Epigenetic regulation of cystatins in cancer

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1. ABSTRACT

Cystatins function as cysteine protease inhibitors, are expressed in numerous cell types, and regulate a number of physiological processes. The cystatin superfamily consists of 12 members that divide into three types based on protein structure, location in the body, and physiological role. Four cystatin family members have been extensively studied: cystatin A, cystatin B, cystatin C, and cystatin M. Aberrant regulation of cystatin family members has been noted in a number of diseases, including cancer and certain neurodegenerative disorders. Recent advances in the understanding of cystatin function suggest that these proteins may regulate promotion or suppression of tumor growth, invasion, and metastasis. Cancer is a disease of abnormal gene expression characterized by inappropriate expression of positive mediators of cell proliferation in conjunction with diminished expression of negative mediators of cell growth. Cancer cells of many different human neoplasms exhibit aberrant epigenetic events (such as DNA methylation), which lead to gene silencing. Members of the cystatin family are epigenetically silenced through DNA methylation-dependent mechanisms in several forms of cancer, including breast, pancreatic, brain, and lung. These findings suggest that DNA methylation-dependent epigenetic mechanisms may play an important role in the loss of cystatin gene expression and protein function during neoplastic transformation and/or tumor progression. This review summarizes the biological processes in which cystatins function, focuses on the neoplastic events that involve aberrant regulation of cystatins, and discusses the possible epigenetic regulation of cystatins in cancer.
2. EPIGENETIC MECHANISMS OF GENE SILENCING IN CANCER

Cancer is a multi-step process driven by changes in gene expression leading to the aberrant growth of cells that characterize the neoplastic phenotype. Cancer has been well characterized as a genetic disease, or more specifically, a disease of abnormal gene expression. The processes of tumorigenesis and invasion are driven by chromosomal aberrations, gene mutations, and epigenetic alterations of DNA that alter the expression of genes that are important for controlling cell proliferation and survival. Cancer cells of many different human neoplasms demonstrate aberrant epigenetic events such as DNA methylation, with global hypomethylation and gene-specific hypermethylation (1). DNA methylation occurs almost exclusively on cytosines within CpG dinucleotides and frequently in regions of CpG density termed CpG islands (2, 3). CpG islands occur in the promoter sequences of many genes (4), and a strong inverse correlation between promoter methylation status and gene expression level has been documented (5, 6). Hypermethylation of promoter CpG islands leads to gene silencing and represents an important mutation-independent mechanism for inactivation of tumor suppressor genes in cancer cells (1, 7, 8), many of which contribute to the hallmarks of cancer (9). Members of the cystatin family have been shown to be epigenetically regulated by DNA methylation in several forms of cancer including breast (10-13), brain (14), and lung (15). These observations suggest that DNA methylation plays a key role in the regulation of cystatins in neoplastic transformation and tumor progression.

This review highlights the biological processes in which cysteine protease inhibitors function, focuses on the neoplastic events that involve abnormal regulation of cystatins, and discusses the possibility of epigenetic regulation of cystatins in cancer. Concepts related to the possible epigenetic regulation of cystatin family members will be introduced and observations from the literature supporting these concepts will be discussed. No attempt was made to comprehensively describe all cysteine protease inhibitors in this paper. Rather, in this work we focus on cystatins A, B, C, and M, discussing their potential roles in neoplastic disease, and possible epigenetic silencing by DNA methylation.

3. CYSTEINE PROTEASE INHIBITORS - CYSTATINS

Cystatins function as cysteine protease inhibitors and were discovered in the 1960s with a report on a factor capable of inhibiting the clotting activity of a thiol-dependent protease in mammalian cells (16). Since that time, other groups identified cystatin proteins that control and regulate physiological processes that range from cell survival and proliferation, to differentiation, cell signaling, and immunomodulation (17, 18). By the early 1980s, it was recognized that cystatins are present in lysosomes of most if not all cell types (19, 20). Aberrant regulation of these important homeostatic factors contributes to a range of pathologies. Cystatins regulate the physiological activities of specific cysteine proteases (cathepsin family members) (21). There is increasing evidence that an imbalance between cysteine proteases and their inhibitors (cystatins) leads to excess protease activity due to high cathepsin levels, which contributes directly to tumor cell invasion (22). Consequently, imbalances in cystatins have been noted in a number of cancers (18).

