# Proton pump inhibitor pantoprazole inhibits the proliferation, self-renewal and chemoresistance of gastric cancer stem cells via the EMT/β-catenin pathways

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**Abstract.** The cancer stem cell (CSC) model suggests that a small subset of cancer cells possess stem cell properties and plays a crucial role in tumor initiation, metastasis and resistance to anticancer therapy. Exploration of the specific therapies targeting at CSCs has been a crucial issue in anti-tumor research. Gastric cancer (GC) cells often exist in an ischemic microenvironment with acidic conditions *in vivo*, thus maintenance of cellular pH homeostasis is important for the survival and function of GC cells. Proton pump inhibitors (PPIs) may prevent intracellular proton extrusions which consequently reduce cancer cell survival under acidic conditions. The effects of PPIs on the suppression of the viability and invasiveness of GC cells have been reported, but the functional role of pantoprazole (PPZ) in GC cells remains unknown. In this study, we found that when cells were treated with PPZ, the 5-fluorouracil (5-FU) chemosensitivity was upregulated, meanwhile the sphere formation ability and the relative expression levels of stem cell markers CD44, CD24, ABCG2, EpCAM and Lgr5 were significantly decreased. It was hypothesized that PPZ inhibits the GC CSCs. Successively a sphere formation culture was performed to establish CSC models and the effect of PPZ on GC CSCs from SGC-7901 and HGC-27 cells was explored. The addition of PPZ reduced the relative expression of CSC markers and anti-drug markers accompanied by a decrease in proliferation, 5-FU chemoresistance and self-renewal capacity via epithelial-mesenchymal transition (EMT)/β-catenin pathways. The study suggests that PPZ could be a promising novel specific therapeutic strategy for targeting GC CSCs.

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*Key words:* gastric cancer, proton pump inhibitors, pantoprazole, cancer stem cells, chemosensitivity, sphere formation

## Introduction

Gastric cancer (GC) is one of the most common malignancies and a leading cause of cancer-related deaths worldwide (1) which remains prevalent in Eastern Asia, South America and Eastern Europe (2,3). Common treatments for advanced GC include surgery, chemotherapy and radiotherapy. Although chemotherapy is effective for suppressing cancer progression and prolonging survival to some extent, chemoresistance represents a predominant obstacle towards the chemotherapeutic treatment of GC (4). It is worth noting that an extremely efficient mechanism of tumor resistance to drugs is the proton pump-mediated acidification of the tumor microenvironment (5).

GC cells often exist in an ischemic microenvironment with acidic conditions which is a consequence of the production of acidic by-products from explosive glycolysis *in vivo* (6,7). Maintenance of cellular pH homeostasis is vital for the survival and function of cancer cells (8,9). Hydrogen/potassium adenosine triphosphatase ($H^+/K^+$-ATPase) plays a vital role in the maintenance of cellular pH homeostasis by exchanging luminal $K^+$ for cytoplasmic $H^+$. This proton pump also participates in the formation of abnormal pH gradients that are typical of GC cells during tumorigenesis (10). Proton pump inhibitors (PPIs) inhibit gastric $H^+/K^+$-ATPase irreversibly which prevents intracellular proton extrusions in GC cells consequently reducing cancer cell survival under acidic conditions (11). It was reported that rabeprazole attenuates the cell viability of human GC cells through inactivation of the ERK1/2 signaling pathway (12). Moreover, pantoprazole (PPZ) could be used to suppress the invasiveness of SGC-7901/ADR cells by targeting epithelial-mesenchymal transition (EMT) (13).

Cancer stem cells (CSCs) play pivotal roles in cancer initiation, progression, recurrence and chemoresistance (14-17). CSCs give rise to tumors through self-renewal and are able to differentiate into multiple cell types (18-21). Chemotherapies that kill the bulk of cancer cells, may ultimately fail as they do not eliminate CSCs that then cause the relapse of tumors (22). Recently, it has been established that CSCs are linked to EMT, metastasis, drug resistance, progression and relapse

of GC (23-26). As a result, exploitation of specific therapies targeting CSCs has been a crucial issue in the chemotherapeutic treatment of GC.

In the present study, we found that the PPI PPZ suppressed the proliferation, sphere formation and GC stem cell-mediated 5-fluorouracil (5-FU) chemoresistance of SGC-7901 and HGC-27 cells by targeting EMT and β-catenin signaling for the first time and our findings suggest that PPIs may serve as a promising CSC-oriented novel antineoplastic agent.

