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Cathepsin F Knockdown Induces Proliferation and Inhibits Apoptosis in Gastric Cancer Cells

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Gastric cancer (GC) is one of the most common cancers in the world. The cathepsin F (CTSF) gene has recently been found to participate in the progression of several types of cancer. However, the clinical characteristics and function of CTSF in GC as well as its molecular mechanisms are not clear. Six GC cell lines and 44 paired adjacent noncancerous and GC tissue samples were used to assess CTSF expression by quantitative polymerase chain reaction (qPCR). We used lentivirus-mediated small hairpin RNA (Lenti-shRNA) against CTSF to knock down the expression of CTSF in GC cells. Western blot and qPCR were used to analyze the mRNA and related protein expression. The biological phenotypes of gastric cells were examined by cell proliferation and apoptosis assays. Microarray-based mRNA expression profile screening was also performed to evaluate the potential molecular pathways in which CTSF may be involved. The CTSF mRNA level was associated with tumor differentiation, depth of tumor invasion, and lymph node metastasis. Downregulation of CTSF expression efficiently inhibited apoptosis and promoted the proliferation of GC cells. Moreover, a total of 1,117 upregulated mRNAs and 1,143 downregulated mRNAs were identified as differentially expressed genes (DEGs). Further analysis identified the involvement of these mRNAs in cancer-related pathways and various other biological processes. Nine DEGs in cancer-related pathways and three downstream genes in the apoptosis pathway were validated by Western blot, which was mainly in agreement with the microarray data. To our knowledge, this is the first report investigating the effect of CTSF on the growth and apoptosis in GC cells and its clinical significance. The CTSF gene may function as a tumor suppressor in GC and may be a potential therapeutic target in the treatment of GC.

Key words: Gastric cancer (GC); Cathepsin F (CTSF); Proliferation; Apoptosis; Microarray

INTRODUCTION

Gastric cancer (GC) is a significant health issue worldwide and is the second most frequent cause of cancer-associated mortality[1]. The tumorigenesis and progression of GC is a multifactorial and multistage process, and thus an increased understanding of gene expression changes during carcinogenesis, particularly the identification of novel biomarkers for carcinoma diagnosis and novel therapeutic targets, will improve the diagnosis, treatment, and prevention of GC.

Cathepsins are a papain family of cysteine proteinases that represent a major component of the lysosomal proteolytic system. In general, cathepsins contain a signal sequence, followed by a propeptide, and then a catalytically active mature region[2]. Cathepsin F (CTSF), also known as CATSF/CLN13, is located on 11q13[3]. The CTSF proregion is very long and is unique within the papain family of cysteine proteases in that it contains an additional N-terminal segment predicted to share structural similarities with cysteine protease inhibitors in the cystatin superfamily. This cystatin-like domain contains some of the elements known to be important for inhibitory activity[4]. The CTSF gene, ubiquitously expressed, encodes a predicted protein of 484 amino acids that contains a 19-residue signal peptide. CTSF also contains five potential N-glycosylation sites, and it may be targeted to the endosomal/lysosomal compartment via the mannose 6-phosphate receptor pathway[5]. High levels of CTSF were detected in cervical cancer[6] in contrast to the low levels observed in different pediatric brain tumors[7]. The

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different expression of CTSF determines its distinct effects in human malignancies.

CTSF transcripts were also found in several cancer cell lines, suggesting that this enzyme could be involved in degradative processes during tumor progression[4]. Recently, Yang et al.[8] found that CTSF could serve as a novel serum biomarker, which is also a noninvasive diagnostic index for GC and may potentially be used as a predictor of overall GC survival rate. Until now, only a small amount of data have been available on CTSF, and the biological role of CTSF in the progress of GC is not thoroughly known.

In the present study, we investigated the effects of downregulated CTSF expression on the proliferation and apoptosis of GC cells and explored the underlying molecular mechanisms by mRNA expression profile screening.

MATERIALS AND METHODS

Cell Culture

Six human GC cell lines (SGC7901, BGC823, MGC803, HGC27, AGS, and MKN45) and one immortalized normal gastric cell line (GES1) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, P.R. China). These cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin–streptomycin (10,000 U/ml penicillin and 10,000 µg/ml streptomycin; SolarBio, Beijing) and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Tissue Samples

Human GC samples and nonmalignant gastric tissues, which were at least 5 cm away from the tumor samples, were collected from 44 patients who underwent a gastrectomy at the Department of Surgery, Shengjing Hospital, China Medical University (Shenyang, P.R. China) between January 2015 and March 2016. All GC cases were pathologically confirmed.

Lentiviral Production and Stable Cell Lines

CTSF Lenti-shRNA and negative control Lenti-shRNA (NC-shRNA) were purchased from Shanghai GeneChem Company (Shanghai, P.R. China). CTSF stable silencing cell lines were selected with puromycin and identified by Western blot in SGC7901, BGC823, and MKN45 cells.

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

The total RNA was extracted from GC cells, human GC tissues, and the adjacent noncancerous tissues from the same patients. Extraction was performed using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and reversely transcribed into cDNA using an Expand Reverse Transcriptase Kit (Takara, Dalian, P.R. China). The expression of CTSF mRNA was detected using qPCR with the following program: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. The reaction mixture contained 10 µl of SYBR Green (Takara), 0.4 µl of each primer, 2 µl of cDNA, and 7.2 µl of diethylpyrocarbonate (DEPC)-treated water. Real-time PCR was performed according to the protocol of SYBR Premix Ex Taq II Kit. Specific primer pairs in the experiment are listed in Table 1. Gene expression levels were calculated relative to the housekeeping gene GAPDH.

