tumor sample. In tumors BrCA-11 and BrCA-12, amplification of the methylated specific amplicon was clearly detectable, whereas BrCA-14 displayed low levels of the methylated specific amplicon. This analysis indicates that, like cultured breast cancer cell lines, CpG methylation of the CST6 gene occurs in primary breast tumors. Moreover, because CST6 gene methylation is not observed in normal breast tissue, CST6 gene methylation in tumor tissues is an aberrant, disease-associated event.

To examine cystatin M gene expression, total RNA was harvested from these frozen tumor/normal tissue samples.
Subsequently, cDNA was synthesized and first-strand reactions used in PCRs with cystatin M–specific or GAPDH-specific primers. This analysis indicated that, in tumors BrCA-11 and BrCA-12, cystatin M transcript was either undetectable or at very low levels when compared with matched normal tissues (Fig. 4B). In tumors BrCA-14 and BrCA-13, cystatin M transcript abundance seemed to be roughly equivalent in tumor and matched normal tissues. To quantify differences in cystatin M gene expression in these matched normal and tumor samples, qPCR analysis was done. Analysis of this data set was conducted by comparing relative cystatin M transcript abundance in tumor samples with transcript abundance in its matched normal tissue sample. The results of this analysis indicated that the BrCA-11 tumor displayed extremely low levels of cystatin M expression. Specifically, we measured that this tumor contains 0.09% (SD, 0.01%; n = 3) of the level of cystatin M transcript measured in the matched normal tissue (Fig. 4C). Similarly, the BrCA-12 tumor sample showed 6.7% (SD, 1.2%; n = 3) of the level of cystatin M measured in its matched normal tissue sample. Analysis of tumor BrCA-13 showed that this tumor expressed slightly higher (109.4%; SD, 12.1%; n = 3), whereas tumor BrCA-14 expressed slightly lower (95.9%; SD, 7.5%; n = 3) levels of cystatin M compared with their respective matched normal tissues. Because MSP analysis clearly detected methylation in the BrCA-11 and BrCA-12 tumor samples, weak amplification of the methylation-specific amplicon in BrCA-14 tumor and no methylation in BrCA-13 tumor, the results obtained from this qPCR analysis indicate that reduced cystatin M expression correlates with aberrant methylation of the CST6 gene in breast tumors.

Figure 4D shows representative MSP data obtained from seven additional IBC tumor samples for which no matched normal samples were available. In tumor samples BrCA-2, BrCA-6, and BrCA-18, we observed amplification with only the unmethylated specific primer sets, indicating a lack of CST6 gene hypermethylation in these tumor samples. Tumor samples BrCA-0, BrCA-4, BrCA-16, and BrCA-17 show amplification with both unmethylated and methylated specific primer sets, indicating the presence of methylated CST6 gene in these tumors. Of the 20 IBC tumors analyzed during this study, we observed that 12 (60%) displayed aberrant methylation of the CST6 gene as judged by MSP, leading us to conclude that methylation of the CST6 gene is a common event in invasive breast tumors.

BGS was again used to independently confirm our MSP results and examine CST6 gene methylation in a more detailed manner than that afforded by MSP. In normal breast tissue, we observed no methylation of any of the 46 CpG dinucleotides within the portion of the CST6 gene examined (Fig. 4E). Similarly, we observed only sparse methylation of the CST6 gene in tumor samples BrCA-13 and BrCA-2. This result is in accordance with MSP results observed on these tumors. In tumor BrCA-4, two of five clones sequenced display extensive CpG methylation. This finding...
suggests that either a subset of cells within this tumor display CST6 gene methylation or there is partial allelic methylation within individual cells of this tumor. In tumors BrCa-12 and BrCa-0, we observed dense, nearly saturating methylation of the CST6 gene in three to four of the five clones sequenced. The results of this experiment clearly indicate that the CpG island present at the 5’ end of the human CST6 gene is subject to dense methylation in breast tumors.

