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CSTB Downregulation Promotes Cell Proliferation and Migration and Suppresses Apoptosis in Gastric Cancer SGC-7901 Cell Line

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This study aimed to investigate the pivotal role of cystatin B (CSTB) in the development of gastric cancer and to explore its possible regulatory mechanism. Human gastric cancer SGC-7901 cells as a model in vitro were transfected with plasmid PCDNA3.1-CSTB and siRNA-CSTB using Lipofectamine 2000. Quantitative real-time PCR (qRT-PCR) and Western blotting were performed to determine the relative expression of CSTB and PI3K/Akt/mTOR pathway-related protein. Moreover, MTT assay, Transwell assay, and flow cytometry were used to assess cell proliferation, migration, and apoptosis, respectively. The results showed that CSTB was significantly downregulated in SGC-7901 cells compared with gastric epithelial cells. CSTB was successfully overexpressed and suppressed after cells were transfected with pc-CSTB and si-CSTB, respectively. Moreover, cell viability and migration were significantly decreased after being transfected with pc-CSTB when compared with the control group, while being obviously increased after transfection with si-CSTB. However, cell apoptosis was significantly induced after being transfected with pc-CSTB, while being obviously suppressed after transfection with si-CSTB. Besides, the expression levels of p-PI3K, p-Akt, and p-mTOR proteins were all significantly decreased in the pc-CSTB transfection group when compared with the control group, while being increased in the si-CSTB transfection group. Our findings suggest that CSTB downregulation may promote the development of gastric cancer by affecting cell proliferation and migration, and the PI3K/Akt/mTOR signaling pathway was activated in this process. CSTB may serve as a potential therapeutic target for gastric cancer.

Key words: Gastric cancer; Cystatin B (CSTB); Proliferation; Migration; Apoptosis; PI3K/Akt/mTOR pathway

INTRODUCTION

Gastric cancer is the fourth most common cancer in the world and has a poor prognosis (1,2). The 5-year survival rate of gastric cancer is reported to be less than 10% (3). Because of only a few symptoms being observed in the early stage, it is usually at an advanced stage when the diagnosis is made and is thus difficult to cure (4). Patients with advanced gastric cancer are also shown to have few efficacious treatment options (5). Although great efforts have been achieved in the past, the molecular mechanisms underlying gastric cancer are largely unclear. Therefore, a better understanding of the molecular mechanism behind this malignancy is crucial in order to develop effective therapeutics.

Cystatin B (CSTB), a member of the cystatin superfamily protein, is a stefin that functions as an intracellular thiol protease inhibitor and has been thought to play a role in protecting against the proteases leaking from lysosomes (6). Increasing evidence has shown the pivotal role of the CSTB homolog in diseases. For example, cystatin C could mediate the p53-induced apoptosis, and cystatin S functions as a cancer diagnosis marker in colorectal cancer and gastric cancer (7,8). Previous studies report that CSTB plays various functions in a variety of diseases, including epithelial ovarian cancer, colon cancer, and myoclonus epilepsy (9–11). In addition, Aggarwal and Sloane reported the diverse roles of CSTB in cancer (12), and Feldman et al. reported that CSTB is considered to be a biomarker in bladder cancer recurrence (13). To date, few researchers have mentioned the mechanism of CSTB in gastric cancer. Only a recent study reports that CSTB, together with two other proteins, including

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triosephosphate isomerase (TPI1) and deleted in malignant brain tumors 1 protein (DMBT1), was downregulated in gastric cancer, and this makes CSTB a biomarker in the clinical diagnosis of gastric cancer (14). However, the correlation between CSTB abnormal expression and the development of gastric cancer, as well as the possible molecular mechanism, still remains unknown.