Western Blot

Total protein was extracted from cells in a lysate buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate, and phenylmethylsulfonyl fluoride (all from Beyotime Institute of Biotechnology, Shanghai, P.R. China). Each sample (60 µg) was electrophoresed in 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) by a BG-blotMiMi transfer machine (Baygene, Beijing, P.R. China). After blocking with 5% nonfat milk for 2 h at room temperature and then incubating with primary antibodies for CTSF (1:500 dilution; Proteintech, Wuhan, Hubei, P.R. China), MDM2 (1:500 dilution; Abcam, Cambridge, MA, USA), RALB (1:200 dilution; Abcam), PCNA (1:2,000 dilution; CST), Smad2 (1:300 dilution; Abcam), FAS (1:1,000 dilution; Abcam), CDK4 (1:1,000 dilution; CST), CDK6 (1:500 dilution; Abcam), BAX (1:1,000 dilution; Abcam), survivin (1:500 dilution; Abcam), Bid (1:1,000 dilution; CST), Bcl-2 (1:500 dilution; Abcam), C-IAP1 (1:1,000

Table 1. Primers Used in the Present Study

Name	Primer Sequence	Distribution	Annealing Temperature (°C)	Product Size (bp)	Extension Time (s)
CTSF	F: 5′-CCCAAAACCATCCAGACAA-3′	NM_003793.3545-704	55.27	160	40
	R: 5′-CGGGCTTCTTCCTTTGACT-3′		57.37		
GAPDH	F: 5′-TGACTTCAACAGCGACACCCA-3′	NM_002046.4273-393	62.18	121	40
	R: 5′-CACCCTGTTGCTGTAGCCAAA-3′		61.09		

dilution; CST), or GAPDH (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The next day, after incubation with a 1:5,000 dilution of secondary antibodies (Santa Cruz Biotechnology) at 37°C for 2 h and after washing, the immunoreactive protein bands were visualized using an electrochemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL, USA). The ratio between the optical density of the protein of interest and GAPDH was calculated as the relative content of the protein detected. Each experiment was repeated three times.

Cell Counting Kit-8 (CCK-8) Assay

The cell proliferative ability was evaluated by a CCK-8 assay (C0037; Beyotime Institute of Biotechnology). The cells were seeded into 96-well plates (1.5×10^3/well). Following culture, CCK-8 solution (10 µl/100 µl medium) was added to each well, and the cells were incubated for 3 h at 37°C. The absorbance was measured at 450 nm using a Synergy2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). The GC cells cultured in RPMI-1640 affected with NC-shRNA and without any infection were used as the controls. The assay was conducted in five replicate wells for each sample, and three parallel experiments were performed.

Apoptosis Analysis by Flow Cytometry (FCM)

To detect the apoptotic variation induced by the effect of CTSF knockdown, the Annexin-V–PE/7-AAD double-labeling kit (KeyGEN, Nanjing, P.R. China) was used. Briefly, cells ($1–2\times10^5$ cells/well) were collected and washed with cold PBS twice. Following the manufacturer's instructions, apoptotic cells were analyzed via FCM. Finally, cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, USA). A proportion of cells were divided into four groups as viable cells ($PE^-/7\text{-}AAD^-$), necrotic cells ($PE^-/7\text{-}AAD^+$), early apoptosis ($PE^+/7\text{-}AAD^-$), and late apoptosis ($PE^+/7\text{-}AAD^+$).

RNA Isolation and GeneChip Hybridization

Total RNA from SGC7901-Lenti-shRNA against CTSF cell lines and negative control SGC7901-Lenti-shRNA cell lines was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA purity was determined using an ultraviolet and visible spectrophotometer (UNIC, Shanghai, P.R. China). Samples with RNA purity were used for GeneChip hybridization and scanning. Labeling, hybridization, and staining of these samples were performed according to the Eukaryotic Target Preparation protocol in the Affymetrix Technical Manual (701021 rev. 4; Affymetrix, Santa Clara, CA, USA). In summary, 1 µg of purified total RNA was used in the synthesis of cDNA, and the cDNA was purified using the GeneChip Sample Cleanup Module (Affymetrix). The purified cDNA was amplified to produce biotin-labeled cRNA using the GeneChip 3′IVT Express Kit (T7; Enzo Life Sciences, Farmingdale, NY, USA). Labeled cRNA was fragmented and hybridized to GeneChip Human Gene 2.0 ST according to the manufacturer's protocol (Affymetrix). The hybridized gene chips were washed and stained for antibody amplification. The gene chips were then scanned using the Gene array Scanner 3000 7G (Affymetrix).

Data Preprocessing and Screening of DEGs

GeneChip® Operating Software (Affymetrix) was used to gather a signal value. Normalization algorithms were used to adjust sample signals by minimizing the effects of variation caused by nonbiological factors. Quantile normalization was performed by the robust multiarray average[9] algorithm with application of the Affy package in R statistical software program[10]. Gene expression values of samples were $\log_2$ transformed and median centered for further analysis. If multiple probes corresponded to the same gene, the mean value was calculated as the expression value of this gene. The Limma package[11] in R language was used to screen DEGs. The DEGs with fold change (FC) ≥1.0 and a value of $p<0.05$ were considered to be significant.

