

Effects of pantoprazole as a HIF-1 α inhibitor on human gastric adenocarcinoma sgc-7901 cells

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Previously, it has been demonstrated that HIF-1 α has close connection with malignant tumor progression, aggressive behavior and prognosis. In addition, proton pump inhibitors (PPIs) have been reported to selectively induce tumor cell apoptosis, thus exerting its anticancer effects. *In vitro* and *in vivo* our study revealed that pantoprazole (PPZ) inhibited tumor cells proliferation, induced apoptosis and decreased the expression of HIF-1 α protein. In summary, PPZ could suppress tumor growth acting as a HIF-1 α protein inhibitor.

Key words: gastric cancer, proton pump inhibitors, HIF-1 α

HIF-1 is a heterodimer composed of two subunits, HIF-1 α and HIF-1 β , and they both have a structure of basic-helix-loop-helix (bHLH)-PAS [1, 2]. HIF-1 β is constitutively expressed whereas HIF-1 α varies following the oxygen density, which is degraded instantly in normal oxygen concentration conditions but is stable in hypoxic conditions [3, 4]. Although malignant tumors tend to form many new vessels, because of severe structural and functional abnormalities of tumor microvessels, deterioration of diffusion geometry, decreasing oxygen transport capacity of the blood and increasing distances of diffusion from capillaries, some parts of tumor regions will be hypoxic [5, 6]. It is suggested that HIF-1 α in tumor cells is stabilized as a result of the hypoxic environment developed in certain regions. In fact, HIF-1 α has been revealed to be overexpressed in more than 70% of solid tumors [7].

Gastric cancer is the fourth most common cancer and the second leading cause of cancer related death in the world [8]. A lot of experiments have showed that HIF-1 α is overexpressed in gastric cancer [9-12] and inhibition of HIF-1 α has proven

anti-tumor effect [13, 14]. The crucial role of HIF-1 α in gastric cancer suggests that identifying the new inhibitors of HIF-1 α should be a promising approach to the treatment of human gastric cancer.

Pantoprazole (PPZ), a type of proton pump inhibitors, acts as an inhibitor of H⁺, K⁺-ATPase of the gastric parietal cells. We hypothesized that the effect of PPZ on HIF-1 α may be related to its inhibitory efficacy on proton pump. Yeo et al. reported that PPZ selectively induced cancer cells apoptosis *in vitro*, and in a xenograft model of nude mice, the isolated tumor from mice with intratumoral administration of PPZ was remarkably smaller than that in the control group [15]. It is suggested that PPZ might provide an effective anticancer effects. However, little data have been focused on the involving mechanisms.

Our study have demonstrated that PPZ refrained proliferation and induced apoptosis selectively in SGC-7901 gastric adenocarcinoma cells, and significantly inhibited tumorigenesis in a tumor xenograft model. We also made an attempt to document the potential mechanism largely through the detection of HIF-1 α protein. Our novel findings suggested that PPIs could be considered as a new important direction to anticancer therapy.

Materials and methods

Cell line, cell culture and experimental design. The human gastric adenocarcinoma cell line, SGC-7901, was kindly

Abbreviations: BCA – biconchoninine acid, bHLH – basic-helix-loop-helix, CCK-8 – cell counting kit-8, DAPI – 4',6-diamidino-2-phenylindole, EDTA – ethylenediamine tetraacetic acid, PBS – phosphate buffer saline, PMSF – phenylmethylsulfonyl fluoride, PPIs – proton pump inhibitors, PPZ – pantoprazole, PVDF – polyvinylidene difluoride, SDS – sodium dodecyl sulphate, SO – sham-operated, TBST – tris buffer saline plus tween 20

provided by Dr. Jing Sun from the Department of Oncology, the Affiliated Drum Tower Hospital of Nanjing University, Medical School. Cells were cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, China) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere with 5% CO₂ at 37 °C (Thermo Direct Heat CO₂, USA).

Pantoprazole sodium salts (Nycomed GmbH, Germany) were resuspended in normal saline (0.85%) at 8 mg/ml immediately before use. Cells were divided into 7 groups when cells fused to 60-70%, in which PPZ was added with the final concentrations of 0, 1, 10, 20, 40, 80 and 160 µg/ml respectively, all cultured for 24 h and used for further experiments.

