Published in final edited form as:

Genes Chromosomes Cancer. 2008 September ; 47(9): 740–754. doi:10.1002/gcc.20576.

Inactivation of the Cystatin E/M Tumor Suppressor Gene in Cervical Cancer

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Abstract

We have previously localized a cervical cancer tumor suppressor gene to a 300 kb interval of 11q13. Analysis of candidate genes revealed loss of expression of cystatin E/M, a lysosomal cysteine protease inhibitor, in 6 cervical cancer cell lines and 9 of 11 primary cervical tumors. Examination of the three exons in four cervical cancer cell lines, 19 primary tumors, and 21 normal controls revealed homozygous deletion of exon 1 sequences in one tumor. Point mutations were observed in six other tumors. Two tumors contained mutations at the consensus binding sites for cathepsin L, a lysosomal protease over-expressed in cervical cancer. Introduction of these two point mutations using site directed mutagenesis resulted in reduced binding of mutated cystatin E/M to cathepsin L. Although mutations were not observed in any cell lines, four cell lines and 12 of 18 tumors contained promoter hypermethylation. Re-expression of cystatin E/M was observed after 5'aza-2-deoxycytidine and/or Trichostatin A treatment of cervical cancer cell lines, HeLa and SiHa, confirming promoter hypermethylation. Ectopic expression of cystatin E/M in these two cell lines resulted in growth suppression. There was also suppression of soft agar colony formation by HeLa cells expressing the cystatin E/M gene. Re-expression of cystatin E/M resulted in decreased intracellular and extracellular expression of cathepsin L. Over-expression of cathepsin L resulted in increased cell growth which was inhibited by the reintroduction of cystatin E/M. We conclude, therefore, that cystatin E/M is a cervical cancer suppressor gene and that the gene is inactivated by somatic mutations and promoter hypermethylation.

Introduction

Cervical cancer is the second most common cancer responsible for cancer related death in women around the world. The incidence is increasing, with 450,000 new cases diagnosed annually worldwide (Bosch et al., 2002; Jemal et al., 2003). The disease is frequently found in

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women having multiple sex partners, smoking habits, and immune system dysfunctions (Roteli-Martins et al., 1998). Cervical cancer is closely linked with human papilloma virus (HPV) infection and HPVs are detected in greater than 90% of cervical cancer lesions (Zur Hausen, 1997). Close to 100 different HPV types are described in cervical cancers with HPV 16 and HPV 18 being commonly associated with these tumors (Chen et al., 1994; Bosch et al., 1995). HPVs play an important role in tumorigenesis by inactivating *TP53* and retinoblastoma (*RB*) tumor suppressor genes. However, detailed studies on a large number of tumors indicate that although HPV is present, viral infection may not be sufficient for tumor development. Studies have also shown that 20-30 million Americans are infected with HPV. However, only a subset of them develops cervical cancer (Bosch et al., 1995). Genomic alterations including promoter hypermethylation and somatic mutations therefore may play a role in tumor development.

Extensive studies from our laboratory have identified a 300 kb minimal tumor deletion on 11q13, between markers *D11S4908* and *D11S5023*, in cervical cancer cell lines and primary cervical tumors (Srivatsan et al, 2002). This region has also been shown to contain a high frequency of repeats, indicating fragility of the locus (Zainabadi et al., 2005). The 11q13 rare fragile site *FRA11A* overlaps with the minimal tumor deletion, indicating the possible role played by *FRA11A* in tumor development. There are at least 11 known candidate genes in the 700 kb tumor deletion/*FRA11A* region including six in the 300 kb tumor suppressor gene (TSG) region and we have previously excluded four of these genes (Zainabadi et al., 2005). Of these genes, cystatin E/M (also known as CST6) seemed to be a promising candidate due to its localization within the 300 kb region of tumor deletion.

Cystatins are protease inhibitors that are specifically active against lysosomal cysteine proteases. Members of this super family have at least one cystatin domain, composed of an approximately 100-amino acid polypeptide that folds into a five-stranded beta sheet partially wrapping around a central alpha helix (Bode et al., 1998). The three different types of cystatins are 1) those without secretory signals (cystatin A and B); 2) those containing the secretory signal (cystatins C, D, M, F, S, SN, and SA); and 3) those representing multidomain kininogens, (plasma proteins) (Abrahamson, 1994; Turk, 1991).

Cystatin E/M was initially identified as a down-regulated transcript in metastatic breast cancers in comparison to corresponding primary tumors (Sotoripoulou et al., 1997). Independently, another group cloned the cDNA from embryonic lung fibroblasts using homologous cystatin sequences and termed the gene cystatin E (Ni et al., 1997). This protein is present as an unglycosylated 14 kD form containing 149 amino acids and a 17 kD form that is glycosylated. There are reports to indicate that this protein plays a significant role in the suppression of breast cancer and skin abnormality (Zeeuwen et al., 2002; Zhang et al., 2004). Further, a null mutation of the mouse cystatin E/M gene seemed to correlate with the development of *ichq* phenotype, characterized by neonatal lethality, abnormal cornification and desquamation (Zeeuwen et al, 2002). Thus, the cystatin E/M gene seems to play an important role in the differentiation of skin epithelial cells.

In this investigation, we present evidence for the loss of cystatin E/M expression in cervical cancer cell lines and primary cervical tumors. We further demonstrate that this loss of expression is correlated to somatic mutations including homozygous deletion of exonic sequences and promoter hypermethylation. Finally, ectopic expression of cystatin E/M is shown to result in growth suppression and diminished expression of extracellular and intracellular cathepsin L, a protein related to tumor invasion and metastasis. Thus, this is the first report documenting cystatin E/M as a cervical cancer suppressor gene and its inactivation by somatic mutations.

