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Original Articles

Proton pump inhibitor pantoprazole inhibits gastric cancer metastasis via suppression of telomerase reverse transcriptase gene expression

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ABSTRACT

The effect of proton pump inhibitors (PPIs) on cancer risk has received much attention recently. Over the last two decades, we and others have disclosed that PPIs exerted anticancer effects. Telomerase reverse transcriptase (TERT) is essential for telomere maintenance. The activation of TERT is considered a crucial step in tumorigenesis; therefore, it is a potential therapeutic target against cancer. However, whether PPIs suppress gastric cancer by targeting TERT remains elusive. Our study demonstrated that PPZ treatment repressed TERT expression in gastric cancer cells via regulating TERT promoter activity by disturbing the interaction of STAT3 with the TERT gene. Additionally, PPZ led to chromatin remodeling within the TERT gene and resulted in a more compacted spatial conformation that is known to be associated with gene silencing. PPZ downregulated the TERT gene to inactivate the Wnt/β-catenin signaling pathway and reverse the EMT process, finally inhibiting gastric cancer metastasis both *in vitro* and *in vivo*. Our results suggest that PPIs may be potentially developed as effective as well as relatively safe and specific anticancer agents.

1. Introduction

Despite the decreasing trend in Western countries in recent years, gastric cancer remains one of the most common malignant tumors and the third-leading cause of cancer-related deaths worldwide [1,2]. In China, gastric cancer represents the second most common type of cancer among males and the third most common type of cancer among females. Gastric cancer is currently the second-leading cause of cancer-related death among both men and women [3]. Early diagnosis of gastric cancer is difficult because most patients are asymptomatic in the early stage; thus, most gastric cancer patients are diagnosed at an advanced stage often accompanied by extensive invasion and lymphatic metastasis. Gastric cancer may be treated with surgery, radiation therapy, chemotherapy, targeted therapy or immunotherapy alone or in combination, which only minimally extend the lifespan of those patients at advanced stage. Therefore, investigations into the molecular mechanisms involved in gastric cancer progression and the identification of a novel therapeutic strategy that can provide highly selective antitumor effects have become imperative and urgent for targeted therapy.

Human telomerase is a ribonucleoprotein complex composed of an RNA template (hTR or hTERC) and telomerase reverse transcriptase (TERT) for telomeric DNA synthesis [4]. TERT re-expression and telomerase activation are present in over 90% of human cancers but not in

Abbreviations: PPIs, proton pump inhibitors; PPZ, pantoprazole; EMT, epithelial to mesenchymal transition; TERT, telomerase reverse transcriptase; STAT3, signal transducer and activator of transcription 3; GFP, green fluorescent protein; H3K9Ac, acetylated histone H3 at lysine 9; H3K4me2, methylated histone H3 at lysine 4; H3K9me3, tri-methylated histone H3 at lysine 9; H3K27me3, tri-methylated histone H3 at lysine 27; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ChIP, Chromatin immunoprecipitation

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normal somatic tissue [5]. In addition to the pivotal role of TERT in chromosome end protection and telomere maintenance, a large number of studies suggest telomere-independent mechanisms by which TERT might contribute to cancer development and progression, including regulation of gene expression, cell signaling, cell cycle regulation, inhibition of apoptosis, modulation of DNA damage response, cell metabolism, and mitochondrial function [6]. Extensive studies suggested that TERT overexpression is not only involved in the initiation of gastric cancer but also promotes its invasion and metastasis, thus making it a promising therapeutic target for the treatment of gastric cancer [7]. Indeed, inhibition of TERT expression suppressed gastric cancer cell proliferation and invasion [8–10]; therefore, the effort to design safe, specific and effective TERT inhibitors for the prevention and curation of gastric cancer is very important.

Proton pump inhibitors (PPIs), such as omeprazole, esomeprazole and pantoprazole (PPZ), have been widely used for the treatment of acid-related diseases, such as gastro-esophageal reflux disease, peptic ulcer disease, and the eradication of *Helicobacter pylori* infection [11]. Although PPIs are generally well tolerated, a growing number of studies have raised concerns relating to the possible adverse clinical consequences of their use in recent years, among which the carcinogenicity induced by PPIs has been mounting [11]. To date, the association of PPI use with the risk of gastric cancer is still controversial [12–14]. Previously, we and others have reported that PPIs function as a promising treatment strategy for gastric cancer because they significantly sensitize gastric cancer cells to antitumor drugs [15–17], directly inhibit cancer cell proliferation and drug resistance-induced metastasis [18,19], and regulate autophagy [20]. In this study, we investigated the role of PPZ in regulating TERT expression and cancer cell invasiveness. Our data suggested that PPZ treatment repressed TERT expression via transcriptional and epigenetic mechanisms. Moreover, the inhibition of TERT following PPZ treatment suppresses gastric cancer cell metastasis both *in vitro* and *in vivo*. Taken together, our results strongly support the feasibility of using PPIs in the treatment of gastric cancer patients.

2. Materials and methods

2.1. Cell lines, drugs and antibodies

Human gastric cancer cell lines AGS and HGC27 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI 1640 medium (Wisent Biotec, Co. Ltd. Montreal, QC, Canada) containing 10% fetal bovine serum (Wisent Biotec, Co. Ltd) in a humidified 5% CO2 atmosphere at 37 °C. Pantoprazole sodium in powder (PANTOLOC) was purchased from Takeda GmbH (Germany) and dissolved 1 mg/ml in saline before use. Antibodies against snail (ab82846) and β-actin (ab8226) were purchased from Abcam (Cambridge, MA, USA). Antibodies against Vimentin (clone VIM 3B4) were purchased from Millipore (Billerica, MA, USA). Antibodies against E-cadherin (BS1098), N-cadherin (W745), and TCF4 (BS6172) were purchased from Bioworld Technology (St Louis Park, MN, USA). Antibodies against β-catenin (610153) were purchased from BD Biosciences. Antibodies against Stat3 (clone 124H6, #9139), phospho-STAT3 (Tyr705) (clone D3A7, #9145), HRP-linked anti-rabbit (#7074) and anti-mouse (#7076) secondary antibodies were purchased from Cell Signaling Technology.

