Proton Pump Inhibitors Could Ameliorate Cachexia of Gastric Cancer by Down-regulating Vacuolar H^+-ATPases and Disrupting PI3K/AKT/mTOR/HIF-1α/PKM2 Signaling Pathway

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Abstract

Objective: Our study aimed to investigate whether PPIs could alleviate cachexia of gastric cancer by inhibiting the expression of V-ATPases, downregulating the PI3K/AKT/mTOR/HIF-1α signaling pathway and then inhibiting PKM2.

Methods: Human gastric cancer cell lines SGC7901, BGC823 and MKN28 were treated by PPIs or downregulated related factors, and then the expressions of PI3K/AKT/mTOR/HIF-1α/PKM2 signaling pathway, cell apoptosis, cell proliferation and cell migration were analyzed, respectively. In addition, *in vivo* experiments were conducted to further strengthen our conclusion.

Results: Omeprazole, pantoprazole and esomeprazole could respectively inhibit the expression of V-ATPases at a concentration of 10 μg/ml in MKN28 whereas it had no significantly inhibitory effects on the expression of PKM2. Esomeprazole could enhance the inhibitory effects of rapamycin on p-mTOR at a concentration of 20 μg/ml , and could enhance the inhibitory effects of rapamycin on HIF-1α at 10 μg/ml (P<0.05). The mRNA expression of V-ATPases, PI3K, AKT, HIF-1α and PKM2 was inhibited in the SGC7901 cells pretreated previously with esomeprazole (20 μg/ml) for 24 hr. LY294002 with 10 μmol concentration could significantly inhibit protein expression of p-AKT and p-mTOR in the BGC823 cells after 48 hr treatment (P<0.05). After the expression of HIF-1α was knocked down by si-RNA in the BGC823 cell line, the expression of HIF-1 significantly decreased, and the expression of PKM2 decreased in the downstream, while the expression of p-mTOR in the upstream also was simultaneously elevated.

Conclusion: PPIs might be a promising agent to alleviate cachexia of gastric cancer according to our *in vitro* and *in vivo* experiments.

Introduction

Gastric cancer (GC) is one of the common malignant tumors in the digestive system, with the fourth incidence and secondary mortality in malignant tumors around the whole world. Altered cancer metabolism is considered as one of new hallmarks of cancer and Warburg effects is characterized as cancer metabolism [1-3]. As the cancer cells activate aerobic glycolysis, glucose is metabolized to lactate[4]. The lactate synthesized in the cytosol is then secreted outside the cells by monocarboxylate transporters, producing and maintaining the acidic microenvironment of tumor[5]. The lower extracellular pH microenvironment also can accelerate the malignant biological behaviors including invasion, metastasis, multidrug resistance(MDR), proliferation and so on [6]. Hypoxia has been found to be associated with tumor progression. Cancer cells are known to adapt to the hypoxic condition via the hypoxia-inducible factor 1 (HIF-1) [7]. Pyruvate kinase (PK) is a key enzyme in the last step of glycolysis that transfers the phosphate group from phosphoenolpyruvate to ADP resulting in the formation of pyruvate and an ATP molecule [8]. PKM2 is expressed in some of tumor cells [9]. According to some studies, the mTOR/HIF-1α/PKM2 signaling pathway could regulate the tumor glycolysis[10-13]. Our previous study has already demonstrated that the regulation of mTOR/HIF-1α/PKM2 signaling pathway

could affect the glycolysis of gastric cancer cells[14]. Moreover, we have found that pantoprazole, one type of PPIs which was widely used to treat gastro-esophageal reflux and peptic ulcer diseases, can inhibit the Vacuolar H^+-ATPases (V-ATPases)/mTOR/HIF-1α/P-gp and MRP1 signaling pathways, and abolish the multidrug resistance (MDR) of SGC7901/ADR cells [15].

In our study, we aimed to investigate whether PPIs could alleviate cachexia of gastric cancer and then affected the PI3K/AKT/mTOR/HIF-1α/PKM2 signaling pathway. Besides, we have already downregulated the expressions of V-ATPases, HIF-1α and PKM2 to investigate the effects on the GC cells. The results demonstrated that PPIs could alleviate cachexia of gastric cancer by inhibiting the expression of V-ATPases, downregulating the PI3K/AKT/mTOR/HIF-1α/PKM2 signaling pathway and then inhibiting PKM2. Moreover, our gene chip assay also indicated that the downstream signaling pathway which was closely related with PKM2 gene might be the MAPK signaling pathway, providing a future research direction.

Materials And Methods

1.1 Cell culture

The human cancer cell lines including MKN28, SGC7901, BGC823 and HELA were provided by the Laboratory of Department of Gastroenterology, the affiliated Drum Tower Hospital of Nanjing University, Medical School, (Nanjing City, Jiangsu Province, P.R. China). The gastric cancer cell lines were originally authenticated in China Center for Type Culture Collection (CCTCC) by Short Tandem Repeat (STR) profiling and passaged less than six months in the lab. The cells were maintained in RPMI-1640 (Hyclone, Logan, U.S.A.) medium with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, Hangzhou, Zhejiang Province, P.R.China) at 37°C in an atmosphere containing 5% CO_2 (Thermo Direct Heat CO_2, U.S.A.). Each cell line was passaged when it reached 70% to 80% confluence. The acid cell culture medium (pH 6.5) was obtained by the addition of 1M HCl solution as previously reported [16, 17]. The pH value was estimated by the use of a FE20 Five Easy plus pH Meter (METTLER TOLEDO Instruments, Shanghai, P.R.China).

1.2 Quantitative real-time polymerase chain reaction

The total RNA in the cells was extracted with Trizol Reagent (Invitrogen Life Technologies, U.S.A.) and subsequently reverse-transcribed using the PrimeScript RT Master Mix according to the manufacturer's instructions. qRT-PCR was determined with the 7500 Real-time PCR System (Applied Biosystems, U.S.A.) using SYBR Premix Ex Taq reagents. The PCR cycling conditions were 40 cycles of 5 seconds at 95°C and 32 to 34 sec at 60°C. All data were normalized to the human β-actin. Fold induction was calculated by using the formula $2^{-\Delta\Delta Ct}$. The data represented were based on at least three independent experiments.