## Meterials and methods

*Cell culture, sphere formation culture and reagents*. Human GC cell lines SGC-7901 and HGC-27 were purchased from Auragene Bioscience Co. (Changsha, China). Under standard conditions, both cell lines were cultured in normal Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) with 10% fetal bovine serum and 100 U/ml penicillin at 37˚C in a humidified atmosphere of 95% air and 5% $CO_2$. Under sphere culture conditions, parental cell lines or the floating spheres obtained from transfections were plated in serum-free defined media (SFDM): low-glucose (1 g/l) DMEM supplemented with L-glutamine, sodium pyruvate, penicillin/streptomycin (Wisent, Inc.), 20 ng/ml basic FGF, 20 ng/ml EGF and B27 (Invitrogen, Grand Island, NY, USA) using 24-well ultra-low attachment plates (Corning, Inc., Tewksbury, MA, USA). Spheres were dissociated with trypsin every 5-7 days and split to 1:3 ratio for next sphere passage if it was necessary. PPI PPZ (H20010032) was obtained from Hangzhou Meidong Pharmaceutical Co., Ltd. (Hangzhou, China). SGC-7901 and HGC-27 parental cells or spheres were treated with 100 $\mu$g/ml PPZ to generate cell models of interest.

*Flow cytometric analysis*. Cells ($1x10^6$) were labeled with PE-conjugated anti-CD44 (BioLegend, Inc., San Diego, CA, USA) and FITC-conjugated CD24 (BD Pharmingen, Mississauga, ON, Canada) for 20 min, washed twice, resuspended in PBS and analyzed on a BD FACSCalibur™ platform (BD Biosciences). Data were analyzed by FCS Express software (BD Biosciences) using floating quadrants to enumerate negative, single- and double-positive populations.

*Drug resistance assay*. Parental cells and spheres of P4 were planted at 2,000 cells/well in 96-well plates and then divided into three groups as follows: treatment with 5-FU (20 $\mu$g/ml), treatment with pantoprazol (PPZ; 100 $\mu$g/ml) and co-treatment with 5-FU and PPZ (5-FU + PPZ). Cell viability was examined. Drug resistance was determined after treatment for 96 h by MTT assay.

*Self-renewal assay*. Sphere formation assay was performed to detect the self-renewal capacity. A total of 4,000 cells/well were seeded in ultra-low attachment 6-well plate (Corning, Inc.) in SFDM medium for 2 weeks, after which sphere formation was assessed by counting the number of spheres (≥3 cells) under a microscope.

*Cell growth assay of parental cells and spheres*. A total of 2,000 cells were plated in 96-well plates and cultured in a $CO_2$ incubator. The cells were harvested at 24, 48, 72 and 96 h. The optical density at 570 nm (OD570) of each well was measured with an ELISA reader (ELx800; BioTek Instruments, Inc.).

Table I. The primer sequences used in real-time RT-PCR.

| Gene | | Primer sequences |
|---|---|---|
| CD44 | Sense | CATCCCAGACGAAGACAGTCC |
| | Antisense | TGATCAGCCATTCTGGAATTTG |
| CD24 | Sense | GACATGGGCAGAGCAATGGTGGC |
| | Antisense | GAGTGAGACCACGAAGAGACTGGC |
| ABCG2 | Sense | CTGAGATCCTGAGCCTTTGG |
| | Antisense | TGCCCATCACAACATCATCT |
| EpCAM | Sense | CGCCATATGCAGGAAGAATGTGT |
| | Antisense | CGCCTCGAGTTATTTTAGACCCTGCATTG |
| Lgr5 | Sense | CCCGGGTTTCAGAGACAACTTC |
| | Antisense | TCCACATGCTTTATTCCAGCAATC |
| BMI1 | Sense | ACGATGCCCAGCAGCAATGACT |
| | Antisense | AAGTGGACCATTCCTTCTCCAGGT |
| ALDH1 | Sense | GATGAAGCTGCGGAATTTG |
| | Antisense | TCTTTGCTCGTTCAATGCTC |
| Tcf4 | Sense | TGCGATGTTTTCACCTCCTG |
| | Antisense | TGCCAAAGAAGTTGGTCCATT |
| β-actin | Sense | AGGGGCCGGACTCGTCATACT |
| | Antisense | GGCGGCACCACCATGTACCCT |

*Real-time RT-PCR*. Total RNA was extracted from the cells with TRIzol reagent (Invitrogen) following the manufacturer's instructions. The relative mRNA levels of CSC markers of CD44, CD24, ABCG2, EpCAM, Lgr5 and drug-resistance markers BMI1, ALDH1, Tcf4 were detected by real-time RT-PCR using the standard SYBR-Green RT-PCR kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's instructions and β-actin was used as an internal control. The specific primer pairs are listed in Table I. The relative expression of target genes was quantified using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA) and the $2^{-\Delta\Delta Ct}$ method (27).