2.2. RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from cultured cells after designated treatments using TRIzol reagent, and reverse transcription was carried out with l μg RNA in a 20 μL total reaction volume using PrimeScript™ RT Master Mix according to the manufacturer's instructions. Quantitative real-time PCR experiments were performed with the 7500 Real-time PCR System (Applied Biosystems, Foster City, Calif) using SYBR Premix Ex Taq reagents. The sequences of primers used in real-time PCR are listed in Supplementary Table 1. All data were normalized to human β-actin. All assays were performed in triplicate.

2.3. Plasmids, DNA transfection and luciferase reporter assay

Vectors expressing TERT (pLV102-TERT) and GFP (pLV102-GFP) were from GeneCopoeia (Rockville, MD). Promoterless (pGL3 basic), SV40 promoter-driven (pGL3-SV40) and pRL-TK luciferase reporter vectors were purchased from Promega (Madison, WI). The TCF/LEF-1 reporter (TOP-FLASH) and mutation vector (FOP-FLASH) were purchased from Addgene (Addgene plasmid 12456 and 12457, Cambridge, MA). The pGL3-3778 and pGL3-2089 reporter vectors were constructed by subcloning the 3778-bp and 2089-bp fragments of the TERT proximal promoter into the pGL3 basic vector, respectively. The mutation in the STAT3 binding site of pGL3-3778 was induced by PCR-based mutagenesis. Transient transfections were performed using GeneJuice (Novagen, Madison, WI). All luciferase reporter assays were performed using a Dual Luciferase Reporter Assay System (Promega).

2.4. Western blot analysis

Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl with pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% NP 40, 1 mM EDTA) supplemented with protease inhibitor cocktail. The lysates were then centrifuged, and the supernatants were collected, and protein concentrations were determined. Equal amounts of protein (at least 30 μg) were resolved by SDS–polyacrylamide electrophoresis and transferred to a nitrocellulose membrane and immunoblotted with antibodies as indicated, followed by appropriate HRP-conjugated secondary antibodies. Signals generated by enhanced chemiluminescence (Millipore, Billerica, MA, USA) were recorded with a CCD camera (CLINX, Shanghai, China). Data are representative of at least three independent experiments.

2.5. Chromatin immunoprecipitation

A chromatin immunoprecipitation (ChIP) assay was conducted as previously described [21]. The primers used in ChIP-qPCR experiments are shown in Supplementary Table 2. Occupancy of p-STAT3 protein or indicated histone markers was normalized to input and expressed as a percentage of the input DNA (% input). IgG was used as a negative control. Data were obtained from at least three independent experiments.

2.6. Cell proliferation and motility assays

The cell proliferation and viability were evaluated by adding Cell Counting Kit-8 (CCK8, Dojindo, Japan) reagent, and the absorbance at 450 nm was measured. Cell migratory abilities were determined by Transwell (Corning Life Sciences, Bedford, MA, USA) migration assays.

2.7. Experimental metastasis assay

Sixteen male BALB/c mice (5 weeks old) were used in this study. Briefly, 5×10^6 HGC27 cells resuspended in 0.25 ml of saline were injected intravenously into the tail vein into each mouse. Four weeks after the tumor cell injection, the animals were randomly divided into two groups (n = 8 each) and treated as follows: the treatment group, PPZ resuspended in saline (15 mg/ml) immediately before use, was orally administered daily by gavage at a dose of 75 mg/kg; and the control group, which received vehicle only (saline). All mice were euthanized 4 weeks later. The presence of tumor nodules was macroscopically determined, and the number of tumor nodules formed on the lung surfaces was counted. The lungs were excised and embedded in paraffin. All animal procedures and care were approved by the Institutional Animal Care and Use Committee of Nanjing Drum Tower

Hospital, Medical School of Nanjing University.

2.8. Statistical analysis

Data were analyzed with GraphPAD PRISM 6.0 (GraphPad Software, La Jolla, CA, USA). Data were expressed as the mean ± SD of at least three independent experiments. Statistically significant differences between the treated and the control groups were identified by Student's t-test, ordinary one-way ANOVA, and two-way ANOVA followed by Dunnett's multiple comparisons test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Identification of TERT as a primary target gene for PPZ

To determine the effect of PPZ on TERT expression in gastric cancer cells, we cultured AGS and HGC-27 cells in medium with PPZ for 24 h over a range of concentrations (0, 20, 40, 60, and 80 μg/ml). TERT gene expression in AGS and HGC-27 cells was evaluated by using qRT-PCR and Western blotting, respectively. Interestingly, our results showed that PPZ markedly decreased the expression of TERT at both the messenger RNA (mRNA) and protein levels in a dose-dependent manner (Fig. 1A and B), suggesting that PPZ may directly regulate TERT expression at the transcriptional level. To further elucidate the role of PPZ in the transcriptional regulation of TERT expression, we cloned 3778 bp (pGL3-3778) and 2089 bp (pGL3-2089) human TERT promoter DNA fragments into a luciferase reporter (Fig. 2A). To explore the role of PPZ in the regulation of TERT promoter activity, HGC-27 cells were co-transfected with each of the luciferase and renilla reporters. While PPZ significantly attenuated the luciferase reporter activity of pGL3-3778, it did not alter the activity of pGL3-2089 or the SV40 promoter (pGL3-SV40) (Fig. 2B). These data suggested that PPZ directly regulates TERT expression via transcriptional regulation at the distal promoter.