Material and Methods

Cell lines

Cervical cancer cell lines, HeLa (D98/AH-2), C41, SiHa, Caski, HT3, C33A were grown in MEM medium with 10% FBS.

Normal and Tumor Tissues

Primary cervical tumors and adjacent normal tissues were obtained from the City of Hope National Medical Center as well as from the co-operative human tissue network of the NIH. Human tissues were obtained after the approval from the IRB committees of the West Los Angeles VA Medical Center and the City of Hope National Medical Center. A total of thirty tumors were analyzed. Eleven tumors and corresponding normal tissues were used for protein expression by Western blotting, nineteen tumors were studied for somatic mutations and sixteen tumors were analyzed for promoter hypermethylation. Sixteen of the corresponding normal tissues and four non-involved lymphocytes were used as controls for the promoter hypermethylation and mutation analyses.

Polymerase Chain Reaction (PCR)

Three exons of the cystatin E/M gene were amplified using primers exon 1 F-5' CGGGCGTCGGCGGGGCGGCC 3' exon 1 R-5' GGGCCGGGTGTCCCTCCAGC 3', exon 2 F-5' GACCCCTGACCTGCCCCTACC 3' exon 2 R-5' GGAGGGCTGGGGCTGGAGGAG 3', exon 3 F-5' GGTCGAGGCTGGGCTCACCCCT 3' exon 3 R-5' GGGGCAGAAGCGAAGCAGTTGG 3'. DNAs were denatured at 94°C for 45 sec; annealed using a step down temperature for 45 sec, and the extension performed at 72°C for 1 min. Step down temperatures from 65°C for 4 cycles, 64°C for 7 cycles, 63°C for 7 cycles and 62°C for 20 cycles for exon 1 and from 58°C to 57°C to 56°C for 5 cycles each followed by annealing at 55°C for 20 cycles for the amplification of exons 2 and 3 were used. A final extension was performed at 72°C for 2 min. After verification of product synthesis, i.e. size of 369 bp, 191 bp, and 268 bp for exons 1, 2 and 3, respectively, on 10% PAGE (polyacrylamide gel electrophoresis) gels, PCR samples were purified, cloned into the pTOPO vector (Invitrogen inc., Carlsbad, CA) and sequenced. At least three different clones were sequenced for each PCR sample.

Reverse Transcription (RT) PCR

RNA was extracted from the cell lines and tissues using Trizol reagent (Invitrogen Inc., Carlsbad, CA). RNA was reverse transcribed using the RT kit (Invitrogen, Inc.). The synthesized cDNA was used in the PCR for the amplification of sequences representing exons 2 and 3 of the cystatin E/M gene. Primers used were; cystatin E/M F-5' GTAATTCCTGACGATGGAGATG 3', cystatin E/M R-5' TAGGAGCTGAGAGGAGTTCTG 3', β actin-F 5' GTCGCCCTGGACTTCGAGCAAGAG 3', and β actin-R 5' CTAGAAGCATTTGCGGTGGACG 3'. For the amplification of cystatin E/M, an initial denaturation at 94°C for 4 min followed by 32 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec were employed with a final extension at 72°C for 2 min. Amplification conditions for β actin were similar to that of cystatin E/M except that the annealing temperature was 58°C and the PCR was performed for 30 cycles. PCR products were separated on 8% TBE (50 mM Tris borate pH 8.0, 1mM EDTA) gels, followed by ethidium bromide staining, and analyzed using the Kodak 1D software.

Site Directed Mutagenesis

Point mutations representing the cathpsin L binding sites were introduced using the Quikchange II site directed mutagenesis kit following manufacturer's protocol (Stratagene, San Diego, CA).

Briefly, cystatin E/M plasmid DNA (10 ng) in p3XFLAG-CMV-10 expression vector (Sigma Chemicals Co., St. Louis, MO) was used in a PCR mixture containing oligonucleotides with point mutations as primers and the PCR was carried out using PfuUltra DNA polymerase (Stratagene). The same sequences in the forward and reverse orientations were used as primers. The primer sequences used in the PCR were, Cystatin E/M M34T F – 5' CGG CCG CAG GAG CGC ACG GTC GGA GAA CTC CGG GAC 3' Cystatin E/M M34T R – 5' GTC CCG GAG TTC TCC GAC CGT GCG CTC CTG CGG CCG 3', Cystatin E/M L131F F – 5' TGT GAC TTT GAG GTC TTT GTG GTT CCC TGG CAG AAC 3' Cystatin E/M L131F R – 5' GTT CTG CCA GGG AAC CAC AAA GAC CTC AAA GTC ACA 3', and Cystatin E/M W135A F – 5' G GTC CTT GTG GTT CCC GCG CAG AAC TCC TCT CAG C 3' Cystatin E/M W135A R – 5' G CTG AGA GGA GTT CTG CGC GGG AAC CAC AAG GAC C 3'. The bold letters in the sequences represent the point mutations. The PCR product was digested with DpnI to digest the parental DNA and the product containing the mutant plasmid was used for the transformation of super competent bacterial cells. The plasmids were purified and sequenced to confirm the presence of point mutations.