In the current study, we detected the expression of CSTB in human gastric cancer SGC-7901 cells and used this cell line as a model in vitro to overexpress and silence CSTB. Various experimental methods including MTT assay, Transwell assay, and flow cytometry were used to explore the effects of CSTB dysregulation on cell proliferation, migration, and apoptosis. In addition, the expression of PI3K/Akt/mTOR pathway proteins was determined after CSTB dysregulation. This study aimed to investigate the pivotal role of CSTB in the development of gastric cancer and to elucidate its possible regulatory mechanism, thus providing a new insight in the identification of potential targets for this disease.

MATERIALS AND METHODS

Cell Culture

Human gastric epithelial cells and gastric cancer SGC-7901 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco) and incubated at 37°C in an incubator with 5% CO_2.

Cell Transfection

Plasmids PCDNA3.1-CSTB and siRNA-CSTB were constructed by the Sangon Biotech (Shanghai, P.R. China) and were respectively transfected into SGC-7901 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the instructions of the manufacturer. Cells transfected with silencing vector with no CSTB sequence were considered as a sham control, and cells transfected with plasmids PCDNA3.1-CSTB and siRNA-CSTB were considered as a negative control. Therefore, SGC-7901 cells in our study were randomly divided into four groups: control group, overexpression group (pc-CSTB), si-CSTB group (si-CSTB), and negative control group (si-CSTB + pc-CSTB).

Cell Viability Analysis

Cell viability was evaluated with the MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide] assay in our study. In brief, SGC-7901 cells (5×10^3) at the logarithmic phase were seeded into 96-well plates after 24, 48, 72, and 96 h of transfection. Cells were then collected by centrifugation, and the supernatant was removed. Then 20 µl of MTT solution was added into each well to incubate cells for 4 h. After terminating the reaction, 150 µl of dimethyl sulfoxide (DMSO) was added to mix with the cells for 10 min. Absorbance (570 nm) was measured with an absorption spectrophotometer (Olympus, Japan). All experiments were carried out independently three times.

Cell Migration Analysis

Cell migration was detected with Transwell migration chambers (8-µm pore size; Corning, USA). In brief, cells (5×10^4 cells) were seeded in the upper chamber containing serum-free medium after 48 h of transfection. DMEM containing 10% FBS as a chemoattractant was added into the lower chamber. After 24 h of incubation at 37°C, cells were put into a new six-well plate, washed by PBS, fixed in 4% paraformaldehyde, and stained with 0.1% crystal violet for 15 min. Olympus optical microscope (Leica, Germany) was then used to count the migrating cells. The migration experiments were repeated at least three times.

Cell Apoptosis Analysis by Flow Cytometry

Cell apoptosis was assessed by flow cytometry using Annexin V-FITC Cell Apoptosis Kit (Invitrogen) based on the recommended protocols of the manufacturer. In brief, cells were harvested after 48 h of transfection, washed by PBS buffer (pH 7.4), and then resuspended in the staining buffer. Afterward, 5 µl of Annexin-V-FITC and 5 µl of propidium iodide (PI) were added. After incubation for 10 min, the apoptotic cells (Annexin-V-positive and PI-negative cells) were determined using the FACSCalibur flow cytometer (BD, USA).

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen). The quality and concentration of total RNA were detected by SMA 400 UVOVIS (Merinton, Shanghai, P.R. China). Reverse transcription for cDNA synthesis was carried out with the Primer Script 1st Strand cDNA Synthesis Kit (Invitrogen). qRT-PCR analysis was then conducted using the SYBR ExScript qRT-PCR Kit (Takara, Japan) with a standard protocol to detect the expression of CSTB. Each reaction was conducted in triplicate, and the $2^{-\Delta\Delta CT}$ method (15) was used to determine the relative gene expression level of CSTB. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. Primers used for gene amplification were as follows: CSTB, 5′-GGGTGTTTCCACCCCTCCCTC-3′ (forward) and 5′-GAGGGAGGGGTGGAAACACCC-3′ (reverse); GAPDH, 5′-TGACTTCAACAGCGACACCCA-3′ (forward) and 5′-CACCCTGTTGCTGTAGCCAAA-3′ (reverse).

