Cystatin SN as a potential marker for gastric cancer and promotes gastric cancer progression

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Abstract

Background

Cystatin SN (cystatin 1, CST1) regulates the progression of many cancers and serves as a diagnostic marker; however, its significance in gastric cancer has not been fully elucidated.

Methods

We first analyzed the expression levels of CST1 in gastric cancer dataset in oncomine and UALCAN databases. We next analyzed the relationship between CST1 and prognosis of gastric cancer patients in KM Plotter database and downloaded TCGA GC data to verify this result. We further divided GC patients into two groups according to the level of CST1 expression for GSEA enrichment analysis. Finally, we validated the role of CST1 in gastric cancer by in vitro functional assays.

Results

We found that CST1 expression levels in gastric cancer tissues were significantly higher than those in normal control tissues and patients with high CST1 expression had a poorer prognosis. GSEA enrichment analysis showed that the main KEGG signaling pathways enriched in the CST1 high expression group were Coagulation, Epithelial-Mesenchymal-Transition, Complement, Apoptosis, Glycolysis. In vitro functional assay confirmed that inhibition of CST1 significantly inhibited the proliferation, colony-formation, migration and invasion ability of gastric cancer cells.

Conclusions

Our study shows that the expression level of CST1 correlates with the development of gastric cancer, and CST1 may be used as a diagnostic marker for poor prognosis of gastric cancer.

Background

Global cancer statistics analysis in 2018 showed that gastric cancer has more than 1 million new cases and about 783,000 deaths per year, making it the fifth most prevalent malignancy and the third most deadly malignancy worldwide(1, 2). Gastric cancer is insidious and difficult to diagnose at an early stage, and some patients are already at an advanced stage when diagnosed, losing the opportunity for radical surgery, while even the recurrence rate is high after surgical resection, resulting in a poor overall prognosis for gastric cancer patients. Finding diagnostic and therapeutic related tumor markers with high specificity and sensitivity is of great significance to improve the survival rate and prolong the survival period of gastric cancer patients.

CST1 is a cysteine protease inhibitor that belongs to the cysteine superfamily(3, 4). CST1 can inhibit the proteolytic activities of cysteine proteases and thus regulate tumor progression. Several studies have been conducted to confirm the role of CST1 or CST1 family members in tumor progression, for example,

Inhibition of CST1 expression inhibits carcinogenesis and stem cell properties of papillary thyroid cancer, The mTOR/HIF1 α / α -Enolase axis promotes thyroid cancer progression by regulating the expression of CST1(5, 6). Constructed an ECM-related signature in esophageal squamous cell carcinoma found that CST1 could be an independent prognostic factor and a potential biomarker of chemotherapy response in patients with Oesophageal Squamous Cell Carcinoma(7). Elevated expression levels of CST1 in breast cancer are closely associated with the progression and prognosis of breast cancer(8). A study confirmed that CST1 promotes invasion of gastric cancer cells through activation of Wnt Pathway(9). Upregulation of CST1 promotes hepatocellular carcinoma progression and predicts a poor prognosis(10). Let-7d inhibited colorectal carcinogenesis through the CST1/p65 pathway(11).

However, the expression level of CST1 in gastric cancer, the changes of the gene and whether it can be used as a prognostic molecular marker in gastric cancer have not been reported yet. We used bioinformatics analysis to elucidate the significance of CST1 in gastric cancer at several molecular levels.

Materials And Methods

Oncomine database extracts data

Oncomine database(<https://www.oncomine.org/>) is a platform based on a gene chip database and integrated data mining. In this database, screening and mining data conditions can be set according to their own needs. Our screening criteria are as follows: 1) "cancer type:gastric cancer"; 2) "gene:CST1"; 3) "Data Type:mRNA"; 4) "Analysis Type:Cancer vs Normal Analysis"; 5) the setting condition of the critical value is: (P value < 1E -4, fold change > 2, and gene rank=top 10%).