Pathway Analysis

Genes showing a onefold and a value of $p<0.05$ in expression changes (microarray; Table S1 available at https://pan.baidu.com/s/1dFeOpcX) were analyzed using Ingenuity Pathway Analysis (IPA).

Statistical Analysis

Statistical analysis was performed with the GraphPad Prism software package (v. 5.0; San Diego, CA, USA) or SPSS 22.0 software (Chicago, IL, USA), and $p<0.05$ was considered to be statistically significant.

RESULTS

CTSF Expression Is Downregulated in GC Cells and Tissues

To investigate the CTSF transcription profile in GC cells, a qPCR was employed. With primers specific to the CTSF gene, qPCR results revealed that transcription of CTSF was found at different levels in various GC cell lines. The mRNA expression of CTSF was substantially downregulated in SGC7901 (0.645±0.077), BGC823 (0.618±0.100), MGC803 (0.275±0.020), HGC27 (0.156±0.023), AGS (0.348±0.0208), and MKN45 (0.501±0.055) cells compared with the normal mucosa line GES1 (onefold as the control; $p<0.01$) (Fig. 1A). Western blot was then performed to assess the level of CTSF protein expression in these cells. The result was also remarkably

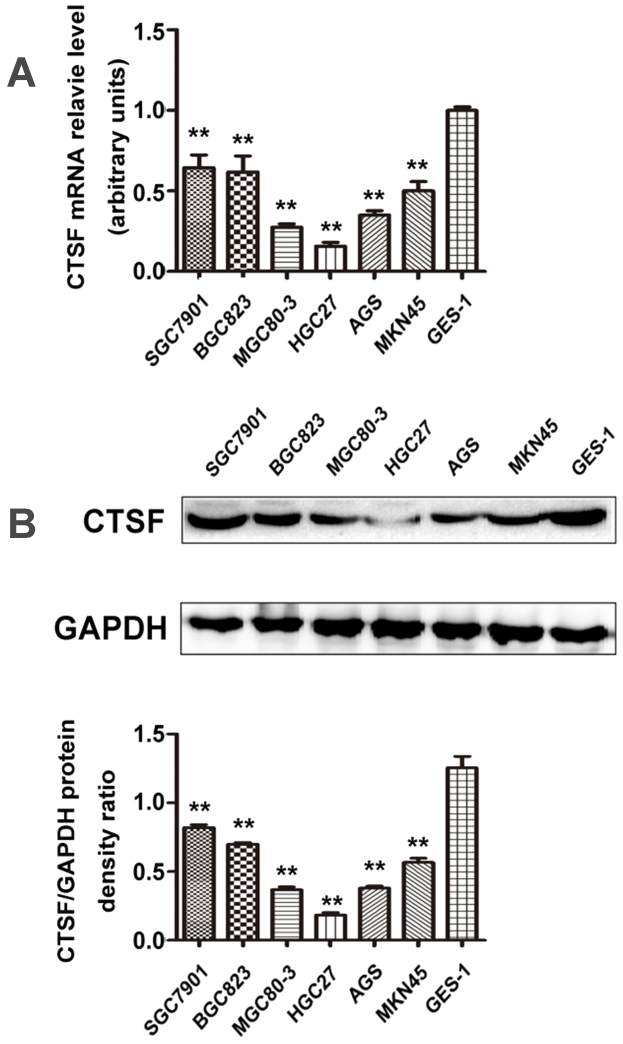


Figure 1. Expression of the cathepsin F (CTSF) gene in gastric carcinoma cell lines and a gastric epithelial cell line. (A) CTSF mRNA was detectable at different levels in gastric carcinoma cell lines (SGC7901, BGC823, MKN45, HGC27, AGS, and MGC803) and a gastric epithelial cell line (GES1). (B) Cell lysate was loaded and probed with anti-CTSF antibody (60 kDa) with GAPDH (36 kDa) as an internal control. CTSF protein expression was comparatively higher in SGC7901, BGC823, and MKN45 cells than in other gastric cancer (GC) cell lines (**$p<0.01$). Results are representative of three different experiments, and data are expressed as mean ± standard deviation.

downregulated in six GC cells compared with GES1 ($p<0.01$) (Fig. 1B). Among the GC cells, CTSF protein expression was found at a comparatively higher level in SGC7901, BGC823, and MKN45 cells and was lowest in HGC 27.

CTSF mRNA expression was also analyzed in 44 paired GC specimens and adjacent normal tissues by qPCR. It was identified that the expression of CTSF was significantly lower in the GC tissues than in their adjacent normal tissues (1.953 ± 1.077 vs. 0.379 ± 0.311; $p<0.0001$) (Fig. 2A, Table 2). Furthermore, the correlation between CTSF mRNA expression and the clinicopathological factors of GC was examined. The CTSF mRNA expression level was associated with tumor differentiation, depth of tumor invasion, and lymph node metastasis (Fig. 2B–D, Table 2).

Knockdown of CTSF Significantly Promotes the Proliferation and Suppresses Apoptosis of Gastric Cancer Cells

To investigate the effects of CTSF suppression on malignant growth potential, we introduced Lenti-shRNA targeting CTSF into SGC7901, BGC823, and MKN45 cells (Fig. 3A, C, and E). Using qPCR, we detected that the efficiency of CTSF knockdown in the Lenti-shRNA1 (KD-1), Lenti-shRNA2 (KD-2), and Lenti-shRNA3 (KD-3) groups was, respectively, 0.827 ± 0.026, 0.817 ± 0.009, and 0.728 ± 0.038 in SGC7901; 0.940 ± 0.008, 0.692 ± 0.011, and 0.384 ± 0.129 in BGC823; and 0.889 ± 0.015, 0.795 ± 0.019, and 0.797 ± 0.008 in MNK45, compared with the group infected with negative control lentivirus (NC; onefold as the control; $p<0.01$) (Fig. 3B, D, and F). Compared with the NC group, the CTSF expression was notably decreased by KD-1 and moderately decreased by the other two shRNAs (KD-2 and KD-3) (Fig. 3B, D, and F). Thus, KD-1 was chosen for further experiments.