*Western blot analysis*. Cells were solubilized in cold RIPA lysis buffer. Subsequently, the proteins were separated with 8% SDS-PAGE and then transferred to a PVDF membrane. The membranes were blocked in 5% non-fat dried milk in PBST for 3 h and then incubated overnight with specific primary antibodies with β-actin as a control. After incubation with the goat anti-rabbit or anti-mouse secondary antibody, immune complexes were detected using an ECL kit (Auragene Bioscience Co.). The primary antibodies against CD44 (1:1,000, ab54037), CD24 (1:1,000, ab113289), ABCG2 (1:1,000, ab63907), EpCAM (1:1,000, ab32392), Lgr5 (1:1,000, ab75732), BMI1 (1:1,000, ab126783), ALDH1 (1:1,000, ab6192), Tcf4 (1:1,000, ab60727), E-cadherin (1:1,000, ab15148), N-cadherin (1:1,000, ab18203), vimentin (1:1,000, ab133260) and Snail (1:1,000, ab82846) were all obtained from Abcam (USA), and

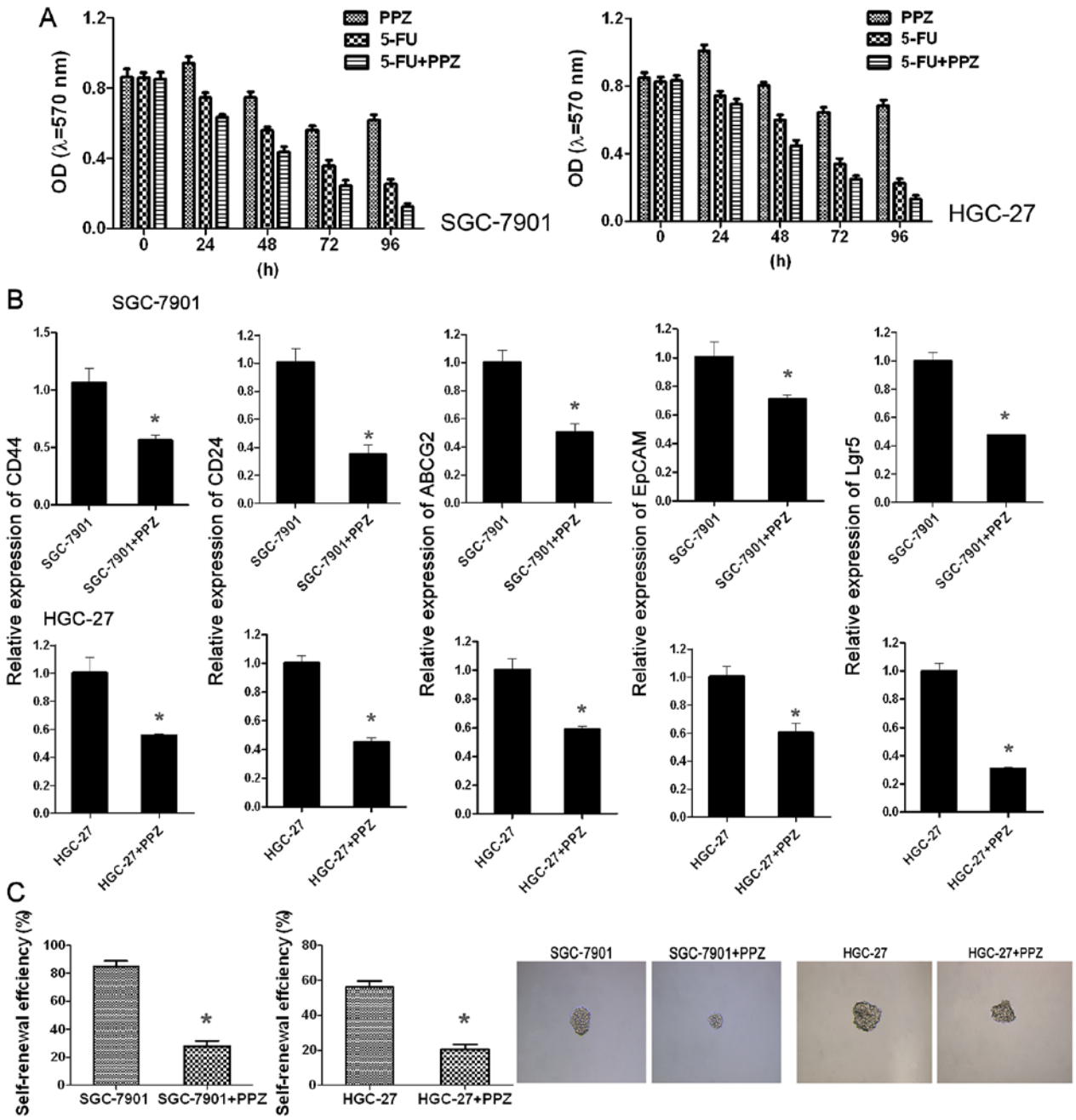


Figure 1. Suppressive effect of PPZ on GC cells involving the mediation of cell stemness of SGC-7901 and HGC-27 cells. (A) MTT assay was performed to measure the inhibition of proliferation of cells treated with PPZ, 5-FU respectively and their combination. (B) qPCR was used to detect the relative mRNA levels of the markers of GC cancer-initiating cells with or without PPZ treatment. (C) Sphere formation was used to measure the self-renewal efficiency of both GC cell lines with or without PPZ treatment. *P<0.05 vs. con (cells without PPZ treatment). PPZ, pantoprazole; GC, gastric cancer; 5-FU, 5-fluorouracil.

total β-catenin (1:1,000, #9562) and active β-catenin (1:1,000, #4270) were purchased from Cell Signaling Technology, Inc. (CST; USA) The images were captured using GeneSnap software from SynGene (Cambridge, UK). The protein levels were normalized to β-actin.