1.3 Western blotting analysis and antibodies

Cells were lysed in RIPA buffer (Beyotime Biotechnology, P0013B, P.R.China) containing EDTA-free protease inhibitor cocktail (Roche, 04693159001, Swiss) on ice for 15 min, and then centrifuged for 15 min (12,500×g, 4℃). The supernatant was collected, and protein was quantified with the BCA kit and denaturated in boiling water for 10 min. Equal amounts of protein (at least 30 μg) were separated on 8%–12% SDS-PAGE for electrophoresis and then electrophoretically transferred onto the PVDF membrane (Millipore, Bedford, M.A., U.S.A.). Then the protein membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 2-4 hr. After the membranes were cut off for demand, they were incubated with specific primary antibodies which were diluted according to the instructions overnight at 4℃. After being probed with the appropriate secondary antibodies (1:3000 dilution), the blots were detected by using a CCD camera (Tanon, Shanghai, P.R.China) with enhanced chemiluminescence (Milipore, U.S.A.). The data were represented at least three independent experiments.

1.4 Transwell assay

The transwell assay was performed by using an 8 μm pore size transwell system. Briefly, the chambers were settled on 24-well plates. The BGC823 cells and SGC7901 cells suspended in 500 μL of RPMI-1640 were seeded in the upper chamber at a density of 5×10^4 cells/well. Then 750 μL of RPMI-1640 containing 15% fetal bovine serum was added into the lower chamber. The cells in the upper chamber were removed after 24 hr. The cells that migrated through the membrane to the underside were fixed with methanol for 15 min and were then stained with 0.5% crystal violet for 15 min. The cell number was counted in five separate fields by using light microscopy at 200×magnification. The experiments were conducted independently at least three times.

1.5 Cell proliferation assay

Firstly, the cells were seeded into 96-well plates (2000 cells/well). Then the medium was removed and 200 μL new medium with 10 μL CCK-8 (Keygen Biotech Co. Ltd.,P.R.China) was added at different time point (24, 48, 72, and 96 hr). Cells were dissolved and incubated for 2 hr at 37°C. The absorbance of each well was determined by a spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, U.S.A.) at 450 nm. The data were presented as MEAN±SEM, derived from triplicate samples of at least three independent experiments.

1.6 Apoptosis assay

After being transfected for 48 hr, apoptosis in cultured cells was evaluated by using annexin V labeling. Alexa Fluor- 647 (APC), 7-ADD viability staining and binding buffer solution were purchased from Biolegend (Biolegend, San Diego, U.S.A.) and were used according to the manufacturer's protocol. Data were collected by multicolor flowing cytometry (Gallios, Beckman Coulter, Brea, C.A.) and the Gallios software (Beckman Coulter, C.A.) and analyzed by the Kaluza software (Beckman Coulter, C.A.).

1.7 Lactate/Glucose colorimetric assay

Firstly, 2 to 50 μL of test samples were added to 96-well plates. Then the lactate/ glucose assay buffer was added to adjust the volume to 50 μL/well. In the fluorometric assay, 10 μL lactate/glucose standard solutions were added to 990 μL of lactate/glucose assay buffer, stirred evenly and diluted to 0.1 nmol/ uL. Thereafter, 20 μL was added to 180 μL of lactate/glucose assay buffer and mixed well. The lactate/glucose assay buffer was used to adjust the volume to 50 μL/ well to generate 0, 0.2, 0.4, 0.6, 0.8, and 1.0 nmol/well of the lactate/glucose standard. Enough reagents were mixed for the number of assays to be performed. For each well, a total of 50 μL reaction mix was prepared containing the following components:

	Reaction mix	Background control mix
Lactate/glucose assay buffer	46 μL	48 μL
Lactate/glucose probe	2 μL	2 μL
Lactate/glucose enzyme mix	2 μL	-

The reaction was incubated at room temperature for 30 min avoiding light. Absorbance (OD 570 nm) was measured in a microplate reader.

1.8 Plasmids transfected cells

The BGC823 and SGC7901 cells were seeded in six-well plates in 2 ml media per well with a quantity of 60 000 per well. After 24 hr, the original medium was removed, 1 ml fresh medium containing 5 μg/mL polybrene was added and appropriate amount of plasmids. The cells were then incubated at 37°C. After 24 hr, the medium containing the plasmids was replaced by 2 ml fresh medium, and the culture was continued until the changes of the cells were detected. The fluorescence expression was clearly observed under the microscope. The plasmids were green fluorescent. In the control group, the cells were transfected by either empty plasmids or untreated. PKM2-knocked down cells, V-ATPases-knocked down cells and HIF-1α-knocked down cells were obtained successfully.

Results

2.1 Effects of PPIs on the expression of PI3K/AKT/mTOR/HIF-1α signaling pathway and downstream factor PKM2 in gastric adenocarcinoma cell lines with different degrees of differentiation

Protein expression of PKM2 in different gastric adenocarcinoma cell lines with different degrees of differentiation (MKN28, SGC7901, and BGC823) was analyzed by western blotting analysis, while the HELA cells were taken as a control. The results showed that the expression of PKM2 increased in human gastric adenocarcinoma cells with high, moderate and poor differentiation (Fig.1A), suggesting that there was a higher level of aerobic glycolysis in the gastric adenocarcinoma cell with higher malignancy.

