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Original Article

Tumor microenvironment interruption: a novel anti-cancer mechanism of Proton-pump inhibitor in gastric cancer by suppressing the release of microRNA-carrying exosomes

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Abstract: Poor prognosis of gastric cancer is related to not only malignancy of gastric cancer cells, but also the tumor microenvironment. Thus drugs, which can inhibit both of them, are urgently needed to be explored. Studies on effect of Proton-pump inhibitors (PPIs) in anti-neoplasms are increasing, but is rare in gastric in gastric cancer. Here we investigated how the gastric cancer microenvironment is regulated by PPIs. The objective response rate of gastric cancer patients in our hospital treated by PPIs is investigated. PPIs' effects were further explored by observing the change of microRNAs, cytokines, cellular apoptosis. Bioinformatic pathway analysis of microarray was used to discover the pathway involved in PPIs' regulation of gastric cancer microenvironments. Immunoblotting assays and qRT-PCR were used to define molecular events with PPIs treatment. We report here that PPIs can improve the prognosis of advanced gastric cancer patients; and inhibit the progress of gastric cancer both in vivo and in vitro. Moreover, high dose of PPIs can regulate the pathway associated with tumor malignancy and microenvironment via inhibiting the release of exosomes, which packed microRNAs. PPIs can inhibit the transformation of CAFs (cancer associated fibroblasts) and cytokines released from CAFs. In addition, PPIs inhibit the malignancy of gastric cancer through regulating HIF-1α-FOXO1 axis. High dose of PPIs can inhibit malignancy of gastric cancer and regulate its surrounding tumor microenvironment. This finding suggests that PPIs maybe of potential value as a therapeutic tool for treatment of gastric cancer.

Keywords: Proton-pump inhibitor, exosome, microRNA, gastric cancer

Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer-related mortality globally [1]. It is often diagnosed at an advanced stage [2] associated with poor survival and efficacy of systemic treatment, which is ascribed to the significant poor biological behavior [3-5]. Mounting evidences indicated tumor biologic behaviors were not only correlated to tumor cells but also to the tumor microenvironment [6]. Tumor microenvironment provides a shelter and supportive soil for tumor cells [7]. Hence, well-tolerated and effective anti-tumor agents, which can not only inhibit the progress of gastric cancer but also interfere with the complicated tumor microenvironment, are eagerly needed.

Gastric cancer cells survive in hypoxic and acid microenvironment [8]. The acid-outside pH gradient of cancer cells originates as a response to the metabolic adaptation to hypoxic tumor milieu. HIF-1α, which is known to regulate proton extrusion and PH homeostasis by enhancing the expression of plasma membrane ion

pumps and transporters under hypoxic conditions [9], is activated. Moreover, extracellular pH affects the amount and characteristic of exosomes. Recent researches on cancer exosomes revealed that acidic microenvironment promotes exosomes traffic and uptake of cancer cells, contributing tumor favorable environment [10]. Previous studies have elucidated that exosomes carrying miRNAs secreted by cells was a new way of cell-cell interaction, which is potentially important in cell microenvironment regulation [11-13].

Clinically, Proton-pump inhibitors (PPIs) are safely used to treat a wide range of gastrointestinal disorders like peptic ulcer, gastritis, etc. [14]. Recently a few studies found PPIs could improve chemosensitivity of gastric cancer cells [15-17] and change the acidity of the tumor microenvironment [18]. A pilot, prospective, randomized, phase II clinical study showed intermittent high dose of PPIs improved the efficacy of chemotherapy in breast cancer patients without obvious toxicity [19]. However, the effects and mechanism of PPIs in the treatment of gastric cancer remain unclear.

In this study, we explored the clinical results of PPIs in treating advanced gastric cancer patients from our hospital, and investigated the mechanism.

Material and methods

Patients in TCGA database and our hospital

RNA expression from TCGA stomach adenocarcinoma were downloaded from the website of THE CANCER GENOME ATLAS (https://cancergenome.nih.gov).

Seven cases of metastatic gastric adenocarcinoma treated in our hospital and enrolled in a single arm pilot study were analyzed. All patients received high dose of esomeprazole (120 mg, qd, for 2 days prior to chemotherapy; or 60 mg, qd, for 6 days, started 3 days before chemotherapy) combined with salvage chemotherapy. The study was approved by Ethical Committee of Tianjin Medical University Cancer Institute and Hospital, and performed in accordance with the Declaration of Helsinki of the World Medical Association. All patients had given written informed consent to the work.

Cell culture and transfection

The gastric cancer cell line SGC7901 was purchased from the Cell Resource Center, Peking Union Medical College (Beijing, China). Human skin fibroblast cell line HFF-1 were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The details of cell culture and transfection was performed as described in Supplementary Materials and Methods.

Ethics, consent and permissions

The study was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice guidelines, the Declaration of Helsinki, and applicable local regulatory requirements and laws. Study procedures were approved by institutional ethical board of Tianjin Medical University Cancer Institute and Hospital. Written informed consent was obtained from all patients.

Animal studies

Female 4-6-week-old BALB/c Nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were maintained in a barrier facility on HEPA-filtered racks. All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and was approved by Animal Care. Mice (approval 2016080) were approved by the Animal Care and Use Committee of Tianjin Medical University Cancer Institute and Hospital, China. Cells were harvested by trypsinization, washed in PBS, resuspended at 2×10^7 cells/ml in PBS, and then injected subcutaneously into the right flank of BALB/c Nude mice. Primary tumors were measured in 2 dimensions (a, b), and volume (mm^3) was calculated as a (mm) × b^2 (mm^2)/2. Primary tumors were harvested from the flank of mice.

Statistical analysis

All of the data were representative of at least 3 independent experiments. The data were expressed as the mean ± S.E. of at least three separate experiments. Statistical significance was considered at $P < 0.05$ using Student's *t*-test. In this study, GraphPad Software was used to conduct the analysis.

Table 1. The baseline characteristics of the seven patients in our hospital

Patient	Gender	Previous Regimen No	Salvage Chemotherapy	Esomeprazole	Cycles	Objective Response
Case 1	Male	1	Irinotecan	120 mg, for 2 days	3	PD
Case 2	Male	2	Docetaxel	120 mg, for 2 days	2	SD
Case 3	Male	1	Irinotecan	120 mg, for 2 days	3	PD
Case 4	Male	1	Docetaxel	120 mg, for 2 days	9	SD
Case 5	Female	1	Docetaxel	120 mg, for 2 days	6	SD
Case 6	Male	2	Irinotecan	60 mg, for 6 days	5	PR
Case 7	Male	1	5FU/LV+Oxaliplatin	60 mg, for 6 days	5	PR