We also identified that the protein expression of CTSF was remarkably decreased in the KD-1 group, compared with the NC and control groups without any infection (CON). To evaluate the potential effects of transfection with KD-1 on the growth of SGC7901, BGC823, and MKN45 cells, growth curves were examined by a CCK-8 assay. The results showed that GC-KD-1 cells grew much more quickly than the other two control cells. Depletion of CTSF promoted growth of GC cells in a time-dependent manner, and the statistical analysis showed a significant difference on the third day (Fig. 4A).

We also examined whether the level of cell apoptosis was altered in CTSF-depleted GC cells. A flow-based annexin V assay was used to determine the effects of Lenti-shRNA1-mediated CTSF silencing on cell apoptosis. The apoptotic rates of the CON, NC, and KD-1 groups were 12.60 ± 0.31%, 12.24 ± 0.73%, and 7.85 ± 0.64% in SGC7901; 9.85 ± 0.61%, 9.63 ± 0.90%, and 6.63 ± 0.26% in BGC823; 8.94 ± 0.20%, 7.79 ± 0.61%, and 6.08 ± 0.07% in MNK45, respectively. The apoptotic rate of the KD-1 group compared with other control groups showed a statistical significance ($p<0.05$). No significant difference was observed among the other two control groups ($p>0.05$). The results indicated that the extent of apoptosis is much lower in the GC-KD-1 group than in the control cells groups, which is in agreement with the proliferation measurement using the CCK-8 approach (Fig. 4B).