Animals and experimental design. All procedures and experiments that involved animals were approved by The National Animal Research Authority. Four-week-old female BALB/c nude mice were obtained from Center of Comparative Medicine, Yangzhou University (Yangzhou, China). Animals were housed in an environment-controlled animal care facility with a constant room temperature and humidity. A 12-h light (started at 7:00 am), 12-h dark cycle was maintained throughout the whole experiment. All animals were fed laboratory chow and water ad libitum. Upon arrival, they were acclimated for at least 1 week and subsequently divided into three groups at random on the basis of body weight, including the sham-operated (SO) group, the control group and the PPZ treatment group.

The SGC-7901 cells were used to induce tumor formation in mice. In brief, on day 1, tumor cells (1×10⁷ cells in 0.2 ml medium) were inoculated subcutaneously into the right subscapular flank of mice in the tumor-bearing animals (the control group and the PPZ treatment group). Mice in the SO group received injection with same amount of culture medium in identical places. General status and tumor size (length and width) were observed every other day. On day 9, mice in the PPZ treatment group were administered intragastrically with 75 mg/ (kg·d) PPZ for 10 days, and mice in the control group and the SO group were given equal volume normal saline in the same way. On day 18, blanking 12 h after the last dose administration, all animals were killed, thereafter tumors were dissected, weighed and frozen at -80°C.

Western blotting analysis. Total cell extracts were prepared on ice for 30 min in lysate (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.2% EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.01% PMSF) supplemented with protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride, sodium orthovanadate; Roche), and centrifugated at 12000 rpm in 4°C (Eppendorf centrifuge 5804R, Germany) for 15 min to remove nuclei and cell debris. In addition, tumor samples were subjected to homogenate ahead of total cell extracts. Protein concentration of the extracts was determined by the BCA Protein Assay Kit (KEYGEN Biotech, China), following the manufacturer's instructions. Fifty micrograms of each protein sample were

subjected to Western blotting on a denaturing 10% SDS-polyacrylamide gel electrophoresis and transferring to PVDF membranes (Immobilon-P, MILLIPORE, USA) using a semidry transfer system (Bio-Rad, USA). Non-specific binding was blocked by incubating the membranes in 1×TBST (Tris Buffered Saline containing 0.05% Tween-20) supplemented with 5% nonfat dry milk for 1 h. Blots were incubated with a monoclonal mouse antibody to HIF-1α (1:1000, Chemicon, USA) and a monoclonal mouse antibody to β-actin (1:3000, Santa Cruz, USA) as an internal control for protein loading. Antibody binding was detected by incubating the blot with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:1000, KPL, USA). Antibody staining was visualized by enhanced chemiluminescence (Santa Cruz, USA). The images of Western blot products were collected and analyzed by Quantity One V4.31 (Bio-Rad, USA).

Immunofluorescence staining analysis. Dispersed single cells (2 × 10⁵ cells per well) were planted on 22 × 22 × 1 mm³ glass coverslips (pretreated with 0.3% gelatin) in 6-well culture plates. After 24 h PPZ treatment, cells were fixed in ice-cold acetone for 10 min at 4 °C. The cells were blocked with 10% normal goat serum (Boster Biotech, China) for 30 min and probed with HIF-1α antibodies (1:100) at 4°C overnight. Alexa Fluor Dye Conjugated secondary antibodies (1:100, Alexa Fluor 488 goat anti-mouse IgG (H + L) highly cross-adsorbed, 2 mg/ml, Invitrogen, USA) were used to incubate for 1 h to be visualized under a fluorescent microscope (Imager A₁, Axio, Zeiss). Tumor tissues were cut into slices with a permanent cold icy slicker, fixed in ice-cold acetone in the same way, and then operated the same as the previous operation of cells. DAPI (2 µg/ml, Invitrogen, USA) was used to stain nuclei.