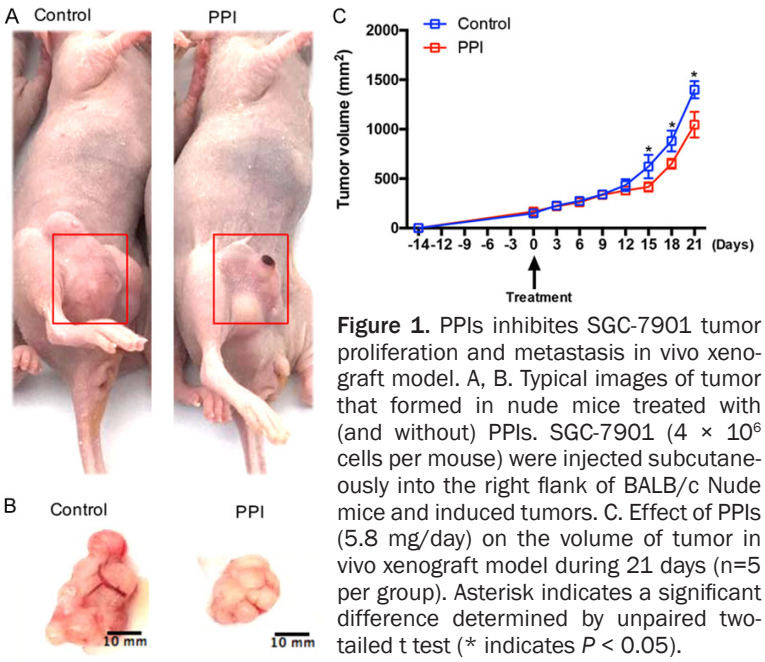


Figure 1. PPIs inhibites SGC-7901 tumor proliferation and metastasis in vivo xenograft model. A, B. Typical images of tumor that formed in nude mice treated with (and without) PPIs. SGC-7901 (4×10^6 cells per mouse) were injected subcutaneously into the right flank of BALB/c Nude mice and induced tumors. C. Effect of PPIs (5.8 mg/day) on the volume of tumor in vivo xenograft model during 21 days (n=5 per group). Asterisk indicates a significant difference determined by unpaired two-tailed t test (* indicates $P < 0.05$).

Other Supplementary Material and Methods were conducted as described in the Supplementary Materials and Methods.

Results

PPIs promoted the prognosis of patients with advanced gastric cancer and inhibited proliferation and metastasis in vivo

Table 1 showed the baseline characteristics of the seven patients in our hospital, including 6 male and 1 female. All patients were previously treated with at least one combination chemotherapy regimen but failed. The company regimens with high dose of PPIs are mainly irinotecan or docetaxel. The addition of esomeprazole to chemotherapy was well tolerated without obvious toxicity. Consequently, two patients had partial response (PR), three achieved stable disease (SD), and two presented with progressive disease (PD) (**Table 1**). The disease control rate was 71.4%, while the response rate was 28.6%. Results of the survival are not matured.

After injecting 5.8 mg PPIs (Omeprazole) or Saline intraperitoneal into the paired tumor-bearing BALB/c Nude mice for 21 days. We found PPIs could limit the tumor volume of mice (**Figure 1A-C**).

PPIs promote the effects of anti-tumor drugs and apoptosis and inhibits proliferation, cell migration and invasion of SGC-7901 gastric cancer cells

To explore whether PPIs (omeprazole) could suppress gastric cancer cell proliferation, we selected SGC-7901 cells, which were treated with different concentrations of PPIs and for various time points, then cell proliferation was evaluated by CCK8 assays. It was observed that the inhibitory role of PPIs on SGC-7901 cells gradually increased with time and change in concentration (**Figure 2A**). We then explored whether PPIs could enhance the chemosensitivity. The relative sensitivity to cisplatin (DDP), paclitaxel (TAX) and 5-FU of gastric cancer cells was determined by the CCK8 assay, with or without pretreatment with PPIs (80 μg/ml). The results of repeated experiments indicated that pretreatment with omeprazole induced the susceptibility of gastric cancer cells to the cytotoxic effect of cisplatin, paclitaxel and 5-FU (**Figure 2B**). To further verify whether PPIs could

