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Cystatin M expression is reduced in gastric carcinoma and is associated with promoter hypermethylation

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ABSTRACT

Cystatin M is a secreted inhibitor of lysosomal cysteine proteases and increasing evidences indicate that it is a novel target for epigenetic silencing during mammary tumorigenesis. Aberrant promoter methylation is a well-known mechanism that participates in cystatin M silencing in breast cancer. However, the role of cystatin M in the gastric cancer remains to be elucidated. Immunohistochemistry was used to investigate the expression of cystatin M in 60 gastric carcinomas. Hypermethylation of cystatin M promoter was evaluated by the methylation-specific PCR (MSP) method in gastric carcinomas (tumor and paired adjacent non-tumor tissues). Reverse-transcriptase PCR and BSP were also performed on gastric cancer cell lines before and after treatment with 5-aza-2′-deoxycytidine (5-Aza-dC). Lost expression of cystatin M was observed in 42 of 60 (70%) gastric carcinomas. 55% (33 of 60) of primary tumors analyzed displayed cystatin M promoter hypermethylation, indicating that this aberrant characteristic is common in gastric malignancies. Moreover, a statistically significant inverse association was found between cystatin M methylation status and expression of the cystatin M protein in tumor tissues ($p = 0.027$). We also found that patients with cystatin M promoter methylation had a significantly shorter survival time than those without this methylation ($p = 0.020$). These results suggest that cystatin M promoter hypermethylation is one of the molecular mechanisms that accounts for reduced cystatin M gene expression in gastric carcinomas.

Introduction

Lysosomal cysteine proteases are involved in the degradation of components of connective tissues and basement membranes *in vitro* and aberrant expression and activity of these proteases accompany cancer invasion and metastasis in vivo [1–3]. Cystatins are inhibitors of lysosomal cysteine proteases that participate in diverse biological functions such as pericellular matrix remodeling, protein catabolism, apoptosis, and antigen processing [4]. Cystatin M is present in both an unglycosylated 14 kDa form containing 149 amino acids and a 17 kDa form that is glycosylated [1]. Cystatin M was initially identified as a downregulated transcript in metastatic breast cancers in comparison to corresponding primary tumors, suggesting a role in suppression of tumor invasion and metastasis, loss of cystatin M expression may be one mechanism that enables tumor cells to spread from the primary site and invade adjacent tissues during breast cancer progression [5,6].

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. Consistent with a role in suppression of metastasis, cystatin M has been suggested to function as a breast tumor suppressor gene [7]. Exogenous expression of cystatin M in human MDA-MB-435S breast cancer cells, cystatin M significantly alters the phenotype *in vitro*, resulting in diminished cell proliferation, loss of cell migration, inhibition of Matrigel invasion, and reduced endothelial cell adhesion. Further, a null mutation of the mouse cystatin M gene seemed to correlate with the development of ichq phenotype, characterized by neonatal lethality, abnormal cornification, and desquamation [8]. Thus, the cystatin M gene seems to play an important role in the differentiation of skin epithelial cells.

CpG islands sited in gene promoters represent a major target for DNA hypermethylation, which contributes to gene silencing by inhibiting the binding of certain transcription factors to their recognition sequence, attracting methylated DNA-binding proteins,

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and/or through chromatin remodeling. Cystatin M promoter contains a large CpG island that spans the proximal promoter and exon 1, encompassing the start site for transcription. Several studies have shown cystatin M to be silenced by methylation in select breast cancer cell lines, primary breast tumors [9]. Furthermore, cystatin M was identified as a methylation-sensitive gene in glioma cell lines and primary brain tumors [10]. These findings combine to suggest that loss of cystatin M expression in multiple tumor systems may be a direct consequence of methylation-dependent gene silencing.

The aim of this study was to detect the expression of cystatin M in gastric cancer, and figure out the potential molecular mechanisms for it. We found that cystatin M was downregulated in gastric cancer compared to the control tissues, and hypermethylation of the promoter of cystatin M was responsible for it. We also explored the correlation between cystatin M methylation status and clinical features.