After PPIs treatment with the concentrations of 0 and 100 μg/ml (pH6.5)，the expressions of V-ATPases and PKM2 were measured, respectively. The results showed that omeprazole, pantoprazole and

esomeprazole could respectively inhibit the expression of V-ATPases at a concentration of 10 μg/ml in MKN28 whereas it had no significantly inhibitory effects on the expression of PKM2. However, after omeprazole or esomeprazole pretreatment with the concentration of 100 μg/ml, the expression of PKM2 was obviously inhibited, consistent with those in the SGC7901 and the BGC823 cells (Fig. 1B).

2.2 PPIs and rapamycin could affect protein expression of PI3K/AKT/mTOR/HIF-1α/PKM2 signaling pathway

SGC7901 cells were treated with PPIs (0, 20, 40, 80, 160 μg/ml) for 24 hr with absence or presence of rapamycin (20 nmol) (Fig.2-1). It was shown that esomeprazole could enhance the inhibitory effects of rapamycin on p-mTOR at a concentration of 20 μg/ml (P<0.05), and could enhance the inhibitory effects of rapamycin on HIF-1α at 10 μg/ml (P<0.05). Nevertheless, at a concentration of 20 or 40 μg/ml, esomeprazole showed the synergistic inhibitory effects with rapamycin on p-mTOR and HIF-1α whereas it showed that inhibition of downstream factor PKM2 was not statistically different from using rapamycin alone (P 0.05). Only when the concentration of esomeprazole was enhanced to 80 μg/ml could the combination be superior to using rapamycin alone. Dramatically, it seemed that esomeprazole did not inhibit p-AKT caused by rapamycin, but promote the expression of p-AKT conversely (P<0.05). We speculated that esomeprazole was similar to rapamycin, inhibiting p-mTOR, but had no effects on the total mTOR expression. In addition, it was indicated in Fig.2-2 (A-D) that both PPIs and rapamycin could change protein localization of PKM2, and when the two drugs combined together, the effects became more significant coefficiently.

2.3 PPIs could inhibit expressions and change the intracellular localization of V-ATPases, PI3K AKT mTOR HIF-1α and PKM2 in the gastric cancer cell lines

It was showed in Fig. 3A that in the condition of pH 6.5, when compared with that in the control group (0 μg/ml), the mRNA expression of V-ATPases, PI3K, AKT, HIF-1α and PKM2 was inhibited in the SGC7901 cells pretreated previously with esomeprazole (20 μg/ml) for 24 hr (P<0.05). The mRNA expression of V-ATPases, PI3K, AKT, HIF-1α and PKM2 decreased conversely as the esomeprazole concentration increased (40, 80, 100 μg/ml).

Correspondingly, in the condition of pH 6.5, compared with those in the control group (0 μg/ml), esomeprazole at relatively lower concentration could not inhibit protein expressions, while the protein expressions of V-ATPases, PI3K, AKT, HIF-1α and PKM2 were significantly inhibited in the SGC7901 cell line pretreated by esomeprazole (40 μg/ml) for 24 hr (P<0.05) concentration-dependently. As the concentration increased, the protein expression decreased conversely (Fig. 3B).

Besides, after pretreating SGC7901 with esomeprazole (0, 10, 40, 100 μg/ml) for 24 hr, we detected V-ATPases, PI3K, AKT, mTOR, HIF-1α and PKM2 expression by the immunofluorescence imaging. The fluorescent images showed that esomeprazole (40 μg/ml) could reduce intracellular expressions of V-ATPases, PI3K, AKT, mTOR, HIF-1α and PKM2 (Fig. 3C).

2.4 PPIs could inhibit cell viability and induce early apoptosis of human gastric adenocarcinoma cell line SGC7901.

Effects of PPIs pretreatment with different concentrations for 24 hr or 48 hr on the cell viability of SGC7901 were then evaluated by CCK-8 cell proliferation assay. The results showed that pretreatment of SGC7901 cells with different concentrations of esomeprazole for 24 hr or 48 hr could inhibit the viability of SGC7901 cells to a certain extent. The viability of SGC7901 cells with esomeprazole (20 μg/ml) pretreatment for 24 hr was (88.97±3.39)%, which was significantly lower than that in the control group simultaneously (P<0.05) (Figure. 4A). With the increasing drug concentration and the prolongation of treating time, cell viability inhibition became more evident, indicating that the inhibitory effects of esomeprazole on the viability of SGC7901 cells was in a concentration- and time-dependent manner.

Furthermore, the SGC7901 cells were treated with esomeprazole at the concentration of 0, 10, 20, 40, 80 and 160 μg/ml for 48 hr. Flowing cytometry analysis showed that the early apoptotic rate in the 20 μg/ml group was significantly higher than in the control group (Figure.4B and 4C) (P<0.05), indicating that esomeprazole could induce early apoptosis of the SGC7901 cells.

2.5 Knockdown V-ATPases could inhibit the protein expressions of intracellular PI3K/AKT/mTOR/HIF-1α/PKM2 signaling pathway

By using plasmid transduction of V-ATPases shRNA, we successfully performed stable transfected SGC7901 cells targeted knockdown of V-ATPases (named pshRNA-V-ATPases) and the negative control cells (named pshRNA-Con). As shown in Fig.5A, the protein expression of V-ATPases significantly decreased by suppressing V-ATPases. Compared with pshRNA-Con group and negative control group, the protein expression of PI3K, AKT, mTOR, HIF-1α and PKM2 was significantly inhibited in the pshRNA-V-ATPases group (Fig.5B) (P<0.05).

2.6 Effects of LY294002, a specific phosphatidylinositol-3-kinase inhibitor, on protein expressions of intracellular PI3K/AKT/mTOR/HIF-1α signaling pathway and PKM2

LY294002 is a specific phosphatidylinositol-3-kinase inhibitor. The BGC823 cells were pretreated with LY294002 (0, 10, 20, 50, 100 μmol/L) or DMSO (0.2%) as a control. As shown in Fig. 6A and 6B, compared with the untreated group and DMSO group, LY294002 with 10 μmol concentration could significantly inhibit protein expression of p-AKT and p-mTOR in the BGC823 cells after 48 hr treatment (P<0.05). When the concentration was elevated to 20 umol/L, the expression of HIF-1α was significantly inhibited (P<0.05), while LY294002 with the concentration of 50 μmol/L could significantly inhibit the expression of PKM2 (P<0.05). The inhibition differed obviouly as the concentration increased. The inhibitory effects were on a concentration-dependent manner (P<0.05). It was indicated that LY294002 could inhibit the activation of PI3K/AKT/mTOR/HIF-1α signaling pathway at a lower concentration, but only at a higher concentration could it exert the inhibitory effects on the expression of PKM2.