4. CYSTATIN SUPER-FAMILY

Cysteine protease inhibitors belong to a cystatin super-family encompassing a large group of homologous proteins that inhibit papain family cysteine proteases (17, 18, 23) (see the MEROPS database at http://merops.sanger.ac.uk) (24). Twelve functional cystatins divide into three types based on protein structure, location in the body, and physiological role. Type 1 cystatins (cystatins A and B) are polypeptides of 98 amino acid residues and are found intracellularly, but occasionally appearing in body fluids at detectable levels (18, 23). The majority of cysteine protease inhibitors encompass type 2 cystatins including cystatin C, D, M, F, G, S, SN, and SA. Type 2 cystatins consist of 120 amino acid residues, two disulphide bridges, and an extracellular signaling peptide (25), and are found in most body fluids (18, 23). Kininogens comprise type 3 cystatins and are large multifunctional proteins with three type 2-like cystatin domains, of which only two are capable of inhibiting cysteine proteases (18). Kininogens are found in blood plasma (18). The tertiary structures of cystatin proteins are conserved and fold into a five-stranded beta-sheet that wraps around a five-turn alpha-helix, termed a ‘cystatin fold’ (17, 26). Cystatins function to protect cells from lysosomal peptidases released during normal cell death, phagocyte degranulation, and/or during cancer cell proliferation (18). Therefore, cystatins are essential in safeguarding against abnormal lysosomal cysteine protease activity that is essential for tumor invasion and metastasis.

5. CYSTATIN A (STEFIN A)

5.1. Structure and function

Cystatin A (encoded by CST4) is a single-chain protein, which possess neither disulfide bonds nor carbohydrate side chains (18, 23, 27). CST4 is located on chromosome 3q21. Although it has been found in some extracellular fluids, cystatin A is located mainly intracellularly (18, 27). Cystatin A forms tight complexes with papain-type proteases and cathepsins B, H, and L. High levels of cystatin A are found in various types of epithelial cells and some blood cell types (27). The protein has been isolated from epidermis, polymorphonuclear granulocytes, liver, and spleen (18, 23).

5.2. Cystatin A in cancer

Cystatin A is expressed in normal human luminal cells and is a known myoepithelial cell marker (28). Thus, expression of cystatin A is lost with the loss of myoepithelial cells during tumorigenesis in most breast and prostate cancers (29-32). Lah et al. found decreased levels of cystatin A mRNA and protein in the majority of breast
neoplasms examined, suggesting a possible role for cystatin A in breast carcinogenesis (31). In contrast, Kuopin et al. demonstrated that breast tumors with positive cystatin A expression are associated with poor outcome, and cystatin A expression is a negative prognostic marker in lymph node negative patients (29). In addition, coexpression of cystatin A and p53 in breast cancer patients is associated with a high risk of death (29). These findings suggest different roles for cystatin A in aggressive breast cancers, and may reflect cystatin A genetic instability during tumor progression, providing growth advantage to tumor cells (29). Cystatin A expression is lost in squamous cell carcinomas of the head and neck (HNSCC), and in brain tumors (21, 33, 34). In patients with low cystatin A expression, the risk of HNSCC disease recurrence is significantly higher than in patients with high cystatin A expression (33). In brain tumors, cystatin A mRNA is only detected in benign neoplasms, but not in malignant tissues, suggesting that cystatin A is lost during the outgrowth of malignant lesions (21). The function of cystatin A in various human neoplasms is not well established. Several investigators have shown that cystatin A can reduce tumor cell motility without affecting viability of the tumor cells (35, 36). This observation suggests that in most tumors cystatin A may be important for cell invasion and metastasis. Loss of cystatin A expression may result in an imbalance in cysteine protease activity. Consistent with this suggestion is the observation that loss of cystatin A accompanies (i) development of invasive neoplasms, and (ii) neoplasms that reoccur and/or spread aggressively. The direct role of cystatin A in tumor cell mobility, invasion, and metastatic spread merits additional study.