3.2. PPZ modulates TERT gene transcription in a STAT3-dependent manner

To explore the potential mechanism involved in the regulation of TERT promoter activity by PPZ, we identified STAT3 as an important mediator between PPZ and TERT. Western blot assays showed that PPZ treatment decreased STAT3 expression and phosphorylation in AGS and HGC-27 cells (Fig. 3A and Supplementary Fig. 1). Bioinformatics analysis using JASPAR [22] revealed putative binding sites of STAT3 located at −3363 ~ -3353 and −1108 ~ -1098 bp of TERT (Supplementary Fig. 2). Our luciferase reporter gene assay suggested that PPZ may regulate TERT gene promoter activity by functioning in a region between −3778 and −2089. Thus, we introduced a site-directed mutation in the region between −3362 and −3354 in the pGL3-3778 construct (Fig. 3B). A luciferase reporter gene assay in HGC-27 cells revealed that mutation of the upstream STAT3-binding site

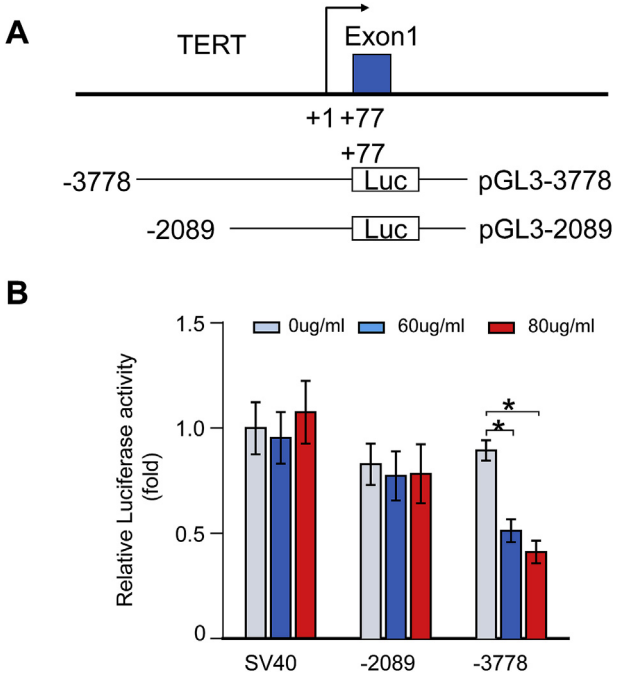

Fig. 2. PPZ inhibits TERT gene promoter activity. (A) A schematic representation of TERT promoter reporter plasmids. The TERT promoter reporter plasmids were generated by inserting the 3778 bp and 2089 bp DNA fragments of the TERT promoter upstream of the initiating ATG into the luciferase (Luc) reporter vector pGL3-Basic in the sense orientation. Arrow, transcription start site. Numbers, the number of bases upstream (−) and downstream (+) of the translational start codon. (B) Normalized luciferase activity of TERT reporter gene constructs. SV40 promoter-driven luciferase reporter vector (pGL3-SV40) was used as a control. The normalized luciferase activity of HGC-27 cells expressing pGL3-SV40 and treated with the mock control was designated as 1. The results are the mean ± S.D. of triplicate measurements from one of three representative experiments with similar results. Statistical analysis was performed with Student's *t*-test. $^{*}P < 0.05$.

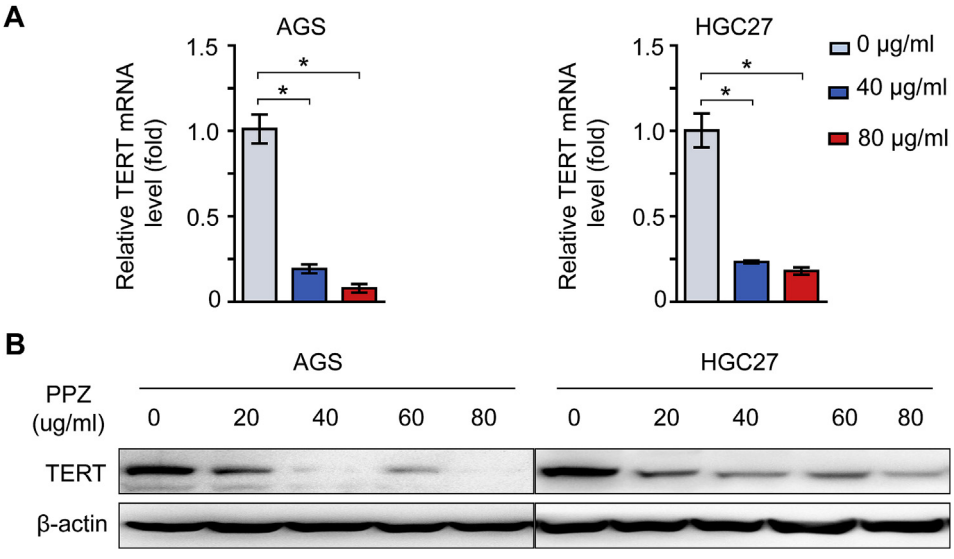

Fig. 1. PPZ inhibits TERT expression. (A) qRT-PCR analysis revealed that PPZ suppresses TERT gene mRNA levels in a dose-dependent manner. Values are the mean ± standard deviation (S.D.) for triplicate determinations from three different cultures. Data obtained from a representative experiment are shown. $^{*}P < 0.05$, Student's *t*-test. (B) Western blotting assay suggested that PPZ suppresses TERT gene protein levels in a dose-dependent manner.