Ualcan and TIMER database Analysis

Ualcan(<http://ualcan.path.uab.edu/index.html>) is an online website for analyzing TCGA data, which can detect gene expression based on different factors in various cancers and normal tissues, such as grade, tumor stage, age, gender, race, and other pathological features. TIMER(<http://timer.cistrome.org/>) allows users to study the differential expression between tumor and adjacent normal tissues for any gene of interest across all TCGA tumors. We used the ualcan and TIMER database to detect the difference in CST1 expression in different types of gastric cancer.

Selection of gastric cancer samples in TCGA

Download mRNA expression information and clinical information of gastric cancer patients from TCGA database (<https://cancergenome.nih.gov/>). The main analysis was the difference in expression of cancer and paracancer CST1 in gastric cancer patients containing complete clinical information. A total of 354 GC patients and 30 normal controls were finally enrolled in the study.

GSEA Analysis of the Hub Genes

We divided GC samples into high-expression and low-expression groups based on the median expression levels of CST1. The effects of the expression of CST1 on multiple gene sets were analyzed in the relevant KEGG pathways using the molecular signatures database (MSigDB) (c2. cp.kegg.all.v7.1. symbols.gmt). The permutation of each analysis was 1,000 times. FDR q-value <0.25, NOM p-value <0.05, and |Normalized enrichment score (NES)| > 1 were used to identify significant differences.

Kaplan-Meier Plotter Database Analysis

The prognostic value of CST1, CST2 and CST4 expression including the overall survival (OS), first progression survival (FP), disease-free survival (DFS), and post progression survival (PPS) was evaluated in Kaplan-Meier Plotter (<http://kmplot.com/analysis/>).

GeneMANIA Database Analysis

We analyzed the genes that interacted most significantly with CST1 on GeneMANIA (<http://genemania.org/>), following the website database guidelines.

cBioPortal database Analysis

A total of 1460 patients with GC was obtained from cBioPortal database (<https://www.cbioportal.org/>) to analyze the genetic alteration of CST1. The mutation frequency and sites of CST1 in GC can be found in the database.

Antibodies and reagents

CST1(16025-1-AP) was purchased from Proteintech. β -actin(A01010) was purchased from Abbkine. The small interfering RNA constructs against CST1 used in this study was from GenePharma. CCK-8 and 0.01% Crystal Violet Staining Solution was purchased from Servicebio. PMSF was purchased from Beyotime Biotechnology.

Western blot

Cells cultured were harvested in 200ul RIPA buffer (Beyotime Biotechnology), The supernatant was obtained by centrifugation at 13000 rpm, 10min, and the protein was cooked at 96°C for 10 min. Protein samples were boiled and separated on 10% SDS-PAGE gels followed by transferring to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk in TBST for 30 min, the membranes were incubated with specific primary antibodies overnight at 4 °C. Finally, antibody-bound proteins were detected by chemiluminescence (BIORAD, USA).

Cell culture

NCI-N87 and AGS cells purchased from China Center for Type Culture Collection (CCTCC). MGC803 cells and SGC7901 cells were obtained from the Laboratory of Medical Translation Platform, Academy of Medical Sciences, Zhengzhou University. The cells were grown in 1640 and F12K, supplemented with 10%

fetal bovine serum (FBS) (Biological Industries) and 0.5% antibiotics, at 37°C with 5% CO₂ incubator. The 1640 was purchased from Servicebio(wuhan). The F12K was purchased from Gbico.

Cell proliferation assay

CCK-8 assay analyzes the effect of silencing CST1 on the proliferative capacity of gastric cancer cells. NCI-N87 cells and AGS cells were seeded into 96-well plates at a density of 5×10^3 cells/well, and incubated for 24 hours, 48 hours and 72 hours, respectively. Add CCK-8 reagent at a ratio of 1:10 each well. Absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Each experiment was performed in triplicate.

Colony-formation assay

NCI-N87 cells and AGS cells (8×10^3 /well) in a 6-well plate cultured in 1ml of 0.33% BME (Eagle basal medium, Sigma-Aldrich Corp.) agar (Sigma-Aldrich Corp.) The cells were maintained in a 37°C, 5% CO₂ incubator for 7-10 days, their colonies were stained with Nitrotertrazolium Blue chloride (NBT) overnight at 37°C then were observed by microscopy, and statistical analysis was performed.