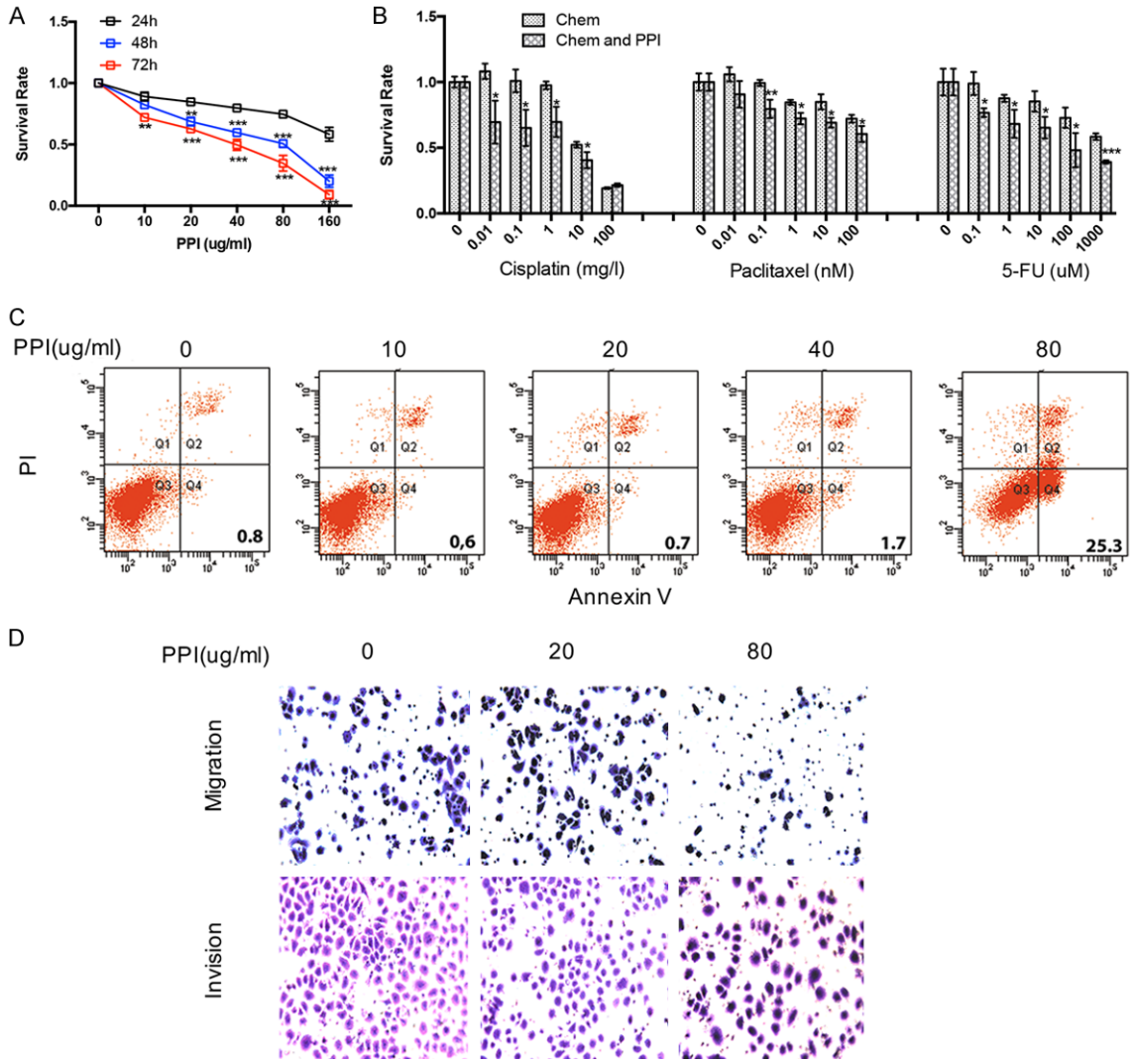


Figure 2. PPIs facilitate the effects of anti-tumor drugs, autophagy and apoptosis and inhibits proliferation, cell migration and invasion of SGC-7901 gastric cancer cells. A. CCK8 assay analysis showing cell viability following PPIs (80 ug/ml) treatment at various concentrations as indicated for 24, 48, and 72 h. Percentages of cell viability is presented as mean ± S.E.M. (n=5). B. SGC-7901 cells were treated with cisplatin, paclitaxel, and 5-FU at indicated concentration combined with (or without) PPIs (80 ug/ml) for 24 h. Percentages of cell viability is presented as mean ± S.E.M. (n=5). C. PPIs effect on apoptosis of SGC-7901 cells at indicated concentration for 24 h (n=3). D. Typical images of migrated and invasive SGC-7901 cells in transwell assays (n=3) following treatment with PPIs at indicated concentration for 24 h. All photographs were taken at a magnification of ×200. Asterisk indicates a significant difference determined by unpaired two-tailed t test (*** indicates $P < 0.001$; ** indicates $P < 0.01$; * indicates $P < 0.05$).

induce apoptosis in gastric cancer. We analyzed cell apoptosis using the annexin-V-FITC and propidium iodide (PI) staining assays. It wasfound that PPIs could enhance apoptosis SGC-7901 cells (**Figure 2C**) at high dose of PPIs. In order to determine whether omeprazole could affect cell motility, transwell assays were performed after incubating with different dose of omeprazole for 24 h. Transwell assays showed that gastric cancer cells treated at higher dose of PPIs showed a lower ratio in migration and invasion (**Figure 2D**).

PPI regulates FOXO1 in SGC-7901 gastric cancer cells

TCGA database showed mRNA of FOXO1 had appositive correlation with AJCC tumor pathologic in gastric cancer patients (**Figure 3A**). Nature, 2014 database also proved this (Sup-

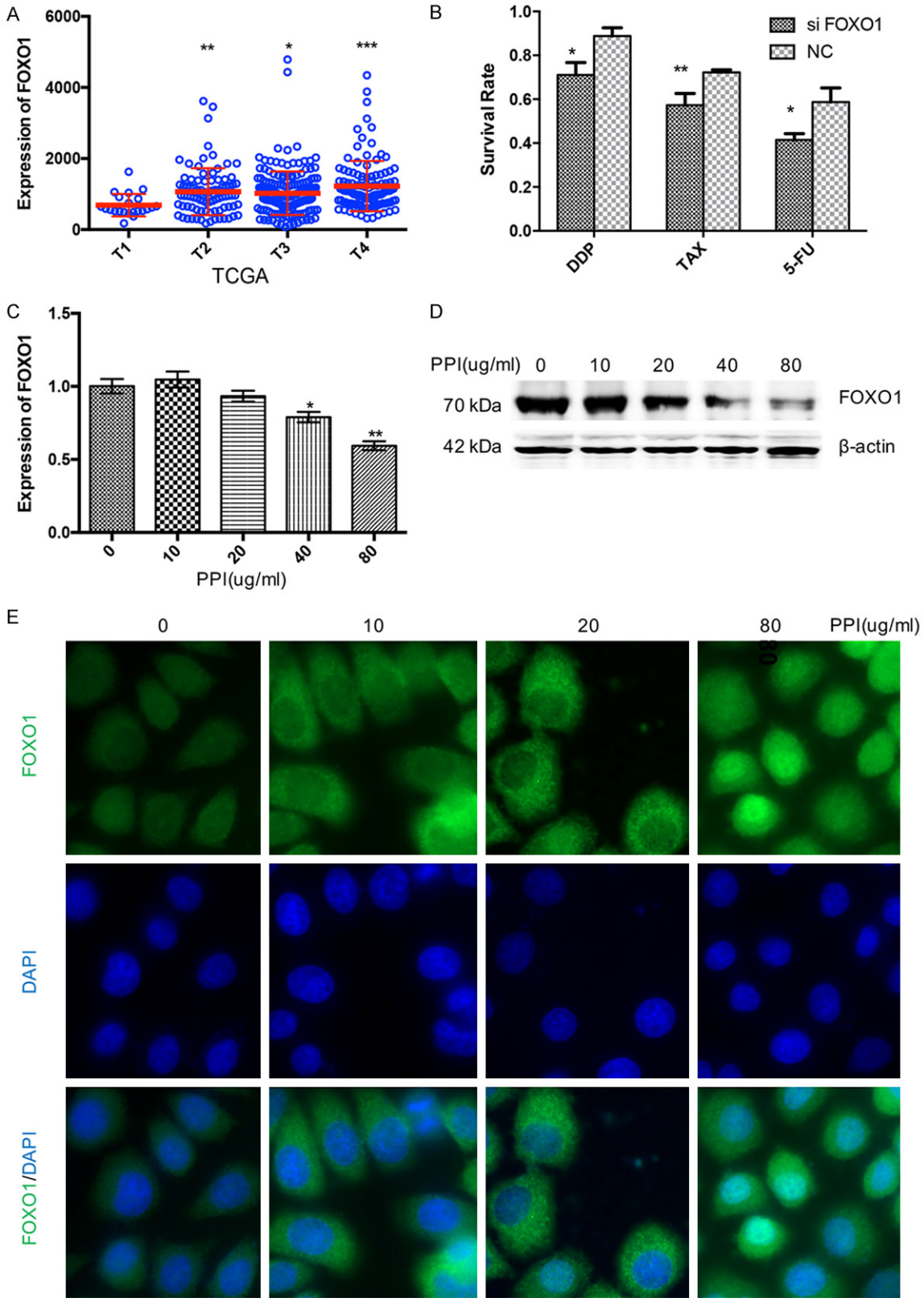


Figure 3. PPIs upregulates FOXO1 in SGC-7901 gastric cancer cells. A. TCGA databases show mRNA of FOXO1 have positive correlation with AJCC tumor pathologic in gastric cancer patients. B. SGC-7901 cells were treated with cisplatin (DDP, 10 mg/l), paclitaxel (TAX, 100 nM), and 5-FU (1 mM) combined with scrambled siRNA or FOXO1 siRNA for 24 h. Percentages of cell viability is presented as mean ± S.E.M. (n=5). C. PPIs effect on the mRNA expression

of FOXO1 of SGC7901 cells at indicated concentration for 24 h (n=5). D. PPIs effect on the expression of FOXO1 and β-actin of SGC7901 cells at indicated concentration for 24 h (n=3). E. SGC7901 cells were treated with PPIs at indicated concentration for 24 h, and the location of FOXO1 were tested by immunofluorescence. And the nucleus of SGC7901 cells was stained by DAPI (n=3). All photographs were taken at a magnification of ×200. Asterisk indicates a significant difference determined by unpaired two-tailed t test (*** indicates $P < 0.001$; ** indicates $P < 0.01$; * indicates $P < 0.05$).