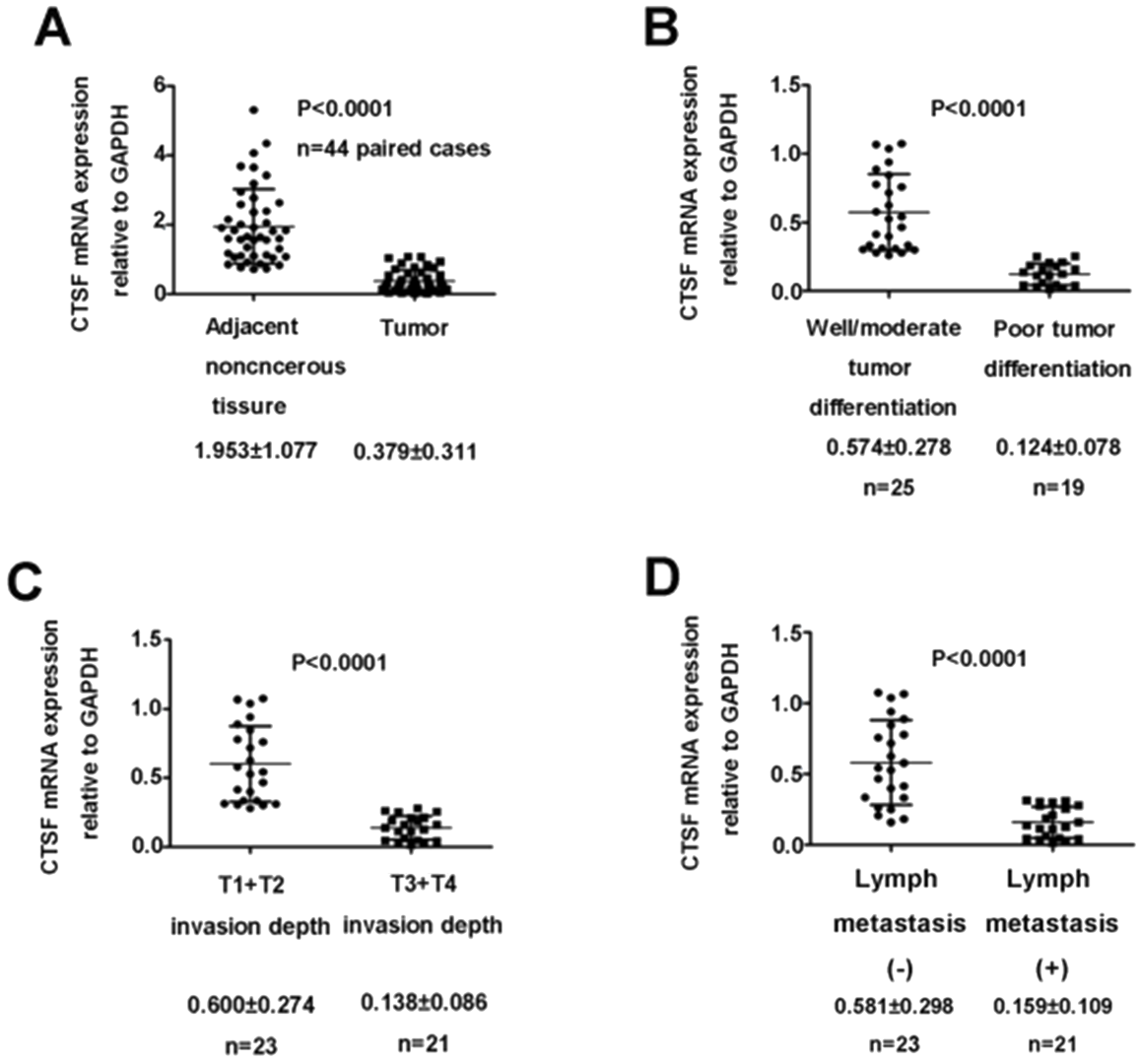


Figure 2. Quantitative polymerase chain reaction (qPCR) analysis of CTSF mRNA expression in human GC tissues. (A) CTSF expression in adjacent noncancerous tissues and gastric cancer tissues. (B) CTSF mRNA expression level in patients with good/moderate tumor differentiation and those in tumors with poor tumor differentiation. (C) Association between increased invasion depth and downregulation of CTSF mRNA. (D) CTSF mRNA expression level in patients without lymph node metastasis and those in tumors with lymph node metastasis.

CTSF Knockdown Induces Major Gene Expression Changes in Gastric Cancer Cells

To investigate the distinct downstream differentiation observed in GC cells with CTSF knockdown in more detail, we sequenced the whole transcriptome of both the KD-1 and NC groups in SGC7901 cells and compared their gene expression profiles. The microarray analysis showed that the DEGs were identified by the FC in filtering (FC≥1 showed statistical significance). The results showed that 1,117 mRNAs were upregulated, whereas 1,143 mRNAs were downregulated (Table S1 available at https://pan.baidu.com/s/1dFeOpcX). The CTSF expression of the sh-CTSF SGC7901 group was decreased 4.24-fold.

Between the two cell groups, we detected 20 genes that mediate regulation of the PI3K/Akt signaling pathway, apoptosis, and transcriptional misregulation in cancer, including NUPR1, ATF4, SERP1, RPUSD4, GDF15, and MZT1, via up- and downregulation. To address how the DEGs in these cells corresponded to changes in biological functions, we analyzed all up- and downregulated DEGs using the IPA software. The top 10 most significantly altered canonical pathways in sh-CTSF SGC7901 cells included p53 signaling, PI3K/AKT signaling, hereditary breast cancer signaling, and molecular mechanisms of cancer. The top 10 most significantly altered disease and function pathways involved cancer, organismal injury

Table 2. Correlation Between the Clinicopathological Features and Cathepsin F (CTSF) mRNA Expression in 44 Gastric Cancer Patients

Variable	Patients (*n*)	CTSF mRNA Expression Relative to GAPDH	*p* Value
Normal	44	1.953 ± 1.077	<0.01
Tumor	44	0.379 ± 0.311	
Age (years)			
<65	24	0.349 ± 0.323	0.483
≥65	20	0.419 ± 0.297	
Gender			
Male	30	0.290 ± 0.278	0.258
Female	14	0.571 ± 0.299	
Tumor differentiation			
Well/moderate	25	0.574 ± 0.278	<0.01
Poor	19	0.124 ± 0.078	
Invasion depth			
T1 + T2	23	0.600 ± 0.274	<0.01
T3 + T4	21	0.137 ± 0.086	
Tumor location			
Upper + middle	18	0.477 ± 0.351	0.949
Lower	26	0.312 ± 0.265	
Size (cm)			
<3	16	0.435 ± 0.251	0.149
≥3	28	0.348 ± 0.340	
Lymph node metastasis			
No	23	0.580 ± 0.298	<0.01
Yes	21	0.159 ± 0.109	

and abnormalities, cellular growth and proliferation, cell death and survival, and gastrointestinal disease.

Validation of Downstream Target Genes in SGC7901-Silenced CTSF Cell Lines

Using Western blot, the present study showed that the expression of MDM2, RALB, PCNA, smad2, FAS, and survivin was significantly increased in the SGC7901-KD-1 group compared with the SGC7901-NC group. Decreased expression of CDK4, CDK6, and Bax in the SGC7901-KD-1 group was also confirmed by Western blot. The results indicated that CTSF may be involved in cellular proliferation, apoptosis, and other processes in GC cells. The relative change in expression was mainly consistent with the microarray data (Fig. 5A).

Western blot assay confirmed the reduced expression of Bid and increased expression of Bcl-2 and C-IAP1 in the SGC7901-KD-1 group compared with the SGC7901-NC group at the protein level in order to affirm the underlying molecular mechanisms on cellular apoptosis in the KEGG pathway database (Fig. 5B).

DISCUSSION

Differential CTSF expression in certain malignant tumors suggests that this gene plays a distinct role in human malignancies[6,7,12]. We found that CTSF expression was significantly reduced in GC cells and tissues compared with normal mucosal cells and adjacent noncancerous tissues in the stomach. CTSF mRNA expression in GC tissues was related to tumor differentiation, invasion depth, and lymph node metastasis. This is in agreement with the result that differential CTSF expression in GC cells may be related to cellular differentiation. CTSF mRNA expression in the human moderately differentiated GC cell line SGC7901 was higher than in the human poorly differentiated GC cell line HGC27, which indicates that the degree of malignancy of GC is inversely proportional to the expression of CTSF.