**A aberrant hypermethylation of the CST6 gene can occur before tumor cell invasion.** During breast cancer progression, tumor cells may acquire an invasive phenotype allowing progression from DCIS to the more serious IBC tumor type. To examine epigenetic silencing of cystatin M at these phases of breast tumor development, we microdissected DCIS and IBC lesions from archival tumor samples where histologic examination revealed contain both lesion types. Nine tumor samples were selected for study based on these criteria, and 10 to 15 individual DCIS and IBC lesions (~ 10,000 cells per lesion type) were microdissected from each tumor using LCM. Following LCM, DNA was extracted from the dissected cells, bisulfite modified, and used in MSP assays. In parallel, anti–cystatin M antiserum was used for immunohistochemical analysis in each of the tumors studied. Sections were sequentially incubated with biotinylated goat anti–cystatin M antibody, strepavidin-conjugated HRP, developed with a buffered solution containing DAB, and stained with hematoxylin. Characterization of this antibody indicates specific detection of cystatin M in immunohistochemical analysis of human breast tumors (see Supplementary Figs. S1B and S1C).

Of the nine tumors studied, four tumors were found to be negative for CST6 gene methylation in both IBC and DCIS lesions (Fig. 5A; Supplementary Fig. S3). As shown for tumor BrCa-101 (Fig. 5B) and other methylation-negative tumors (Supplementary Fig. S3), we observed intense staining of normal ductal epithelium within the surgical margin of this tumor, in clear agreement with the epithelial expression pattern observed previously for cystatin M (46, 47). We also observed strong staining of both DCIS and IBC lesions within the tumor mass, consistent with the lack of gene methylation observed in this tumor sample. A general lack of chromagen in tissue sections stained with nonspecific biotinylated goat IgG confirmed the specificity of DAB staining. Of note, no or limited staining was observed within the fibrous stroma of this tumor. We have conducted MSP analysis on cultured normal human fibroblasts and found them to be negative for CST6 methylation (data not shown). This suggests that mechanisms other than gene methylation may control cystatin M expression in this cell type.

One tumor, BrCA-107, presented weak methylation in DCIS lesions, but a strong methylated specific amplicon was detected in IBC lesions from the same tumor (Fig. 5C), suggesting that CST6 methylation occurred during or following stromal invasion. By immunohistochemistry, we observed strong staining of normal ductal epithelium and DCIS lesions in BrCA-107 (Fig. 5D), consistent with the general lack of CST6 gene methylation in normal mammary tissue in general and DCIS lesions in this tumor in particular. In this sample, we observed areas of the tumor containing IBC lesions that stain strongly and areas that show weak staining. This finding is in agreement with our MSP analysis, which indicates the presence of both unmethylated and methylated CST6 gene within microdissected IBC lesions taken from BrCA-107 and moreover indicates that cystatin M silencing can be heterogeneous within a single breast tumor.

Finally, four tumors displayed CST6 gene methylation in both IBC and DCIS lesions (Fig. 5E; Supplementary Fig. S3). The results of this experiment indicate that CST6 methylation may precede the escape of tumor cells from the ducts of the mammary gland and invasion of surrounding normal stroma. In tumor BrCa-109, which displayed CST6 methylation in both DCIS and IBC lesions, we also observed prominent staining of normal ductal epithelium but did not observe strong staining in either DCIS or IBC lesions within this tumor (Fig. 5F). In general, CST6 methylation-positive tumors, such as BrCa-109, display low-level immunohistochemical staining, indicating a sharp reduction in cystatin M expression in neoplasms of this type. Of note, the cystatin M–positive DCIS lesion shown in BrCa-101 (Fig. 5G) is a low-grade lesion, whereas the cystatin M–negative DCIS lesion in BrCa-109 (Fig. 5F) is a high-grade lesion. Although our study was not designed to specifically examine cystatin M expression in various classifications and grades of DCIS lesions, this result may suggest that more aggressive types of cancer lesions have a higher likelihood of silencing cystatin M. Clearly, a broader study is required to adequately address this issue. Nevertheless, these results support the conclusions we have drawn using other approaches to investigate cystatin M expression in cultured breast cancer cell lines and surgically obtained breast tumors; specifically, aberrant methylation of the CST6 gene correlates with reduced cystatin M expression.