2.7 Knockdown of HIF-1α had some effects on upstream and downstream molecules.

After the expression of HIF-1α was knocked down by si-RNA in the BGC823 cell line, the expression of HIF-1 significantly decreased, and the expression of PKM2 decreased in the downstream, while the expression of p-mTOR in the upstream also simultaneously increased (Fig.6C). It could be speculated that it might be related to the existence of a feedback mechanism. Over-suppressing on HIF-1α might lead to feedback up-regulation of p-mTOR and thus slowed down PKM2 reduction in the downstream.

2.8 Effects of knocking down PKM2 on human gastric adenocarcinoma cells.

The BGC823 and SGC7901 cells were treated with PKM2 plasmid or empty plasmid for 48 hr (named pShRNA-PKM2/pShRNA-Con), and then the messenger RNA (mRNA) and protein expressions of PKM2 were detected by RT-PCR and Western blotting analysis. And the protein localization also was detected by immunofluorescence imaging (Fig.7A). The results showed that PKM2 mRNA and protein expressions significantly decreased in the PKM2 transfection group when compared with the control group (P<0.05) (Fig.7B and 7C).

The cell viability was then evaluated by the CCK-8 assay. It was indicated in Fig.8A that compared with those in the pshRNA-Con group and negative control group, knockdown of PKM2 inhibited cell viability in the SGC7901and BGC823 cells. The transwell assay revealed that the number of migrated cells significantly decreased in the PKM2 transfection group compared with the control group in the BGC823 cells (P<0.05), while there was no statistical difference between the PKM2 transfection group and the control group in SGC7901 cells (Fig. 8B).

We further detected the effects of the transfection of PKM2 plasmids on cell aerobic glycolysis. SGC7901 cells were transfected with PKM2 siRNA (PKM2 siRNA group) or empty plasmids (con siRNA group) and we used SGC7901 cells with no treatment as the control group. SGC7901 cells in the control group and the transfection group were cultured for 24 hr, and the total protein was extracted, as determined by Western blotting analysis. The protein expressions of GLUT-1 and LDHA in the transfected group were lower than those in the control group (Fig.8C). Furthermore, the cells of the control group and the transfection group were cultured for 24 hr, and the extracellular glucose, as well as lactic acid contents, were detected respectively. The results showed that the contents of glucose and lactate were lower in the transfection group than that in the control group (Fig. 8D-F). These results indicated that knockdown of PKM2 had negative effects on aerobic glycolysis of gastric adenocarcinoma cells.

2.9 In vivo experiments corresponded with experiments *in vitro*

The athymic nude mice were randomly divided into five groups, accordingly. In the MOCK group SGC7901 cell suspension was inoculated subcutaneously into the back of the mouse and the mouse were then untreated. The NS group: SGC7901 cell suspension was inoculated subcutaneously into the back of the mouse and the mouse were treated by gavage with 5 ml NS every two days. In the PPI group: SGC7901 cell suspension was inoculated subcutaneously into the back of the mouse and the mouse were treated by gavage with pantoprazole (75 mg/kg) in 50 ml NS every two days. The PKM2 knocked down group: PKM2 knocked down SGC7901 cell suspension was inoculated subcutaneously into the back of the

mouse and the mice were then untreated. The NC group: empty plasmids injected SGC7901 cell suspension was inoculated subcutaneously into the back of the mouse and the mouse were then untreated. Weight of the mouse was measured and food or water intake was then recorded. After 5 weeks treatment, the mice were sacrificed and the tumors were resected for weighing and western blotting assay.

The results suggested that tumor growth was suppressed and weight loss of mouse was alleviated in the PPI group and the PKM2 knocked down group. It can be seen that the PKM2 knocked down group and the PPI group have the smallest tumor size compared with the MOCK group and the NC group (P<0.05, as shown in Fig. 9A and Fig. 9B). In the pantoprazole group, the tumor weight was significantly lower than that in the NS group (P<0.05, as shown in Fig.9C). The tumor weight was also significantly lower in the PKM2 interference group than that in the NC group (P< 0.05, as shown in Fig. 9D).

The body weight of the mouse in the pantoprazole group was significantly higher than that in the NS group and in the MOCK group (P<0.05, as shown in Fig. 9E). The body weight of the mouse in the PKM2 knocked down group was significantly higher than that in the NC group and the MOCK group, (P<0.05, as shown in Fig. 9F).

The average daily food intake of mouse in the PPIs group was lower than that in the MOCK and the NS group, but the difference was not statistically significant (P>0.05) (as shown in Fig.9G). The average daily intake of mouse in the PPIs group was lower than that in the MOCK group and the NS group, but the difference also was not statistically significant (P>0.05) (as shown in Fig. 9H).

As shown in Fig.10, PPZ treatment might inhibit the expression of PI3K, p-AKT, p-mTOR, HIF-1α and PKM2 of tissues of tumor-bearing mice accordingly.

2.10 MAPK signaling pathway is the most relevant downstream of PKM2.

To further verify the downstream of PKM2, PKM2 were knocked down in GC cells and untreated cells played the role of the control. Then we performed gene chip experiment to find out downstream of PKM2. Total RNA was extracted from the cells and RNA samples that passed the quality test were used for gene chip experiment.