Cell proliferation assay. Cells were seeded (10, 000/well) into 96-well plates in 100 µl medium per well and treated with PPZ for 24 h. The antiproliferative effect of PPZ was assessed by Cell Counting Kit-8 (CCK-8) (KEYGEN Biotech, China) according to manufacturer's instruction, and expressed as relatively cell viability, using the following formula: Percent cell viability (% = OD of drug-treated sample/OD of untreated sample) × 100%. The experiment was repeated for three times.

Apoptosis analysis by flow cytometry. SGC-7901 cells were seeded into 6-well plates. After the PPZ administration according to the means mentioned above, the cells were trypsinised, washed twice with PBS, resuspended and then stained with an Annexin V-FITC Apoptosis Detection kit (KEYGEN Biotech, China). Apoptosis of cells was analyzed by a flow cytometer (BD Biosciences, USA). The experiment was repeated for three times.

Statistical analysis. Statistical analysis of the data was performed using the software package SPSS 13.0, the data were expressed as mean ± SD of the three independent experiments at least. The comparisons among different groups were evaluated by one-way ANOVA, using SNK, LSD and Dunnett's methods. Results were considered statistically different when the analysis returned a *P* value of <0.05.

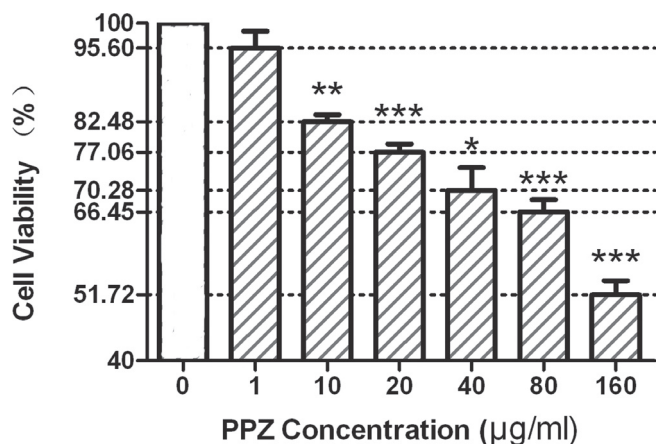


Figure 1. Comparison of the cell viability of SGC-7901 after the treatment of PPZ on different concentrations for 24 h. Significance is shown compared with viability of SGC-7901 cells without PPZ (PPZ concentration is 0 µg/ml) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The experiment was repeated for three times ($N = 3$).

Results

PPZ suppresses SGC-7901 cell proliferation in a dose-dependent manner. As shown in Fig. 1, PPZ could inhibit proliferation of the gastric cancer SGC-7901 cells in a dose-dependent manner. The cell viability in 10, 20, 40, 80 and 160 µg/ml PPZ groups was significantly lower than that in the control group (0 µg/ml PPZ) (-17.52%, -22.94%, -29.72%, -33.55% and -48.28% respectively vs. the control group). Meanwhile, the cell viability in 1 µg/ml PPZ group was also lower than the control group, whereas no significant difference was found between the two groups ($P > 0.05$).

PPZ induces apoptosis in SGC-7901 cells. A quantitative analysis of the fluorescent signals was performed by Fluorescence Activated Cell Sorting (FACS). The results are summarized in Fig. 2A and B. As displayed in Fig. 2B, PPZ could induce the early apoptosis and total apoptosis of SGC-7901 cells dose-dependently, which is as follows: the early apoptosis rate in 1, 10, 20, 40, 80 and 160 µg/ml PPZ groups was markedly higher than that in the control group (+1.64%, +8.17%, +12.00%, +18.97%, +49.10% and +71.24% respectively vs. the control group, $P < 0.001$); the total apoptosis rate in 1, 10, 20, 40, 80 and 160 µg/ml PPZ groups was evidently higher than that in control group (+7.53%, +21.76%, +24.10%, +44.73%, +56.20% and +77.26% respectively vs. the control group, $P < 0.001$). PPZ could also induce late apoptosis of SGC-7901 cells, but act not dose-dependently, which was as below: the late apoptosis rate in 1, 10, 20, 40, 80 and 160 µg/ml PPZ groups was evidently higher than that in the control group (+5.90%, +13.60%, +12.10%, +25.77%, +7.10% and +6.04% respectively vs. the control group, $P < 0.001$).