**Discussion**

The central focus of this study was to examine the CST6 gene, which encodes the cysteine protease inhibitor cystatin M, as a potential target for epigenetic silencing in breast cancer. The rationale behind this hypothesis is that expression of cystatin M is decreased in breast cancer cells and that available evidence implicates this molecule as a tumor suppressor in breast cancer. The first evidence in support of our hypothesis came from inspection of CST6 genomic architecture. This analysis showed a large (~ 500 bp) and dense CpG island superimposed on the 5’ end of this gene. This CpG island engulfs the entirety of the first exon of the CST6 gene, which encodes both transcriptional and translational start sites as well as a significant portion of region upstream of the CST6 coding region. Such molecular architecture is entirely consistent with other genes known to be targets for epigenetic silencing during tumorigenesis (41). We subsequently determined that cystatin M expression was low in many cultured breast cancer lines and that this expression could be markedly increased by culturing the cells on the global DNA demethylating agent 5-aza-dC. Further, analysis of the methylation status of the CpG island in the CST6 gene showed that this region of the genome contains multiple methylated CpG in many tumor cell lines, that often this pattern of methylation is quite dense, and that methylation of this CpG island corresponds to reduced cystatin M expression. In primary breast tumors, we also observed methylation of the CST6-associated CpG island and observed that expression correlates with methylation and that methylation of this gene is encountered quite frequently in this disease. Moreover, because dense methylation was not observed in normal breast tissue, we conclude that this epigenetic event is aberrant and is disease associated. Finally, we used LCM to dissect noninvasive DCIS and IBC cells from the same tumor and subjected DNA from these lesions to methylation analysis. We observed in many of the tumors that methylation of the CST6-associated CpG island occurs...
in noninvasive DCIS cells, suggesting that epigenetic silencing of this gene may contribute to acquisition of an invasive cellular phenotype during breast cancer progression. When taken together, the conclusion drawn from these studies is that the \textit{CST6} gene is a novel and frequent target for epigenetic silencing in breast cancer and that this event has a high likelihood of affecting disease progression.

Analysis of the \textit{CST6} locus determined that this gene possesses compact genomic architecture. The coding region of the gene is contained within a ~1.5-kb genomic interval on chromosome 11.q13.1, in agreement with previous mapping studies conducted using a hybridization approach (37). Our analysis indicated that the \textit{CST6} gene is composed of three coding exons of modest size (294, 12, and 188 bp) separated by two moderately sized introns (541 and 365 bp). Further, the genomic region flanking the \textit{CST6} gene contains several other functionally unrelated genes. Using gene-specific FISH probes, Dickinson et al. (48) mapped a 905-kb region that contains the genes encoding most members of the family 2 cystatins (i.e., C, D, S, SN, and SA) and two pseudogenes within 20p11.2. Thus, the \textit{cystatin M} gene is decidedly distinct from the majority of family 2 cystatin genes that exist within a tight gene cluster on human chromosome 20.