Gene set enrichment analysis (GSEA) was performed based on the selected differential genes. According to the genetic information of all pathways in KEGG and BIOCARTA, the differential genes were analyzed by GSEA (Fig. 11A and 11B). After sorting according to the P value, we selected the top ten to display (Fig.11B). We can see from Fig.11A and 13B that MAPK pathway is the downstream of PKM2.

Discussion

Various reports have confirmed that proton pump inhibitor (PPIs) pretreatment can serve as an attractive anticancer therapeutic strategy in numerous types of cancer, including gastric cancer [15, 16, 18]. Besides,

our previous work identified that pantoprazole, a PPI which is widely used to treat gastro-esophageal reflux and peptic ulcer diseases, can inhibit the Vacuolar H^+-ATPases (V-ATPases)/ mTOR/HIF-1α/P-gp and MRP1 signaling pathways, and abolish the multidrug resistance (MDR) of SGC7901/ADR cells[15]. In this study, we aimed to explore effects of PPIs pretreatment and knockdown of factors in PI3K/AKT/mTOR/HIF-1α signaling pathway or PKM2 on cell proliferation, survival, migration and aerobic glycolysis.

We found that in human gastric adenocarcinoma cells with high, moderate and poor differentiation, the expression of PKM2 increased gradually (Fig. 1A), suggesting that gastric adenocarcinoma cells with higher malignancy had higher level of aerobic glycolysis, as PKM2 was considered as an important factor in tumor cell aerobic glycolysis[19]. Then we pretreated GC cells with or without PPIs and found that PPIs could suppress protein expression of PKM2 in the GC cells (Fig. 1B), indicating that PPIs may interference with cell aerobic glycolysis via down regulate PKM2.

PI3K/Akt/mTOR/HIF-1α signaling pathway is a classical signaling pathway which has an important role in intracellular signaling transduction and is significant to cell proliferation, survival and angiogenesis [20, 21]. Besides, PKM2 is a downstream factor of the pathway. Therefore, we pretreated the GC cells with or without PPIs with the absence or presence of rapamycin to evaluate whether PPIs had synergistic effects on rapamycin. As we have predicted previously, PPIs could strengthen the suppressing effects of rapamycin on the expressions of p-mTOR and HIF-1α. Interestingly, the synergistic effects of with rapamycin on PKM2 suppressing seemed of no statistical significance at lower PPIs concentration and esomeprazole did not inhibit the increase of p-AKT caused by rapamycin, but promotes the increase of p-AKT instead (Fig.2A). We speculated that esomeprazole is similar to rapamycin, inhibiting p-mTOR, but has no effects on the total mTOR expression.

For further exploration, we pretreated GC cells by using PPIs with various concentrations and found that PPIs could inhibit expression and change the intracellular localization of V-ATPases、PI3K、AKT、mTOR、HIF-1α and PKM2 in the GC cell line SGC7901 (Fig.3). Furthermore, our study also confirmed that PPIs could inhibit cell viability and induce early apoptosis of human GC cell line SGC7901. Thus we were interested in the relationship between the signaling pathway, PKM2 and malignant biological behavior.

We knocked down related factors respectively and confirmed that knockdown of V-ATPases could inhibit the protein expressions of intracellular PI3K/AKT/mTOR/HIF-1α signaling pathway and PKM2 (Fig.5), which indicated that V-ATPases are closely connected with the PI3K/AKT/mTOR/HIF-1α signaling pathway and could be a potential target of GC therapy. We knocked down upstream PI3K by a specific P13K inhibitor, LY294002 and found that LY294002 could inhibit the activation of PI3K/AKT/mTOR/HIF-1α signaling pathway at a lower concentrations, but only at a higher concentration could it show the inhibitory effects on PKM2 (Fig. 6). Then HIF-1α played an important role in cellular response to systemic oxygen levels, cell proliferation and survival, as well as glucose and iron metabolism[22]. Besides, HIF-1α was a member of PI3K/AKT/mTOR/HIF-1α signaling pathway and might be an upstream factor of

PKM2[23]. The results showed that the expression of HIF-1α significantly decreased, and the expression of PKM2 also decreased in the downstream, while the expression of mTOR in the upstream increased at the same time (Fig. 6C). We considered that it might be due to the existence of a feedback mechanism. Furthermore, we knocked down PKM2 in the GC cells and found that cell viability was then inhibited, cell migration weakened and cell aerobic glycolysis was inhibited (Fig. 7 and Fig.8). According to those above results, it could be summarized that inhibitory activity of PI3K/AKT/mTOR/HIF-1α signaling pathway had a negative effect on PKM2, and thus contributing to weakened cell proliferation and cell aerobic glycolysis, together with up regulated cell apoptosis.

In addition, we conducted *in vivo* experiments to further verify our conclusion. Based on our findings, PPIs treatment suppressed tumor growth and weight loss of tumor bearing mouse, so did knockdown of PKM2 (Fig. 9). The decrease in food intake was observed, but there was no statistical significance (Fig. 9). It was indicated that PPIs treatment or knockdown of PKM2 could rescue the mouse from cachexia to a certain degree. Protein levels of related factors were measured, suggesting that PPIs treatment could downregulate the PI3K/AKT/mTOR/HIF-1α signaling pathway in bearing-tumor tissue to some extent (Fig.10).

Morover, we conducted gene chip assay and found that MAPK signaling pathway was the downstream of PKM2, indicating that anti-tumor effects of knockdown of PKM2 might not only be related to cell aerobic glycolysis, which provided us with a potential target for our further study (Fig.11).

There was no doubt that there were some limitations in our *in vivo* and *in vitro* experiments. The main problem was that we hadn't determined whether other down-regulated factors in the signaling pathway were involved in cell proliferation, survival, migration and metabolism, etc. Besides, it would be better if we detected or controlled pH value after PPIs treatment.