5.3. Epigenetic regulation of CSTA

Mechanisms that account for the loss of CSTA in cancer have not been elucidated. Numerous mechanisms may contribute to the downregulation of CSTA in human neoplasms, including chromosomal alterations (deletions), inactivating gene mutations, and epigenetic gene silencing. However, no deletions or structural rearrangements of CSTA have been characterized, which suggests that these mechanisms may not represent the major pathway for loss of CSTA in cancer cells. The CSTA gene is located on chromosome 3, which has been shown to be subject to loss of heterozygosity (LOH) in human breast cancers (37), and others. Thus, LOH events may result in loss of CSTA in specific forms of cancer. DNA methylation is a well-known epigenetic mechanism, and a number of different genes have been shown to be inactivated in cancer through methylation-dependent gene silencing (38). Genes that are subject to methylation-dependent silencing typically have dense regions of CpG dinucleotides (CpG islands) sited in their gene promoter regions. CSTA lacks a promoter CpG island or any regions of CpG density (Figure 1A). This suggests that the CSTA gene may not be a target for DNA methylation. However, direct evidence for methylation-dependent regulation of genes lacking CpG islands has emerged from a few investigations (10, 39-41). Well-characterized examples of methylation-sensitive genes lacking CpG islands include E-cadherin (38, 42), RAR-β2 (43), APC (44), and LAMB3 (45-48). In genes lacking well-defined CpG targets, methylation of specific CpG dinucleotides in critical sequence locations can result in diminished expression of gene silencing. Methylation of specific CpG dinucleotides may (i) recruit methylated DNA binding proteins to the promoter region resulting in a blockade of transcription factor access to crucial recognition sequences, or (ii) directly inhibit transcription factor binding to the promoter. Therefore, the loss of CSTA expression in many cancer types could be the result of transcriptional silencing by DNA methylation. However, no evidence for methylation-dependent regulation of CSTA has appeared in the literature. Therefore, establishment of DNA methylation-dependent mechanisms of gene silencing for this gene will require direct examination using sensitive bisulfite sequencing methods.

6. CYSTATIN B (STEFIN B)

6.1. Structure and function

Cystatin B is very similar to cystatin A. Cystatin B (encoded by CSTB) maps to chromosome 21q22.3 and is expressed intracellularly (18). Cystatin B is ubiquitously expressed, and is more abundant than cystatin A with expression levels varying two-fold to six-fold across different tissue and cell types (17, 18, 49). Cystatin B is a tight-binding reversible inhibitor of papain-type proteases and cathepsins B, H, and L, and protects the cell against leakage of lysosomal enzymes into the cytoplasm (18). Loss of cystatin B has been reported in specific forms of pathology, but only involving a few cell types. This observation suggests that other cystatins may act (at least in part) to compensate for loss of cystatin B function (18).

6.2. Cystatin B in cancer

Cystatin B is required for maintenance of normal neuron structure and survival (18). Mutations in the gene encoding cystatin B are primarily responsible for Unverricht-Lundborg disease, which causes progressive myoclonus epilepsies (50, 51). In various cancers, an imbalance in cystatin B expression levels has been noted in tumor tissues when compared to matched normal samples (17, 52, 53). Cystatin B serves as a biological marker for prognostic assessment of low-risk and high-risk non-small cell lung cancer patients (52). In patients with non-small cell lung cancer, high levels of cystatin B correlate with increased probability of survival (52). Cystatin B is downregulated in many breast cancers (31, 54, 55). The loss of cystatin B expression in more aggressive neoplasms, suggests that its loss may contribute to tumor progression. In fact, cystatin B protein is expressed at significantly lower levels in invasive metastatic breast cancer cell lines when compared to less aggressive breast cancer cells (53). This observation is consistent with the suggestion that loss of cystatin B may contribute to tumor cell invasion, and metastasis.