PPZ inhibits protein expression of HIF-1α in SGC-7901 cells. The expression of HIF-1α in the SGC-7901 cells was ex-

amined by western blot. The expressions of HIF-1α were much lower in PPZ treatment groups (10-160 µg/ml; 24h) than that in control group (Fig. 3). During different concentrations of PPZ treatment groups, 10 µg/ml PPZ showed the most powerful inhibitory effects on HIF-1α expression.

PPZ influences the intracellular expression and distribution of HIF-1α. As an inhibitor of HIF-1α, PPZ treatment for 24 h on various concentrations caused a different intracellular expression and localization of HIF-1α vesicles (Fig. 4). After 10 µg/ml PPZ treatment, the fluorescent intensity of HIF-1α expression significantly decreased compared to the control and other PPZ groups (Fig. 4B). After 20 µg/ml PPZ treatment, HIF-1α was rather more dispersive than the control and other groups, instead of perinuclear accumulation phenomenon (Fig. 4C). Through PPZ administration with different concentrations, the fluorescent intensity of HIF-1α expression represented varying degrees of decline (some data not shown).

In a xenograft model of nude mice, administration of PPZ significantly inhibited tumorigenesis and down-regulated HIF-1α protein. In a human gastric cancer xenograft model, we evaluated also the inhibition effects of PPZ in tumor growth and HIF-1α expression (Fig. 5). Intragastric administration of PPZ significantly inhibited tumorigenesis in mice of PPZ treatment group, with a drop in tumor volume at day 9 after the first PPZ administration compared with mice in control group ($P < 0.05$, $n = 11$ for each group, Fig. 5A and B), without severe side effects. The isolated tumors from mice with administration of PPZ were remarkably smaller than that of mice treated with normal saline (Fig. 5C). The expression of HIF-1α in the PPZ treatment group was significantly lower than that in the control group (Fig. 5D and E).

Discussion

In a variety of human tumor cell lines or tissues, the role of HIF-1α in cancer development has been described. However, whether HIF-1α is able to promote or inhibit tumor growth remains controversial. Between the two opponent points of view, one is that HIF-1α is a positive factor in tumor growth, and its positive adjustment effect seems to be achieved by the expression of VEGF [16-18]; on the contrary, a slice of experiments elucidate that HIF-1α may inhibit tumor growth through its capacity of association with p53, which is a mediator of genotoxic apoptosis [19, 20]. Our conclusions based on this study seem to be more supportive of the former, which is HIF-1α can promote tumor growth, in spite of absence of detecting VEGF. To get this judgment for the purpose of clarifying the issue, we made use of gastric adenocarcinoma cell line SGC-7901, intervened with the PPZ *in vitro* and *in vivo* respectively, observing the tumor cells proliferation and apoptosis, and simultaneously detecting the levels of HIF-1α expression.

This study showed that the expression of HIF-1α could be inhibited effectively under various concentrations of PPZ,