Transwell assay

Cell-invasion and migration ability was evaluated using the transwell assay. Next, 200 µL of serum-free 1640 and F12K medium at a density of 5×10^5 cells/mL suspension was added into the upper layer of the transwell chamber (Corning) covered with Matrigel (BD Biosciences, San Jose, CA, USA), while 600 µL of 20% FBS medium was added to the lower chamber. The transwell chamber was cultured for 48 h. Migration assay with 5×10^4 NCI-N87 cells and AGS cells. after 12 h, the cells were fixed with 4% Paraformaldehyde for 30 min. Next, cells were stained with 0.01% crystal violet solution for 30 min, PBS wash 3 times, 5 min each time. Wipe off the upper layer of cells with a cotton swab. The invaded cells were observed and images taken under an inverted microscope (Leica), and statistical analysis was performed.

Statistical analysis

The expression of CST1 and other clinicopathological features were analyzed by student's, t test, χ^2 test with Spss21.0 software. Kaplan-Meier method and log-rank, t-test were used to make OS curves.

Result

Expression Analysis of CST1

CST1 are expressed in a wide range of cancers according to The Cancer Genome Atlas (TCGA) UALCAN database. CST1 expression is lowest in the Brain lower grade glioma (LGG) dataset (median value 0) and highest in the Pancreatic adenocarcinoma (PAAD) dataset (median value 7.181), CST1 expression in gastric cancer(GC) is second only to PAAD(Fig1A).compared with normal tissues, most tumor types show

significantly higher expression of CST1, and is most evident in GC (Figure 1B). Next, we used OncoPrint database data to analyze the expression level of CST1 in cancer. The results showed that there were 388 studies involving in cancer, 38 of them were statistically different, and 37 of them had increased expression of CST1 in tumors (Figure 2A). The results of 4 studies on gastric cancer suggested that the expression of CST1 in cancer tissues was significantly higher than that in normal tissues. There was significant difference in Cho gastric (Fig 2B, $p < 0.0001$), Cui gastric (Figure 2C, $p < 0.0001$), Derrico gastric (Fig 2D, $p < 0.005$), and Wang gastric (Fig 2E, $p < 0.0001$).

The expression of CST1 in different types of gastric cancer

We used a UALCAN online database to detect the expression level of CST1 in the classification of different influencing factors of gastric cancer. The results suggested that the expression of CST1 in primary tumor vs normal (Figure 3A), age vs normal (Figure 3B), gender vs normal (Figure 3C), grade vs normal (Fig 3D), stage vs normal (Figure 3E), histological subtypes vs normal (Fig 3F) was higher than that in normal tissues ($p < 0.05$).

Promoter methylation levels of CST1 in different types of gastric cancer

Next, we used a UALCAN online database to detect the promoter methylation levels of CST1 in the classification of different influencing factors of gastric cancer. The results suggested that the promoter methylation levels of CST1 in primary tumor vs normal (Figure 4A), gender vs normal (Figure 4B), age vs normal (Figure 4C), grade vs normal (Figure 4D), TNM vs normal (Figure 4E), stage vs normal (Figure 4F) was lower than that in normal tissues ($p < 0.05$).

Correlation Between the Expression of CST1 and Prognosis

We employed the KM plotter database to explore the effect of CST1 expression on the survival of patients of GC, we detected a significant correlation between prognosis and CST1 expression (Figure 5). We revealed that a higher CST1 expression level was significantly related to a poorer prognosis in GC (OS, HR = 1.7, 95% CI = 1.42–2.02, $p = 2.9 \times 10^{-9}$; First progression survival (FPS), HR = 1.63, 95% CI = 1.32–2.01, $p = 3.9 \times 10^{-6}$; post progression survival (PPS), HR = 1.92, 95% CI = 1.51–2.43, $p = 4.4 \times 10^{-8}$) (Figures 5A, Figure 5B, Figure 5C).