Material and methods

Tissue microarray and immunohistochemistry. After screening H&E-stained slides for optimal tumor tissue and tissue adjacent to tumor (TAT) with a distance of 2 cm from the tumor, we constructed TMA slides (Shanghai Biochip, Shanghai, China). Two cores were taken from each formalin-fixed, paraffin-embedded HCC and TAT sample by using punch cores that measured 1.0 mm in greatest dimension from the non-necrotic area of tumor foci and TAT. Immunohistochemistry was performed by a 2-step method using primary antibody including heat-induced antigen-retrieval procedures. Sections were incubated overnight at 37 °C with primary antibody; after the primary antibody was washed off, the components of the Envision detection system were applied with an antimouse polymer (EnVision1/HRP/Mo, Dako, Glostrup, Denmark). Reaction products were observed by incubation with diaminobenzidine. The primary antibodies used were all mouse-antihuman monoclonal antibodies against cystatin M (1:10 dilution; American Research Products, Belmont, Mass). Negative controls were treated identically but with the primary antibody omitted.

Scoring of expression of cystatin M. Immunoreactivity was evaluated independently by three researchers who were blinded to patient outcome. The percentage of positive tumor cells was determined by each observer, and the average of 3 scores was calculated. We randomly selected 10 high-power fields; and counted 1000 cells in each core. When the mean of percentage of positive cells is close to 0% or 100%, the standard deviation (SD) is close to 0, and when the mean is approximately 50% the SD is approximately 5%. Thus, the SD does not increase with the mean. The following categories were used for scoring: intensity of staining, none (0), mild (1), moderate (2), strong (3); percentage of the positive staining, <5% (0), 5–25% (1), 25–50% (2), >50% (3). Combining intensity and percentage staining resulted in the following score: 0–1, negative (−); 2–6, positive (+) [11].

DNA extraction from paraffin block. The DNA extraction was performed as previously described [12]. Briefly, formalin-fixed, paraffin wax-embedded tissues were cut into 10 mm thick sections. Before DNA extraction, the sections were placed on slides and stained with hematoxylin–eosin to evaluate the admixture of non-tumorous tissues. Areas corresponding to tumor or surrounding normal gastric tissue were microdissected separately. Microdissected tissues were collected in 15 ml centrifuge tubes, and deparaffinized overnight at 63 °C in xylene. After centrifugation at full speed for 5 min, the supernatant was removed. Ethanol was added to the pellet to remove residual xylene, and then removed by centrifugation. The genomic DNA was isolated using DNeasy Tissue Kit according to the manufacturer's instruction.

DNA methylation analysis of the cystatin M gene. Genomic DNA (2 μg) was modified with sodium bisulfite using EpiTect Bisulfite kit (Qiagen). Methylation status was analyzed by bisulfite genomic sequencing of the CpG islands. The fragment covering 46 CpG sites from cystatin M promoter region was amplified from bisulfite-modified DNA. The primers used were 5′-GGTTTTTTGG GTTTTTTGAATTT-3′ (sense) and 5′-CTACCCATATTATAACTAACC-3′ (antisense) [13]. Amplified bisulfite-sequencing PCR products were cloned into pMD18-T simple vector (Takara). Methylation status of human gastric normal tissues, tumor samples was examined by methylation-specific PCR (MSP) analysis. Primers for methylated reaction were 5′-GGTTTTTTGGGTTTTTTGAATTTTG-3′ (sense), and 5′-TACCAAACTTACAACCACACAACT-3′ (antisense), and for the unmethylated reaction were 5′-GGTTTTTTGGGTTTTTTGAATTTTG G-3′ (sense), and 5′-TACCGAACTTACGACCGCGCAACT-3′ (antisense) [14].

5-Aza-dC treatment and reverse transcription, polymerase chain reaction (RT-PCR). We investigated the effect of a demethylating agent, 5-Aza-dC (Sigma) on the expression of five gastric cancer cell lines (AGS, SGC-7901, MKN28, MKN45 and BCG-823) cells, which were all preserved in our laboratory and maintained in PRIM 1640 with 10% FBS. Cells were plated at a density of 2×10^5 per well in 6-well plates 18 h before the treatment at the concentration of 10 μM/L. After treatment for 48 h, cells were harvested. Reverse transcription reaction was performed using 2 mg of total RNA with a first strand cDNA kit (Promega). The mRNA expression levels of the cystatin M were determined by PCR.