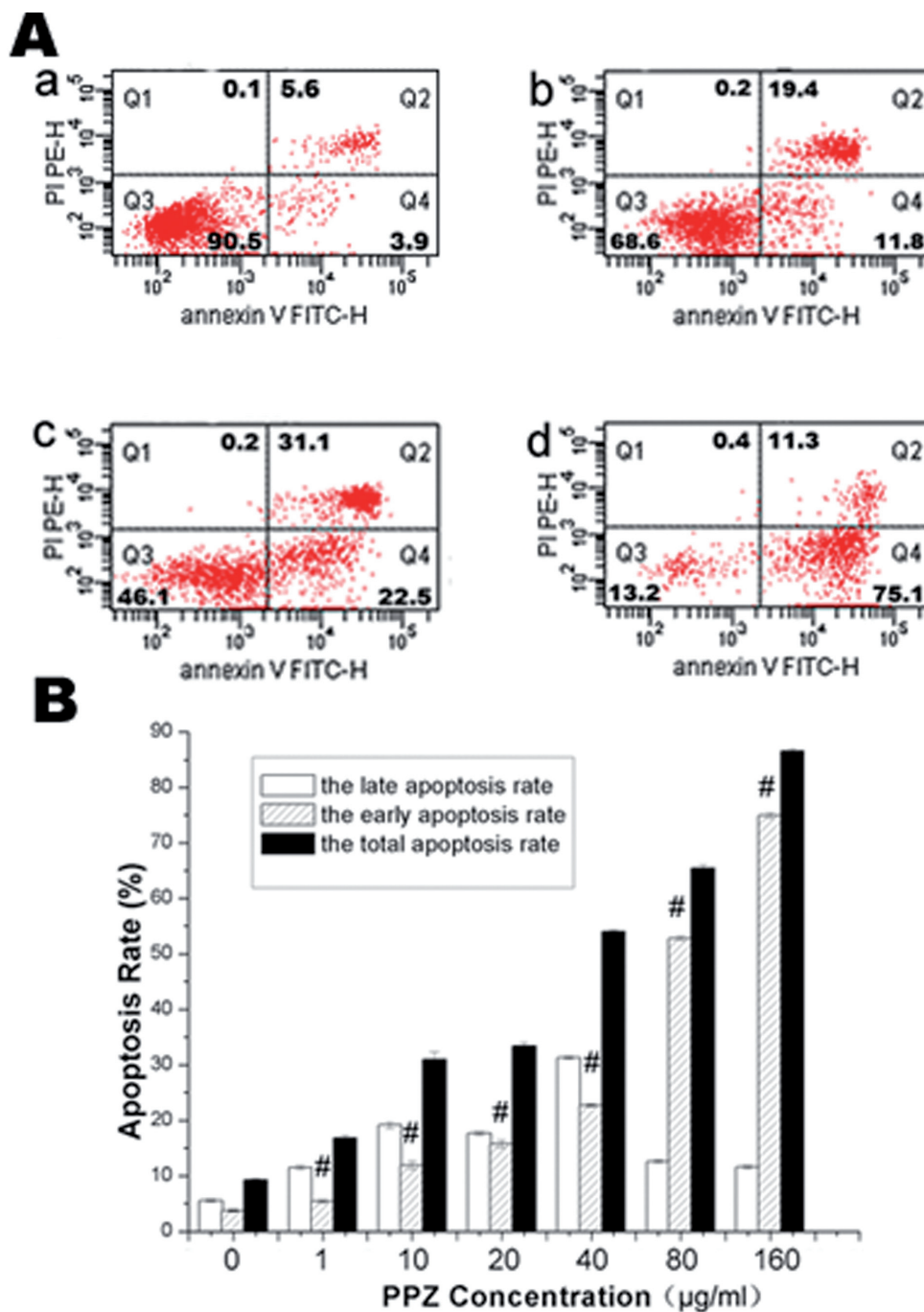


Figure 2. A) Comparison of the apoptosis rate of SGC-7901 cells after the administration of PPZ, a) the control group: PPZ concentration is 0 µg/ml, b) PPZ concentration is 10 µg/ml, c) PPZ concentration is 40 µg/ml, d) PPZ concentration is 160 µg/ml. B) Comparison of the apoptosis rate of SGC-7901 cells after the treatment of PPZ. # $P < 0.001$, the significance is shown compared with the control group (PPZ concentration is 0 µg/ml). The experiment was repeated for three times (N = 3).

but the interesting point is that acting on the most obvious inhibition effects is not the densest group, rather a relatively low concentration (10µg/ml), which is not consistent with the effects of tumor cell proliferation and apoptosis. Contrary to

a majority of reports [21-24], our study demonstrated that PPZ inhibited protein expression of HIF-1α in an optimal dosage manner, or else a dramatic wording of “break point”, instead of dose-dependent mode. This may be because the effects of