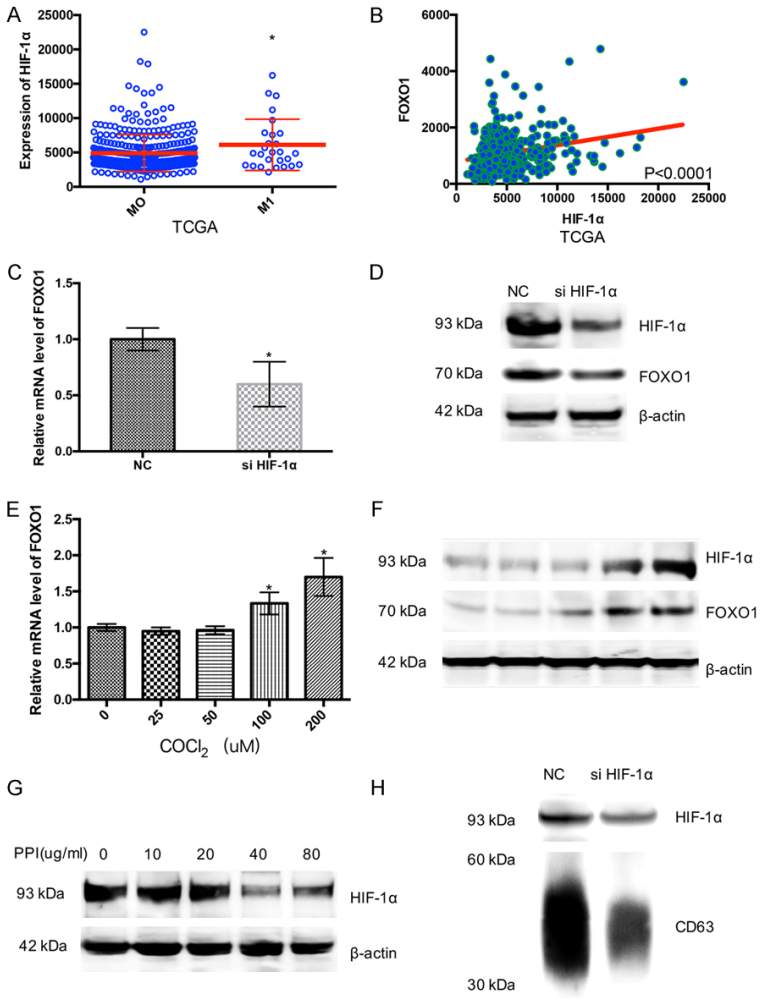
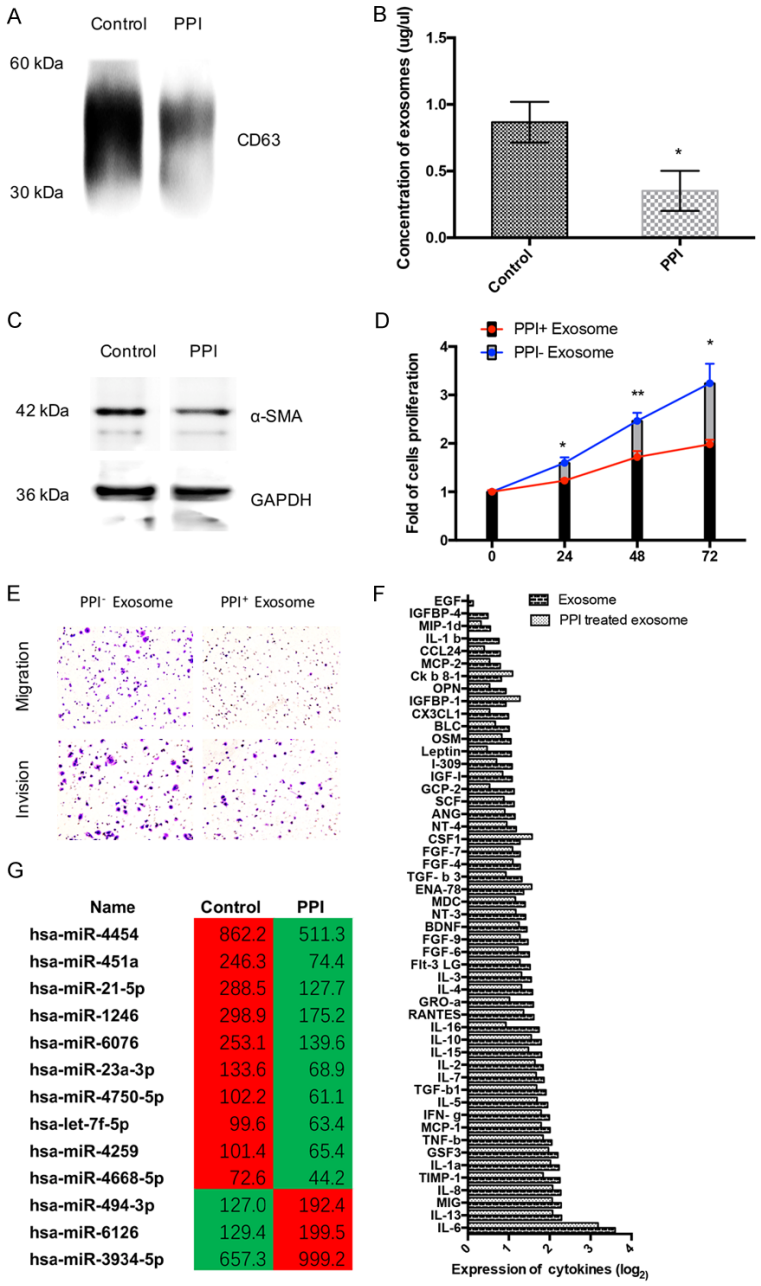


Figure 4. PPIs upregulates FOXO1 though HIF-1α in SGC-7901 gastric cancer cells. A. TCGA databases show mRNA of HIF-1α have positive correlation with AJCC metastasis pathologic in gastric cancer patients. B. TCGA database reveal the positive correlation of mRNA between FOXO1 and HIF-1α. C. Scrambled siRNA or HIF-1α siRNA effect on the expression of FOXO1 of SGC7901 cells for 24 h (n=3). D. Scrambled siRNA or HIF-1α siRNA effect on the mRNA expression of FOXO1 of SGC7901 cells for 24 h (n=3). E. $COCl_2$ effect on the Mrna expression of FOXO1 of SGC7901 cells at indicated concentration for 24 h (n=3). F. $COCl_2$ effect on the expression of HIF-1, FOXO1 and β-actin of SGC7901 cells at indicated concentration for 24 h (n=3). G. PPIs effect on the expression of HIF-1α and β-actin of SGC7901 cells at indicated concentration for 24 h (n=3). H. Scrambled siRNA or HIF-1α siRNA effect on the expression CD63 and HIF-1α in exosome of SGC7901 cells for 24 h (n=3). Asterisk indicates a significant difference determined by unpaired two-tailed t test (*** indicates $P < 0.001$; ** indicates $P < 0.01$; * indicates $P < 0.05$).

plementary Figure 1A). These data showed FOXO1 had correlation with the progression of gastric cancer, and PPIs might regulate FOXO1 in gastric cancer.