CST1 expression and prognosis in TCGA GC dataset

The data of 354 patients with gastric cancer expressing CST1 were downloaded from the TCGA database, and the expression of CST1 in patients with gastric cancer was analyzed. CST1 expression level was most significantly upregulated (Fold-change = 20.94, $p < 0.001$) (Figure 6A, Figure 6B). The patients were divided into two groups according to the median expression of CST1 (35.107): high expression of CST1 ($n = 177$) and low expression of FBXO22 ($n = 177$). The clinicopathological features of patients with gastric cancer were shown in Table 1. The results showed that the expression of CST1 was positively correlated with the OS (Figure 6C).

Functional Annotations and Predicted Signaling Pathways

To identify the CST1-related pathways activated in GC, we conducted GSEA between low and high CST1 expression datasets. Significant differences (false-discovery rate <0.25 , $p < 0.05$) in the enrichment of the Molecular Signature database Collection are shown in Figure 7. The results showed that CST1 was mainly involved in Coagulation(Figure7A) and Epithelial-Mesenchymal-Transition(Figure7B), Complement(Figure7C), Apoptosis(Figure7D), Glycolysis(Figure7E). These results showed that high expression of CST1 was closely associated with malignancy in GC.

Enrichment Analysis of CST1-Related Genes and CST1,CST2 and CST4 Genetic Alterations in Patients with GC

We investigated the interaction network for CST1 by the GeneMANIA database. Figure 8A shows the top 20 genes and their relationship with CST1. Among them, CST2 and CST4 of the same family of CST1 are extremely correlated with it. Next, we investigated the frequency of alterations CST1,CST2 and CST4 in five groups of GC cases via the cBioPortal web resource(Figure8A). Overall, 1460 samples from TCGA GC database were studied (Figure8B,Figure8C), and the mutation rate was found to be low (1.36–3.16%) in Gastric Cancer (OncoSG, 2018),Stomach Adenocarcinoma (TCGA, Nature 2014),Stomach Adenocarcinoma (TCGA, Firehose Legacy),Stomach Adenocarcinoma (TCGA, PanCancer Atlas),Stomach Adenocarcinoma (Pfizer and UHK, Nat Genet 2014). The type as well as the location of distinct mutations are indicated in Figure8D.

KM plotter database analysis of the relationship between CST2 and CST4 expression and prognosis in gastric cancer

We employed the KM plotter database to explore the effect of CST2 and CST4 expression on the survival of patients of GC, we detected a significant correlation between prognosis and CST1 expression (Figure9). We revealed that a higher CST2 expression level was significantly related to a poorer prognosis in GC (OS, HR = 1.82, 95% CI = 1.53–2.18, $p = 1.8e-11$; First progression survival (FPS), HR = 1.84, 95% CI = 1.5–2.25, $p = 2.7e-09$; post progression survival (PPS), HR = 2.07, 95% CI = 1.65–2.61, $p = 2.2e-10$;) (Figures9A, Figure9B, Figure9C). Higher CST4 expression level was significantly related to a poorer prognosis in GC (OS, HR = 1.52, 95% CI = 1.28–1.8, $p = 1.5e-06$; First progression survival (FPS), HR = 1.54, 95% CI = 1.24–1.9, $p = 5.9e-05$; post progression survival (PPS), HR = 1.56, 95% CI = 1.23–1.99, $p = 0.00025$;) (Figure9D, Figure9E, Figure9F).

Knockdown of CST1 expression inhibited the proliferation, colony formation, migration and invasion ability of gastric cancer cells.

We first examined the protein expression level of CST1 in four gastric cancer cell lines and found that CST1 was expressed at a higher level in AGS and NCI-N87 than in MGC803 and SGC7901 (Figure10A). Secondly, we knocked down the expression of CST1 in AGS and NCI-N87 cells (Figure10B). Anchorage-independent colony-formation assay showed that knockdown of CST1 significantly inhibited the

clonogenic ability of AGS and NCI-N87 cells (Figure10C, Figure10D). We then used Transwell assay to detect the effect of CST1 on the migration and invasion ability of gastric cancer cells, and found that inhibition of CST1 expression significantly inhibited the migration and invasion ability of AGS and NCI-N87 cells (Figure11A, Figure11B).