Promoter Assay

The putative promoter sequence +8 to -243 of the cystatin E/M gene identified by the promoter program (http://www.ualberta.ca/~stothard/javascript/cpg_islands.html) was amplified from a lymphocyte genomic DNA using primers containing SacI and XhoI restriction enzyme sequences at the 5' end. The primers REST MET F1-5' CTGAGCTCTGCTGGGGACTGTCGCTGTCCTCT 3' and REST MET R1-5' ATCTCGAGCGGAGCCCTCAGAGCCGTGAGTGC 3' were used. The PCR product was digested with SacI and XhoI, purified using a microcon 100 filter and cloned into the SacI – XhoI site of the firefly luciferase plasmid pGL4.16 (Promega Corporation, Madison, WI). Plasmid DNA was sequenced to confirm that the promoter was in the correct read through orientation of the luciferase gene. The promoter construct and the promoterless control pGL4.16 vector were mixed in different ratios to a final DNA amount of 5µg and transfected into a semi-confluent culture (70-80%) of HeLa cells. The promoter assay was then carried out on cell extracts with the luciferase reagent using the manufacturer's protocol (Promega Corporation, Madison, WI).

Sodium Bisulfite DNA Modification and PCR

DNA samples were treated with sodium bisulfite using the Chemicon CpG genome fast modification kit (Chemicon International, Temecula, CA). Primer sequences identified by the primer 3 program and spanning -14 to -151 of the promoter were used for the unmethylation and methylation specific PCR. While a single forward primer, Unmet F-5' GGTTTTTTGGGTTTTTTGAATTTTG 3' was used for both the PCRs, the reverse primer for the unmethylation specific PCR was Unmet R-5' TACCAAACCTTACAACCACACAAC 3' and the reverse primer for the methylation specific PCR was Met R-5' TACCGAACTTACGACCGCGCAACT 3'. For the PCR analysis, 40 ng of bisulfite treated DNA was denatured for 94°C for 5 min and subjected to a 35 cycle amplification with denaturation at 94°C for 45 sec, annealing at 59°C for 45 sec and extension at 72°C for 1 min. A final extension was performed at 72°C for 2 min and the PCR products were cloned and sequenced. At least three different clones were sequenced for each sample.

Treatment With 5'Aza 2-deoxycytidine (5-AzaCdR) or Trichostatin A (TSA)

HeLa and SiHa cells were grown overnight to a 70-80% confluency and treated with 5 µM, 10 µM, 50 µM of 5-AzaCdR and/or 150 nM of TSA for up to 72 h. Treated cells were used for immunofluorescence or for the extraction of DNA for PCR and total RNA for RT PCR analyses.

Western Blotting

Proteins were extracted from the cells and tissues using RIPA lysis buffer containing a complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Proteins secreted into the media were concentrated using the published protocol (Shridhar et al, 2004). Western blotting was performed using 20-30 µg of denatured proteins on 4-20% SDS acrylamide gels (Invitrogen, Inc.). Proteins transferred onto nitrocellulose were hybridized to the antibodies (cystatin E/M, cathepsin L from R & D systems, MN, and β-tubulin from Santa Cruz Biotechnology, CA) using the established protocol (LoTempio et al., 2005). Immunoprecipitations were carried out using 500 µg of the total protein lysates in RIPA lysis buffer, using the anti-flag antibody (Sigma/Aldrich Chemicals, St. Louis, MO). Antibody (10 µg) and protein lysates were incubated overnight at 5° C with protein G conjugated beads (Santa Cruz biotechnology, Santa Cruz, CA). After the incubation, beads were washed four times with lysis buffer and the immune-complexes were eluted using 70 µl of sample buffer. Samples were denatured at 100°C for five min and used for the Western blotting.

Transfection

Cells (1.6×10^5) were grown in 6-well plates overnight to achieve a 70-80% confluency for transfection with pCMV control vector or the cystatin E/M containing plasmid using the lipofectamine reagent (Invitrogen, Inc.). Cells were also transfected with cathepsin L cDNA in pCMV vector (Origene Technologies, Inc., Rockville, MD). Twenty-four hours after transfection, cells were used for the cell viability assays at different time periods. Stable transfectants were isolated using zeocin as the selection reagent to a maximum of 800 µg/ml. Cells grown in selection medium for 10 days (mass culture) were used for soft agar colony assay. Individual clones isolated after extensive selection protocol were used to study the expression levels of cystatin E/M and cathepsin L.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] Assay for Cell Viability

Control and transfected cells (4×10^4) were grown in 24 well tissue culture dishes for 24, 48, 72, and 96 h. The MTT assay was carried out following a previously established protocol (LoTempio et al., 2005).

Immunofluorescence

Cells were grown overnight on cover slips to semi-confluency (70-80%) and treated with 5-AzaCdR or TSA, or transfected with plasmid DNAs. Immunofluorescence was performed using the anti cystatin E/M antibody (R & D systems, MN) following the established protocol (LoTempio et al., 2005).

Soft Agar Colony Assay

Cystatin E/M and control vector transfected mass cultures (grown in zeocin selection medium for 10 days) were trypsinized, and suspended in MEM medium containing 0.1% lukewarm agar at a cell concentration of 5×10^3 cells/ml. The suspension was spread on top of 0.5% solidified agar plates. The agar plates were incubated for 21 days at 37°C. Colonies were stained with 0.001% crystal violet blue, counted, and photographed using a Zeiss microscope.

Results

Loss of Cystatin E/M Expression in Cervical Cancer Cell Lines and Primary Tumors

We have previously mapped a cervical cancer tumor suppressor gene to a 300 kb interval of 11q13, between the markers *D11S4908* and *D11S5023* (Fig. 1A). At least six genes, SART1 (squamous cell carcinoma antigen recognized by T cells 1), *CST6* (a lysosomal protease inhibitor), *CATSPER1* (cation channel sperm-associated protein 1), *GAL3ST3*

(Galactose1-3GalNAC 3'-sulfotransferase 3), *SF3B2* (a splicing associated protein) and *PACSI* (phosphofurin acidic cluster sorting protein 1) are mapped to the 300 kb TSG region. Three of these genes, cystatin E/M (*CST6*), *SF3B2* and *PACSI* and three other genes in proximity to the TSG region, *BRMS1* (breast cancer metastatic suppressor 1), *RIN1* (Ras inhibitor protein 1) and *RAB1B* (Ras binding protein 1) related to tumorigenesis were analyzed. Of these, cystatin E/M expression was not observed in any of the cervical cancer cell lines and thus was considered the potential cervical cancer tumor suppressor gene at 11q13 (Fig. 1B). The different genes analyzed in the mapping of cystatin E/M as the suppressor gene are also shown Figure 1.