To explored whether FOXO1 was associated with drug resistance in gastric cancer, siRNA was used to knock down the expression of FOXO1. Following transfection, cells were treated with cisplatin, paclitaxel, 5-FU or PBS. Cell proliferation was evaluated by CCK8 assays. Transfection of siRNA of FOXO1 could enhance the cytotoxicity of the anti-tumor drugs cisplatin, paclitaxel and 5-FU in 24 h (**Figure 3B**). Furthermore, to verify whether PPI could regulate the expression of FOXO1 in gastric cancer, qRT-PCR was conducted and demonstrated omeprazole could inhibit expression of FOXO1 mRNA in high dose (**Figure 3C**). Western blotting showed that the expression of FOXO1 decreased at high dose of PPIs (**Figure 3D**). These data suggesting that high dose of PPIs could inhibit the expression of FOXO1 and enhance the effect of antitumor drugs though FOXO1.

Researches have proved that FOXO1 could promote apoptosis signaling through the activation or repression of apoptosis-related genes in the nucleus [45], and cytosolic FOXO1 is essential for the induc-



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Name	Control	PPI
hsa-miR-4454	862.2	511.3
hsa-miR-451a	246.3	74.4
hsa-miR-21-5p	288.5	127.7
hsa-miR-1246	298.9	175.2
hsa-miR-6076	253.1	139.6
hsa-miR-23a-3p	133.6	68.9
hsa-miR-4750-5p	102.2	61.1
hsa-let-7f-5p	99.6	63.4
hsa-miR-4259	101.4	65.4
hsa-miR-4668-5p	72.6	44.2
hsa-miR-494-3p	127.0	192.4
hsa-miR-6126	129.4	199.5
hsa-miR-3934-5p	657.3	999.2

Figure 5. PPIs inhibited the release of exosomes and exosomes related miRNA and regulated tumor microenvironment though regulating exosomes. A. PPIs (80 ug/ml, 24 h) effect on the expression CD63 in the exosome of SGC7901 cells (n=3). B. PPI (80 ug/ml, 24 h) effect on the concentration of exosomes were measured by BCA protein assay (n=5). C. HFF-1 cell were treated with exosome of SGC7901 cells (80 ug/ml of PPI or PBS (control) treated for 24 h), and the expression of α-SMA and β-actin of HFF-1 cells were tested by Western Bloting (n=3). D. SGC7901 cells were cultured with the medium of HFF-1 cell, which were treated with exosome of SGC7901 cells (80 ug/ml of PPI or PBS (control) treated for 24 h). CCK8 assay was performed to analyze cell proliferation of SGC-7901 cells (n=5). E. SGC7901 cells were cultured with the medium of HFF-1 cell, which were treated with exosome of SGC7901 cells (80 ug/ml of PPI or PBS (control) treated for 24 h). Migration and invasion of SGC-7901 cells were tested by transwell assays (n=3). F. HFF-1 cell were treated with exosome of SGC7901 cells (80 ug/ml of PPI or PBS (control) treated for 24 h), and secreted cytokine were tested by Human Cytokine Array (n=3). G. The unsupervised hierarchical clustering analysis (microarray) of de-regulated miRNAs of exosome. The left is control group and right is the PPIs group. (|Fold change| ≥ 1.5 and P < 0.05) (n=3). All photographs were taken at a magnification of ×200. Asterisk indicates a significant difference determined by unpaired two-tailed t test (*** indicates *P* < 0.001; ** indicates *P* < 0.01; * indicates *P* < 0.05).

tion of autophagy [44]. We then explored whether PPIs affects the cellular distribution of FOXO1 in gastric cancer cells by immunofluorescence. FOXO1 was primarily localized to the cytoplasm. When we treated SGC-7901 cells with relative low dose of PPI, FOXO1 stayed the same. However, FOXO1 was shuttled into the nucleus when we treated gastric cancer cells with PPIs at 80 ug/ml (**Figure 3E**). It was revealed that high dose of PPIs induced apoptosis though regulating the cellular distribution of FOXO1.

PPIs regulates FOXO1 though HIF-1α in SGC-7901 gastric cancer cells

TCGA database showed mRNA of HIF-1α had positive correlation with AJCC metastasis pathologic in gastric cancer patients (**Figure 4A**). Nature, 2014 database also proved this (Supplementary Figure 1B). Then we explore whether FOXO1 had a relation with HIF-1α in gastric cancer. TCGA databases and Nature, 2014 database reveal the positive correlation of mRNA between FOXO1 and HIF-1α (**Figure 4B** and Supplementary Figure 1C). And these data showed HIF-1α had a correction with the malignancy of gastric cancer and PPIs might inhibit the progress of gastric cancer by affecting HIF-1α-FOXO1 axis.

siRNA was used to knock down the expression of HIF-1α, and qRT-PCR showed the expression of FOXO1 decreased in SGC7901 cells with the transfection of si-HIF-1α (**Figure 4C**). Western blotting suggested that both of HIF-1α and FOXO1 decreased in SGC7901 cells (**Figure 4D**). SGC7901 cells were treated with different dose of $CoCl_2$ which could maintain a high level of HIF-1α in cancer cells. qRT-PCR showed the RNA expression of FOXO1 increasedin hypoxia (**Figure 4E**). Western blotting showed that the expression of FOXO1 was markedly highas the expression of HIF-1α increased in SGC7901 cells (**Figure 4F**). These results showed HIF-1α could promote FOXO1 transcription and expression and highdose of omeprazole could decrease FOXO1 through inhibiting the expression of HIF-1α to restrainthe progress of gastric cancer.

It was revealed that the expression of HIF-1α experienced an opposite tendency with the concentration of PPIs enrichment (**Figure 4G**). siRNA was used to knock down the expression of HIF-1α, Western blotting showed siRNA could inhibit the HIF-1α expression in exosomes, and could also suppress exosomes release from gastric cancer cells (**Figure 4H**).

PPIs regulated tumor microenvironment by inhibiting the release of exosomes and exosomes carrying miRNAs

We explored whether PPIs could inhibit the exosomes release. Firstly, we confirmed the exosome by electronic speculum (Supplementary Figure 1D). SGC-7901 cells were treated with 80 ug/ml PPIs for 24 h, and then exosomes were isolated and resuspended in the PBS. The expression of CD63, a well-known marker of exosomes, was confirmed by means of Western blotting, which showed a lower level in the PPIs treated group (**Figure 5A**). And BCA protein assay proved that omeprazole could inhibit exosomes release (**Figure 5B**). What's more, to explore whether PPIs could affect tumor microenvironment through exosomes, we added exosomes derived from SCG-7901 cells treated with or without PPIs into medium of HFF-1 cells, respectively (**Figure 5C**). Western blotting showed exosomes could induce CAFs transformation, however less CAFs transformation was induced by exosomes treated with PPIs. Furthermore, PKH67 was used to label exosomes. 30 ul exosomes was added in the medium of HFF-1 cells for 3 h, and fluorescence microscope showed exosomes could effectively enter into HFF-1 cells. However, less exosomes from SGC-7901 cells treated with PPIs could enter into HFF-1 cells (Supplementary Figure 1E).

After cultured with exosome derived from SGC7901 cells (80 ug/ml of PPIs or PBS (control) treated for 24 h), the media of HFF-1 cells were collected and then replaced the normal media of SGC7901 cells for 24 h. To determine the impact of cytokines secreted from CAFs on gastric cancer cells, CCK8 and transwell assays were conducted. It showed that PPIs could also inhibit the proliferation (**Figure 5D**), migration and invasion (**Figure 5E**) of SGC7901 cells through the transportation of exosomes in tumor microenvironment.