Discussion

Gastric cancer is one of the most malignant cancers worldwide and a large number of patients lose their lives every year. The reason for the high mortality rate of gastric cancer is the lack of early markers.

The molecular role of CST1 in several cancers has been elucidated, for example ,CST1 acts as an oncogene in ER + breast cancer, and CST1 contributes to cancer development by regulating the ER α /PI3K/AKT/ER α loopback pathway in ER + breast cancer(12).CST1 was confirmed as a prognostic marker for cholangiocarcinoma by weighted gene correlation network analysis(13, 14). CST1 family members can regulate metastasis in triple-negative breast cancer(15). We analyzed the differential genes by downloading TCGA GC data and found that CST1 was most significantly upregulated in gastric cancer. We analyzed the relationship between CST1 and gastric cancer prognosis and genetic alterations by Oncomine,Ualcan ,Kaplan-Meier Plotter and cBioPortal database, and clarified the role of CST1 as a prognostic biomarker in gastric cancer, gastric cancer patients with high CST1 expression have poor prognosis. This is consistent with previous studies that confirmed the role of CST1 as a proto-oncogenic molecule in gastric cancer(9). In contrast, our study focused on confirming the role of CST1 in gastric cancer through TCGA cancer database and analyzed the expression and gene mutation of CST1 in gastric cancer.CST1 is an independent prognostic factor for non-small cell lung cancer (NSCLC) patients(16).CST1 is a prognostic marker and therapeutic target for colorectal cancer patients(17). In vitro functional assay confirmed that inhibition of CST1 significantly inhibited the proliferation, migration and invasion ability of gastric cancer cells.The above study illustrates that CST1 acts as a proto-oncogenic factor in several cancers, and through cancer database analysis we confirmed that CST1 is closely associated with poor prognosis in gastric cancer patients and can be used as a prognostic marker in gastric cancer, the only drawback is that we did not do molecular biology experiments to confirm the role of CST1 in gastric cancer.

Conclusions

In conclusion, CST1 was highly expressed in gastric cancer and patients with high CST1 expression had a poorer prognosis, and CST1 was found to promote the proliferation and metastatic ability of gastric cancer cells. Our data confirm the prognostic value of CST1 as a biomarker for gastric cancer.

Abbreviations

GC Gastric cancer

EMT Epithelial–mesenchymal transition

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Raw data of GC cohort was acquired from TCGA portal (<https://portal.gdc.cancer.gov/>). Analyzed data is available within the materials and results.

Competing interests

All authors declare there are no any competing interests with regard to the publication of the study.

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No.

Authors' Contributions:

XTT and FWJ collected data,performed data analysis and wrote the manuscript. XTT, FWJ, LHY, JST, GXX and DYW cultured cells and performed functional experiments,FWJ and YBZ performed statistical analysis. XTT contributed to data interpretation, and manuscript revision.

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Contributor Information

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Tables

Table1. Patient characteristics and associations with CST1 expression in TCGA data

Clinical characteristics	patients 354		CST1	
	Number	%	High	low
Age				
>60	237	67	126	111
≤60	117	33	51	66
Gender				
Male	224	63	113	111
Female	130	37	64	66
Stage				
NA	17	5	13	4
Stagel	42	12	19	23
Stagell	112	32	51	61
Stagelll	147	42	77	70
StagelV	36	9	18	18
TNM stage				
T				
TX	8	2	7	1
T1	16	5	4	12
T2	75	21	36	39
T3	163	46	85	78
T4	92	26	45	47
N				
NX	16	5	11	5
N0	105	30	44	61
N1	95	27	43	52
N2	64	18	35	29
N3	74	20	44	30
M				
MX	13	4	7	6
M0	317	90	159	158

Figures

A

B

Figure 1

Expression of CST1 in cancer. (A) Expression of CST1 among various cancer types. (B) Comparison of the expression of CST1 between tumor and normal tissues. The images and significance are from ULCAN database.

