Cystatin 2 leads to a worse prognosis in patients with gastric cancer

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Despite the amazing progress in the treatment of gastric cancer (GC), it is still the third leading cause of cancer death in the world. This study explored the key genes that are related to the prognosis and pathogenesis of GC. Data from the cancer genome atlas (TCGA) and Oncomine were applied to evaluate the expression of cystatin 2 (CST2) in GC samples. Kaplan-Meier plotter was carried out to detect the overall survival of GC patients with different expression levels of CST2. Gene Set Enrichment Analysis (GSEA) was carried out to investigate the functions and pathways connected with CST2 expression. Quantitative real-time polymerase chain reaction (qPCR) and Western blot assays were used to assess CST2 expression. The biological properties of GC cells were assessed with the support of cell proliferation and Transwell assays. Important proteins involved in the regulation of CST2 in GC cell behaviors were evaluated by Western blot. Through analysis of the database, we found that CST2 expression was significantly upregulated in GC samples and actively related to GC patients’ poor outcomes. Importantly, the analysis of GSEA showed that GST2 expression was closely connected with the proliferation and migration of cells, as well as the TGF-β1 signaling pathway. In addition, biological assays illustrated that over-expression of CST2 strengthened the activity and metastasis of GC cells. After the upregulation of CST2, the expression of cyclin D1, N-cadherin, vimentin, TGF-β1, and Smad4 increased, and E-cadherin expression decreased. Our findings revealed that over-expression of CST2 enhanced the growth, migration, and invasion of GC cells through modulating the epithelial-mesenchymal transition (EMT) and TGF-β1 signaling pathway, affording a possible biomarker for the treatment of GC.

Gastric cancer (GC), one of the most common malignant tumors worldwide, has a complex onset, and is the result of the combined action of internal and external causes (1). Although the incidence of GC is decreasing, it still accounts for more than 1 million newly diagnosed cases and 850,000 deaths worldwide each year (2). The high mortality rate is primarily the result of the late manifestation; the early stages of GC are asymptomatic or have nonspecific symptoms. The survival rate at diagnosis relies on the phase of GC (3). While surgery is currently the primary treatment option, more than 50% of patients still relapse after

Key words: gastric cancer; CST2; cyclin D1; E-cadherin; TGF-β1; Smad4
radical resection. Excellent screening methods for early detection are the best approach to decreasing the mortality rate. Endoscopy is uncommon in the community due to its side effects, including perforation, aspiration pneumonia, and bleeding (4). Therefore, there is an urgent need to find biomarkers for the early detection of GC.

Cystatin 2 (CST2) is an endogenous protease inhibitor and belongs to the superfamily of cystatins. CST2 plays a crucial role in epithelial barrier and immunomodulatory processes (5). A previous study revealed that CST2 might be as a crucial factor in the relapse of eosinophilic chronic rhinosinusitis and allergic rhinitis through the interplay with several factors, including infection, fibroblasts, and the suppression of allergen-related histamine release (6). In addition, CST2 was highly upregulated in subjects with both mild and moderate asthma compared with healthy subjects (7). Interestingly, CST2 was shown to be overexpressed in patients with hepatocellular carcinoma (8). Through analysis of the TCGA database, Wang et al. revealed that CST2 was highly expressed in GC (9). Although CST2 was discovered more than 30 years ago, research on its function has been limited (10), especially in gastric cancer; therefore, further exploration into the effects of CST2 in GC is urgent.

Our results revealed that CST2 was profoundly upregulated in the GC samples and was connected with poor prognosis in GC patients. Importantly, CST2 upregulation strengthened the viability and aggressiveness of the GC cells by elevating the progression of the epithelial-mesenchymal transition (EMT) and the TGF-β1 signaling pathway. Our findings offer a potential novel biomarker for the treatment and prognosis of gastric cancer and revealed the underlying mechanism of CST2 in GC development.