Western blot analysis of a number of normal tissues, cervical cancer cell lines, and primary tumors revealed expression of the 14 kD protein in the normal tissues, including those of the cervix (Fig. 1C). The glycosylated 17 kD protein was seen in lung tissue (Fig. 1C). In contrast, cystatin E/M expression was absent in four of the cervical cancer cell lines, and 9 of 11 primary tumors examined in the present investigation (Fig. 1D). Two tumors (tumors # 6 and # 9) showed low levels of protein expression, possibly representing normal cell contamination. However, all the normal adjacent tissues had high level expression of cystatin E/M.

Somatic Mutation of the Exonic Sequences in Primary Tumors

To determine whether loss of cystatin E/M expression was due to mutations in the exonic sequences of the gene, the three exons were examined in four cervical cancer cell lines and 19 primary tumors including the two tumors, # 11 and # 23 that have shown loss of expression in the western blot analysis (Fig. 1D). Corresponding normal endometrium for 16 of the tumors, four non involved normal lymphocytes, and a normal lung tissue (a tissue with high level expression of cystatin E/M), totaling 21 normal tissues, were used as controls. There was homozygous deletion of exon 1 sequences in one of the tumors and a point mutation in six other tumors (Table 1, Fig. 2A). The homozygous deletion for exon 1 was observed by the absence of the 369 bp PCR product in tumor # 54 (Fig. 2B). Although we could not determine the deletion breakpoints, additional primer sets showed the deletion to extend at least 260 bp upstream of the translation start site (data not shown). PCR products were seen for the 191 bp, and 268 bp sequences of the exons 2 and 3, respectively (Fig. 2C). Since this tumor was previously shown to contain heterozygous alleles for probes in the 300 kb deletion interval (Srivatsan et al., 2002), a probe from this interval, AW167735, was used as a control to examine the copy number status of exons 2 and 3. A semi-quantitative comparative analysis with the normal tissue revealed the presence of a single copy of these two exons in the tumor sample (data for exon 3 are shown in Fig. 2C). Thus, this tumor has undergone a loss of heterozygosity (LOH) for exonic sequences as a first event and the homozygous loss of exon 1 sequences as a second event in the tumor cells indicating a two hit mechanism for the inactivation of the cystatin E/M gene.

The point mutations in six other tumors were missense in nature resulting in the replacement of aspartic acid (D) to glycine (G) in tumor # 18, alanine (A) to threonine (T) in tumor # 23, phenylalanine (F) to tyrosine (Y) in tumor # 53, histidine (H) to arginine (R) in tumor # 55, methionine (M) to threonine (T) in tumor # 59, and leucine (L) to phenylalanine (F) in tumor # 62 (Table 1 and Fig. 2A). Three of these mutations were mapped at or near the consensus sites for binding to the lysosomal protease cathepsin L. For confirming the role of these mutations in the functional inactivation of cystatin E/M, mutant cystatin E/M plasmids were used in the transfection studies. Two of the tumor specific mutations (M34T in tumor # 59 and L131F in tumor # 62) and a third cathepsin L binding site mutation (W135A) identified in the in vitro studies (Cheng et al., 2006) were used. Western blot analysis showed equal level expression of cystatin E/M in the wild type and the mutants (Fig. 2D). However, high level expression of cathepsin L was observed in the mutant transfected cells indicating functional

inactivation of cystatin E/M in these cells (Fig. 2D). Immunoprecipitation with the plasmid tag, the FLAG antibody, followed by Western blot analysis showed the presence of similar levels of cystatin E/M in the wild type and mutant transfected cells (Fig. 2E). However, there was reduced binding of cystatin E/M protein to cathepsin L in the mutant transfected cells (Fig. 2E). These results again confirmed functional inactivation of cystatin E/M by cathepsin L binding site mutations.

Deletions or point mutations were not observed in any of the 21 normal tissues. Presence of mutations only in tumor samples in comparison to the matched normal tissues indicated that the mutations are tumor specific. Further, in two of the cases, functional inactivation of the cystatin E/M/E gene with the introduction of these mutations clearly pointed out that point mutations and the homozygous deletion represented tumor specific events and not polymorphic changes. Of the six tumors showing somatic mutations or deletions, four (67%) contained HPV 16 sequences (data not shown), indicating a possible association between HPV 16 and somatic mutations. However, a large tumor set will be required to confirm this relationship, as well as any association that might exist between the HPV type and the expression of cystatin E/M and/or cathepsin L.

Hypermethylation of the Promoter in Cell lines and Primary Tumors

Exonic mutations were not detected in the cervical cancer cell lines and in 12 of the primary tumors. Also, sequencing of the exons has indicated the presence of wild type allele in the six tumors that contained point mutations (Table 1). Therefore, we explored the promoter region for hypermethylation of CpG islands as an additional reason for gene inactivation.

The +8 to -243bp sequence of the gene (Fig. 3A) was amplified from normal lymphocyte DNA and cloned into a pGL4.16 firefly luciferase vector. HeLa cells were transfected with the vector or the promoter construct, in combination with an internal renilla luciferase control plasmid DNA. The relative luciferase activity with respect to that of renilla luciferase was measured. The results showed a 30 and 60 fold increased luciferase activity with 2 µg and 5 µg of the promoter DNA respectively (Fig. 3B). Thus, there was a direct relationship between the amount of promoter construct used and the luciferase activity.