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic morphological changes and death[13]. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay[14]. Growth disorders can occur at the cellular level, and these consequently underpin much of the subsequent course in cancer, in which a group of cells display uncontrolled growth and division beyond the normal limits[15]. To investigate further tumor-suppressive functions of CTSF, SGC7901, BGC823, and MKN45 cells were treated with CTSF-Lenti-shRNA to knock down CTSF expression. Cell growth was markedly enhanced, and the level of cellular apoptosis was reduced

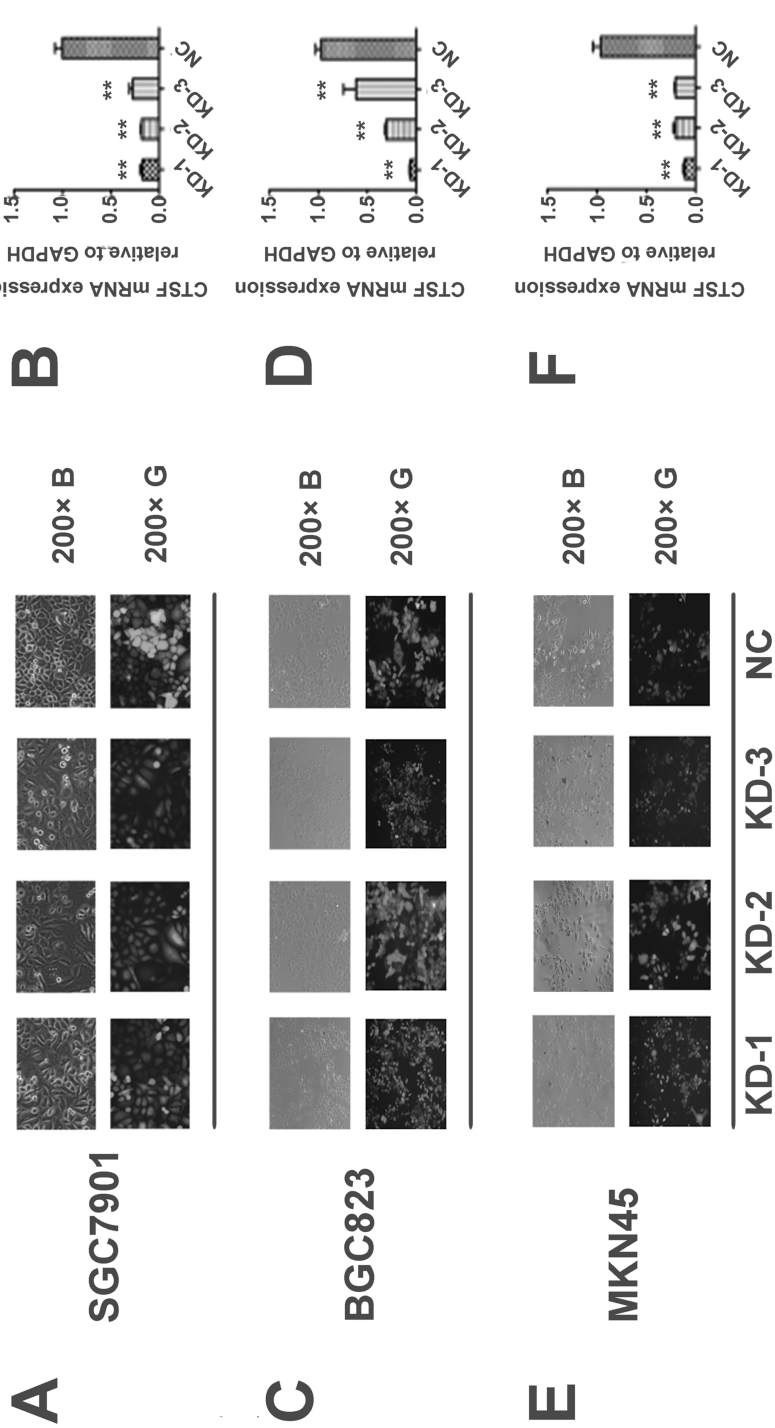


Figure 3. Transfection efficiency tested by GFP-Lenti-shRNA under a fluorescence microscope and knockdown efficiency examined by qPCR. (A, C, E) Fluorescent particles within the cells indicated that Lenti-shRNA was successfully transfected into GC cells. (B, D, F) qPCR results showed that KD-1 has a higher efficiency of silencing CTSF expression than the other two shRNAs (KD-2 and KD-3). The ratio of CTSF knockdown was compared in the three groups (KD-1, KD-2, and KD-3). Each bar indicates the mean silencing rate ± standard deviation per group. All experiments were done three times, and data are presented as mean ± SD. $^{**}p < 0.01$. (B) Visualization of the cells under common light. (G) Visualization of the cells under GFP (green fluorescent protein) fluorescence light. Lenti-shRNA, lentivirus-mediated small hairpin RNA; KD-1, Lenti-shRNA1; KD-2, Lenti-shRNA2; KD-3, Lenti-shRNA3. Magnification: ×200.