*Statistical analysis*. The data are shown as the mean ± SD. The Student's t-test was used to analyze the differences between the experimental and control groups. Statistical analyses were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA), and P<0.05 was considered to indicate a statistically significant difference.

## Results

*PPZ enhances 5-FU chemosensitivity and suppresses sphere formation and the expression of stem cell markers in GC cell lines*. In order to ascertain whether PPZ exerts synergistic action with 5-FU to inhibit cell proliferation, SGC-7901 and HGC-27 cells were divided into three groups: 5-FU-treated, PPZ-treated and 5-FU combined with PPZ-treated. The results showed that the inhibition of proliferation by PPZ appeared after 48 h and until 96 h, inhibition was still slight. After addition of 5-FU (24 h), inhibition of proliferation appeared; at 48 until 96 h, it became significant. When cells

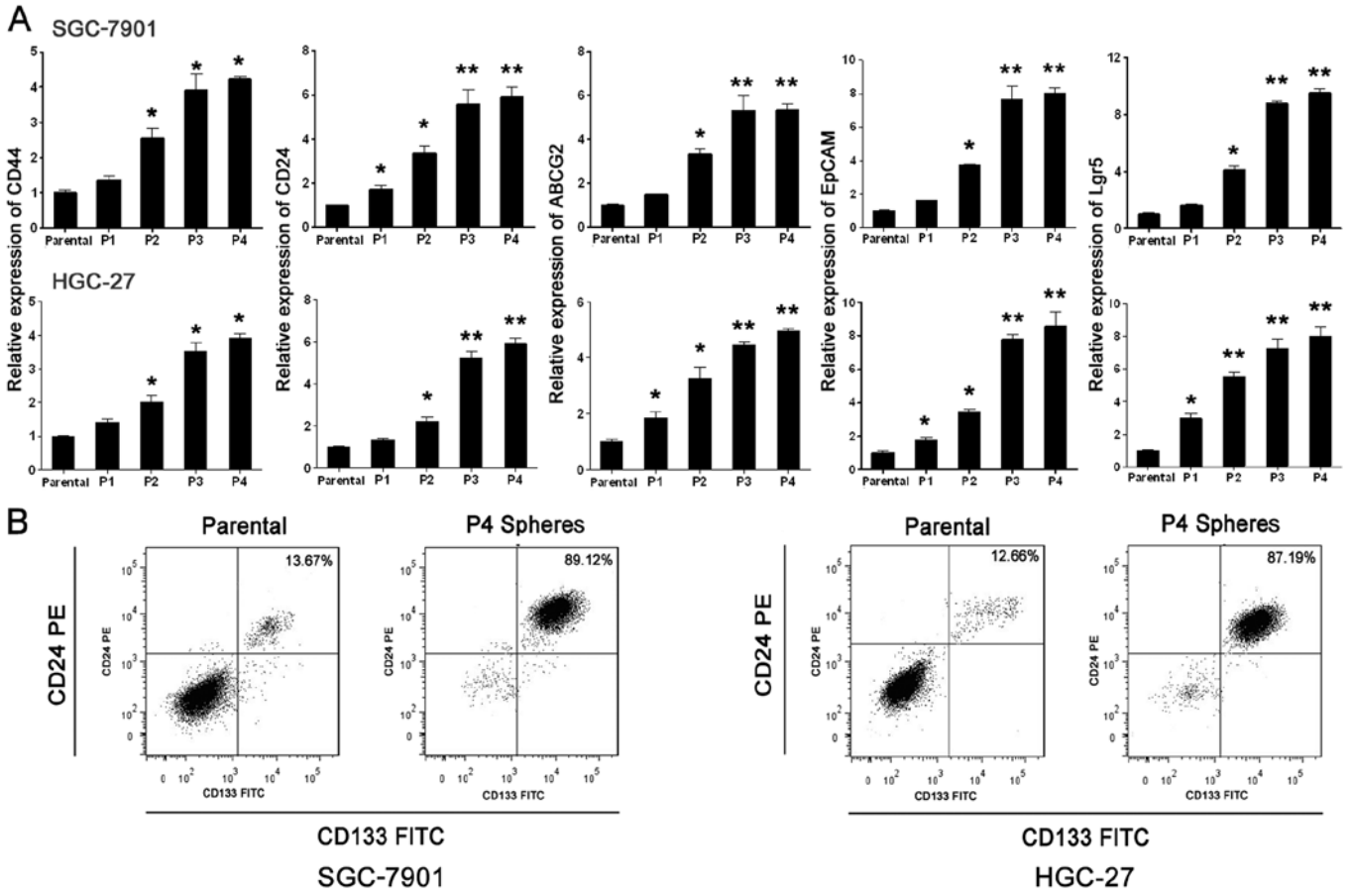


Figure 2. GC CSCs are enriched in the successive sphere formation population. (A) Parental SGC-7901 and HGC-27 cells and their P1-P4 sphere derivatives established by successive culture in SFDM were subjected to qPCR and flow cytometric analysis to detect CSC markers. qPCR was used to detect the relative mRNA levels of the markers of GC CSCs. $^{*}$P<0.05 and $^{**}$P<0.01 vs. the parental cells. (B) CSC surface protein $CD133^{+}/CD24^{+}$ ratio was significantly higher in the P4 spheres. P4 spheres showed a further increase in the double-positive cell population. GC, gastric cancer; CSCs, cancer stem cells; SFDM, serum-free defined media.

were co-treated with 5-FU + PPZ, the inhibition of proliferation was most obvious. This suggested that PPZ enhanced 5-FU chemosensitivity of both the SGC-7901 and HGC-27 cells (Fig. 1A). Then the sphere formation of SGC-7901 and HGC-27 cells was assessed and the results showed that after PPZ treatment, the self-renewal efficiency of both cell lines declined obviously, not only the sphere numbers but also the size (Fig. 1C). CSCs play a role in chemoresistance, thus real-time RT-PCR and western blot analysis were performed to detect the relative expression of stem cell markers. In both cell lines, after PPZ treatment, the mRNA levels and protein expression of CD44, CD24, ABCG2, EpCAM and Lgr5 were reduced significantly (Fig. 1B). All of these results suggest that the antitumor effect of PPZ may be related to the mediation of GC cell stemness.