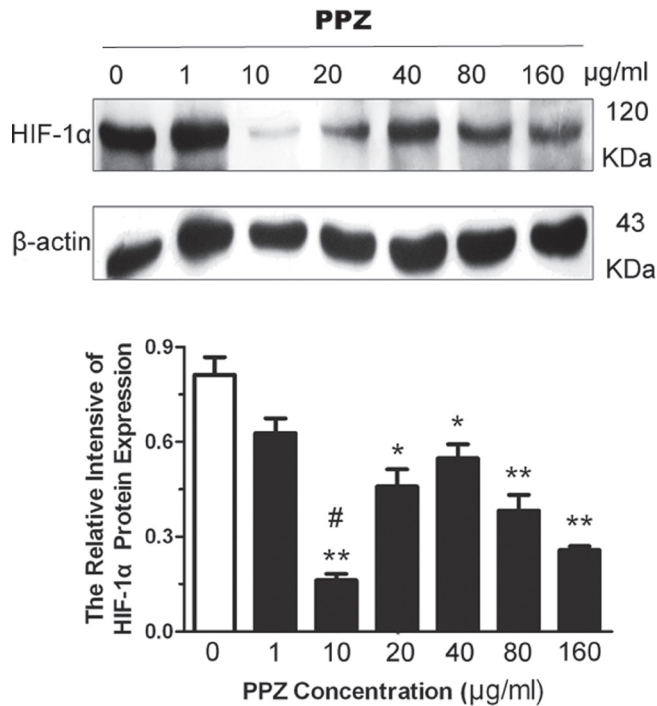


Figure 3. Effects of PPZ treatment on HIF-1 α expression of SGC-7901 cells on different concentration points. * $P < 0.05$, ** $P < 0.01$, significant differences were revealed when compared to that in the 0 $\mu\text{g/ml}$ PPZ group. # $P < 0.05$, there were significant differences between one group and other five groups among six PPZ treatment groups.

PPZ on tumor cells are not only through the changing levels of HIF-1 α ; as a matter of fact, the concrete mechanism is not yet clarified. One previous research has found that cancer cells were much more susceptible to growth inhibition of PPZ at a low pH [15], and the value of pH may also influence the protein expression related to tumor cells growth. However, all steps of our experiment were carried out at a neutral pH, so the effects of PPZ at lower pH on the tumor cells growth need to be further studied. Immunofluorescence observations supported the results of Western Blot, in which the weakest fluorescent intensity was observed under the same concentration (10 $\mu\text{g/ml}$); however, when administered with 20 $\mu\text{g/ml}$ PPZ, HIF-1 α was dispersive without perinuclear accumulation phenomenon, indicating that pantoprazole had not only the inhibitory effect to HIF-1 α , but also could affect its distribution in certain concentrations. As mentioned earlier [3], HIF-1 α accumulated, entered into the nucleus and bound with HIF-1 β to play a role, so its distribution could influence the activity indirectly.

The mechanisms of how PPZ affects the levels of HIF-1 α remain unknown. Activation of HIF-1 α involved a large number of intracellular signal transduction processes, such as mTOR [25, 26], EGFR [27], BCR/ABL [28] and Her2/neu [29] pathways, and the compounds acting on various signaling pathways mentioned above are likely to have the role of HIF-1 α inhibition. In post-translation phase, HIF-1 α also undergoes hydroxylation, acetylation, phosphorylation,