Western Blotting Analysis

Cells were washed with ice-cold PBS and lysed with radioimmunoprecipitation (RIPA; Sangon Biotech) solution containing phenylmethanesufonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was measured using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Then an equal amount of protein per lane was separated on a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The bands were then transferred onto a polyvinylidencefluoride (PVDF) membrane (Millipore). After being blocked in Tris-buffered saline Tween (TBST) containing 5% nonfat milk, the membrane was incubated with primary antibodies overnight at 4°C. GAPDH served as the internal control. The antibody against CSTB was purchased from Novus International Inc. (Saint Charles, MO, USA). Antibodies against p-PI3K (pY-458), p-Akt (pS-473), p-mTOR (pS-2448), and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Then the membrane was incubated with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. After being washed three times with 1× TBST buffer, the bands were detected with a chromogenic substrate using the enhanced chemiluminescence (ECL) method and further quantified by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

All measurement data from multiple experiments were expressed as mean ± SD, and statistical analysis was then performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical differences between groups were analyzed with *t*-test or post hoc Tukey test in one-way ANOVA and considered as statistically significant with a value of $p < 0.05$.

RESULTS

Analysis of CSTB Expression in Human Gastric Cancer SGC-7901 Cells

We first detected the expression of CSTB in human gastric cancer SGC-7901 cells. The results showed that CSTB expression at both mRNA and protein levels was significantly downregulated in human gastric cancer SGC-7901 cells when compared with gastric epithelial cells ($p < 0.05$) (Fig. 1A and B).

In addition, we measured the expression of CSTB in different transfected groups. Expected results were obtained that, in comparison with the control group, the expression of CSTB at both mRNA and protein levels was successfully overexpressed and suppressed in pc-CSTB and si-CSTB groups, respectively, and significant differences existed between them ($p < 0.05$) (Fig. 1C and D).