*The establishment of GC CSC models*. Successive sphere formation culture was performed to establish the CSC models using SGC-7901 and HGC-27 cell lines. The relative mRNA levels of classic CSC marker genes were used as a measurement of the CSC proportion. It was found that the relative mRNA levels of classic CSC markers CD44, CD24, ABCG2, EpCAM and Lgr5 were significantly increased from P1 to P3, but there was no significant difference between P3 to P4 (Fig. 2A). These results suggest that when spheres were cultured to P4, the CSC proportion reached a relative peak amplitude using these methods. Flow cytometric analysis was used to detect the CD133- and CD24-positive cell ratio of parental cells and P4 spheres and the results showed that compared with the parental groups the ratio of $CD133^{+}/CD24^{+}$ cells was enhanced obviously in the P4 sphere groups (Fig. 2B). It was concluded that during the successive sphere formation culture to P4, the GC CSCs were effectively enriched and the P4 spheres were able to be used as the GC CSC models in the following experiments.

*PPZ inhibits the expression of CSC markers and drug-resistance markers*. P4 spheres of both SGC-7901 and HGC-27 cells were treated with PPZ (100 $\mu$g/ml), and the expression of CSC markers CD44, CD24, ABCG2, EpCAM, Lgr5 and drug-resistance markers BMI1, ALDH1 and Trf4 was detected. Not only CSC marker genes but also anti-drug genes were all significantly inhibited at both the mRNA and protein levels when compared with the spheres without PPZ treatment (Fig. 3). The results suggest that PPZ exerts its tumor inhibition effect by reducing the stemness of GC cells.

*PPZ enhances 5-FU chemosensitivity, and suppresses cell proliferation and sphere formation of GC-initiating cells*. P4 spheres of both SGC-7901 and HGC-27 cells were used as GC initiation cell models which were treated with PPZ (100 $\mu$g/ml).