6.3. Epigenetic regulation of CSTB

The promoter and exon 1 region of CSTB contains two large CpG islands (figure 1B). The first CpG island (334 bp) contains 20 CpG dinucleotides and is found in the promoter region approximately 900 bp from the start site of transcription. The second CpG island (546 bp) contains 69 CpG dinucleotides and spans the proximal
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Figure 1. CpG dinucleotide diagram of the proximal promoter and exon 1 in \textit{CSTA}, \textit{CSTB}, \textit{CST3}, and \textit{CST6}. The distribution of CpG dinucleotides proximal to the transcription start site in the promoter (0 to -1400 nucleotides) and exon 1 is depicted schematically (vertical lines indicate the relative position of individual CpG dinucleotides). (A) \textit{CSTA} contains no CpG islands or other regions of CpG density. (B) \textit{CSTB} contains two large CpG islands. The first CpG island is 334 bp and is found in the promoter region. The second CpG island is 452 nucleotides from the first, consisting of 546 bp encompassing the proximal promoter and exon 1 regions. (C) \textit{CST3} contains a 435 bp CpG island that spans the proximal promoter and exon 1, encompassing the transcriptional start site. (D) \textit{CST6} has a large CpG island covering the proximal promoter region and exon 1, harboring 64 CpG dinucleotides.

promoter and exon 1, encompassing the transcriptional start site. The promoter region of \textit{CSTB} contains 14% CpG dinucleotide content in the 1400 bp upstream of the transcription start site, with a much more extensive region of CpG density (24%) in the first 500 bp of the proximal promoter. DNA methylation-dependent gene silencing can result from methylation events occurring at CpG dinucleotides within a CpG island and represents a normal mechanism for regulation of gene expression (56). However, in cancer cells methylation-dependent epigenetic gene silencing represents a mutation-independent mechanism for inactivation of genes (8). Promoter CpG hypermethyltion contributes to gene silencing by inhibiting the binding of certain transcription factors to their recognition sequence (44, 57), attracting methylated DNA binding proteins (58), and/or through chromatin remodeling (59). Loss of \textit{CSTB} expression in human neoplasms may be a direct consequence of methylation-dependent gene silencing. Multiple targets sequences for CpG methylation exist in the \textit{CSTB} promoter, including the recognized CpG islands. However, no evidence for methylation-dependent regulation of \textit{CSTB} has appeared in the literature. Thus, an investigation to explore \textit{CSTB} CpG island methylation and expression status will be required to directly determine if aberrant DNA methylation represents a mechanism for the loss of \textit{CSTB}.

7. CYSTATIN C

7.1. Structure and function

The gene that encodes cystatin C (encoded by \textit{CST3}) is located on chromosome 20p11.21 within a cystatin multigene locus composed of all type 2 cystatins (with the exception of cystatin M) (18). Similar to cystatin B, cystatin C is expressed across a variety of tissues (17, 18) and most cells are able to secrete cystatin C (60, 61). Cystatin C is found in all body fluids and is considered the most important extracellular inhibitor of cysteine proteases.
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(18, 62). Due to its widespread distribution, cystatin C is described as an ‘emergency’ inhibitor of cysteine proteases found in body fluids (63), and is a reversible inhibitor of cathepsins B and L (17). Cystatin C is synthesized as a preprotein containing a 26-residue signal peptide and in its mature form is composed of 120 amino acids, and has a low molecular weight of 15,000 (18). Therefore, cystatin C is eliminated efficiently from serum through the glomerular filtration system (17). Based on this and other properties, cystatin C has become an important marker for kidney function (64, 65).