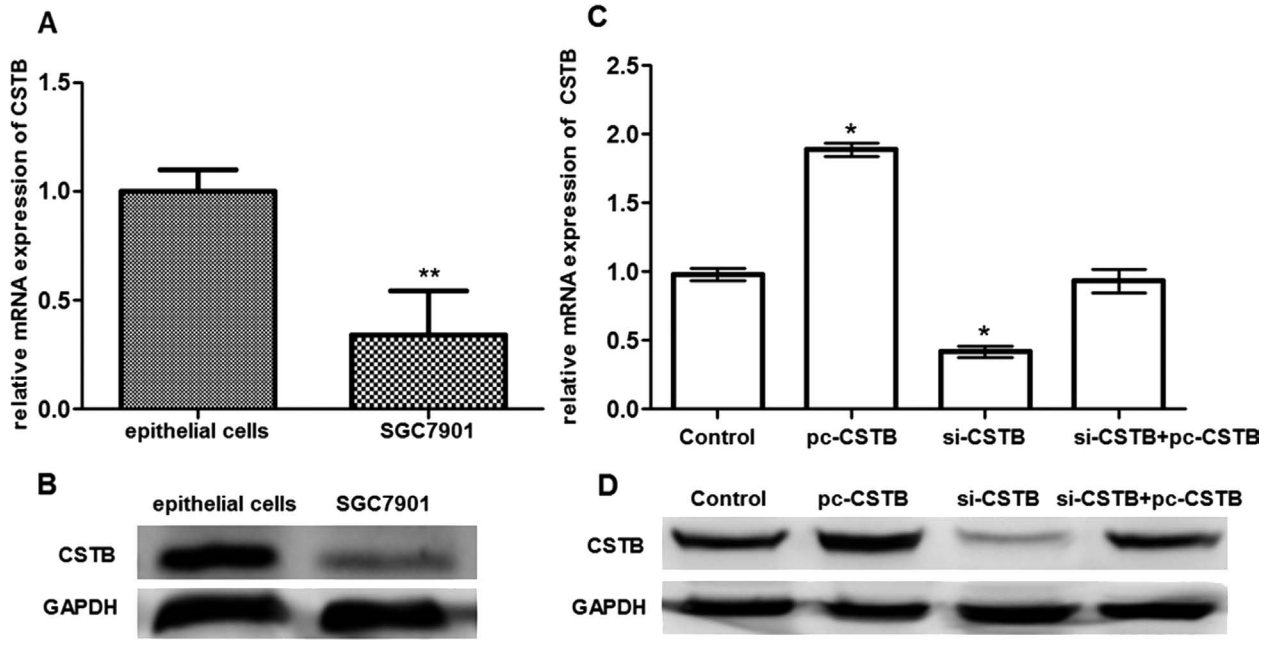

Figure 1. Expression of CSTB in human gastric cancer SGC-7901 cell. (A) qRT-PCR showed the mRNA expression of CSTB in human gastric cancer SGC-7901 cells. (B) Western blot showed the protein expression level of CSTB in human gastric cancer SGC-7901 cells. (C) qRT-PCR showed the mRNA expression level of CSTB in different transfected groups. (D) Western bolt showed the protein expression level of CSTB in different transfected groups. Error bars indicate means ± SD. *$p < 0.05$, **$p < 0.01$.

However, after transfection with both pc-CSTB and si-CSTB at the same time, there was no significant difference in the expression level of CSTB when compared with the control group ($p > 0.05$).

Overexpression of CSTB Suppressed Cell Viability

Cell viability of different transfected groups in an experimental period of several days after transfection was determined using MTT assay (Fig. 2). The results showed that cell viability of SGC-7901 cells in the pc-CSTB group was significantly decreased with the increase in transfection time when compared with the control group, while cell viability of the si-CSTB group was markedly increased ($p < 0.05$).

Overexpression of CSTB Suppressed Cell Migration

The results of the Transwell assay showed the ability of cells to migrate (Fig. 3). By counting the number of migrated cells (Fig. 3A), we found that the number of migrated cells in the pc-CSTB transfection group was significantly decreased when compared with that in the control group, while it was markedly increased in the si-CSTB transfection group ($p < 0.05$). These results were in accordance with the migrated cells by Transwell assay (Fig. 3B).

Overexpression of CSTB Induced Cell Apoptosis

Cell apoptosis of different transfected groups was detected by flow cytometry (Fig. 4). We found that the percentage of apoptotic cells significantly increased after being transfected with plasmid pc-CSTB when compared with the control group while obviously being decreased after transfection with plasmid si-CSTB ($p < 0.05$) (Fig. 4A). The apoptotic cells in each group by flow cytometry analysis are shown in Figure 4B.

Overexpression of CSTB Reduced the Activation of PI3K/Akt/mTOR Pathway

To further explore the possible regulatory mechanism of CSTB in gastric cancer, the protein expression levels of key molecules involved in the PI3K/Akt/mTOR pathway were detected by Western blot and qRT-PCR (Fig. 5). The results showed that the relative mRNA expression (Fig. 5A) and protein (Fig. 5B) levels of p-PI3K, p-Akt, and p-mTOR proteins were all significantly downregulated in the pc-CSTB transfection group when compared with the control group while being upregulated in the si-CSTB transfection group ($p < 0.05$), indicating the suppressive effect of CSTB overexpression on the activation of the PI3K/Akt/mTOR pathway.

DISCUSSION

Gastric cancer is still one of the most common malignant tumors whose molecular mechanism and effective treatment are unclear. CSTB is found to be dysregulated in several cancers, such as ovarian cancer and breast cancer (9,16). Previous data showed that CSTB was downregulated in gastric cancer and may function as a biomarker for the diagnosis of gastric cancer, but the regulatory mechanism of CSTB in gastric cancer is still unknown. In this study, we analyzed the expression of CSTB in a