Statistical analysis. Pearson Chi-Square test and one-ANOVA were used for statistical analysis of group differences. Pearson Chi-Square tests were performed to evaluate the significance of the differences between the frequencies of cystatin M promoter hypermethylation status of the various tissue categories and comparisons with clinical characteristics. With regard to survival analysis, we analyzed 60 patients with gastric carcinoma using Kaplan–Meier analyses. We used log-rank tests in order to compare the survival curves between groups. Univariate and multivariate survival analyses were then conducted using the Cox regression model. *p*-Values less than 0.05 was considered significant.

Results

Expression profile of cystatin M in gastric lesions

Tissues from patients with gastric cancer and other gastric lesions were retrospectively identified from the Department of Pathology, Henan tumor Hospital. The 60 gastric cancers comprised 18 early cases and 42 advanced cases, the clinicopathologic characteristics were analyzed according to tumor size, histological grading and presence of nodal metastasis. Cytoplasmic cystatin M expression in normal gastric epithelium and carcinomas detected by immunohistochemistry were semiquantitated. Overall, cystatin M was absent in 42 of 60 carcinomas (70%) and 6 of 60 (10%) in normal gastric samples. Representative examples of cystatin M protein expression in gastric cancer samples are shown in Fig. 1.

Promoter methylation status in gastric carcinomas

To elucidate the mechanism of cystatin M downregulation in gastric carcinoma, we examined the methylation status of the promoter region using MSP (see Fig. 2A). We found that in 33 of the 60 (55%) gastric carcinomas analyzed, the cystatin M promoter was hypermethylated. Meanwhile, the aberrant methylation was only detected in 3 of 6 normal samples without cystatin M expression. 12 of the 27 unmethylated carcinoma samples (44.4%) demon-

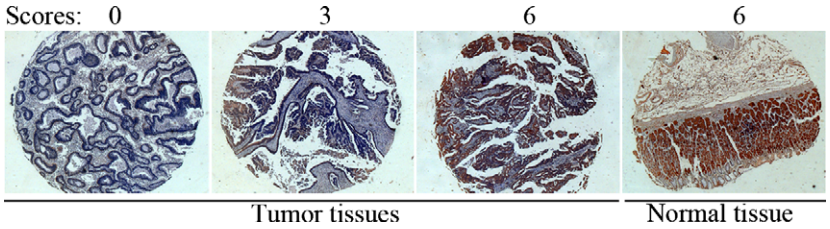

Fig. 1. Immunohistochemical staining for cystatin M with anti-cystatin M in the cancerous and normal tissues. The nuclei were countered stained with hematoxylin. The scores of cystatin M expression graded according to the number of stained cells and the staining intensity of staining were shown on the top of the pictures.