We added exosomes, which were released from PPIs-treated (or untreated) SCG-7901 cells into medium of HFF-1 for 24 h. We measured cytokines via the Human Cytokine Array G5. The markedly changes of respectable cytokines were found in medium (**Figure 5F**), which could also promote the progression of gastric cancer. Our results inferred that PPIs could affect the CAFs transformation by regulating the exosomes derived from gastric cancer cells.

We performed microarray analysis to compare the signature difference of microRNA within normal SGC-7901 secreted exosomes and PPIs-treated exosomes (**Figure 5G**). Overall, we detected 13 miRNAs out 4774 arrayed miRNAs. The screening criteria was $|\text{Fold change}| \geq 1.5$ and $P < 0.05$. Where in 3 miRNAs showed a significant upregulation and 10 miRNAs showed a significant down-regulation. In order to determine the biological processes regulated by up-regulated and down-regulated miRNAs, we performed Bioinformatics pathways analysis using DIANA-mirPath program. Our data demonstrated 30 significantly enriched KEGG pathways ($P < 0.01$, FDR corrected), which are probably under the control of aforementioned miRNAs (**Table 2**). Among those, tumor invasion and metastasis related pathway, adherence junction and focal adhesion pathway, malignancy of tumor related pathway, FoxO and HIF-1 pathway and TGF-beta pathway (tumor microenvironment related pathway) were involved in the pathway regulated by the exosomes contained miRNAs.

Table 2. Top 30 cellular pathways influenced by dysregulated exosomes miRNAs

KEGG pathway	*p*-value	Genes	miRNAs
MicroRNAs in cancer	4.70E-42	94	11
Proteoglycans in cancer	5.98E-12	110	11
Renal cell carcinoma	2.70E-09	45	11
Hepatitis B	4.18E-08	74	11
Pancreatic cancer	3.70E-07	46	11
Prion diseases	8.29E-07	13	8
Adherents junction	8.29E-07	43	9
Pathways in cancer	8.29E-07	189	11
Thyroid hormone signaling pathway	1.18E-06	65	11
Colorectal cancer	2.01E-06	41	11
Glioma	2.01E-06	39	11
Chronic myeloid leukemia	6.23E-06	46	11
Protein processing in endoplasmic reticulum	1.20E-05	91	11
Fatty acid biosynthesis	1.27E-05	6	6
FoxO signaling pathway	2.28E-05	75	11
Endometrial cancer	2.28E-05	34	11
Prostate cancer	2.28E-05	54	11
Non-small cell lung cancer	2.45E-05	36	11
Hippo signaling pathway	3.24E-05	76	11
ErbB signaling pathway	6.07E-05	48	12
Phosphatidylinositol signaling system	6.10E-05	45	10
Cell cycle	9.15E-05	65	10
Lysine degradation	0.000108681	25	8
TGF-beta signaling pathway	0.000118299	40	10
Viral carcinogenesis	0.000428011	80	11
Focal adhesion	0.000587465	104	11
p53 signaling pathway	0.000610132	42	6
Bacterial invasion of epithelial cells	0.000740575	41	9
HIF-1 signaling pathway	0.001817139	56	10
Neurotrophin signaling pathway	0.002456577	63	11

Discussion

Several preclinical studies have elucidated that PPIs can modulate tumor acidification and restore chemotherapeutic sensitivity in drug-resistant cancer cells [20-24]. But only two recent small sample size clinical studies had reported the promising value of PPIs in treating osteosarcoma [25] and breast cancer patients [19] yet.

Based on our pilot study, high dose of PPIs (esomeprazole) had promising DCR and response rate, considering the extremely poor efficacy in advanced gastric cancer second or third line treatment [27, 28]. It is interesting that all the patients with PR were given higher accumulated dose of esomeprazole (three days before chemotherapy, and three days concurrent with chemotherapy). No partial response was seen among the rest of patients who received esomeprazole only two days prior to chemotherapy. The dose of esomeprazole we used was lower than HU's regimen [19]. Because our study was only a non-controlled pilot study, the optimal regimen of high dose esomeprazole still needed to be further verified. We didn't observe obvious side reactions induced by esomeprazole. Previous studies had also shown PPIs had no significant side effects [26], even at high dosages (as in patients with Zollinger-Ellison syndrome) [29, 30]. Furthermore, our study confirmed high dose of PPIs could inhibit the tumor size in tumor-bearing BALB/c Nude mice compared to placebo. All of these results indicated PPIs had great potential in treating gastric cancer patients. However, the biological and molecular changes PPIs treatment gastric cancer cells were not well studied.

Previous studies demonstrated tumor exosomes release was increased by acidic PH [31] and PPIs selectively accumulate in acidic spaces and target the H^+, K^+-ATPase of cell to regulate cellular PH gradient [14]. But whether PPIs could regulate the gastric cancer derived exosomes was rarely known. In this study, we found that PPIs could inhibit exosomes release at the concentration of 80 ug/ml. Exosomal miRNAs have emerged as micro-communicators of pathologic conditions including cancer. Cancers can educate the tumor microenvironment in favor of metastasis [32]. In this research, we

purified exosomes from SCG-7901 cells which was treated with PPIs, and analyzed by microarray. We detected 13 significantly changed miRNAs out of 4774 arrayed miRNAs. According to bioinformatics pathway analysis, we found TGF-β signaling pathway was correlated with tumor microenvironment and the transformation of CAFs [33]. CAFs have recently received attention because of their pivotal roles in tumor growth, angiogenesis, invasion, and metastasis by interacting with tumor cells. The contribution of CAFs to tumor cell proliferation and motility include cytokines, chemokines, growth factors [34]. In this study, we demonstrated exosome from gastric cancer cells treated with PPIs induced less CAFs transformation and cytokines including IL-6, IL-8, TIMP-1 and TGF-β1 release than that in the control group. Studies have demonstrated that cytokines including IL-6 [35], IL-8 [36], TIMP-1 [37], and TGF-β1 [38] contribute to the progress of gastric cancer. These results implied that PPIs could play an effective role by the form of exosome in tumor microenvironment. PPIs could suppress the transformation into CAFs and the secretion of cytokines, consequently. Thereby, PPIs could restrain the progression of malignant behavior of gastric cancer cells by means of exosome to some extent. Western blotting showed siRNA of HIF-1α could inhibit the HIF-1α expression exosomes, and could also depress exosomes delivery from gastric cancer cells. These results indicated the inhibition of exosomes release by PPIs at least partially through HIF-1α.