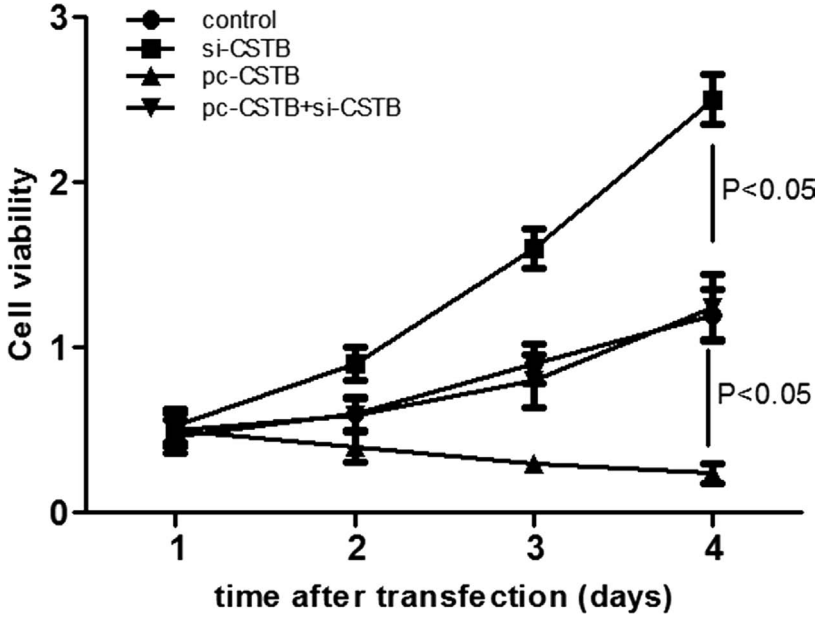

Figure 2. Effect of CSTB expression on the cell viability of different transfected groups in an experimental period of 4 days after transfection.