Having established the promoter activity, four different cell lines and 18 primary tumors were examined for CpG methylation using the sodium bisulfite protocol. Normal endometrium from 16 of the tumors, four different non involved normal lymphocytes and a lung tissue were used as controls. The analysis was performed using the sequences spanning the -14 to -151 bp region of the promoter. Amplification of the 138 bp product was observed with the unmethylation specific PCR in 18 of the normal tissues (data is included for a lymphocyte sample in Fig. 3C). Similarly, the PCR product was seen for the unmethylation specific primers in 5-AzaCdR treated HeLa, SiHa, and HT3 cell lines and in six primary tumors (three of the tumors #11, #23, and #36 are shown in Fig. 3C). Untreated HeLa cells yielded the 138 bp product with the methylation specific PCR. Cell lines Caski, C4I, C33A, three of 21 normal tissues (14 %) and seven of sixteen tumors (44 %) gave the PCR product with both the unmethylation and methylation specific PCRs. Five other tumors (31 %) contained only the methylation specific product. Thus, there was a significant frequency of methylated promoter in the cervical tumors (12 of 18, 67 % in tumors versus 3 of 21, 14 % in normals, $P < 0.01$, Student's t-Test). Methylation in the three normal endometrial tissues could also represent tumor cell contamination. Four of the tumors contained methylation and point mutation (Table 1) indicating possible two hits for the functional inactivation of the cystatin E/M gene.

To confirm promoter hypermethylation in the cell lines, sequencing was carried out on the PCR products cloned into the pTOPO plasmid vector. The sequence showed absence of C residues in the lymphocyte, 5-AzaCdR treated HeLa, SiHa and HT3 DNAs (data are shown

for the lymphocyte – top row, and 5-AzaCdR treated HeLa DNA – third row in Fig. 3D). All the C residues were converted into T residues indicating the presence of an unmethylated promoter in these samples. However the untreated HeLa cell DNA showed retention of all C residues in the CpG sequences (second row in Fig. 3D), implying promoter hypermethylation in this cell line. Partial methylation, retention of some of the C residues of the CpG sequences, was observed for the methylation specific PCR product of the Caski DNA (bottom row in Fig. 3D). Thus the sequencing results confirmed the presence of promoter methylation in two different cell lines, HeLa and Caski. Further, the data indicated demethylation of the C residues in the HeLa cells after treatment with 5-AzaCdR.

To determine whether 5-AzaCdR treatment resulted in the expression of the gene, RT PCR analysis was performed. The RT PCR, representing exons 2 and 3, showed expression of the 169 bp cystatin E/M product in 24 and 72 h post treatment of HeLa cells with either 5-AzaCdR or TSA or in combination (Fig. 4A). SiHa cells containing unmethylated CpG sequences did not show expression with lower concentrations of 5-AzaCdR (Fig. 4A). Treatment with 50 μ M resulted in a low level expression of the gene. High level RNA expression was observed in SiHa cells treated with TSA (Fig. 4A), indicating gene inactivation through a histone deacetylation pathway in this cell line.

To verify that the gene expression was not limited to RNA synthesis and to identify the cellular location of protein expression, immunofluorescence (IF) was used. Data representing DAPI alone, Alexa Fluor 568 alone (secondary antibody used to detect cystatin E/M), merged DAPI/Alexa Fluor and expression in a single cell respectively are shown in the four columns of Figure 4. Cystatin E/M expression reflected by red fluorescence (Alexa Fluor 568 signals) was absent in the untreated HeLa cells (panels ii – iv in the top row of Fig. 4B). However, the red signals, representing cystatin E/M expression, were observed in the HeLa cells treated with 5-AzaCdR (panels vi – viii in the middle row of Fig. 4B). There was uniform expression in the cytoplasm of all cells. The gene transfected cells on the other hand, showed an increased cytoplasmic expression (panels x – xii in the last row of Fig. 4B). TSA treatment showed a uniform cytoplasmic protein expression in the SiHa cells (bottom panel in Fig. 4C). Thus, gene reactivation by 5-AzaCdR and TSA in HeLa and SiHa cells respectively confirmed promoter methylation and/or histone deacetylation playing a role in the inactivation of cystatin E/M in these two cell lines.

Ectopic Expression of Cystatin E/M Results in Reduced Cell Growth

To demonstrate that cystatin E/M expression resulted in cell growth control, the gene was transfected into HeLa and SiHa cells and assayed for gene expression and cell growth. Expression was assayed by RT-PCR and Western blot analyses. Expression was observed in both the cell lines up to 96 h post-transfection (Fig. 5A-B). Although there was some cell killing seen with the control vector, introduction of the cystatin E/M gene resulted in markedly increased cell growth inhibition in the HeLa cells (Fig. 5B). In SiHa cells, the control vector had no effect on cell growth, while cystatin E/M expression slowed down growth. In both cell lines, very few cells survived 96 h post-transfection indicating a strong growth suppressive effect of cystatin E/M.

Cystatin E/M Expression Reduces Soft Agar Colony Forming Potential of HeLa Cells

For cystatin E/M to be a tumor suppressor, we would expect reduced soft agar colony formation. Western blot studies confirmed the expression of cystatin E/M protein in the culture used for the soft agar colony assay (Fig. 5C and 6B). Since there was extensive cell death upon cystatin E/M expression, it was difficult to perform soft agar colony assays on isolated clones. Therefore, the assays were carried out using transfected cells grown in selection medium for ten days. Spherical and irregular colonies were apparent after five days in CMV transfected

cells. However, visible colonies appeared in cystatin E/M gene transfected cells after 12 days. Examination of soft agar plates after 21 days showed 60% reduction in the number of colonies ($P < 0.01$, Student's t-Test) in the cystatin E/M transfected cells (Fig. 5C). The size of the individual colonies was also reduced in gene transfected cells. The results thus confirmed the growth suppressive effect of cystatin E/M in cervical cancer cell lines.