Previous studies demonstrated HIF-1 [39-42] and FOXO1 [43-46] had a correlation with malignancy of cancer. In this study, we found HIF-1α and FOXO1 had positive correlation with AJCC metastasis pathologic in gastric cancer patients from TCGA database. We proved that high dose of PPIs could suppress cell proliferation and motility, enhance the effects of anti-carcinoma drugs, and induce apoptosis in gastric cancer cells. However, whether FoxO1 and HIF-1 pathway involved in these biological changes induced by PPIs remains unclear. We found PPIs could inhibit the expression of FOXO1 and induce cellular translocation of FOXO1. Studies have illuminated that FOXO1 could promote apoptosis signaling through the activation or repression of apoptosis-related genes in the nucleus [47, 48], and cytosolic FOXO1 is essential for the induction of autophagy [46]. Consistent with previous studies, we discovered that high dose of PPIs induces nucleus translocation of FOXO1 to induce apoptosis. Meantime PPIs inhibit the tumor metastasis and enhance the effects of anti-cancer drugs by depressing the expression of FOXO1. Study has proved HIF-1α could lead to drug resistance in gastric cancer [49]. We found high-dose PPIs could also depress the expression of HIF-1α. We have proved HIF-1α could promote transcription of FOXO1 in gastric cancer. As a consequence, we demonstrated HIF-1α acted as upstream gene of FOXO1 and regulated the expression of FOXO1 with the administration of high-dose PPIs. These results indicate that PPIs can exert the most effect to inhibit gastric cancer at a high dose, and reasonable dose of PPIs should be formulated in clinical applications.

In this study, we found for the first time that high dose of PPIs could not only improve the prognosis of advanced gastric cancer patients, but also inhibit the progress of gastric cancer in vivo and in vitro. Furthermore, we showed that high-dose PPIs could not only inhibit the release of exosome and its packed microRNA to regulate gastric cancer and its microenvironment, but also enhance the effects of anti-tumor drugs, induce apoptosis, inhibit cell migration and invasion through regulating HIF-1α-FOXO1 axis in gastric cancer. Hence, high dose of PPIs will be used as a promising agent to relieve malignant progress of gastric cancer and regulate its surrounding tumor microenvironment in the future.

Data sharing statement

Microarray data deposited into the Gene Expression Omnibus (G.E.O.) with Accession NO. GSE87152 (https://www.ncbi.nlm.nih.gov/geo/). All the other supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author (D.H.) upon request.

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Disclosure of conflict of interest

None.

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Supplementary material and methods

Cell culture and transfection

SGC7901 cells were cultured in RPMI-1640 medium (Gibco, 11875093) supplemented with 10% fetal calf serum (Gibco, 10099141), and HFF-1 were cultured in DMEM medium (Gibco, 11965092) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. For details of confocal microscopy and morphological analysis, see Supplementary Materials and Methods.

Cells were allowed to grow overnight in 6-well plates (5×10^5 cells per well). On the following day, the small interfering RNA (siRNA) against human FOXO1 or HIF-1α and control scrambled siRNA (GenePharma) were individually transfect into cells using Lipofectamine 2000 reagent (Invitrogen, 11668019) according to the manufacturer's protocol and then cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 24-48 h. The siRNA sequences were as follows:

FOXO1 sense: 5'-CCAGGCAUCUCAUAACAAATT-3' and antisense: 5'-UUUGUUAUGAGAUGCCUGGCT-3'; HIF-1α sense: 5'-CCAGUUAUGAUUGUGAAGUUATT-3' and antisense: 5'-UAACUUCACAAUCAUAACUGGTT-3'.

RNA isolation and real-time reverse transcription-polymerase chain reaction

For total RNA of exosomes extraction, RNA was isolated using the Total RNA Purification Kit (Norgen, 17200) according to the manufacturer's instructions from 200 μL of frozen exosome in PBS. The volume of the obtained RNA solution was 60 μL.

Total RNA for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was extracted from cell lines using Trizol reagent (Invitrogen, 15596026) as specified by the manufacturer's instructions for FOXO1 and HIF-1α messenger RNA (mRNA) expression quantification. cDNA was prepared from 500 ng of total RNA using the Primescript RT Master Kit (Takara RR036A) according to the manufacturer's instructions. A PCR bulk reaction mixture was prepared as described in the SYBR Premix Ex Taq™ IIKit, and the thermal cycling parameters included initial denaturation at 95°C (30 s), followed by 40 cycles at 95°C (5 s) and 60°C (34 s). qRT-PCR was performed in triplicate and was repeated in at least three separate experiments using ABI Prism 7500 (Applied Biosystems). Data analysis was using the $2^{-\Delta\Delta CT}$ method with β-actin serving as the comparator. The results were presented as the fold-change relative to control. The primer sequences for FOXO1 and β-actinwere, respetively:

5'-TGGACATGCTCAGCAGACATC-3' and 5'-TTGGGTCAGGCGGTTCA-3', 5'-ACACCTTCTACAATGAGCTG-3' and 5'-CATGATGGAGTTGAAGGTAG-3'.

Hypoxia induced by $CoCl_2$ solution

25-mM $CoCl_2$ (Amresco, J297) stock solution was prepared in sterile double-distilled water. $CoCl_2$ was used at the final concentration of 12.5 μM-200 μM in culture media to induce hypoxia. $CoCl_2$ containing media was added to cells, and the cultures were incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2.

Western blot analysis

Proteins (25 μg/lane) were resolved by 10%-15% SDS-PAGE and electro transferred to a PVDF membrane using standard procedures. After blocking with 5% BSA in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h, the blots were probed with primary antibodies at 4°C overnight, reacted with a peroxidase-conjugated secondary antibody for 1 hat room temperature, followed by detection of the proteins with ECL reagents (Pierce). And quantification of protein levels was performed using image J software. The primary antibodies were as follows: Rabbit-anti-FOXO1, 1:1000 (Cell Signaling Technology, 2880), Rabbit-anti-α-SMA, 1:1000 (Abcom, ab5694), Mouse-anti-HIF-1α, 1:1000 (Abcom, ab113642), Mouse-anti-β-actin (Santa Cruz, 1:1000). The secondary antibodies were as follows: goat-anti-mouse IgG and goat-anti-rabbit, 1:500 (Santa Cruz).

Cell survival assay

Cells were seeded in 96-well plates and treated with different treatments. Next, 10 μl of CCK-8 (Dojindo, CK04) was added to each well, and the samples were incubated at 37°C for 4 h in a humidified CO_2 incubator. Absorbance was measured at a wavelength of 450 nm. Omeprazole (AstraZeneca), cisplatin (Haosen, China), paclitaxel (Xiehe, China) and 5-FU (Haixin, China) were used in our research.

Cell apoptosis determination

Cells were seeded at 2×10^6 cells per well in 6-well plates for 24 h. Then, omeprazole or paclitaxel was added at the indicated concentrations following the scheduled groups to culture the cells. Cells were digested by 0.25% pancreatin without EDTA and were collected for analysis subsequently. The determination of cell apoptosis was performed by annexin-V-FITC and propidium iodide (PI) staining following the manufacturer's instructions (BD, 556547), and the samples were analyzed by flow cytometry.

Immunofluorescence

Gastric cancer cells were grown on glass coverslips, washed twice in phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min. Permeabilization was with 0.1% Triton X-100 for 10 min at room temperature prior to blocking in 5% BSA for 30 min. FOXO1 was detected by reacting with antibody (1:100) overnight at 4°C, followed by Alexa Fluor 488 conjugated (Beyotime, P0176) or Cy3-conjugated (Beyotime, P0193) secondary antibodies at 1:200 for 1 h in the dark. Coverslips were mounted on glass slides using ProLong® Diamond AntifadeMountant with DAPI (Life Technologies, P36971) mounting medium prior to imaging with a fluorescence microscope.