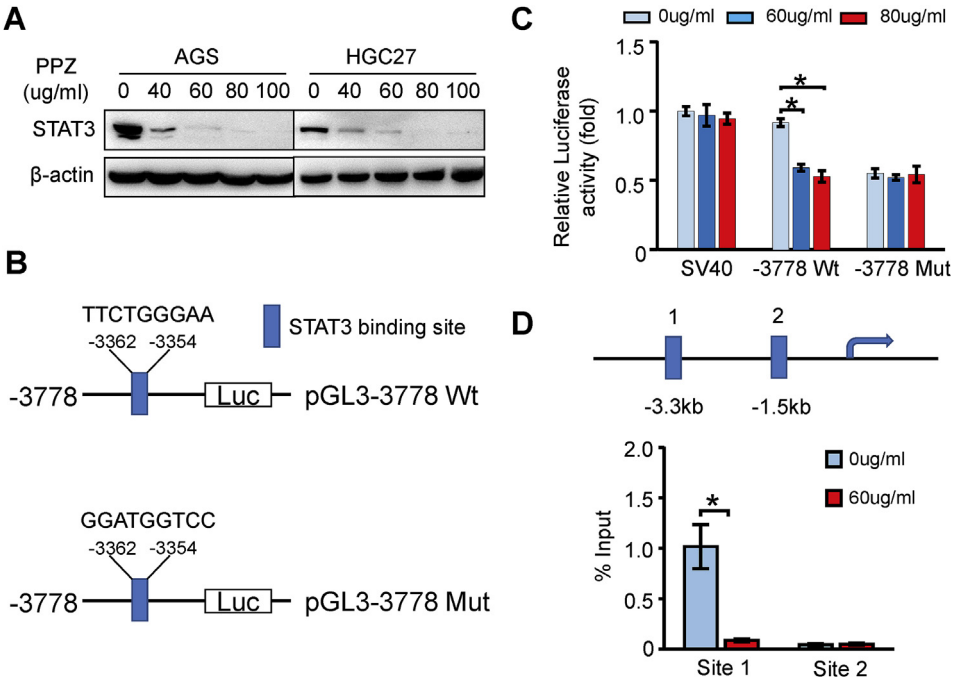

Fig. 3. PPZ inhibits TERT gene expression by disturbing the interaction of STAT3 with the TERT gene promoter. (A) Western blotting assay results suggested that PPZ suppresses STAT3 expression in a dose-dependent manner. (B) Diagram of the TERT promoter with the location of consensus STAT3-binding sites and the corresponding site mutations indicated. (C) Normalized luciferase activity of the wild-type and STAT3 binding site mutated TERT reporter gene constructs. pGL3-SV40 was used as a control. The normalized luciferase activity of HGC-27 cells expressing pGL3-SV40 and treated with the mock control was designated as 1. The results are the mean ± S.D. of triplicate measurements from one of three representative experiments with similar results. Statistical analysis was performed with Student's t-test. $^{*}P < 0.05$. (D) ChIP-qPCR analyses of STAT3 occupancy of the TERT gene of HGC-27 cells. $^{*}P < 0.05$, one-way ANOVA. The results show the mean ± standard deviation from triplicate experiments.

reduced TERT promoter activity by approximately 45%. More importantly, the mutation of the nine nucleotides of the STAT3-binding site eliminated the inhibition by PPZ (Fig. 3C), showing that the distal STAT3-binding site in the TERT promoter is functional. To determine the *in vivo* binding of STAT3 to the TERT promoter, we performed ChIP-qPCR assays in HGC-27 cells. The data showed that the TERT promoter DNA fragment spanning the distal STAT3-binding site was immunoprecipitated by an anti-STAT3 antibody, but it was abolished by PPZ treatment. In contrast, no significant enhancement of signal was observed in the region within −1.5 kb, where there is no putative STAT3-binding site around this locus (Fig. 3D). Considered together, these data are in support of PPZ modulating the expression of TERT via regulation of the interaction of STAT3 with its distal promoter.

3.3. PPZ caused chromatin remodeling at the TERT gene

In addition to the interaction of various transcription factors as the primary mechanism of transcriptional control, histone acetylation and methylation also play important roles in TERT gene regulation. Specifically, hyperacetylated histones and histone H3 methylation at lysine 4 are common markers of active chromatin, whereas hypoacetylated histones and methylation of histone H3 at lysines 9 and 27 are generally associated with inactive genes [23]. To explore whether these chromatin markers affect TERT gene expression in response to PPZ treatment, we performed ChIP assays followed by quantitative PCR analysis of three amplicons spanning the distal regulatory regions (−3.3 kb and −1.5 kb) and transcriptional start site of the TERT gene (Fig. 4A). PPZ treatment led to a significant decrease in acetylated histone H3 at lysine 9 at the most distal locus and transcriptional start site in HGC-27 cells. Similarly, it also led to a marked reduction in histone H3 methylation at lysine 4 at the transcription start site. In contrast, PPZ treatment led to a profound enrichment of trimethyl histone H3 at lysines 9 and 27 at the distal 5' regulatory region (Fig. 4B). Collectively, these results suggest that PPZ may modulate TERT transcription through chromatin remodeling primarily through alterations of methylation and acetylation in specific histone residues across the TERT gene.