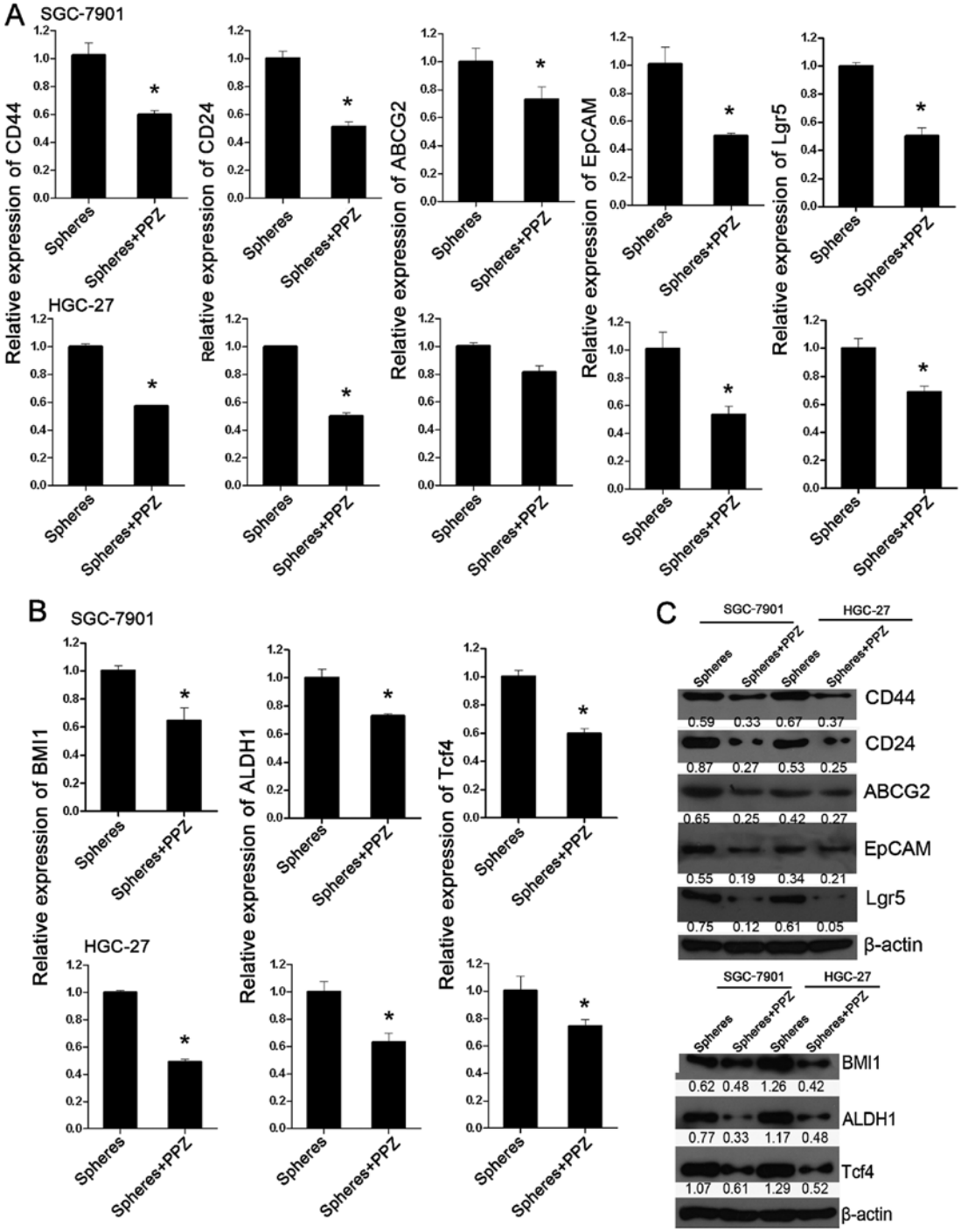


Figure 3. PPZ inhibits the expression of CSC markers and drug-resistance markers of GC cancer-initiating cells. Both GC P4 spheres were used as cell models of cancer-initiating cells. (A) PPZ treatment inhibited the relative mRNA levels of CSC marker genes of CD44, CD24, ABCG2, EpCAM and Lgr5. (B) qPCR was used to detect the relative mRNA levels of drug-resistance genes with or without PPZ treatment. (C) Western blot analysis was used to detect the expression of above gene markers with or without PPZ treatment. *P<0.05 vs. con (spheres without PPZ treatment). PPZ, pantoprazole; CSC, cancer stem cell; GC, gastric cancer.

Then the cell proliferation was measured by MTT assay. Compared with the P4 spheres without PPZ, the cell proliferation capacity was obviously decreased after 48 h and became even more significant after 72 h in both cell lines (Fig. 4A). P4 spheres of both cell lines were treated with 10-50 $\mu$g/ml 5-FU combined with or without PPZ under normal culture conditions. The cell viability assay showed that the cell viability was significantly inhibited when cells were co-treated with 5-FU + PPZ which indicated that PPZ enhanced the 5-FU sensitivity of the GC-initiating cells at least to some extent (Fig. 4B). Then the sphere formation of both P4 spheres was measured. After PPZ treatment, the self-renewal efficiency of both sphere types was obviously declined, not only sphere numbers but also the size (Fig. 4C). These results demonstrated that PPZ could exert its tumor inhibitory effect by reducing the stemness of GC cells at the cell functional level.