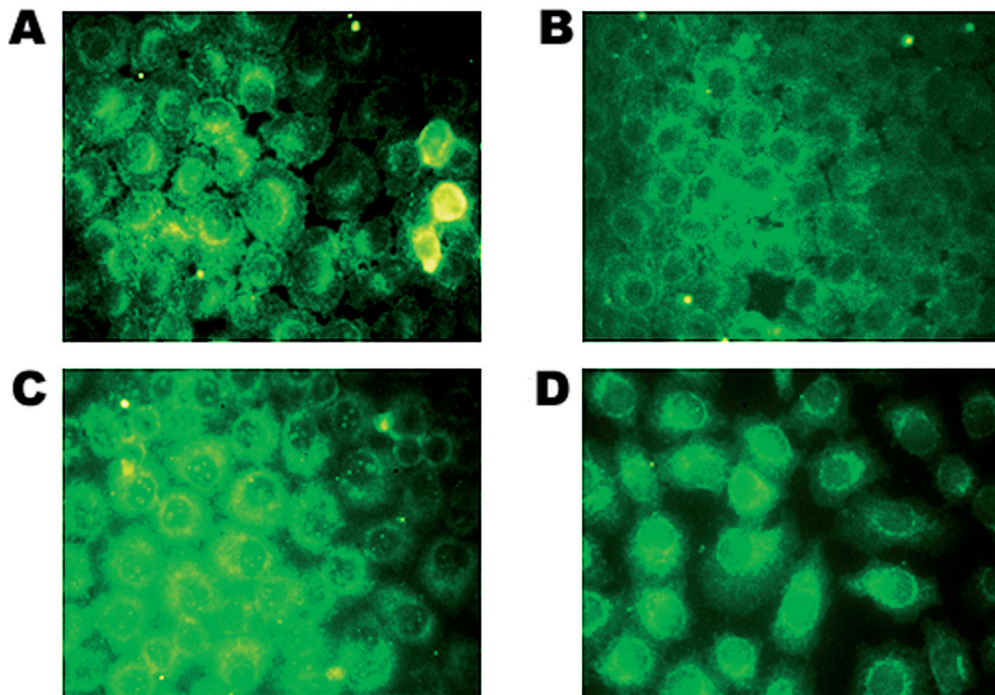


Fig. 4. Effects of PPZ treatment on the intracellular distributions of HIF-1 α in SGC-7901 cells. A) Intracellular distributions of HIF-1 α in SGC-7901 cells without PPZ treatment ($\times 200$ magnification). B) Intracellular distributions of HIF-1 α in SGC-7901 cells after 10 $\mu\text{g/ml}$ PPZ treatment ($\times 200$ magnification). C) Intracellular distributions of HIF-1 α in SGC-7901 cells after 20 $\mu\text{g/ml}$ PPZ treatment ($\times 200$ magnification). D) Intracellular distributions of HIF-1 α in SGC-7901 cells after 160 $\mu\text{g/ml}$ PPZ treatment ($\times 200$ magnification).