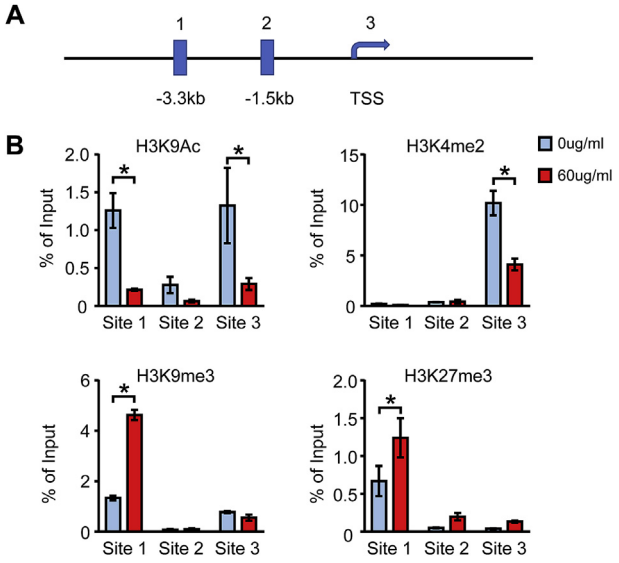

Fig. 4. Alterations of histone modification of the TERT gene following PPZ treatment in HGC-27 cells. (A) Three regions were investigated by ChIP-qPCR analysis. The arrow represents the transcription start site. (B) Occupancy of each of the chromatin marks, acetyl-H3K9 (H3K9Ac), dimethyl-H3K4 (H3K4me2), trimethyl-H3K9 (H3K9me3) and trimethyl-H3K27 (H3K27me3), are normalized to input and shown as the fold difference relative to that in control cells. Each sample was analyzed in triplicate by qPCR. All results are representative of one of three independent experiments with similar results. $^{*}P < 0.05$ control cells by Student's *t*-test.

3.4. PPZ inhibited gastric cancer cell motility but not growth via downregulation of the TERT gene

Previously, we demonstrated that PPZ treatment leads to the inhibition of gastric cancer cell viability and motility [17,19]. We then wanted to determine whether the ectopic expression of TERT is capable of preventing inhibition of viability and motility induced by PPZ. The consequences of TERT inhibition on cell growth and migration resulting from PPZ treatment were then analyzed comparatively in TERT- and GFP-expressing cell lines with or without PPZ treatment. HGC-27 cells

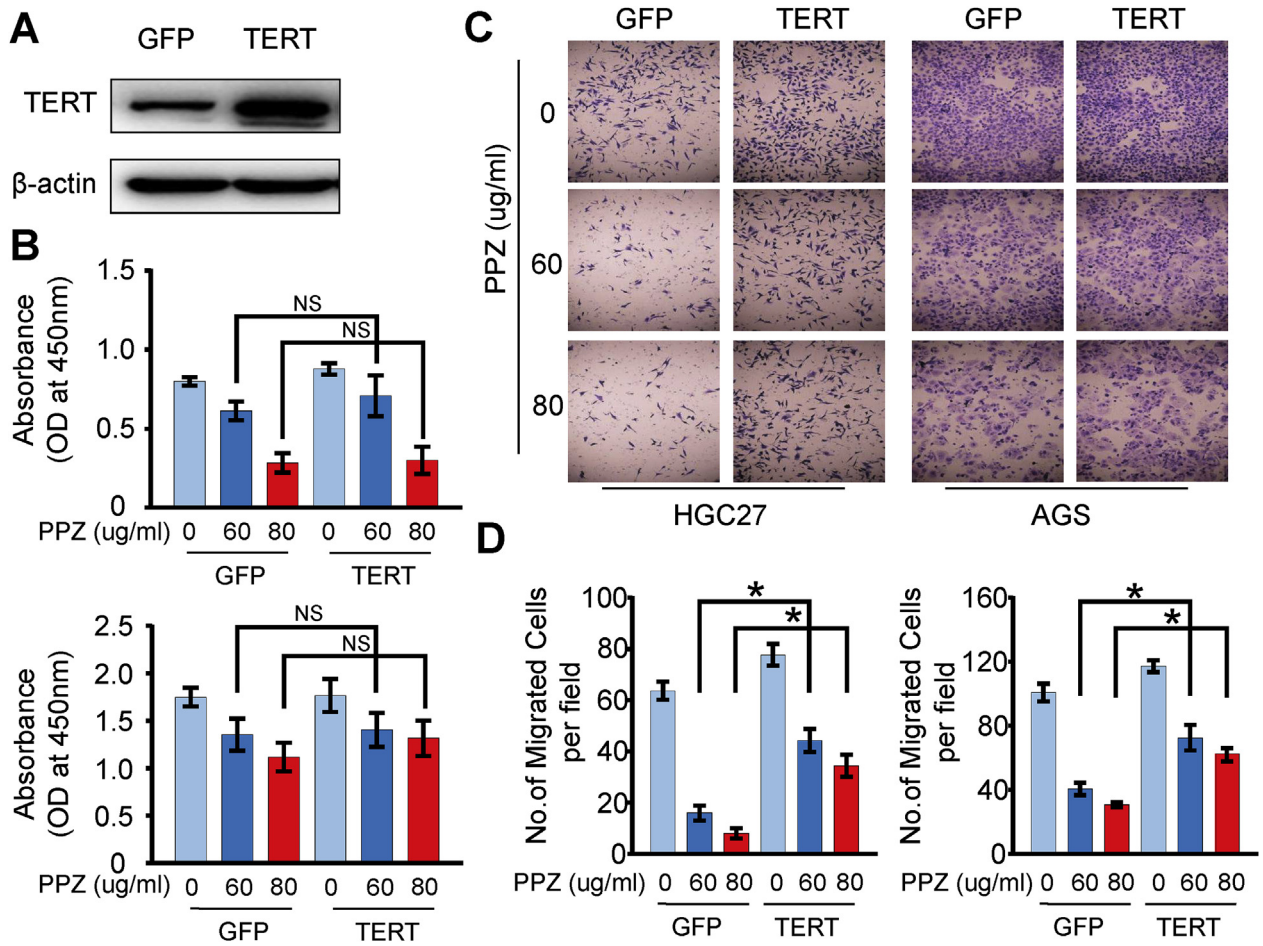

Fig. 5. PPZ inhibited gastric cancer cell migration by downregulating TERT gene expression. (A) Results of Western blot analysis showing the induction of TERT by transient transfection of TERT-expressing plasmid. (B) Results of CCK-8 analysis suggested that TERT ectopic expression failed to rescue the decreased growth speed of HGC-27 cells following treatment with PPZ. (C) Transwell migration assays revealed that TERT overexpression significantly rescued the reduced migratory ability of AGS and HGC-27 cells following treatment with PPZ. (D) Quantification of cell migration under different conditions. For each condition, five different fields were counted. $^{*}P < 0.05$.