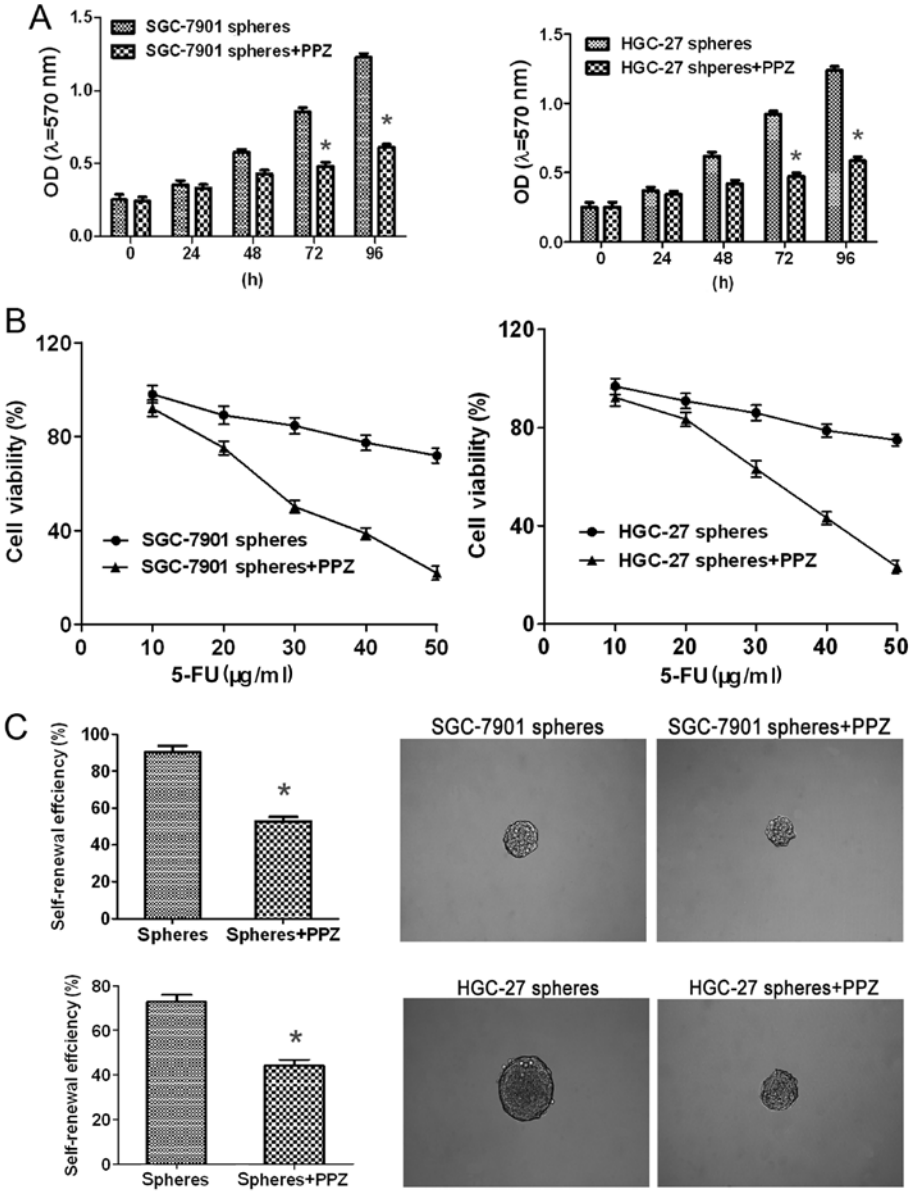


Figure 4. PPZ enhances 5-FU chemosensitivity, and suppresses cell proliferation and sphere formation of GC cancer-initiating cells. Both GC P4 spheres were used as cell models of cancer-initiating cells. (A) The proliferation of P4 spheres was measured using MTT assay at 24, 48, 72 and 96 h with or without PPZ treatment. (B) Cell viability was measured to evaluate the 5-FU sensitivity of P4 spheres with or without PPZ treatment. (C) Self-renewal capacity was evaluated using sphere formation assay of both GC P4 spheres with or without PPZ treatment. *P<0.05 vs. con (spheres without PPZ treatment). PPZ, pantoprazole; 5-FU, 5-fluorouracil; GC, gastric cancer.

*PPZ suppresses expression of EMT-related genes and the activation of β-catenin signaling.* To explore the potential downstream molecular pathways underlying the targeting of stemness inhibition by PPZ, we assessed the expression of genes involved in EMT and classic CSC-related signaling pathways including E-cadherin, N-cadherin, vimentin, Snail and total/active β-catenin by western blot analysis in both types of P4 spheres with or without PPZ treatment. A significant reduction in the expression of N-cadherin, vimentin, Snail and total/active β-catenin proteins and upregulation of E-cadherin were observed in the spheres treated with PPZ in both types of GC spheres (Fig. 5). It was demonstrated that the inhibitory effect associated with the PPZ targeting of GC CSCs was mediated by EMT and activation of the β-catenin signaling at least partly.

## Discussion

Current antineoplastic strategies are aimed at promoting the efficiency and specificity of therapies for GC. Intracellular proton extrusion in GC cells has been reported to promote cancer cell survival under acidic conditions via $H^+/K^+$-ATPase. PPZ is a frequently used second-generation PPI that irreversibly inactivates gastric $H^+/K^+$-ATPase. Early as 2004, relative research found that PPI selectively induced *in vivo* and *in vitro* apoptotic cell death in GC and could be used for a selective anticancer effect (28).