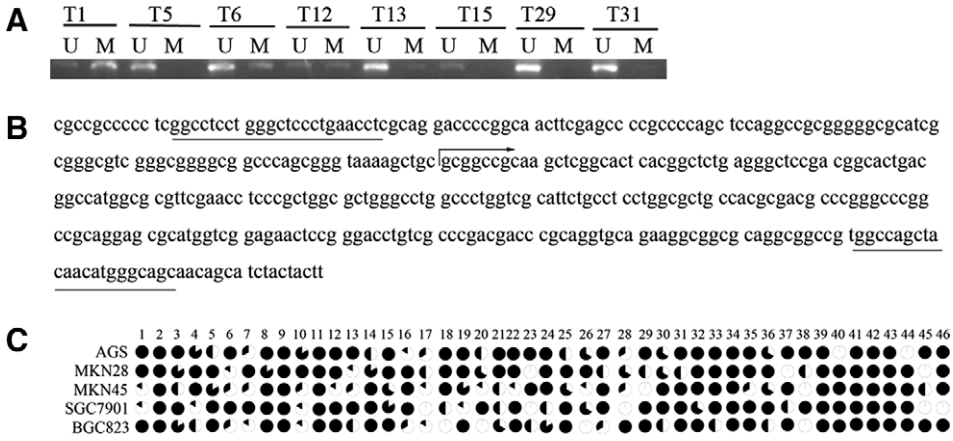

Fig. 2. (A) Representative MSP results of cystatin M hypermethylation in primary gastric cancer tumors. Case numbers are shown on top. *M*, methylated primers; *U*, unmethylated primers. (B) A map of the CpG islands in relation to the promoter of the cystatin M. The locations of sense and antisense primers used for bisulfite-sequencing PCR were indicated by underlining. The translation start site is shown by a horizontal arrow. (C) Demonstration of cystatin M promoter methylation by sequencing of sodium bisulfite-modified DNA from the indicated gastric cancer cell lines. Methylated and unmethylated CpG dinucleotides are shown by closed and open circles, respectively. Each line of circles represents analysis of a single cloned allele.

strated positive cytoplasmic staining and 12 of 33 methylated carcinoma samples (18.2%) showed loss of expression of cystatin M. Thus, the immunostaining results were strongly correlated ($p = 0.027$) with cystatin M methylation status (Table 1).

Table 1
Clinical characteristics of gastric cancer patients according to hypermethylation status of cystatin M.

Group	Cystatin M methylation		*p*-Value
	U	*M*	
Normal tissues	55	5	
Cancer tissues	27	33	<0.001[a]
Differentiation			
Well	12	4	
Moderate	9	11	
Poor	6	18	0.008
Metastasis			
Yes	13	29	
No	14	4	0.001
Size (cm)			
<2	7	11	
⩾2	20	22	0.533
Gross type			
Early	10	8	
Advanced	17	25	0.282
Cystatin M expression			
Yes	12	6	
No	15	27	0.027

[a] Statistically significant when compared with the normal tissues.

Cystatin M expression could be restored with 5-Aza-dC treatment in gastric cancer cell lines

The area of the CpG-rich region around the transcription initiation site of cystatin M gene between the nucleotides −118 and +242 which spanned 46 CpG site was sequenced (Fig. 2B) [9,13]. As shown in Fig. 2C, most CpG dinucleotides were methylated in gastric cancer cell lines. As shown in Fig. 3A, cystatin M was down-regulated in all five human gastric cancer cell lines. We examined the role of methylation in the silencing of cystatin M. To confirm that CpG methylation is indeed responsible for the silencing of cystatin M, we treated these heavily methylated and silenced cell lines with 5-Aza-dC, a methyltransferase inhibitor. Cystatin M expression was markedly induced after the treatment in all the cell lines (Fig. 3B). Bisulfite DNA sequencing of the gastric carcinoma cell lines confirmed the promoter methylation status with or without treatment of 5-Aza-dC (Fig. 3B).

Hypermethylation of cystatin M is associated with gastric cancer differentiation and metastasis

To characterize the correlation between hypermethylation of cystatin M promoter and clinical features of gastric cancer, several clinicopathological characteristics including age, gender, gross type, cell differentiation, tumor size and long distant metastases were compared between patients with normal and hypermethylation of cystatin M promoter (Table 1). The result found that hypermethylation cystatin M promoter was not associated with tumor size ($p = 0.533$), gross type of gastric cancer ($p = 0.282$), patient's age and gender (data not shown). However, hypermethylation of