Inverse Relationship Between the Expression of Cystatin E/M and Cathepsin L

Previous studies have shown that purified cystatin E/M inhibits both cathepsin L and cathepsin L2/V (Cheng et al., 2006). Interestingly, cystatin E/M seems to target only the single- but not the two-chain form of cathepsin L. Single-chain forms of cathepsins are believed to be secretory and/or endosomal active enzyme forms (Waguri et al., 1995; Sato et al., 1997; Linebaugh, et al., 1999). These authors have also suggested that the secreted form of cystatin E/M might play a role in the control of cathepsin mediated cell growth. We, therefore, analyzed cervical cancer cell lines and primary tumors for the expression of cathepsins. There was high level expression of cathepsin L in some of the cell lines (Fig. 6A). Cathepsin B was expressed at a reduced level and the expression of cathepsin L2/V was undetectable (data not shown). Re-expression of cystatin E/M in HeLa cells resulted in lower steady state levels of cathepsin L (Fig. 6B). Further, cathepsin L expression was undetectable in the media of cells expressing cystatin E/M (Fig. 6B).

To determine whether cathepsin L is indeed involved in growth promotion, over-expression studies were performed. Cathepsin L over-expression increased the cell growth and an intermediate growth was observed with the transfection of both the cathepsin L and cystatin E/M genes (Fig. 6C). Introduction of cystatin E/M alone resulted in increased growth suppression. These results indicated that cystatin E/M down-regulates cathepsin L protein levels resulting in growth inhibition. We therefore analyzed primary tumors for the expression of cathepsin L. Two tumors (# T21 and # T22) that did not express cystatin E/M (Fig. 1D) contained higher levels of cathepsin L in comparison to that of the corresponding normal tissues (Fig. 6D). These results indicated an inverse relationship between the expression of cystatin E/M and cathepsin L. Also, expression of a lower molecular weight form of cathepsin L was observed in some tumors (Fig. 6D) indicating more extended processing of this form in the absence of Cystatin E/M.

Discussion

Chromosomal aberrations are a common event in human tumors, including cervical cancer. Cytogenetic studies of cervical cancer cell lines and primary tumors have shown non-random structural rearrangements in chromosomes 11 and 14 (Stanbridge et al., 1982; Srivatsan et al., 1991). We and others have confirmed the involvement of chromosome 11 in cervical cancer by suppression of the tumorigenic phenotype with the transfer of chromosome 11 into cervical cancer cell lines (Saxon et al., 1986; Koi et al., 1989). Detailed cytogenetic and molecular genetic analysis of cervical cancer cell lines, non tumorigenic and tumorigenic HeLa cell hybrids, and primary tumors localized the gene to a 300 kb interval of 11q13 (Jesudasan et al., 1995; Srivatsan et al., 2002; Mendonca et al., 2004). CGH (comparative genomic hybridization) and SKY (spectral karyotyping) studies have identified translocations and amplifications at chromosome 11q13 in cervical cancer lines (Harris et al., 2003; Rao et al., 2004). We have also previously reported on the amplification of 11q13 sequences in the cell lines (Jesudasan et al., 1995). Of the eight cell lines, HeLa cells showed the presence of three copies of 11q13 and Caski cells contained multiple 11q13 sequences. However, we did not observe amplification of the *cyclin D1*, *INT2*, *HST1* or *SEA* genes in the cell lines. The different studies have also observed deletion of 11q13. We are of the opinion that 11q13 deletions have resulted in the amplification of distal sequences.

Extensive analysis of genes mapped to the deletion interval by our group has for the first time resulted in the identification of cystatin E/M as a cervical cancer suppressor gene. Although there are a number of microarray reports on the expression analysis of cervical cancer cell lines and primary cervical tumors, none of them have identified cystatin E/M as a differentially expressed gene (Cheng et al., 2002; Fujimoto et al., 2004; Bachtiary et al., 2006; Chao et al., 2006).

Again for the first time, we have provided evidence for the presence of somatic mutations including homozygous deletion of exonic sequences in the primary cervical tumors. Knudson's two hit model of gene inactivation (Knudson, 1996) observed in a tumor exhibiting homozygous deletion and four other tumors containing somatic mutation and promoter hypermethylation clearly pointed to cystatin E/M as a true tumor suppressor gene. Point mutations mapping to the consensus cathepsin L binding sites and decreased binding of the mutant cystatin E/M to cathepsin L confirmed the possible inverse relationship between cystatin E/M and its substrate cathepsin L. This relationship was also seen in cell lines and tumors lacking cystatin E/M expression, resulting in overexpression of cathepsin L and this overexpression was lost upon re-expression of cystatin E/M. Finally, overexpression of cathepsin L confirmed the role of cathepsin L in growth promotion and expression of both cystatin E/M and cathepsin L reflected the role of cystatin E/M in the suppression of cathepsin L mediated cell growth.

Although our finding that promoter hypermethylation is also a cystatin E/M inactivation event is confirmatory to recent reports of promoter hypermethylation in the cancers of the breast, lung and brain (Ai et al., 2006; Kim et al., 2006; Schagdarsurengin et al., 2007), we have provided functional evidence for the presence of the promoter sequences upstream of the transcription start site. In four tumors, we have identified both promoter methylation and a point mutation indicating a possible two hit mechanism of gene inactivation. Further, the SiHa cell line studies showing cystatin E/M expression after treatment with TSA, pointed to yet another mechanism of gene inactivation - histone deacetylation - in cervical cancer.