In conclusion, PPIs could inhibit the expression of V-ATPases and then reversed cachexia of the gastric cancer via inhibiting PKM2, which contributed to weakened cell proliferation and cell aerobic glycolysis, together with up regulated cell apoptosis. The underlying mechanism was closely related to the inhibition of the PI3K/AKT/mTOR/HIF-1α signaling pathway *in vitro* and *in vivo*. PPIs might be the promising drug for alleviating cachexia of gastric cancer in the future.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All the authors agreed to signed the consent form for publication of our manuscript in your journal.

Availability of data and materials

All the original data and materials were available and trackable. If need, we can provide them.

Competing interests

All the authors declare that they have no conflicts of interest.

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Authors' contribution

Prof. Chen Min contributed to the manuscript by conceiving and designing the study; approving the final version of the manuscript.

Dr. Wang Zhuoting, Dr. Wang Yunrong and Prof. Wei Wei made the contributions to the work in collecting the data, analyzing and interpreting the data and writing the manuscript. Dr. Sun Wenqi and Dr. Wu Xinrong contributed to the work of data analysis and statistics as well as collecting references. Prof. Wei Wei mainly provided critical revisions that were important for the intellectual content.

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Figures

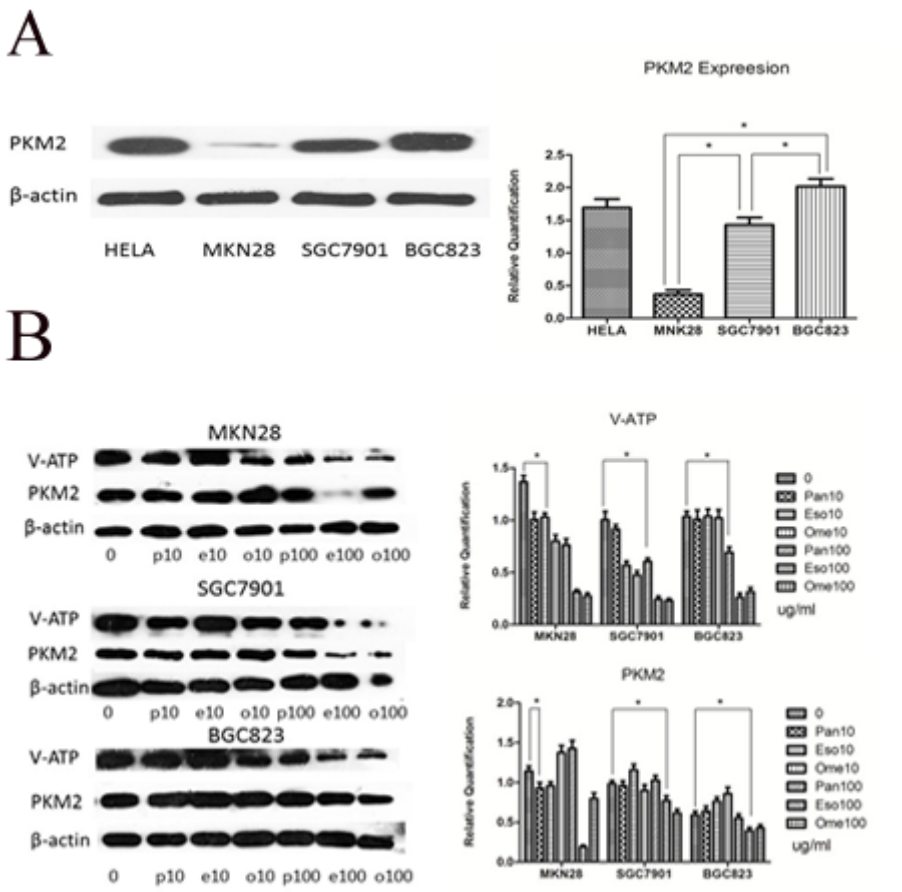


Figure 1

Inhibitory effects of PPIs on protein expression of PKM2 in gastric adenocarcinoma cell lines with different degrees of differentiation. (A) Protein expression of PKM2 in gastric adenocarcinoma cell lines with different degrees of differentiation(*P 0.05) . (B) MKN28, SGC7901 and BGC823 cells were pretreated with PPIs (omeprazole pantoprazole esomeprazole)(0, 10, 100 μg/ml) for 24 hr and then protein expressions of V-ATPases and PKM2 were analyzed by Western Blotting assay.