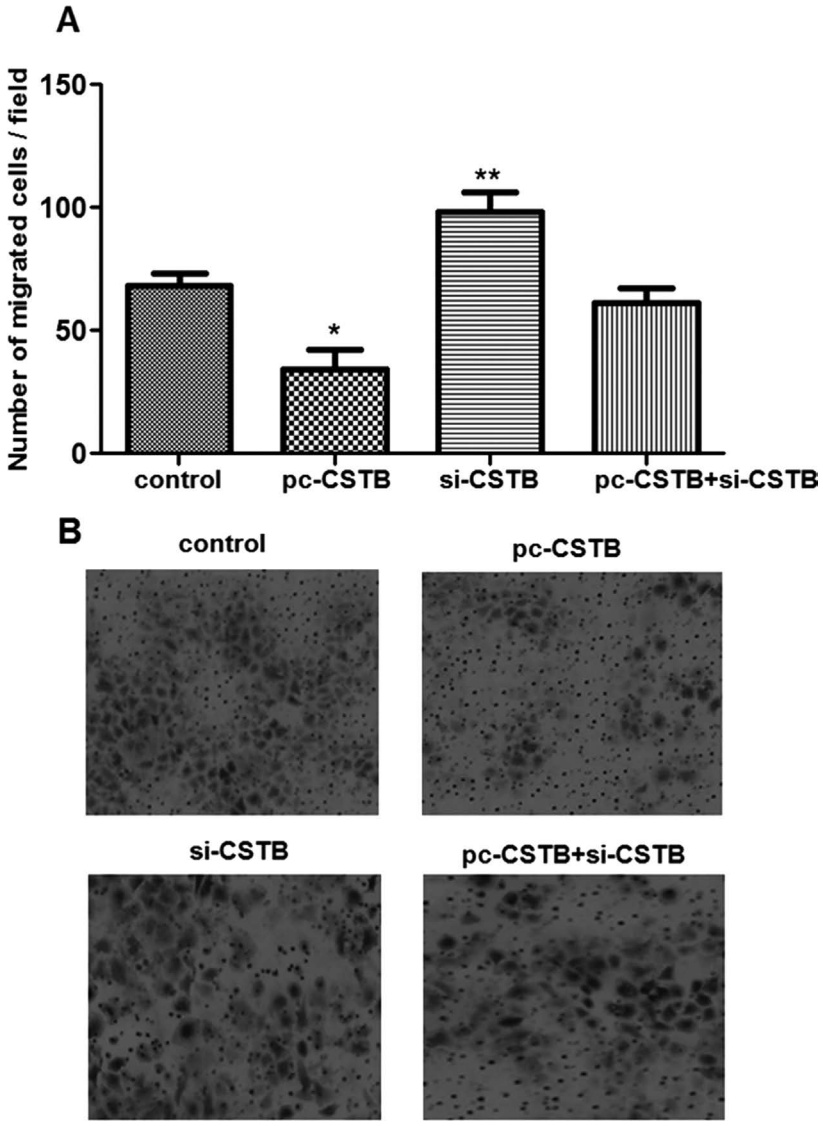

Figure 3. CSTB upregulation suppressed the cell. (A) The number of migrated cells in each group. (B) Transwell assay showed the migrated cells in each group. Error bars indicate means ± SD. *$p < 0.05$, **$p < 0.01$.

gastric cancer cell line in vitro and further investigated the possible mechanisms. In accordance with previous results (14), our data showed that CSTB was significantly downregulated in human gastric cancer SGC-7901 cells when compared with gastric epithelial cells (Fig. 1), indicating the pivotal correlation between CSTB dysregulation and gastric cancer.

Meanwhile, cell viability and migration ability of gastric cancer SGC-7901 cells were inhibited, while apoptosis was induced, by the overexpressed CSTB (Figs. 2–4). All these findings merit further discussion. The role of CSTB in gastric cancer cell apoptosis and migration has not been fully discussed. However, it has been proven that apoptosis was increased in CSTB-deficient mice with a neurological disorder (17), and CSTB functioned as an intracellular modulator of bone resorption via regulating bone cell migration (18). On the basis of our results, we speculated that CSTB upregulation may prevent the development and metastasis of gastric cancer through involvement in the biological processes of proliferation, apoptosis, and migration.

Furthermore, accumulating evidence has confirmed that the PI3K/Akt/mTOR pathway is widely involved in the regulation of cell processes, including angiogenesis, cell proliferation, and metabolism (19–21). In a previous study, activated PI3K was found to be involved in a number of cellular processes, including tumor growth (22). Yoda et al. proved that a variety of dysregulated