Cystatins are inhibitors of lysosomal cysteine proteases that participate in diverse biological functions like pericellular matrix remodeling, protein catabolism, apoptosis, and antigen processing (Abrahamson, 1994; Turk, 1991). Cystatins control proteases by the formation of reversible high affinity complexes (Barrett and McDonald, 1986; Alvarez-Fernandez et al., 1999). The cystatin family comprises of at least seven different members i.e., the cystatins C, D, E/M, F and the salivary cystatins S, SA and SN. Of these, cystatin E/M acts on both papain- and legumain-type proteases (Cheng et al., 2006; Alvarez-Fernandez et al., 1999; Bromme, 2002; Puente et al., 2003).

Cathepsins with a cysteine residue in their active site belong to the class of papain-type cysteine proteases. These proteins are present in lysosomes as well as in glycosylated forms in the extracellular space (Zhang et al., 2004). The secreted forms are involved in the degradation of extracellular matrix and basement membrane proteins. They also participate in the conversion of pro-uPA (urinary plasminogen activator/urokinase) into active uPA. Overexpression of cathepsin B has been reported in aggressive human cancers including that of the cervix (Makarewicz et al., 1995; Keppler, 1996; Häckel et al., 2000; Iacobuzio-Donahue CA et al., 2003; Kruszewski et al., 2004). Overexpression of cathepsin L is also reported in human tumors, and in inflammatory arterial diseases, aortic aneurysm and atherosclerosis (Sever et al., 2002; Potts et al., 2004; Hashimoto et al., 2006; Stabuc et al., 2006).

Biochemical studies have shown that cystatin E/M binds to cathepsins L and V as a non competitive inhibitor thereby preventing membrane digestion by cathepsins (Cheng et al., 2006). Cystatin E/M is also shown to be involved in keratinocyte differentiation. Further,

cystatin E/M plays an important role in the control of infection, inflammation and carcinogenesis through potential regulation of cathepsin L. The secreted form of cathepsin L could be inhibited by cystatin E/M, and thus prevent the proteolysis of the basement membrane.

Previous gene transfer studies in conjunction with an orthotopic mouse model have shown that cystatin E/M suppresses breast cancer cell growth at both primary and secondary sites such as the lung and liver (Zhang et al., 2004). Several other groups, in contrast, have shown overexpression of cystatin E/M during progression of tumors (Iacobuzio-Donahue et al., 2003; Vigneswaran et al., 2003, 2005; Haider et al., 2006). As a follow up to these intriguing gene expression profiling results the authors, however, did not analyze the cystatin E/M gene sequences for somatic mutations that could explain overexpression.

In conclusion, we have provided evidence that: 1) cystatin E/M is a house keeping gene, as seen by expression in several normal tissues; 2) expression is lost in primary cervical tumors; 3) somatic mutations including homozygous deletions are present in primary tumors; 4) the cystatin E/M promoter is silenced by hypermethylation and/or histone deacetylation; 5) re-expression of cystatin E/M results in growth suppression; and 6) cystatin E/M expression leads to a decrease in intracellular and extracellular levels of mature cathepsin L, a potential physiological target of this protease inhibitor. Thus, we strongly believe that cystatin E/M is a novel tumor suppressor gene for cervix.

Acknowledgments

We thank Dr. Vatche Agopian for help with fluorescence microscopy.

Supported by: VAGLAHS West Los Angeles Surgical Education and Research Program, and by a Merit grant from the VA administration to Eri S. Srivatsan.

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Figure 1. Loss of cystatin E/M expression in cervical cancer cell lines and primary cervical tumors. (A) Physical map of the 300 kb tumor suppressor gene (TSG) locus in relation to the centromeric and telomeric markers of 11q13, the location of microsatellite markers and the candidate tumor suppressor genes examined are also shown. (B) Physical map of cystatin E/M indicating the size of introns and exons. HD refers to the original 5.7 kb homozygous deletion that led to the mapping of minimal tumor deletion (Srivatsan et al., 2002). (C) Cystatin E/M is expressed in normal tissues and normal cervix. However, expression is lost in (D) cell lines and in 9 of 11 primary cervical tumors. All the uninvolved normal tissues have high level expression of cystatin E/M. LCK-SCC, SmCC, SCC, and AC represent large cell and small cell keratinizing squamous cell carcinoma, squamous cell carcinoma, and adeno carcinoma respectively.

Figure 2.

Somatic mutation of the cystatin E/M gene in primary tumors. (A) Schematic representation of cystatin E/M was derived from the protein sequence alignment to cystatin F reported by Schüttelkopf et al, (2006). Numbers underneath the arrows represent the location of amino acids affected by point mutations in tumors and the amino acid conversions are listed in brackets. Tumors # 59 and # 62 have mutations in the cathepsin L binding domains and tumor # 53 is mutated one amino acid proximal to the consensus site. Homozygous deletion of sequences from exon 1 in tumor # 54 is also presented. (B) While sequences of the 369 bp product of exon 1 are homozygously deleted in tumor # 54, (C) sequences of exons 2 and 3 are present in the tumor and the absence of exon 1 (indicated by the stars) is again observed in tumor # 54 in the multiplex PCR with exons 1 and 3. A semi-quantitative PCR using AW167735, mapped 200 kb proximal to cystatin E/M, as a control probe indicates the presence of a single copy of exon 3 in tumor # 54 demonstrating a two hit model of homozygous deletion of exon 1 sequences. (D) Hybridization of wild type and mutant cystatin E/M transfected HeLa cells shows equal level expression of cystatin E/M in all four transfectants. However, the level of cathepsin L expression is higher in the mutant transfectants. (E) Immunoprecipitation with the FLAG tag antibody followed by hybridization to cystatin E/M again shows equal level expression of cystatin E/M in all four transfectants. However, the mutant transfectants show reduced level of cathepsin L, indicating low level, if any, of cathepsin L binding to the mutated cystatin E/M protein. Thus, the results demonstrate that cathepsin L binding site mutations in the primary tumors play an important role in the functional inactivation of cystatin E/M. Dashed lines in 2A represent homozygous deletion of exon 1 in tumor 54.