MATERIALS AND METHODS

Data acquisition and analysis

Data from the TCGA (https://cancergenome.nih.gov/) dataset, which included 375 GC tumor samples and 32 normal samples, were used to assess the expression of CST2 in GC. Data from the Oncomine (http://www.oncomine.org) database, which covered 26 cases of GC tissues and 31 cases of normal samples, were applied to detect CST2 expression in GC. A total of 324 cases of GC samples with complete clinical data were used to analyze the correlation between CST2 expression and clinical features. Analysis of the 208555_x_at database by the Kaplan-Meier (KM) plotter revealed that the overall survival and the first progression and post-progression survival differed among GC patients with different expression of CST2. Gene Set Enrichment Analysis (GSEA) was used to explore the functions and pathways related to CST2 expression.

Cell culture and treatment

Human GC cell lines AGS, SGC-7901, and BGC-823 were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China), and the normal cell GSE-1 was acquired from ATCC (USA). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C, 5% CO₂.

si-CST2#1 (5’-GAGTATAACAAGGCCACTGAA-3’), si-CST2#2 (5’-TAATCGAGGGTGGCATCTATG-3’), si-con (5’-CGAACUCACUGGUCUGACC-3’), pcDNA3.1-CST2, and pcDNA3.1 were commercially provided by Sangon (Shanghai, China) and transfected into GC cells with the support of Lipofectamine 2000 (Invitrogen, USA) to regulate CST2 expression.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated from GC cells using Trizol (Invitrogen, USA) and then reverse transcribed into cDNA using a GoScript™ RT kit (Promega Corporation). qPCR was carried out utilizing SYBR Fast qPCR mix (Invitrogen, USA) to evaluate the CST2 expression level. The qPCR program was run using the following steps: 95°C for 5 min; 40 cycles, including 9°C for 30 sec, 60°C for 45 sec, and 72°C for 1 min and with extension at 72°C for 10 min. The relative expression level of CST2 was evaluated via the 2⁻ΔΔCT method and normalized to GAPDH. All sequences used are shown below:

CST2: forward: 5’-CTCATCAGCCACTGGAAAGGCA-3’, reverse: 5’-GACTCGTGAGGCTGACCTGAAAC-3’;
GAPDH: forward: 5’-TGTGTCCGTCGTGGATCTGA-3’, reverse: 5’-CCTGCTTCACCACCTTCTTGA-3’.
Cultured GC cells were lysed in radio immuno precipitation assay (RIPA) buffer. Proteins were isolated on a 12.5% SDS-PAGE and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, the membrane was incubated at 4°C overnight with primary antibodies, including anti-cyclin D1 (1:500), E-cadherin (1:1000), N-cadherin (1:1000), vimentin (1:1000), TGF-β1 (1:1000), and Smad4 (1:1000). After rinsing with TBST, the membrane was incubated with secondary antibody at room temperature for 1.5 h. Visualization was performed using ECL reagents and developed on a film.

Western blot

Cell proliferation analyses

The proliferation of GC cells was examined with the help of the cell counting kit-8 (CCK-8). Firstly, the treated cells were seeded in 96-well plates and cultured under the above condition. The activity of the cells was calculated every 24 h in line with the supplier’s recommendations. Before each test, 15 μL of CCK-8 reagent was loaded into the well and incubated with the cells for 1.5 h at 37°C in an incubator. Afterward, the optical density was measured at a wavelength of 450 nm, and GraphPad Prism 6.0 was used to draw the proliferation curve.

Cell invasion and migration assays

Transwell chambers were applied to evaluate the invasion and migration abilities of the GC cells. For the invasion assay, chambers were pre-coated with matrigel matrix (RPMI 1640: matrigel matrix=6:1). A total of 5,000 cells were seeded in the top well with serum-free medium, while complete medium supplemented with 10% FBS was loaded in the lower well. After a 24-h incubation, the cells in the top well were obliterated lightly with a cotton swab. The cells that had invaded the bottom surface of the membrane were fixed with paraformaldehyde and stained with crystal violet. The invading cells were counted under a light microscope. The steps in the migration assay were similar to the invasion experiment, except that matrigel was not required.

Statistical analysis

Results were processed with SPSS software (version 22.0; SPSS, Inc.). The survival rates of GC patients were assessed under the support of the Kaplan-Meier (KM) plotter and the log-rank test was used for comparison. Numerical data are shown as the mean ± standard deviation (SD). Statistical comparisons between the two groups of normalized values were analyzed utilizing the Student’s t-test. Statistical comparisons among multiple groups of normalized data were analyzed using one-way analysis of variance followed by Dunnett’s post hoc test. P-value less than 0.05 indicated a statistically significant difference.