expressing ectopic TERT contained approximately 2- to 3-fold the TERT protein level compared with the GFP-expressing counterparts (Fig. 5A). Upon exposure to PPZ, TERT-expressing AGS and HGC-27 cells still exhibited decreased growth speed in a dose-dependent manner, which is similar to that observed in the GFP-expressing cells (Fig. 5B). Consistently, the cell cycle analysis revealed that TERT overexpression does not rescue the G2/M arrest of HGC-27 cells induced by PPZ, suggesting that TERT overexpression does not attenuate PPZ-induced cell viability inhibition (Supplementary Fig. 3). We then further sought to determine whether TERT overexpression was capable of antagonizing PPZ-induced migration inhibition. Transwell migration assays revealed that PPZ significantly reduced the migratory ability of GFP-expressing AGS and HGC-27 cells in a dose-dependent manner. However, PPZ decreased the migratory ability of TERT overexpressing cells to a lesser extent (Fig. 5C and D). Consistently, PPZ treatment led to decreased cell invasion, which was reversed by ectopic expression of TERT (Supplementary Fig. 4). These data collectively indicate that the TERT gene is a key component in the process of PPZ suppressing gastric cancer cell motility.

3.5. PPZ downregulates TERT gene to inhibit Wnt/β-catenin and epithelial-mesenchymal transition

Earlier, we found that PPZ suppresses the invasiveness of chemoresistant gastric cancer cells by targeting the **epithelial-mesenchymal transition** (EMT) and Wnt/β-catenin signaling [19]. In the present study, PPZ treatment significantly decreased the transactivation of the TCF reporter as determined by TOP/FOP flash luciferase reporter analysis (Fig. 6A). Furthermore, Western blotting showed that PPZ caused a significant reduction in β-catenin and its downstream targets, such as TCF4, in both AGS and HGC-27 cells (Fig. 6B), suggesting that PPZ also inhibits Wnt/β-catenin signaling activity in parental gastric cancer cells. The Wnt/β-catenin signaling pathway serves as an important regulator of EMT in cancer cells. Indeed, PPZ treatment resulted in dramatic activation of E-cadherin and concurrent inhibition of N-cadherin, Vimentin and Snail proteins in both cell lines (Fig. 6B), suggesting that the PPZ-induced suppression of cell migration is associated with inactivating the Wnt/β-catenin signaling pathway and reversal of the EMT process. TERT has been proven to act as a transcriptional modulator of the Wnt/β-catenin signaling pathway [24]. Our data consistently revealed that TERT overexpression upregulated β-catenin and TCF4 in HGC-27 cells and decreased E-cadherin but increased N-cadherin, Vimentin and Snail proteins (Fig. 6C). This prompted us to investigate whether TERT re-expression could restore the reversed EMT process induced by PPZ. As expected, while PPZ dramatically reduced TERT, N-cadherin and Vimentin as well as increased E-cadherin proteins in GFP-expressing HGC-27 cell lines, it failed to modulate TERT, E-cadherin and Vimentin proteins in TERT-expressing cells and downregulated N-cadherin to a lesser extent compared with the changes in GFP-expressing cells (Fig. 6D). Taken together, these data suggest that PPZ downregulates the TERT gene to inhibit Wnt/β-catenin and EMT and consequently suppress gastric cancer cell migration.