Figure 2

Expression of CST1 in oncomine database. (A) The number of data sets presented in this graph belongs to the over-expression (red) or down-expression (blue) of target genes (cancer and normal tissues), which is of statistical significance. (B–E) The CST1 mRNA levels were significantly higher in GC than in Normal according to the Cui gastric Statistics, Derrico gastric Statistics, Cho gastric Statistics and Wang gastric Statistics datasets.

Figure 3

Box plots showing CST1 expression levels in GC via the UALCAN database analysis. (A) Box plots show CST1 protein expression levels in normal tissues vs. GC tissues (B) in normal tissues vs. GC tissues in different age. (C) in normal tissues vs. GC tissues in different gender. (D) in normal tissues vs. GC tissues in different grade. (E) in normal tissues vs. GC tissues in different stage. (F) in normal tissues vs. GC tissues in different histological subtypes.

Figure 4

Box plots showing CST1 promoter methylation levels in GC via the UALCAN database analysis. (A) Box plots show CST1 protein expression levels in normal tissues vs. GC tissues (B) in normal tissues vs. GC tissues in different gender. (C) in normal tissues vs. GC tissues in different age. (D) in normal tissues vs. GC tissues in different grade. (E) in normal tissues vs. GC tissues in different TNM. (F) in normal tissues vs. GC tissues in different stage.

Figure 5

CST1 expressions were closely related to the prognosis of GC patients. (A) Overall survival rate was assessed in TCGA GC patients. (B) First progression survival rate was analyzed in TCGA GC patients. (C) Post progression survival rate was analyzed in TCGA GC patients. Abnormal expression and low expression were identified with the auto best cutoff.

Figure 6

CST1 expression and prognosis in the TCGA GC dataset. (A) Heat map of differential genes in TCGA GC dataset. (B) Expression levels of CST1 in GC tissue vs. normal tissue. (C) Survival differences between high and low CST1 expression in GC patients

Figure 7

The Gene Set Enrichment Analysis (GSEA) of the relationship between the expression level of CST1 in the TCGA GC dataset. The most involved significant hallmark pathways which were closely correlated with CST1 in GC obtained by GSEA. (A) Coagulation. (B) Epithelial-Mesenchymal-Transition. (C) Complement. (D) Apoptosis. (E) Glycolysis. NES: normalized enrichment score; FDR: false discovery rate.

Figure 8

Enrichment Analysis of CST1-Related Genes and CST1,CST2 and CST4 Genetic Alterations in Patients with GC (A) Using the GeneMANIA approach, top 20 CST1-correlated genes were analyzed. (B) Alteration frequency in GC. (C) Mutation types of CST1,CST2 and CST4. (D) Mutation sites of CST1.

Figure 9

CST2 and CST4 expressions were closely related to the prognosis of GC patients. (A) Overall survival rate of CST2 was assessed in TCGA GC patients. (B) First progression survival rate of CST2 was analyzed in TCGA GC patients. (C) Post progression survival rate of CST2 was analyzed in TCGA GC patients. (D) Overall survival rate of CST4 was assessed in TCGA GC patients. (E) First progression survival rate of CST4 was analyzed in TCGA GC patients. (F) Post progression survival rate of CST4 was analyzed in TCGA GC patients. Abnormal expression and low expression were identified with the auto best cutoff.

Figure 10

CST1 promotes the proliferation ability of gastric cancer cells.(A) Protein levels of CST1 in four gastric cancer cell lines.(B) Knockdown of CST1 expression in AGS and NCI-N87 cells.(C) Cell proliferation assay of AGS and NCI-N87 after knockdown of CST1.(D) Colony formation proliferation assays of AGS and NCI-N87 after knockdown of CST1.

Figure 11

CST1 promotes the migration and invasive ability of gastric cancer cells.

(A) Cell migration assays of AGS and NCI-N87 after knockdown of CST1.(B) Cell invasion assays of AGS and NCI-N87 after knockdown of CST1.