Figure 3.

Promoter methylation of cystatin E/M in cell lines and tumors. (A) Sequence of the region indicating the primers (underlined sequences) used for the cloning of the promoter and for methylation specific PCR (sequences in bold letters). (B) Promoter assay showing an increased relative luciferase activity in the HeLa cells containing the promoter DNA. (C) Bisulfite treated DNAs show 138 bp amplification product with the unmethylation specific PCR in 5-AzaCdR treated HeLa, SiHa, and HT3 cells and in primary tumors #11, #23 and #36. Product with methylation specific PCR is seen in the untreated HeLa cells. The amplification product is observed for both the unmethylation and methylation specific PCR in Caski, C4I, and C33A cells and in primary tumor #18. (D) Sequence analysis of PCR products shows retention of all

the C residues of the CpG sequences in the bisulfite treated HeLa DNA (peaks in blue for a representative region) and partial retention of C residues in the methylation specific product of Caski DNA indicating promoter methylation in these two cell lines. B40, a lymphocyte DNA is included for comparison. In figure A above, the methylated Cs in HeLa cells are shown in red and the underlined Cs represent methylated Cs in Caski cells.

Figure 4.

Re-expression of cystatin E/M with 5-AzaCdR or TSA treatment. (A) RT-PCR shows robust expression of the transcript in 5-AzaCdR and/or TSA treated HeLa cells. However, in SiHa cells, high level expression is seen only with TSA treatment indicating histone deacetylation as a possible gene inactivation mechanism. (B) Immunofluorescence representing DAPI alone, Alexa Fluor 568 alone, merged DAPI/Alexa Fluor and expression in a single cell respectively is shown. Absence of cystatin E/M protein is visualized in the untreated HeLa cells (absence of red Alexa Fluor 568 signal- panel ii of the top row). On treatment with 5-AzaCdR, there is uniform cytoplasmic expression in all the cells (panel vi of the middle row). Gene transfected cells show higher intensity signal (panel x of the bottom row). (C) Although lesser in intensity

to that of HeLa cells, SiHa cells also show protein expression with TSA treatment. All the photographs represent 100× magnification.

Figure 5.

Cystatin E/M inhibits cell growth. (A) RT PCR shows gene expression both in HeLa and SiHa cells up to 96 h post transfection. (B) Western blotting confirmed translation of RNA in the transfected cells (data are shown for HeLa cells). (C) Although there is an effect on cell growth by vector control in the HeLa cells; transfection with cystatin E/M results in enhanced growth inhibition. This growth inhibition is again visualized in gene transfected SiHa cells. Cells transfected with lipofectamine (L) alone were also used as controls. (D) Cystatin E/M transfected mass culture expressing the non glycosylated (14 kD) and glycosylated (17 kD) forms of cystatin E/M was used for growth in soft agar. Vector (CMV) transfected cells were used as control. After 21 days of incubation, cystatin E/M transfected cells show growth reduction in size and number of colonies (20 $\times$ magnification). A single colony shown in the inset (100 $\times$ magnification) demonstrates a significant reduction in colony size of cystatin E/M expressing cells. The bar graph representing average number of colonies from 5 plates again shows a greater than 50% growth reduction for cystatin E/M expressing cells.

Figure 6.

Expression of cystatin E/M results in degradation of cathepsin L. (A) Cell lines express cathepsin L in varying quantities. However, (B) re-expression of cystatin E/M leads to a reduced level of intracellular cathepsin L in the gene transfected cells as well as in the extracellular space, media collected from these cells. (C) Over expression of cathepsin L results in enhanced cell growth. Expression of cystatin E/M leads to reduced growth and co-expression of both cystatin E/M and cathepsin L results in an intermediate cell growth. (D) Comparison of normal tissues and tumors show overexpression of cathepsin L in tumor samples, and the 30 kD protein shows faster mobility in some tumor samples indicating the possible presence of the non phosphorylated form in these samples. The higher molecular weight bands in A and B represent cathepsin L precursor proteins.

Table 1

Cystatin E/M Mutations in Cervical Tumors

Tumor Sample	11q13 status	Presence of wild type sequence	Methylation status of promoter	Mutation in the second allele (nucleotide change)	Amino acid change (codon)
18	Heterozygous	Yes	U/M	D131G (GAC to GGC)	Asp to Gly (44)
23	ND	Yes	U	A37T (GCC to ACC)	Ala to Thr (13)
53	Heterozygous ^a	Yes	ND	F383Y (TTT to TAT)	Phe to Tyr (128)
54	LOH ^b	No	ND	HD (exon 1)	
55	Heterozygous	Yes	U/M	H320R (CAC to CGC)	His to Arg (107)
59	Heterozygous	Yes	U/M	M101T (ATG to ACG)	Met to Thr (34)
62	Heterozygous	Yes	U/M	L391F (CTT to TTT)	Leu to Phe (131)

Numbers in the mutation column correspond to the nucleotides altered by mutations;

^aPolymorphism near the TSG locus;

^bLOH, loss of heterozygosity identified in the present investigation;

U, unmethylated; M, methylated; ND, not determined