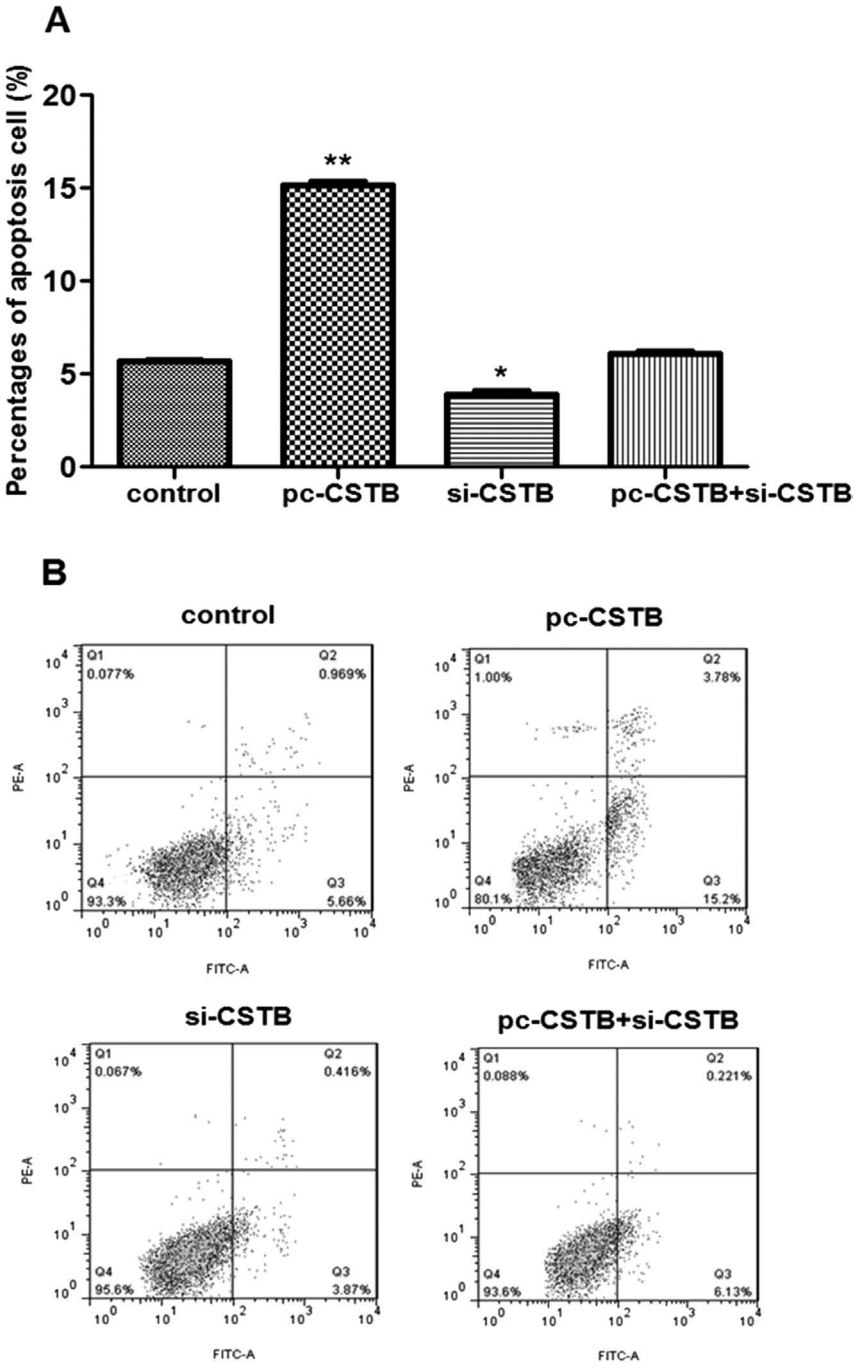

Figure 4. CSTB upregulation induced the cell apoptosis. (A) The percentage of apoptotic cells in each group. (B) Annexin V-FITC/PI of apoptotic cells in each group, which was in accordance with the result in (A). Error bars indicate means ± SD. $*p < 0.05$, $**p < 0.01$.

signaling pathways were correlated to gastric cancer, in which the PI3K/Akt/mTOR signaling pathway was included (23). Suppression of the mTOR pathway is also reported to play a key role in inhibiting the proliferation of gastric cancer cells (24). In addition, BMP2 is shown to enhance tumor metastasis in gastric cancer associated with the activation of the PI3K/Akt pathway (25). Also, p-mTOR expression in pT2b gastric cancer is found to be associated with lymph node metastasis, which is strongly correlated with poor disease-free survival (26). It is also reported that the PI3K/Akt pathway is implicated in bufalin-induced apoptosis in gastric cancer MGC803 cells (27). β-Ionone can initiate the apoptosis of gastric cancer SGC-7901 cells through involvement in the