7.2. Cystatin C in cancer

Cystatin C mRNA and immunostaining studies show that expression does not change significantly in human premalignant and malignant cells in cancers of the brain, pituitary, endocrine gastro-entero-pancreatic system, kidney, breast, and colon (66-70). Other studies report high levels of cystatin C in serum, pleural effusions, and ascites fluids collected from cancer patients (71-75). However, it has not been established if these high levels of cystatin C correspond to a reactive condition related to the proliferation and invasiveness of malignant tumors (17). Cystatin C binds to the cysteine protease cathepsin B, forming a specific enzyme-inhibitor complex (17). A few studies have shown decreased expression of cathepsin B/cystatin C complexes accompanied by increased levels of cathepsin B in serum of patients with malignant tumors, when compared to benign diseases or healthy controls (73, 76). These results suggest that during tumor progression the cathepsin B/cystatin C complex is disturbed, resulting in escape of cathepsin B from the control mechanisms provided by cystatin C (76). Cystatin C efficiently inhibits in vitro tumor cell-mediated degradation and invasion of extracellular matrix (77-80). This finding suggests that cystatin C is important in the molecular regulation of tumor cell invasion/metastasis, and aberrant expression of this protein could contribute to tumor progression. Cystatin C has been shown to be regulated by TGF-beta in mouse cells (80, 81). TGF-beta has multiple functions within tumor cells, and is able to function as a tumor promoting and tumor-suppressing protein (17). Cystatin C has been shown to have these same properties within tumor cells, possibly reflecting its relationship with TGF-beta.

7.3. Epigenetic regulation of CST3

Numerous mechanisms may contribute to the aberrant regulation of CST3 including, chromosomal alterations, gene mutations, and epigenetic alterations of DNA. CST3 contains a large CpG island (435bp) including 46 CpG dinucleotides that spans the proximal promoter and exon 1, encompassing the start site for transcription (figure 1C). The promoter region of CST3 contains a 5% CpG dinucleotide content 1400 bp upstream of the transcription start site, and a 9% CpG dinucleotide content in the first 500 bp. A CpG island situated in promoter regions represents a major target for DNA methylation, and suggests that CST3 could potentially be transcriptionally silenced by hypermethylation. However, direct evidence for DNA methylation-dependent silencing of CST3 has not been reported to date. Identification of DNA methylation-dependent mechanisms of CST3 gene regulation will require direct examination of the CST3 promoter CpG island using sensitive bisulfite sequencing methods.

8. CYSTATIN M

8.1. Structure and function

Cystatin M is encoded by the CST6 gene. This gene was originally identified in breast cancer cell lines isolated from a metastatic lesion and matched primary breast tumor by differential RNA display (82). Subsequently, cystatin M was independently cloned from cDNA in an investigation of EST-libraries of amniotic and fetal skin epithelial cells (83). The biochemical properties, chromosomal localization (chromosome 11q13), and biological distribution of cystatin M is significantly different from the other cystatins (84). Cystatin M is expressed in a variety of normal human tissues including brain, lung, heart, liver, pancreas, spleen, thymus, small intestine, prostate, ovary, peripheral blood cells, and placenta (82, 83). Cystatin M is a reversible inhibitor of cathepsins B and L, and consists of 121 amino acids and unlike other type 2 cystatins, is found in two different protein forms: (i) glycosylated (17kDa), and (ii) non-glycosylated (14.4 kDa) (83).

8.2. Cystatin M in cancer

Cystatin M expression is diminished or lost in various forms of cancer including, (i) basal and squamous cell carcinomas of the skin (87), (ii) squamous cell carcinomas of the head and neck and lung regions (88), (iii) non-small cell lung cancer (15), (iv) metastatic oral cancer cell lines (84), (v) malignant glioma (14), (vi) melanoma cell lines (60), (vii) prostate cancer cell lines (60), and (viii) breast cancer (11-13, 60, 82, 89, 90). Cystatin M has been suggested to function as a breast tumor suppressor gene (89). The majority of human breast cancer cell lines derived from metastatic breast tumors lack cystatin M expression, whereas normal and premalignant cells express abundant levels of cystatin M (82, 89). Exogenous expression of cystatin M in MDA-MB-435S breast cancer cells results in the suppression of cell proliferation, migration, matrix invasion, and tumor-endothelial cell adhesion in vitro (60). These observations combine to suggest that loss of cystatin M correlates strongly with the invasive/metastatic phenotype in various forms of cancer. Cystatin M functions in the regulation of cathepsin B and cathepsin L activity, and an imbalance between these proteases and cystatin M is important in driving tumor progression (76, 85, 86). Thus, loss of cystatin M expression may contribute significantly to tumor invasion secondary to elevations in cathepsin B and L activities in the microenvironment of the neoplasm.