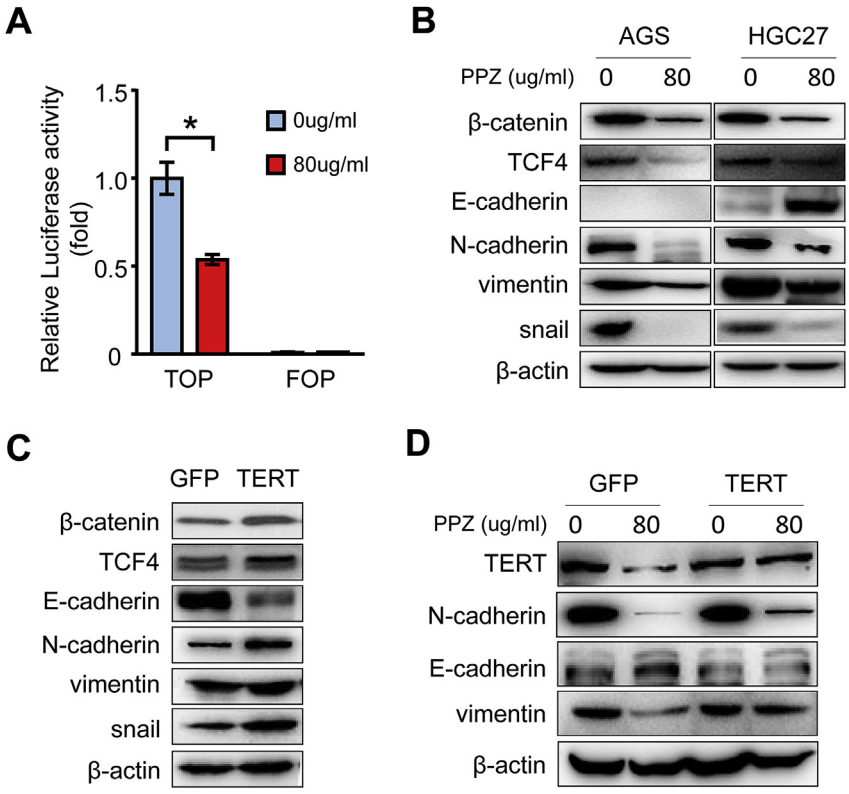

Fig. 6. PPZ downregulates the TERT gene to inhibit Wnt/β-catenin and EMT. (A) β-Catenin/TCF4 transcription activity was determined by using a TOP/FOP luciferase activity assay. (B) The protein levels of β-catenin, TCF4, E-cadherin, N-cadherin, Vimentin and Snail in AGS and HGC-27 cells following treatment with PPZ were assayed by Western blotting. (C) The protein levels of β-catenin, TCF4, E-cadherin, N-cadherin, Vimentin and Snail in HGC-27 cells following TERT-expressing or GFP-expressing plasmid transfection were assayed by Western blotting. (D) The protein levels of TERT, E-cadherin, N-cadherin and Vimentin in HGC-27 cells under different conditions were assayed by Western blotting.

3.6. PPZ inhibits tumor metastasis in an HGC-27 xenograft mouse model

To assess the potential clinical relevance of the *in vitro* results, we performed *in vivo* experiments in a mouse model to further evaluate the antimetastatic effect of PPZ. After the mice were sacrificed, the metastatic nodules at the lung surfaces were counted. As shown in Fig. 7A, the incidence of lung metastasis was significantly lower in mice treated with PPZ than that in mice treated with vehicle. H&E staining suggested that the nodules on the surfaces of mouse lungs were metastatic tumors and showed a marked reduction in the tumor mass in the lungs of the PPZ-treated animals (Fig. 7A and B). Moreover, PPZ treatment markedly reduced the mRNA levels of TERT in the lung (Fig. 7C). These results indicate that PPZ exerts antimetastatic activity *in vivo*.

4. Discussion

Here, we show that gastric cancer cells have active TERT that can be inhibited by proton pump inhibitors (PPIs), leading to inhibition of metastasis, as shown by decreased invasive potential and lung metastasis. We report the first *in vivo* study in which PPZ treatment suppresses gastric cancer metastasis. Mechanistically, we demonstrated that PPZ treatment repressed TERT expression via transcriptional and epigenetic mechanisms, which inactivate the Wnt/β-catenin signaling pathway and reverse the EMT process. The present study demonstrated for the first time that TERT can be targeted by proton pump inhibitors, which has been previously proven to suppress gastric cancer cells by our group and others [17,18].

PPIs act by irreversibly binding to the H^+/K^+-ATPase pump on parietal cells, effectively suppressing acid secretion into the gastric lumen. As such, they are indicated for several acid-related disorders, including gastro-esophageal reflux disease, peptic ulcer disease and treatment of *Helicobacter pylori* infection. During the last two decades, PPIs have become the most commonly used drugs in the world. With the increasing use of PPIs, a series of important therapeutic challenges, adverse effects and complications have emerged. In recent years, an increased risk of gastric cancer with PPI use has often been discussed but is still controversial. Although several investigations revealed that the incidence of gastric cancer increased among PPI users [25,26], they are only a few retrospective observational studies, and the data accuracy cannot be guaranteed. Therefore, the causal relationship between PPI use and gastric cancer development might be confounded. Moreover, in a pooled analysis of four RCTs, PPIs were not associated with gastric atrophy or other premalignant changes [27]. In the SOPRAN and LOTUS trials, 812 adults were randomized to antireflux surgery versus PPIs and followed with serial study biopsies. After up to 12 years of follow-up, there was no difference between groups in gastric premalignant changes and gastric cancers [28]. Thus, future investigations assessing the risk of gastric cancer associated with PPI use should be conducted prospectively in selected groups of patients free of risk factors, including long-term monitoring strategies.

The peculiar anaerobic or aerobic metabolism of glucose by cancer cells leads to the accumulation of acid byproducts resulting in an acidic environment that strongly affects tumor cells and their host; this is called the “Warburg Effect,” representing the ability of cancer cells to ferment sugars with lactate production, independent of the oxygen levels within the tumor mass [29]. The acidic extracellular environment favors tissue damage, activation of destructive enzymes in the extracellular matrix (ECM), and the increased metastatic potential as well as the acquisition of multidrug resistance (MDR) cell phenotypes. PPIs exert antitumor effects by targeting the tumor acidic microenvironment [15,17]. We and others reported that PPIs sensitize tumor cells and tumors to the action of chemotherapeutics [15,17,30] and showed that PPIs exert potent antitumor activity *per se* [18,31]. Recently, two clinical trials in osteosarcomas and metastatic breast cancer patients provided proof of concept that PPIs may be associated with current anticancer therapies. In the first study, the investigators reported that pretreatment with PPIs increased the effectiveness of neoadjuvant chemotherapy in osteosarcoma patients [32]. In the second study, researchers suggested that women with metastatic breast cancer undergoing PPI treatment experienced the highest response rates and the longest survivals [33]. Falk et al. confirmed that PPIs plus high-dose aspirin prevented esophageal adenocarcinoma in patients with Barrett's