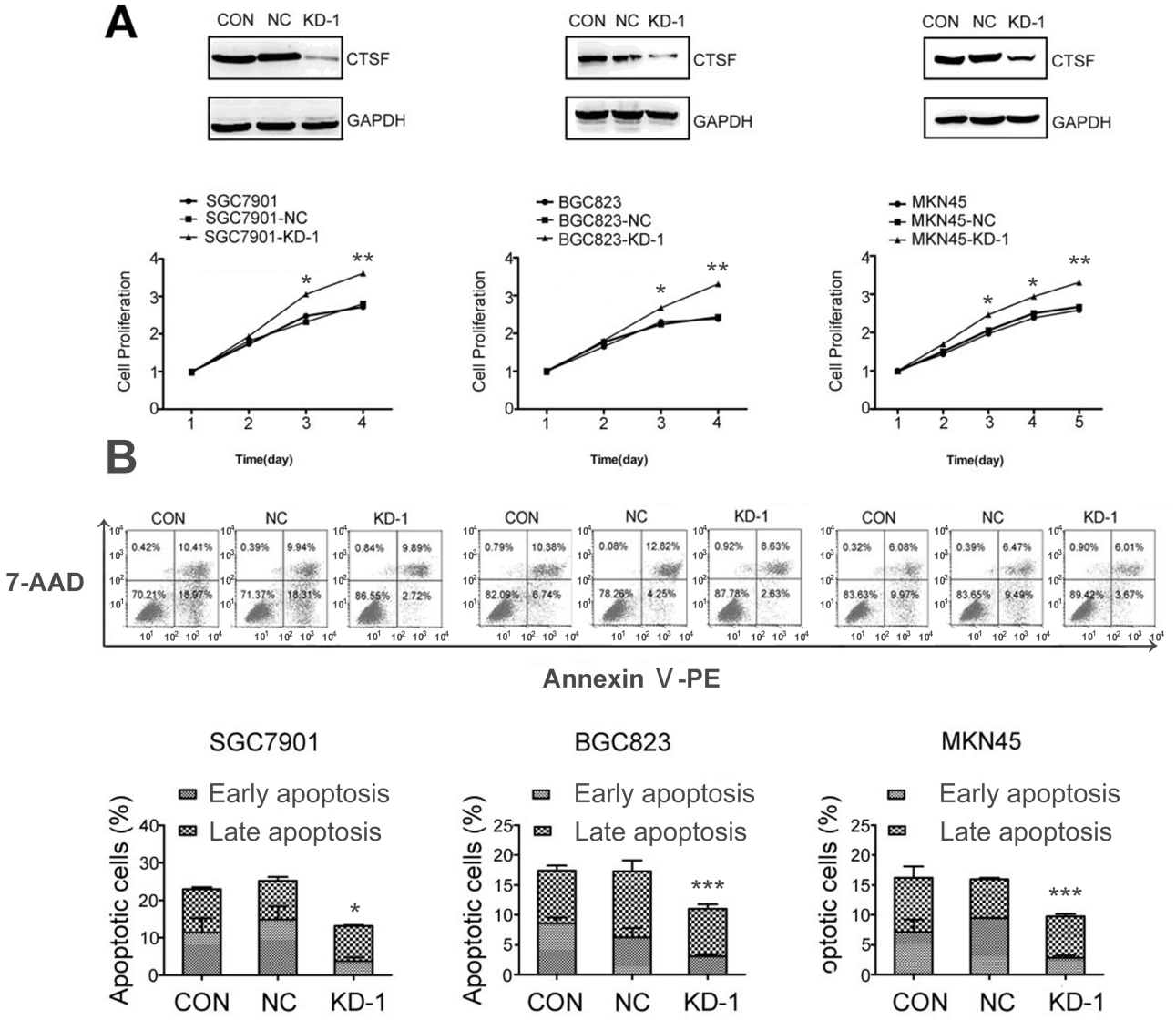
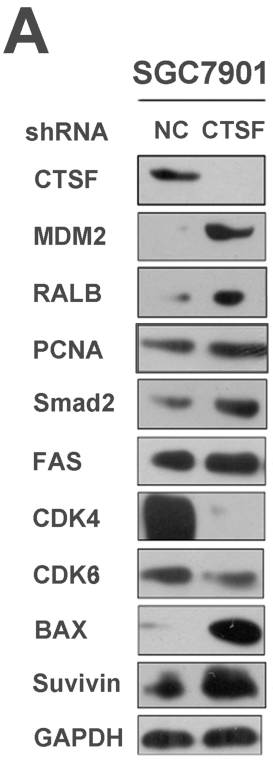


Figure 4. Knockdown of CTSF increases proliferation and inhibits the apoptosis of GC cells. (A) Growth curves for the indicated GC cells (CON, NC, and KD-1) were evaluated using a cell counting kit-8 assay. (B) The ratio of apoptosis in the cells of the indicated GC cells (CON, NC, and KD-1) was detected by flow cytometry. The ratio of apoptosis was compared in the three groups. Each bar indicates the mean apoptosis rate ± standard deviation per group. All experiments were done three times, and data are presented as mean ± SD. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

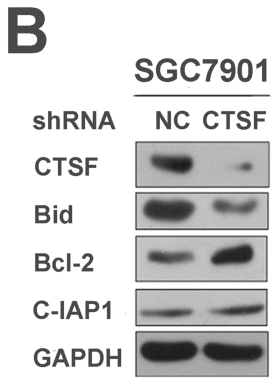
simultaneously. This provides further evidence that CTSF may function as a tumor suppressor in GC.

To illuminate the fact that CTSF has a critical role in possible cellular processes required for proliferative development, we analyzed microarray data by IPA and identified DEGs affected by CTSF knockdown. Interestingly, consensus genes that were differentially regulated following CTSF alteration were significantly linked to ATM signaling, p53 signaling, transcriptional regulation, and other cancer-related cellular processes. We then used IPA to further analyze the disease and function pathways altered in our models. Some genes that showed altered expression form part of cancer, cellular growth, and proliferation, as well as cell death and survival pathways. These pathways have important roles in GC processes, but a deeper mechanistic analysis is required to achieve more conclusions from these data.