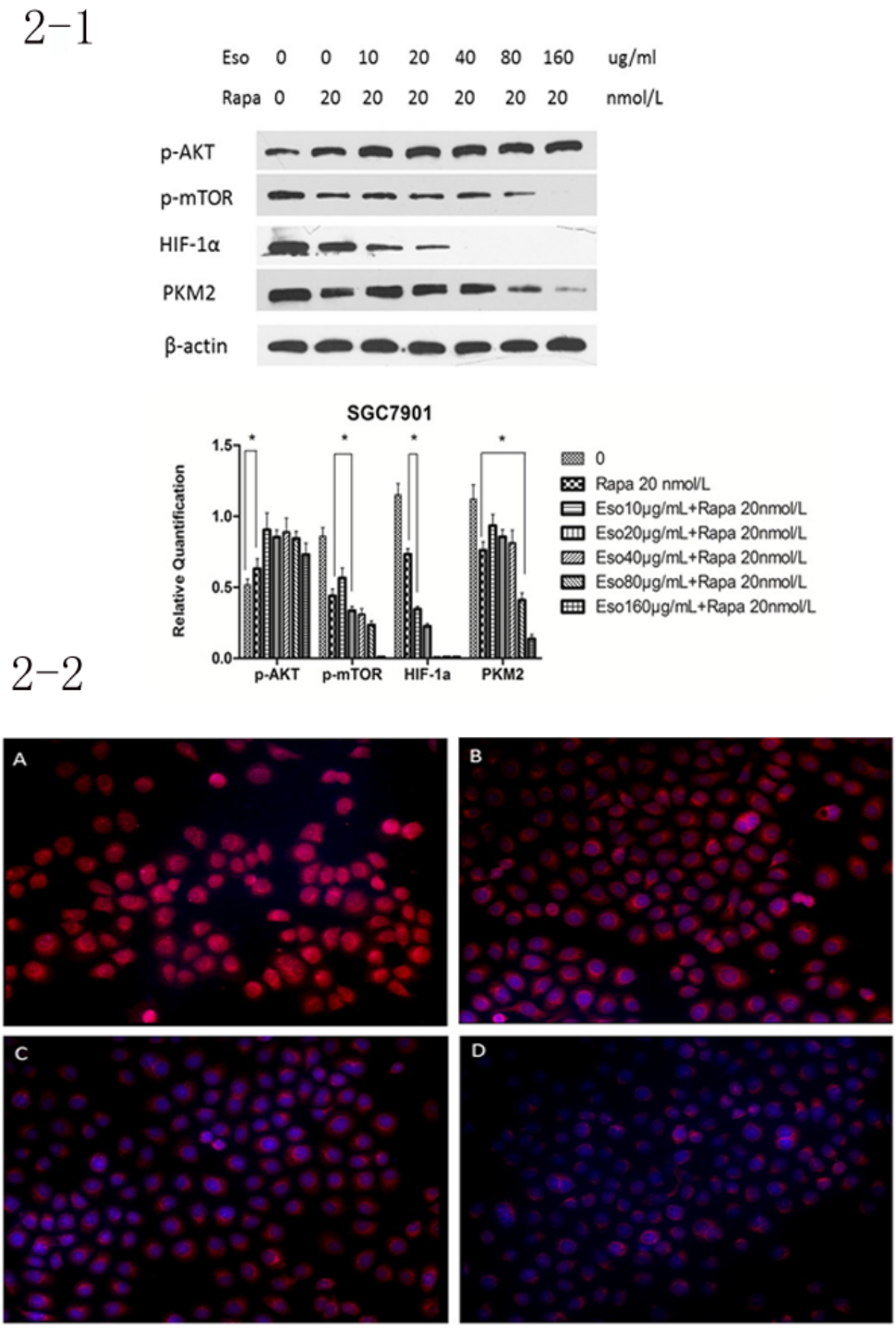


Figure 2

Esomeprazole could enhance the inhibitory effects of rapamycin on PI3K/AKT/mTOR/HIF-1α signaling pathway and downstream PKM2. (1) SGC7901 cells were treated with PPIs (0, 20, 40, 80, 160 μg/ml) for 24 h in the absence or presence of rapamycin (20nmol) and indicated protein levels were analyzed by WB assay.

(2) SGC7901 cells were either untreated or treated with PPI (80 μg/ml) for 24 hr in the absence or presence of rapamycin (20 nmol) and PKM2 expression was measured by immunofluorescence staining. A. Control group; B. SGC7901 cells were treated with PPIs (80 μg/ml); C. SGC7901 cells were treated with rapamycin (20 nmol); D. SGC7901 cells were treated with PPIs (80 μg/ml) in the presence of rapamycin (20 nmol).