We observed that the CpG island present within the 5' end of the human \textit{CST6} gene often undergoes methylation in breast cancer cells. Further, this aberrant molecular event correlates with reduced \textit{cystatin M} expression, leading us to the straightforward conclusion that \textit{cystatin M} is targeted for epigenetic silencing in breast tumors. Although the mechanisms that lead to gene silencing during the process of tumorigenesis remain unclear, it is well recognized that epigenetic gene silencing is an often encountered event in breast and other tumor types (4, 5). For example, our group recently showed that the \textit{ATM} gene, which encodes a DNA damage-activated protein kinase critical in the activation of cellular response to genotoxic insult and maintenance of genomic integrity (49, 50), is epigenetically silenced in breast tumors (11). Indeed, several other genes are subject to epigenetic silencing in breast cancer, such as \textit{BRCA1}, \textit{p16\textsuperscript{INK4a}}, \textit{14-3-3\gamma}, \textit{E-cadherin}, \textit{WTH3}, \textit{estrogen receptor-\alpha}, \textit{TIMP-3}, and \textit{death-associated protein kinase} (6–10, 51–54). Moreover, silencing of genes, such as these, will, for example, result in the relaxation of cellular controls that serve to restrain acquisition and fixation of mutations, control cellular growth, restrict invasion of normal tissue, and maintain drug sensitivity. Because alterations, such as these, can provide a powerful stimulus to cancer development and/or progression, it is abundantly clear that epigenetic gene silencing is a critical aberrant event during breast tumorogenesis.

\textit{Cystatin M} is a characterized inhibitor of lysosomal cysteine proteases (27, 29). This class of proteases is capable of degrading extracellular matrix components (20–22); thus, cysteine proteases have long been considered of potential importance during tumor cell invasion and metastasis (55, 56). \textit{Cystatin M} is a secreted protein and, when expressed at appropriate levels, functions to constrain extracellular cysteine protease activity (27). Consistent with this notion, restoration of \textit{cystatin M} expression in breast cancer cells resulted in reduced cell proliferation and cellular
invasion in vitro (32). Thus, silencing of cystatin M expression has clear implication in the acquisition of an invasive cellular phenotype. Similarly, the TMP-3 gene, which encodes another inhibitor of extracellular proteolytic activity, also undergoes epigenetic silencing in breast cancer (54), indicating that genes with functions similar to cystatin M are silenced by this mechanism. Beyond a theorized role in tumor suppression, extracellular proteolytic activity can result in inactivation of growth-inhibiting molecules or the production of molecular fragments with growth-promoting properties (16), suggesting that silencing of cystatin M and other secreted protease inhibitors could potentially affect diverse aspects of the tumorigenic process.

Dysregulation of mechanisms that constrain cellular invasive- ness have a distinctly important role in breast cancer. The majority of breast cancers are adenocarcinomas that develop from the ductal epithelium of the mammary gland. Although the cells that comprise a DCIS lesion are frank tumor cells free to infiltrate within the ductal structures, this type of lesion is physically constrained by the myoepithelium and basement membrane that comprises the outer layers of the duct. However, should cells of a DCIS lesion acquire an invasive capability, they can break through these barriers and invade surrounding stroma resulting in an IBC. We have documented that methylation of the CST6-associated CpG island and diminished expression of cystatin M can occur in DCIS lesions. Considering the biochemical functions of the cystatin M protein, this result suggests that silencing of cystatin M could facilitate escape of tumors from the duct structure. Similarly, epigenetic silencing of E-cadherin, a molecule that promotes homotypic cell-cell adhesion (57), is often observed in microdissected DCIS lesions (58), leading these authors to similarly propose that E-cadherin silencing allows tumor cells to dissociate from the primary tumor and invade surrounding tissue.

In summary, we document that the CST6 gene is targeted for epigenetic silencing in human breast cancer. We observed that hypermethylation of the CST6 gene occurs in 60% of the primary breast tumors investigated and can occur in noninvasive DCIS lesions as well as IBC lesions. In light of the function of the encoded cystatin M protein in inhibiting extracellular protease activity, this often-encountered aberrant epigenetic event is likely to be of importance in the progression of breast malignancies.

Acknowledgments

Received 2/14/2006; revised 5/16/2006; accepted 6/20/2006.

Grant support: NIH grants R1 CA102229 (K.D. Brown) and B10CA114229 (K.D. Robertson).

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We thank Drs. William Cance and Martha Carmell-Thompson (University of Florida) for providing tumor samples and Ms. Marsha Scott-Jorgensen for expert technical assistance.

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