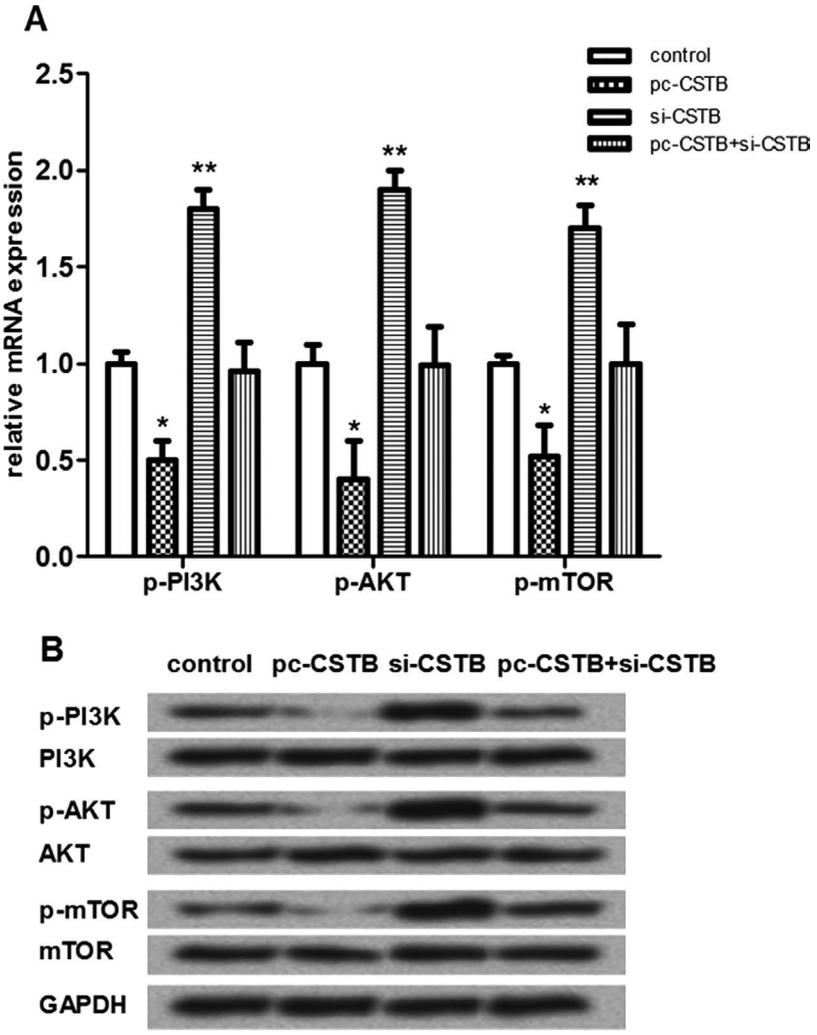

Figure 5. Effects of CSTB expression on the PI3K/Akt/mTOR signaling pathway-related protein expression. (A) The relative mRNA expression levels of p-PI3K, p-Akt, and p-mTOR were all decreased by the upregulated CSTB. (B) The protein expression levels of p-PI3K, p-Akt, and p-mTOR were all decreased in cells treated with pc-CSTB. Error bars indicate means ± SD. *$p < 0.05$, **$p < 0.01$.

PI3K/Akt pathway (28). Activation of the PI3K/Akt/mTOR pathway is shown to have potential prognostic and predictive significance in gastric cancer (29). From another point of view, the association between CSTB and PI3K/Akt/mTOR signaling pathway has not been fully investigated in gastric cancer. However, Wang et al. proved that CSTB was dysregulated by the transforming growth factor-β (TGF-β) signaling pathway in ovarian cancer (9). Additionally, from the study of Malla et al., cathepsin B, which was inhibited by CSTB, could regulate cell apoptosis by inhibiting the PI3K/Akt pathway in glioma (30). This evidence suggests that there is a potential correlation between CSTB and the PI3K/Akt pathway. In our study, the p-PI3K, p-Akt, and p-mTOR expression were all decreased by the overexpressed CSTB in SGC-7901 cells, but were all increased by the silenced CSTB (Fig. 5), suggesting that CSTB upregulation could block the activation of the PI3K/Akt/mTOR signaling pathway and thus affect the malignant behaviors of gastric cancer.

In conclusion, our study indicates that CSTB expression is reduced in gastric cancer cells and may contribute to the development of gastric cancer via promoting cell proliferation, enhancing cell metastasis, but inhibiting cell apoptosis through activating the PI3K/Akt/mTOR pathway. CSTB may serve as a potential target in the management of gastric cancer. However, only one human gastric cancer cell line, SGC-7901, was used to explore the potential roles of CSTB in vitro. Further experimental validations with more cell lines in vitro and experiments in vivo to investigate the correlation between CSTB abnormal expression and PI3K/Akt/mTOR signaling pathway activation in gastric cancer are still needed to verify our observations.

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