8.3. Epigenetic regulation of CST6

CST6 contains a large CpG island (424 bp) that spans the proximal promoter and exon 1, and includes 54 CpG dinucleotides, encompassing the start site for transcription (figure 1D). The promoter region of CST6 contains a 8% CpG dinucleotide content 1400 bp upstream of the transcription start site, with the most CpG density (12%) occurring in the proximal 500 bp of the promoter. No deletions or structural rearrangements of CST6 have
CpG island hypermethylation and loss of proximal promoter was strongly associated with the lack of (that express methylation of the proximal promoter region of its CpG island (10). In a subsequent study, methylation analysis of CST6 in cystatin M-negative breast cancer cells and cells that express CST6 revealed a direct relationship between CpG island hypermethylation and loss of CST6 expression (11). Furthermore, the extent of regional methylation in the proximal promoter was strongly associated with the lack of expression of CST6 (11). Consistent with these findings, several other studies have shown that CST6 is epigenetically regulated by DNA methylation-dependent silencing in breast cancer cell lines and primary invasive ductal carcinomas (11-13). Ai et al. showed that 12/20 (60%) primary breast tumors exhibit CST6 promoter hypermethylation, and microdissection of individual cells from select tumors revealed that methylation occurs in both DCIS and IDC cells (12). In a similar study, Schagdarsurengin et al. showed that 24/40 (60%) breast carcinomas exhibited CST6 promoter hypermethylation, and that estrogen-receptor positive tumors were more frequently methylated than estrogen-receptor negative tumors (13). In addition, CST6 was identified as a methylation-sensitive gene in glioma cell lines and primary brain tumors (14). Overall, these observations suggest strongly that methylation-dependent epigenetic silencing of CST6 represents an important mechanism for loss of CST6 in multiple tumor systems. Likewise, methylation-dependent CST6 silencing may represent an informative clinical marker for cancer cells that predicts invasive potential. Additional studies will be required to establish the temporal order of events that accompany methylation-dependent silencing of CST6 in tumorigenesis.

9. CONCLUSIONS AND PERSPECTIVES

The field of cysteine protease inhibitors has grown exponentially since the 1960s and a large group of proteins that are important in tumor promotion, suppression, invasion, and metastasis to date have been identified. Currently, there are ongoing research efforts to characterize the mechanisms that account for the aberrant regulation of cystatins in neoplastic transformation and tumor progression. Epigenetic events such as DNA methylation are seen in cancer cells of many different neoplasms. CpG islands are found in the promoter regions of many cysteine protease inhibitors, making these genes targets for DNA hypermethylation. Sodium bisulfite sequencing can be used to directly examine methylation of CpG dinucleotides within the promoter sequence of these genes. Several members of the cystatin family have been shown to be epigenetically regulated, suggesting that epigenetic mechanisms play a role in the regulation of cystatins in neoplastic transformation and tumor progression. Lysosomal cysteine proteases are involved in the degradation of components of connective tissues and basement membranes, and aberrant expression and activity of these proteases accompany cancer invasion and metastasis. Thus, loss of cysteine protease inhibitors that control the degradation of stromal components might contribute to increased proteolysis of tissue architecture, leading to the spread of cancer cells. Therefore, it is essential to the understanding of cancer invasion/metastasis to characterize the mechanisms that account for abnormal regulation of cystatins. Methylation-dependent epigenetic gene silencing represents a novel mechanism for selectively altering cystatin gene expression in malignant cancers.

10. ACKNOWLEDGMENTS

This work was supported by grants from the Susan G. Komen Breast Cancer Foundation (BCTR0100575) and the National Cancer Institute (NIH grant CA78343).

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Abbreviations: 5-aza: 5-aza-2’-deoxycytidine

Key Words: Cancer, Cystatins, Epigenetic, DNA Methylation, Cysteine Protease Inhibitor, Review

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