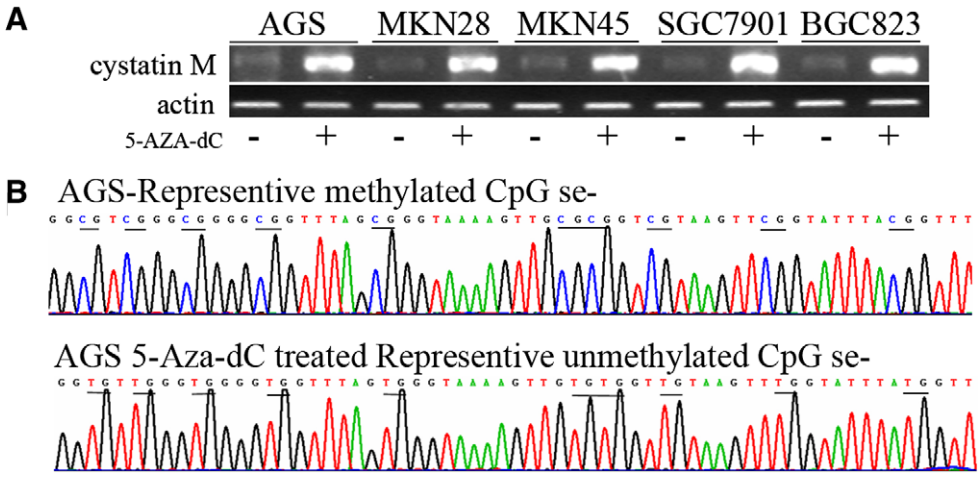

Fig. 3. (A) The mRNA expression of cystatin M in gastric cancer cell lines (AGS, MKN28, MKN45, SGC-7901, and BCG-823) treated with or without demethylation agent 5-Aza-dC as determined by RT–PCR. Pharmacologic treatment with 5-Aza-dC restored the expression of cystatin M in tumor cell lines. (B) An illustrative fragment of the sequencing electropherogram is shown for AGS cells treated with or without 5-Aza-dC, the CpG sites are underlined.

cystatin M was significantly correlated with distant metastasis ($p = 0.001$) and poor differentiation ($p = 0.008$).

Hypermethylation of cystatin M is associated with poorer prognosis of gastric cancer

The potential correlation between hypermethylation of cystatin M promoter and gastric cancer prognosis was also addressed in the present study. Visual inspection of the Kaplan–Meier curves suggested that overall survival time of patients without cystatin M promoter hypermethylation was significantly longer than that of patients with hypermethylation of cystatin M promoter ($p < 0.001$) (Fig. 4). Univariate Cox regression analysis showed that downregulation of cystatin M expression, diffuse type, and late tumor stage were significantly associated with patient survival. Multivariate analysis illustrated metastasis, hypermethylation of cystatin M promoter and downregulation of cystatin M were independent prognostic variables of gastric cancer survival (Table 2).

Discussion

Gastric cancer is a heterogeneous disease that results from the accumulation of a complex series of genetic and epigenetic events

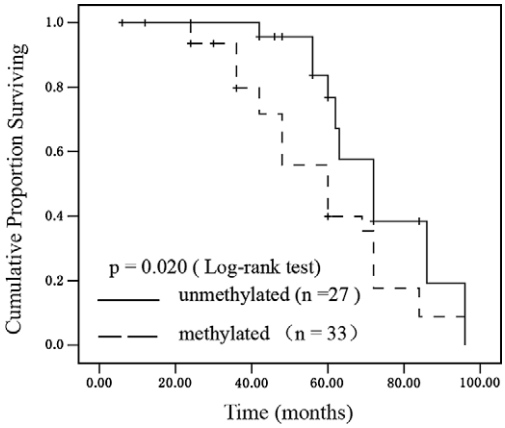

Fig. 4. Kaplan–Meier survival analysis of cystatin M methylation in 52 advanced gastric cancer patients. Patients with cystatin M methylation had a significantly poorer outcome than those without methylation ($p = 0.020$).

driving divergent pathways that ultimately convey varying phenotypic properties to individual lesions. Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumor suppressor genes is believed to be a mechanism involved in human carcinogenesis. In gastric cancer, a growing number of genes have been identified as undergoing aberrant promoter hypermethylation, suggesting that promoter hypermethylation is an important mechanism involved in gastric cancer.