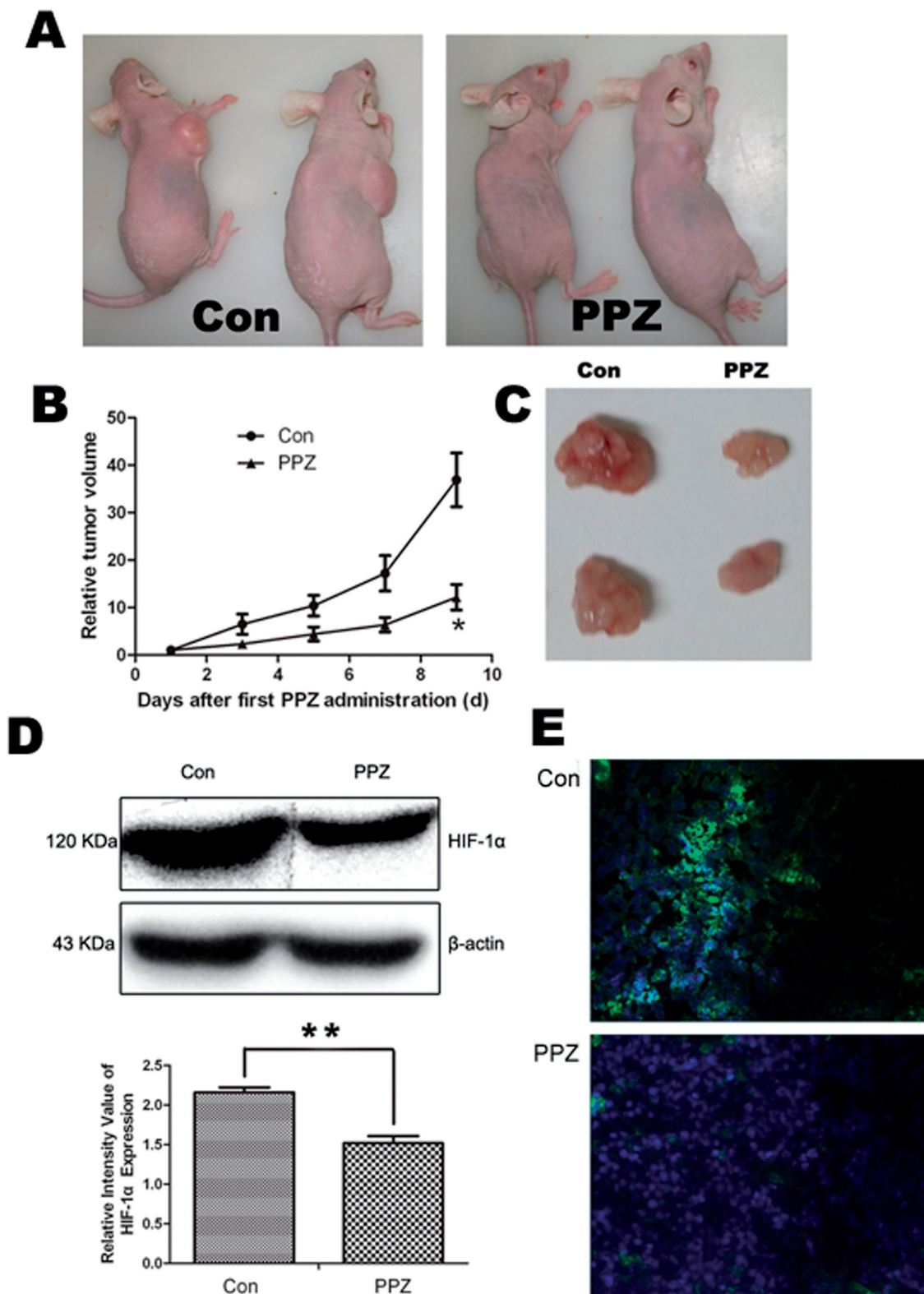


Fig. 5. Inhibition of tumorigenesis and HIF-1α expression with PPZ in xenograft model. A) Comparison of the tumor volume after the treatment of PPZ for 9 days. B) Dynamic observation of the relative tumor volume after the first PPZ administration. C) Comparison of the isolated tumor volume after the sacrifices of nude mice. D) Effects of PPZ treatment on HIF-1α expression of tumor samples. E) Effects of PPZ treatment on the levels of HIF-1α protein in tumor tissues (×200 magnification). Con: tumor-bearing mice treated with normal saline; PPZ: tumor-bearing mice treated with PPZ. (* $P < 0.05$; ** $P < 0.01$)

ubiquitination and SUMO modification, and in recent years a large number of non-specific/specific inhibitors of HIF-1 α have been discovered [30]. HIF is activated by hypoxia, but undergoes degradation by the VHL (von Hippel-Lindau) tumor suppressor protein in the presence of oxygen [31, 32]. An increase in hydrogen ions elicits a transient and reversible loss of VHL function by promoting its nuclear sequestration [33]. Because of the abnormal cell metabolism, the hypoxic state in solid tumors often associated with tissue acidification, the extracellular pH value may reduce to below 6.0. The PPZ could inhibit tumor cell proton pump, change the acidification of tumor microenvironment [34] and increase VHL expression, which by the way so that increased the degradation of HIF-1 α . On the other hand, benzimidazole analogue was found to regulate the stability of HIF-1 α through the Hsp90-Akt pathway, leading to the degradation of HIF-1 α [35], and we concluded that PPZ might inhibit HIF-1 α expression as a kind of benzimidazole compounds. In a word, the inhibitory effects of PPZ on HIF-1 α protein may involve several different factors.

Recent years witnessed considerable progress in numerous related experimental studies targeting HIF-1 α , such as inhibiting HIF-1 α protein on gene level by RNA interference [36], suppressing the expression of HIF-1 α by various drugs (mostly on post-transcriptional level, equaling the protein level) [35], and hydroxylating HIF-1 to induce the degradation of HIF-1 α under normoxia [37]. As a result, the research on anticancer therapy is getting more and more intensive. In summary, our findings pointed out the novel anticancer mechanism of gastric proton pump inhibitor, and extended the clinical application as an anticancer drug by inhibiting the expression of HIF-1 α in cancer cells.

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