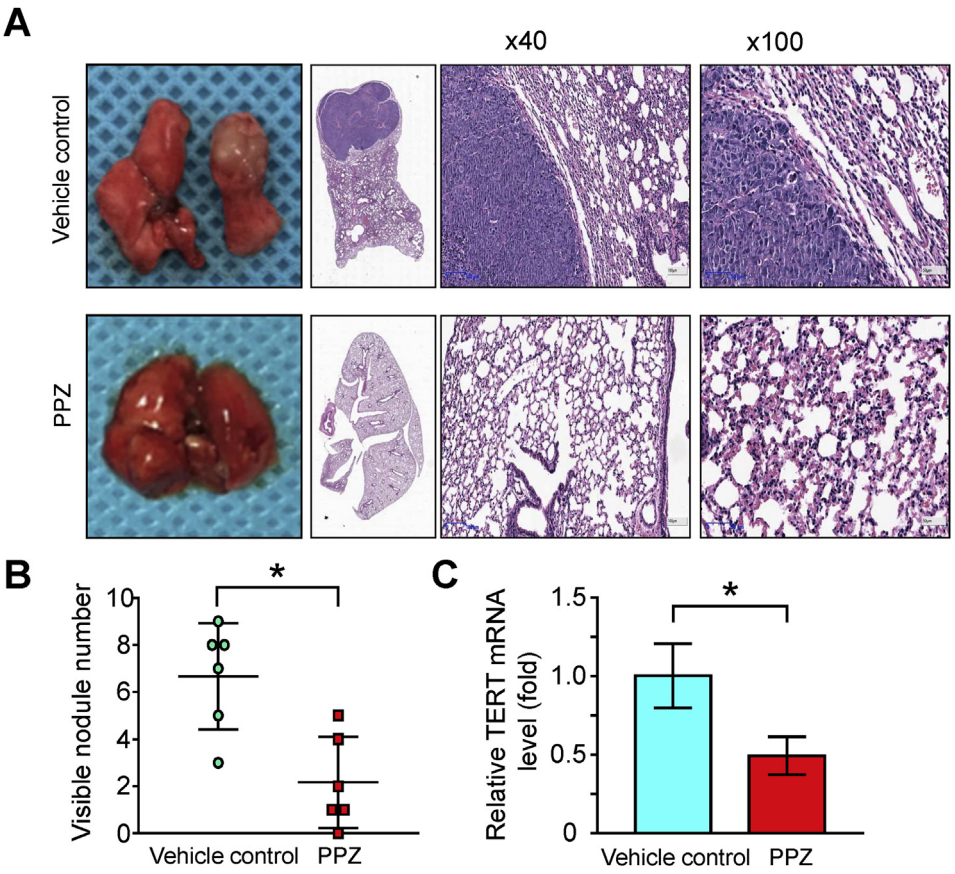

Fig. 7. PPZ exerts antimetastatic activity *in vivo*. (A) Representative metastatic nodules on the surface of the lung and the tissue with H&E staining. (B) The number of nodules was quantified in the lungs of mice (n = 8 per group). (C) TERT mRNA levels were significantly lower in the lung tissue of mice treated with PPZ.

esophagus [34]. It is desirable that in the future, PPIs will be recognized as a novel anticancer therapy, opening a nonmainstream avenue to the clinical approach of cancer.

Telomerase reverse transcriptase (TERT) is the catalytic component of telomerase, especially the rate-limiting determinant of telomerase activity. To date, TERT has been reported to be overexpressed in more than 90% of cancers, thereby playing a critical role in sustained proliferation and survival potentials of various cancer cells [5]. In addition, studies suggest telomere-independent mechanisms by which TERT might contribute to cancer development and progression, including modulation of Wnt/β-catenin signaling and mitochondrial RNA processing [24,35]. TERT has thus been suggested as a promising target for cancer therapeutics. Indeed, inhibition of the TERT gene by RNAi suppresses cancer cell proliferation, leading to apoptosis and enhances chemosensitivity [20,36,37]. Much effort has been devoted to the identification of TERT inhibitors as anticancer drugs. Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables, inhibited TERT gene expression in a dose- and time-dependent manner to suppress cellular growth and induce apoptosis in human breast cancer cells, making it an attractive target for chemoprevention in varying cancer cell types [38]. Our group has reported that piperlongumine, a biologically active component of the long pepper, dramatically inhibited the TERT gene and suppressed gastric cancer both *in vitro* and *in vivo* [39]. In the present study, our data suggested that PPZ suppressed gastric cancer metastasis by targeting the TERT gene. To the best of our knowledge, this is the first report describing the novel role of PPZ in regulating the TERT gene. Given the common use of PPIs during the last two decades, they may represent a novel TERT inhibitor and a safe and specific anticancer agent.

Gastric carcinogenesis is a multistep and multifactorial process. Previous studies have documented the importance of genetic and epigenetic alterations of oncogenes, tumor suppressor genes and mismatch repair genes in the development of gastric cancer. Some studies demonstrated that TERT expression increases with the sequential steps of intestinal-type gastric carcinogenesis, suggesting that TERT deregulation represents an important step in the carcinogenesis progress [40–43]. Since TERT is a direct target of PPZ, we hypothesize that PPIs can also reverse these precancerous lesions and thus interrupt malignant transition. This may broaden the indication for their clinical use. However, a prospective randomized controlled trial should be conducted to test this hypothesis. In addition, our study revealed that PPZ directly alters the chromatin conformation of TERT in gastric cancer cells to a more closed state. It will be interesting to determine whether PPZ alters the expression of epigenetic enzymes and their activity, as well as the conformation change for access of various transcription factors to the TERT promoter. However, the PPZ-induced epigenetic alterations observed in this study make it an attractive target for chemoprevention in varying cancer cell types.

In the present study, we demonstrated the PPZ-induced downregulation of the TERT gene in gastric cancer cells and explored possible transcriptional and epigenetic mechanisms. We further identified the functional role of PPZ in suppressing cancer metastasis via regulating the TERT gene. It is important to point out that TERT gene control is unique, and the proposed mode of action is not the only way PPZ inhibits cancer cell growth. Mechanistic studies should examine whether TERT downregulation by PPZ treatment inhibits metastasis by a telomere-dependent or -independent mechanism. Our results, together with previous work, suggest that PPIs may be potentially developed as effective as well as relatively safe and specific anticancer agents.

Conflicts of interest

The authors declare no potential conflicts of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

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