Cystatin M is a member of a family of proteins that function as physiological inhibitors of lysosomal cysteine proteases, and control target proteases by forming high-affinity reversible complexes [6]. Loss of cystatin M expression might contribute to increased proteolysis of tissue architecture, facilitating the spread of cancer cells [4]. Cystatin M expression has been reported to be diminished or lost in various forms of cancer including basal and squamous cell carcinomas of the skin, squamous cell carcinomas of the head/neck and lung, non-small cell lung cancer, metastatic oral cancer cell lines, malignant glioma, prostate cancer, and breast cancer [3,8,10,14–17]. Similarly, we found that cystatin M expression was downregulated in gastric cancer tissues compared with normal tissue, which was kept in line with its role of tumor suppressor gene. More recently, it has been shown that cystatin M is epigenetically regulated by DNA methylation-dependent silencing in breast cancer cell lines. Ai et al. showed that 60% primary breast tumors exhibit cystatin M promoter hypermethylation, and methylation also occurs in both DCIS and IDC cells [9]. Similarly, Schagdarsurengin et al. showed that 60% breast carcinomas exhibited cystatin M promoter hypermethylation, and that estrogen-receptor positive tumors were more frequently methylated than estrogen-receptor negative tumors [18]. Furthermore, the differential CpG island methylation of cystatin M promoter between primary breast tumors and lymph node metastases indicates that certain individual methylation events occur during or following stromal invasion and tumor spread and evidence from breast cancer cell lines suggests that cystatin M promoter hypermethylation leading to gene silencing may represent one major mechanism for loss of cystatin M in breast cancer. Cystatin M has a typical CpG island around its transcription start site (Fig. 3) [9,19]. We first analyzed cystatin M CpG island hypermethylation in 60 patients with primary gastric cancer by use of MSP. We observed that cystatin M CpG island hypermethylation was a common event in gastric cancer tissues (33 of 60, 55%), whereas only three case with hypermethylation were detected in adjacent non-tumor tissues.

Table 2
Results of univariate and multivariate analyses of the overall survival of patients with gastric carcinoma using Cox-proportional hazards regression.

Predictor	Univariate Relative risk (95% confidence interval)		Multivariate Relative risk (95% confidence interval)	
Hypermethylation	4.241 (1.231–14.611)	$p = 0.022$	7.185 (2.661–19.398)	$p < 0.001$
Differentiation	1.670 (0.841–3.315)	$p = 0.143$		
Metastasis	0.153 (0.055–0.437)	$p < 0.001$	0.143 (0.050–0.404)	$p < 0.001$
Size	1.422 (0.563–3.590)	$p = 0.457$		
Gross type	0.716 (0.294–1.745)	$p = 0.457$		
Cystatin M expression	4.802 (1.420–16.244)	$p = 0.012$	6.693 (2.066–21.688)	$p = 0.002$

We then determined the cystatin M CpG island methylation status of a panel of four human gastric cancer cell lines by bisulfite genomic sequencing of multiple clones. Similarly, cystatin M CpG island hypermethylation was found in all gastric cancer cell lines tested. We examined a further link between cystatin M CpG island hypermethylation and its gene silencing by the treatment of these cancer cell lines with 5-Aza-dC (a DNA demethylating agent). After the treatment of 5-Aza-dC, the expression of cystatin M mRNA was restored. The clinical significance of cystatin M downregulation was also addressed in this study. The results indicated that the hypermethylation of cystatin M was significantly associated with many clinicopathologic characteristics of gastric cancers, including distant metastasis and metastasis. In this study, we also demonstrated that downregulation of cystatin M was significantly associated with poor prognosis of gastric cancers ($p = 0.020$).

Cystatin M is located in the chromosomal region 11q13, which is subject to amplification or LOH (loss of heterozygosity) in several cancers, such as gastric cancer, gastric neuroendocrine tumors, cervical cancer [20–24]. Epigenetic silencing due to DNA hypermethylation often leads to inactivation of the wildtype allele at sites of LOH that introduces one hit in the well-known Knudson's model for tumorigenesis that accounts for loss-of-function of tumor suppressor genes. It was reported that LOH at 11q13 occurs in gastric cancers, suggesting that LOH is a potential mechanism for loss of cystatin M in gastric cancers [20]. No deletions or structural rearrangements of cystatin M have been characterized, suggesting that loss of gene expression maybe the result of transcriptional silencing in breast cancer cells.

In summary, we document that the cystatin M gene is targeted for epigenetic silencing in human gastric cancer. We observed that hypermethylation of the cystatin M gene occurs in 55% of the primary gastric tumors investigated. 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 gastric malignancies.

Competing interests

The author(s) declare that they have no competing interests.

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