RESULTS

High expression of CST2 correlated positively with a poor prognosis of GC patients

Firstly, we evaluated the expression of CST2 in GC samples based on the data from the TCGA and Oncomine databases. The data from TCGA containing 375 tumor samples and 32 normal samples indicated that CST2 was highly expressed in GC tissues compared with normal tissues (Fig. 1A, P<0.0001). Consistent with the results from TCGA, CST2 expression in GC tissues from the Oncomine database was also profoundly upregulated (Fig. 1B, P<0.0001). By KM plotter analysis, we discovered that the overall survival of GC patients was poor in the group with high CST2 expression (Fig. 1C, P=0.026). Analysis of the 208555_x_at database by the KM plotter revealed that the overall survival, first progression, and post-progression survival of GC patients in the high CST2 expression group were lower than the low expression group (Fig. 1 D–F, P<0.01). The clinical correlation analysis showed that CST2 expression was connected with the grade and lymph node metastasis in patients with GC (Table I). The findings illustrated that high expression of CST2 appeared in patients with GC, and was positively correlated with GC patients’ poor prognosis.

Identification of the function and pathways closely related to CST2 expression

To investigate the function of CST2 in GC, GSEA was used to explore the functions and pathways related to CST2 expression. The information in Fig. 2 shows that CST2 was involved in the processes of cell migration (2A) and proliferation (2B). In addition, the expression of CST2 was closely associated with
cytokine receptor interaction (2C) and the TGF-β signaling pathway (2D). These findings provided direction for our following research.

CST2 expression correlated positively with the activity and aggressiveness of GC cells. Based on the analysis from GSEA and the literature review, we discovered that CST2 was closely related to cell proliferation and migration. We assessed the expression of CST2 in the GC cell lines. The PCR result presented that the expression of CST2 was elevated in the GC cell line compared with normal cell GSE-1 (Fig. 3A, P<0.01). To discover the function of CST2 in GC, we employed AGS and SGC-7901 cells for gain and loss-of-function assays due to their significant difference in the expression of CST2. Firstly, si-CST2 and pcDNA3.1-CST2 were employed to regulate CST2 expression in GC cells. As Fig. 3B–C shows, both si-CST2#1 and si-CST2#2 were used to knock down CST2 expression in AGS cells; the interference with si-CST2#2 was the most effective. Therefore, in the subsequent experiments, si-CST2#2

Table I. Relationship between CST2 expression and clinical pathological characteristics of patients with gastric cancer.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Expression of CST2</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>Low</td>
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<tr>
<td>Age</td>
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<tr>
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<td>M1</td>
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*P<0.05; T: tumor; N: lymph nodes; M: metastasis. Data from the TCGA database.
CST2 expression was related to cell proliferation. A) CST expression was related to cell migration. B) CST expression was connected with cell proliferation. C) CST expression was associated with cytokine-cytokine receptor interaction. D) CST expression was related to the TGF-β1 signaling pathway.

was selected for interference-related experiments. In addition, the expression of CST2 in SGC-7901 cells was significantly upregulated after treatment with pcDNA3.1-CST2 (Fig. 4 A–B, P<0.01). Next, the data from the CCK-8 and Transwell assays revealed that depletion of CST2 reduced the activity of AGS cells, as well as the number of invaded and migrated AGS cells (Fig. 4 D–F, P<0.01). Upregulation of CST2 increased the activity of SGC-7901 cells, as well as the number of invaded and migrated SGC-7901 cells, compared with the control group (Fig. 3 C–E, P<0.01). These results suggested that the upregulation of CST2 enhanced the growth and metastasis of the GC cells.