RNA labeling and microRNA microarray hybridization

The Human microRNA Microarray Kit (Agilent Technologies, Agilent-G4870C) was used for labeling and hybridization according to the manufacturer's protocol. In brief, equal amount of small RNA (defined by Agilent bioanalyzer pico assay) was labeled with Cyanine3 (Cy3), re-suspended in hybridization buffer and hybridized to the array platform overnight (20 hours) at 55°C in a rotating Agilent hybridization oven using Agilent's recommended hybridization chamber. Subsequently, the microarrays were washed with the Agilent Gene Expression Wash Buffer 1 for 5 min at room temperature. A second washing step was performed with Agilent Gene Expression Wash Buffer 2 warmed to 37°C for 5 min. Fluorescence signals after hybridization were detected with a DNA microarray scanner G2505C (Agilent Technologies) using one color scan setting for 8 × 60 K array slides (Scan Area 61 × 21.6 mm, Scan resolution 2 μm, Dye channel is set to Green and Green PMT is set to 100%).

Microarray data analysis

In order to obtain background subtracted and outlier rejected signal intensities, the scanned microarray images were analyzed and processed with the Agilent feature extraction software (v10.7.3.1) using default parameters (Grid: 046064_D_20121223). The resulting raw Signal intensities (gMedianSignal) were exported to R software and normalized by Quantile normalization method. The pairwise expression fold change and p value were calculated via Student's paired t-test after merging the spots with same Agilent probe ID. Differential expressed genes were defined when fold change > 1.5 ($|\log2 \text{ ratio}| > 0.585$) and p value < 0.05.

The hybridization protocol, raw and normalized data are provided in NCBI's Gene Expression Omnibus (GEO, Series accession number: GSE87152).

Isolation and identification of SGC-7901 exosomes

All experiments were performed with exosome-free FBS. Exosome-free FBS was prepared by ultracentrifugation at 110,000 × g for 16 h. 10 ml SGC-7901 cells culture media were collected and centrifuged at 3000 g for 15 min. Supernatant was added 2 ml ExoQuick-TC exosome Precipitation Solution (System

Bioscience, EXOTC50A-1). This was mixed well and refrigerated overnight. After that, the mixture was centrifuged at 1500 g for 30 min, and the supernatant was removed. The residual solution was centrifuged at 1500 g for 5 min and aspirated. Due to omeprazole could also affect the proliferation of SGC-7901, CCK8 was used to detect the proliferation of SGC-7901 treated with omeprazole. Absorbance at a wavelength of 450 nm was used to determinate the volume of PBS to resuspend the exosome pellet and the exosome was stored at -80°C for the use in the experiments described later.

By using a BCA protein assay kit (Pierce), the amount of exosome was detected by measuring total protein content. Further, CD63, 1:500 (Santa Cruz, sc-5275) in exosomes was also detected with Western blot analysis.

Internalization of labelled exosomes

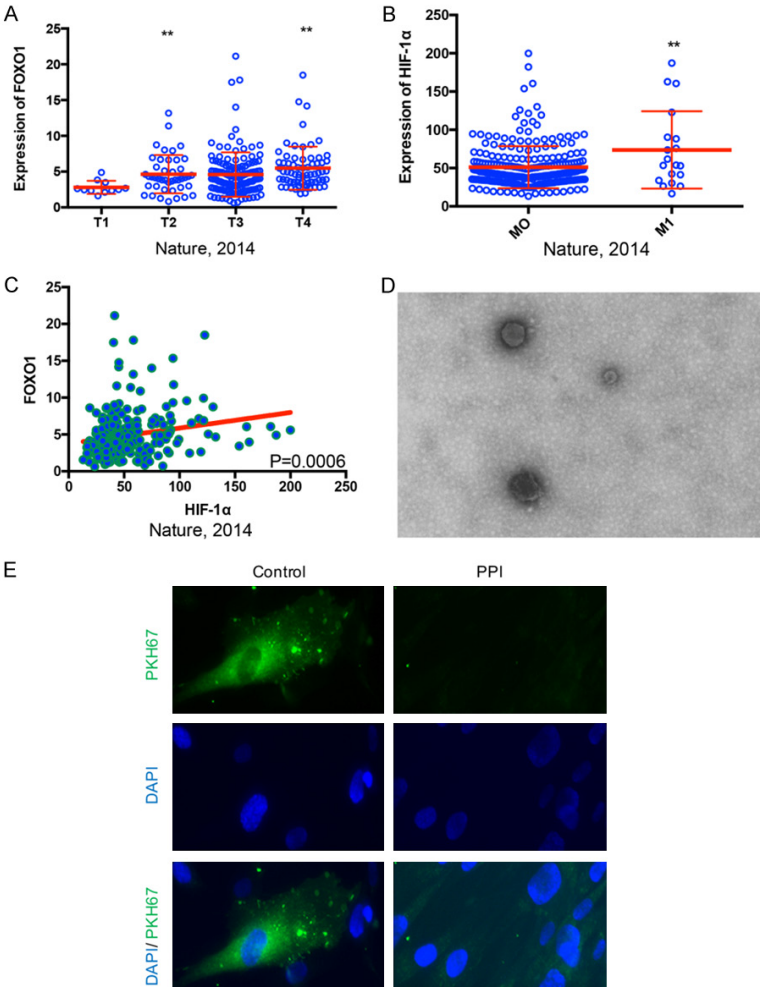
HFF-1 were seeded in a glass coverslips inserted in 12-well plate and cultured overnight. Cells were treated with 30 ul PKH67 (Sigma, miNi67)-labelled SCG-7901 exosome for 3 h at 37°C. After washing, Coverslips were mounted on glass slides using ProLong® Diamond AntifadeMountant with DAPI (Life Technologies, P36971) mounting medium prior to imaging with a fluorescence microscope (Zeiss).

Migration and invasion assays

Cell migratory and invasive abilities were assessed by way of transwell (Corning) and Matrigel invasion (BD Biosciences), respectively. For transwell migration assay, 1×10^5 cells were seeded, whereas 5×10^4 cells were seeded for the invasion assay. Cells that migrated to the underside of the membrane were fixed and stained with 0.1% crystal violet and were enumerated by counting four random fields per transwell. Mean values of migrating or invading cells were expressed as percentages relative to control. Each experiment was performed in replicate inserts, and mean value was calculated from three independent experiments.

Cytokine array

HFF-1 cells were treated with exosome from gastric cancer cells and supernatants were harvested and examined for differential cytokine expression using the Human Cytokine Array G5 (RayBiotech) per the manufacturers using the Imagequant TL software (GE Healthcare Life Sciences).



Supplementary Figure 1. A. Nature, 2014 databases show FOXO1 mRNA has positive correlation with AJCC tumor pathologic in gastric cancer patients. B. Nature, 2014 data bases show that HIF-1α mRNA have positive correlation with AJCC metastasis pathologic in gastric cancer patients. C. Nature, 2014data bases reveal the positive correlation of mRNA between FOXO1 and HIF-1α. D. Typical image of electronic speculum showed the morphology of exosome. E. HFF-1 cells were treated with PKH67 labelled exosome of SGC7901 cells (80 ug/ml of PPI or PBS (control) treated for 24 h) and the uptake of exosome by HFF-1 cells was tested by immunofluorescence, and the nucleus of HFF-1 cells was stained by DAPI (n=3).