In regards to GC chemotherapy, the results are not satisfactory, and among the tested chemotherapeutic agents, only a limited number of compounds [5-FU, adriamycin (doxorubicin) and cisplatin] have demonstrated response rates ranging

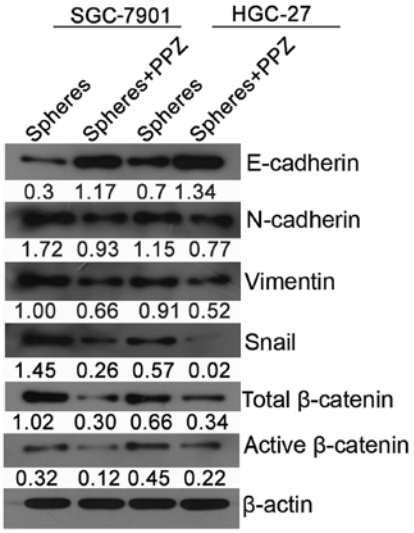


Figure 5. PPZ suppresses EMT-related gene expression and the activation of β-catenin signaling. With or without PPZ treatment, western blot analysis was used to detect the expression of E-cadherin, N-cadherin, vimentin, Snail and total/active β-catenin protein in both GC cell line P4 spheres. PPZ, pantoprazole; EMT, epithelial-mesenchymal transition; GC, gastric cancer.

from 15 to 50% selectively. Recent studies have elucidated the presence of CSCs that have the exclusive ability to regenerate tumors. These CSCs share many characteristics with normal stem cells, including self-renewal, differentiation and drug-resistance (29). The presence of CSCs has already been associated with chemotherapeutic failure in a variety of solid tumors including GC. Thus, revealing the underlying molecular mechanisms responsible for maintenance and chemoresistance of CSCs has become a crucial issue in the clinical treatment of GC. PPZ was reported to suppress proliferation and restore the chemosensitivity of GC SGC-7901 cells by inhibiting the STAT3 signaling pathway (30). It was found that PPZ can effectively reverse the aggressiveness and EMT marker expression of SGC-7901/ADR (adriamycin-resistant) cells and EMT was a typical feature of CSCs. Our research showed that associated with the proliferation, and 5-FU resistance inhibition, administration of PPZ decreased the expression of GC CSC markers. Thus, we hypothesized that PPZ could play a role in targeting CSCs.

The CSC model suggests that a small subset of cancer cells possesses stem cell properties and plays a crucial role in tumor initiation, metastasis and resistance to anticancer therapy (31). The characteristic features of CSCs include: i) self-renewing (tested by sphere formation in SFDM); ii) high tumorigenicity in xenograft-based model; and iii) ability to differentiate to the cell types of the tumor of origin (32,33). To date, although the purity is not 100%, there are three relatively satisfactory methods by which to establish CSC models in tumor cells: side population isolation, sphere culture and immunomagnetic bead isolation. No matter what isolation method is used, the relative expression levels of stem cell surface markers are favourable indices to measure the abundance of CSC models. In our study, we adopted a successive sphere culture method to establish our GC CSC models. Using qPCR, we found that the relative mRNA levels of stem cell markers were significantly upregulated from P1 to P3, but were not further upregulated from P3 to P4. The results suggested that, using the successive sphere culture method, we received a comparatively high proportion of GC CSCs from P4. Thus, in our following experiments, the P4 spheres were used as our CSC models.

Upon pre-treatment with PPI, ERK1/2 phosphorylation was completely inhibited; the inhibitory action on the phosphorylation of ERK1/2 might contribute to the induction of apoptosis in GC cells by PPI (34). Furthermore, PPZ treatment resulted in a profound reduction in both total and phosphorylated forms of Akt and GSK-3β, which in turn suppressed the adriamycin-induced Wnt/β-catenin signaling in SGC-7901/ADR cells. It is possible to suppress the invasiveness of SGC-7901/ADR cells by PPZ which targets the EMT and Akt/GSK-3β/β-catenin signaling (13). It is worth mentioning that EMT and Wnt/β-catenin signaling are classic relative signaling pathways that mediate the stemness of CSCs. EMT is an important way to induce CSC formation in a number of solid tumors. Moreover, EMT is associated with increased expression of stem cell-related transcription factors and with increased tumorigenic ability (31). In our experiment, when spheres were treated with PPZ, the EMT course and the activation of β-catenin were both inhibited to some extent which demonstrated that PPZ exerts its antitumor effect by targeting CSCs via the EMT/β-catenin pathways.

Our research for the first time demonstrated that PPZ could be used to promote a selective anticancer effect targeting GC CSC inhibition, but the underlying molecular mechanism should be further explored. PPZ may be a promising breakthrough in GC therapy.

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