**CST2 expression regulated the biological behavior of the GC cells by affecting the proliferation/invasion/TGF-β signaling**

According to the analysis from GSEA, we discovered that CST2 was not only related to proliferation and invasion but also closely connected with the TGF-β1 signaling pathway. Therefore, we detected the protein expression of cyclin D1, E-cadherin, N-cadherin, vimentin, TGF-β1, and Smad4 in GC cells with different CST2 treatments. After silencing CST2, the expression of cyclin D1, N-cadherin, vimentin, TGF-β1, and Smad4 were reduced, while E-cadherin expression was increased. These phenomena demonstrated that depletion of CST2 attenuated the growth, invasive, and migratory abilities of AGS cells via blocking the EMT/TGF-β1 signaling pathway. In SGC-7901 cells, after treatment with pcDNA3.1-CST2, the expression levels of cyclin D1, N-cadherin, vimentin, TGF-β1, and Smad4 were elevated, and E-cadherin expression was decreased. These data suggested that overexpression of CST2 strengthened the proliferative, invasive, and
Fig. 3. Depletion of CST2 limited the activity and aggressiveness of AGS cells. A) The expression of CST2 was obviously elevated in the GC cell lines in contrast with the normal cell GSE-1. B–C) The protein expression of CST2 was profoundly decreased after si-CST2 treatment. D) The activity of AGS cells was reduced with CST2 depletion. E–F) The invasion and migration number of AGS cells were lower with si-CST2 treatment. **P<0.01 vs control.

Fig. 4. Upregulation of CST2 increased the growth and metastasis of SGC-7901 cells. A–B) The protein expression of CST2 was increased after pcDNA3.1-CST2 treatment. C) The activity of SGC-7901 cells was elevated with CST2 upregulation. D–E) The number of invaded and migrated SGC-7901 cells was increased with pcDNA3.1-CST2 treatment. **P<0.01 vs control.
amino acid substitution in one of the most conserved domains is in charge of the cysteine proteinase inhibitory activity (11). Moreover, research has revealed that high expression of salivary cystatin CST2 promotes bone metastasis in vivo (12). To date, little research has concentrated on the function of CST2 in any type of tumor, apart from the discovery that CST2 responded to the anti-growth activity of triptolide in ovarian cancer cells (13), and that CST2 is involved in the development of breast cancer (14). In our study, by bioinformatics analysis, CST2 was observably upregulated in gastric cancer and associated with worse outcomes in GC patients. It was also related to cell proliferation, invasion, and the TGF-β1 pathway. Consistent with the bioinformatics analysis, experiments in vitro confirmed that GST2 was highly expressed in GC cell lines and accelerated the proliferation and metastasis of the GC cells.

EMT is regarded as one of the crucial processes in GC cell invasion and metastasis (15). During EMT, epithelial cells drop their characteristic marker E-cadherin and acquire mesenchymal markers, including N-cadherin and vimentin (16). To investigate whether the function of GST2 on GC is related to the EMT process, we detected EMT-related proteins after up- and down-regulation of CST2. The data showed that E-cadherin expression was elevated, and the expressions of N-cadherin and vimentin were reduced after CST2 depletion. Inversely, upregulation of CST2 attenuated E-cadherin expression and elevated the expression of N-cadherin and vimentin. These findings suggest that CST2 increases the biological characteristics of GC cells by promoting EMT.

DISCUSSION

In the present study, we provide evidence that CST2 is highly expressed in human GC cells. The bioinformatics analysis showed that CST2 expression is closely related to proliferation and migration, as well as a poor prognosis of GC patients. In addition, consistent with the results from the bioinformatics research, we also found that CST2 elevates the growth and metastasis capacities of GC cells partly through mediating the EMT and TGF-β1 signaling pathway.

The CST2 gene, belonging to the CST family, has been reported as a specific allele, indicating that
we examined the expression of TGF-β1 and Smad4 after CST2 aberrant expression. The findings suggest that the expression trends of TGF-β1 and Smad4 were consistent with that of CST2, speculating that CST2 has a positive regulatory effect on the TGF-β/Smad4 signaling pathway.

While our study has made some findings, the results are not yet applicable in clinical diagnosis. More animal studies are needed to confirm our conclusions.

In short, our outcomes state that high expression of CST2 was actively associated with a worse outcome in GC patients. Moreover, upregulation of CST2 promoted the proliferation, invasion, and migration abilities by activating EMT and the TGF-β1 signaling pathway.

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