The protein expression levels of nine differentially expressed mRNAs (MDM2, RALB, PCNA, smad2, FAS, CDK4, CDK6, Bax, and survivin) in the cancer-related pathways were verified by Western blot analysis. The relative change in protein expression was mainly consistent with the microarray data. Several human tumor types have been shown to have an increased level of murine



DEGs	change (Genechip)	change (WB)	positive or negative
MDM2	up	up	positive
RALB	up	up	positive
PCNA	up	up	positive
Smad2	up	up	positive
FAS	up	up	positive
CDK4	down	down	positive
CDK6	down	down	positive
BAX	up	up	positive
Suvivin	down	up	negative



Downstream genes	change (apoptosis pathway in kegg database)	change (WB)	positive or negative
Bid	down	down	positive
Bcl-2	up	up	positive
C-IAP1	up	up	positive

Figure 5. Western blot validation of downstream genes indicated by the microarray and KEGG pathway data in SGC7901 cell lines. (A) Expression change of the KD-1 group versus the NC group was verified by calculating Western blot results. The result showed that the change in gene expression by Western blot was mainly consistent with the microarray data. (B) Western blot analyses of Bid, Bcl-2, and C-IAP1 protein levels, which are apoptosis-related genes in the KEGG pathway database, between the two groups. GAPDH was used as the loading control.

double minute (MDM2), as an oncogene, including soft tissue sarcomas and osteosarcomas as well as breast tumors[16]. MDM2 antagonizes p53 but may also carry out p53-independent functions. Removing MDM2 can arrest cell proliferation[17]. Ras-related protein (RalB) has been associated with the progression of several cancers, including bladder and prostate cancers[18]. RalB, involved in cellular apoptosis and motility, promotes tumor invasion and metastasis. Consequently, inhibition of RalB inhibits further progression of cancer[19]. Proliferating cell nuclear antigen (PCNA) is a DNA clamp that acts as a processivity factor and is essential for replication. PCNA protein, which is ubiquitinated, is involved in the RAD6-dependent DNA repair pathway. PCNA is also a potential therapeutic target in cancer therapy[20]. Mothers against decapentaplegic homolog 2 (SMAD2) mediates the signal of transforming growth factor-β (TGF-β) and thus regulates multiple cellular processes, such as cell proliferation, apoptosis, and differentiation[21]. This protein is recruited to the TGF-β receptors through its interaction with the SMAD anchor for receptor activation (SARA) protein[22]. Fatty acid synthase (FAS) has been investigated

as a possible oncogene, which is upregulated in breast cancer[23]. It is also an indicator of poor prognosis and acts as a chemotherapeutic target[24]. Cyclin-dependent kinase inhibitor 2A (p16) acts as a tumor suppressor protein, which is a cyclin-dependent kinase (CDK) inhibitor that slows down the cell cycle by prohibiting progression from the G_1 phase to the S phase[25]. Normally, CDK4/6 binds cyclin D and forms an active protein complex that phosphorylates retinoblastoma protein (pRB)[26,27]. Bcl-2-associated X protein (Bax) is regulated by the tumor suppressor p53 and is involved in p53-mediated apoptosis. Drugs that activate Bax act as anticancer treatments by inducing apoptosis in cancer cells[28]. Survivin protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. Overexpression of survivin, which is regarded as an oncogene, decreases cellular apoptosis and increases in tumor growth[29–31]. The Western blot results indicated that MDM2, RALB, PCNA, smad2, FAS, and survivin were upregulated; on the contrary, CDK4, CDK6, and Bax were downregulated in the KD-1 group compared with the NC group in SGC7901. As a result, we have reason to believe that silencing CTSF can promote cellular growth and inhibit apoptosis in GC-KD-1 cells due to the potent roles of these DEGs, which were validated by Western blot analysis.

By analyzing the KEGG pathway database, we found that CTSF enhanced Bid and reduced Bcl-2 and C-IAPs in the cellular apoptosis pathway. The expression of Bid is upregulated by the tumor suppressor p53, and Bid has been shown to be involved in p53-mediated apoptosis[32]. The antiapoptotic Bcl-2 can bind Bid and inhibit Bid's ability to activate Bax. As a result, the antiapoptotic Bcl-2 proteins may inhibit apoptosis by sequestering Bid, leading to reduced Bax activation. Simultaneous overexpression of Bcl-2 and the proto-oncogene Myc may produce aggressive B-cell malignancies including lymphoma[33]. C-IAP1 is important for the activation of MAPK signaling, which is involved in directing cellular processes that regulate cell functions including proliferation, gene expression, differentiation, cell survival, and apoptosis[34]. Therefore, CTSF may be useful for cellular growth and apoptosis in many malignant diseases. By Western blot analysis, the present study also indicated that Bcl-2 and C-IAPs were upregulated and Bid was downregulated in the KD-1 group compared with the NC group in SGC7901. However, further research on whether CTSF could also be a prognostic factor for GC patients remains to be explored.

In summary, we believe our study to be the first to report on the tumor suppressor functions of CTSF and that the silencing of CTSF after transfection with Lenti-shRNA promotes GC progression. We also identified the differentially expressed downstream genes in CTSF that downexpressed SGC7901 GC cells by microarray analysis, which provides clues for further experiments on the molecular mechanisms of CTSF in tumorigenesis. The present study provides new targets for prognosis and pharmacological intervention in human GC. Therefore, further studies, in vitro and in vivo, are required to determine the precise mechanism of CTSF in the progression of GC.

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