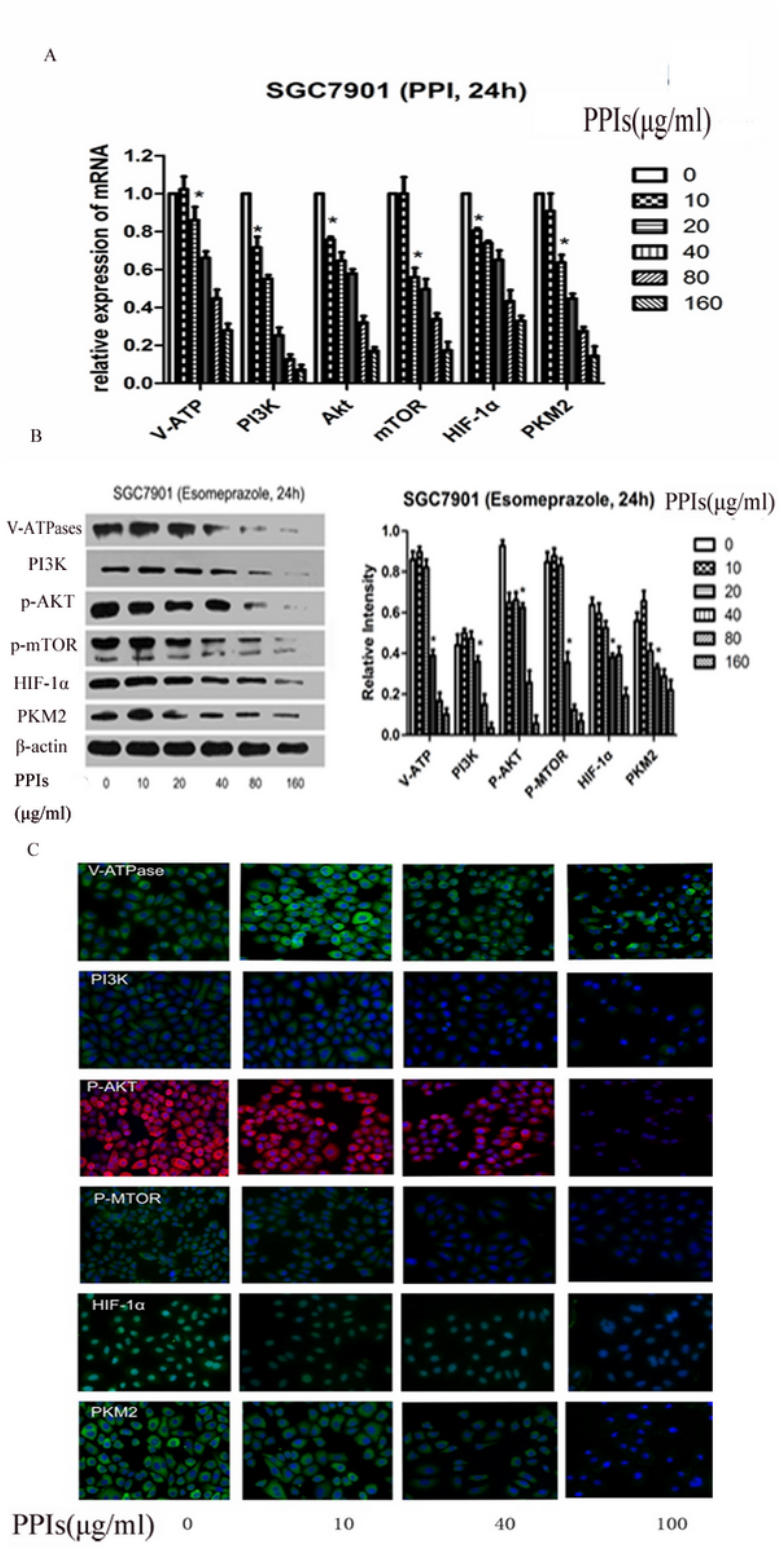


Figure 3

Effects of esomeprazole on expressions of V-ATPases, PI3K, AKT, mTOR, HIF-1α and PKM2 in gastric cancer cell line SGC7901.

(A) The mRNA levels of V-ATPases, PI3K, AKT, HIF-1α and PKM2 in SGC7901 cells treated or untreated with esomeprazole (0, 10, 20, 40, 80, 160 μg/ml) were assayed by qRT-PCR analysis (* P<0.05).

(B) SGC7901 cells were either untreated or treated by increasing concentration of esomeprazole for 24 h in the condition of pH 6.4. The indicated protein levels were analyzed by western blotting analysis (*P<0.05). (C) Immunofluorescence staining of indicated factors in the SGC7901 cells treated or untreated with esomeprazole (0, 10, 40, 100 μg/ml).

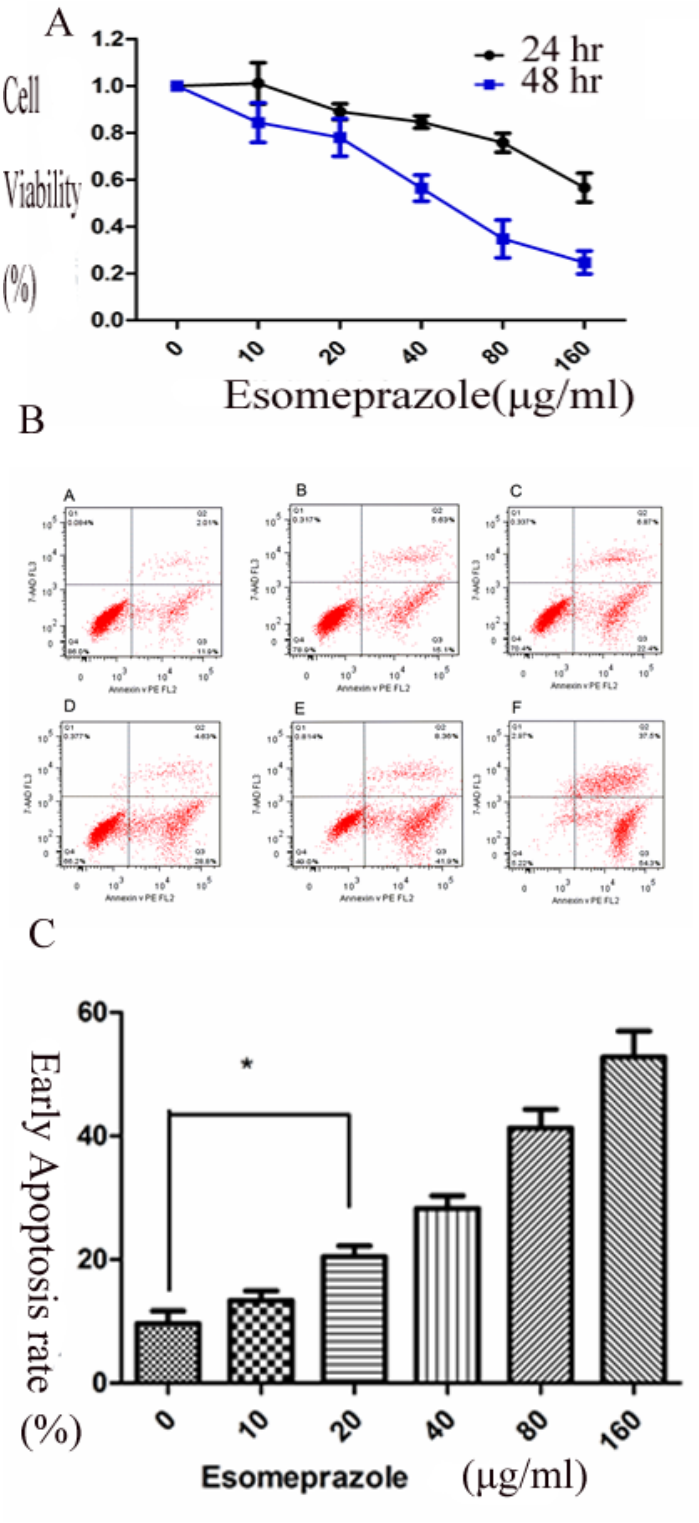


Figure 4

PPIs inhibited cell viability and induced early apoptosis of human gastric adenocarcinoma cell line SGC7901.

(A) SGC7901 cells were pretreated with esomeprazole(0, 10, 20, 40, 80, 160 μg/ml) for 24hr or 48hr and CCK-8 assay were used to assess the effects of esomeprazole on cell proliferation.

(B) SGC7901 cells were either untreated or treated with PPIs for 48 hr and apoptosis analysis was conducted by using flow cytometry.

(C) SGC7901 cells were either untreated or treated with PPIs for 48 hr and early apoptosis analysis was calculated by using flow cytometry analysis.

(*P<0.05)

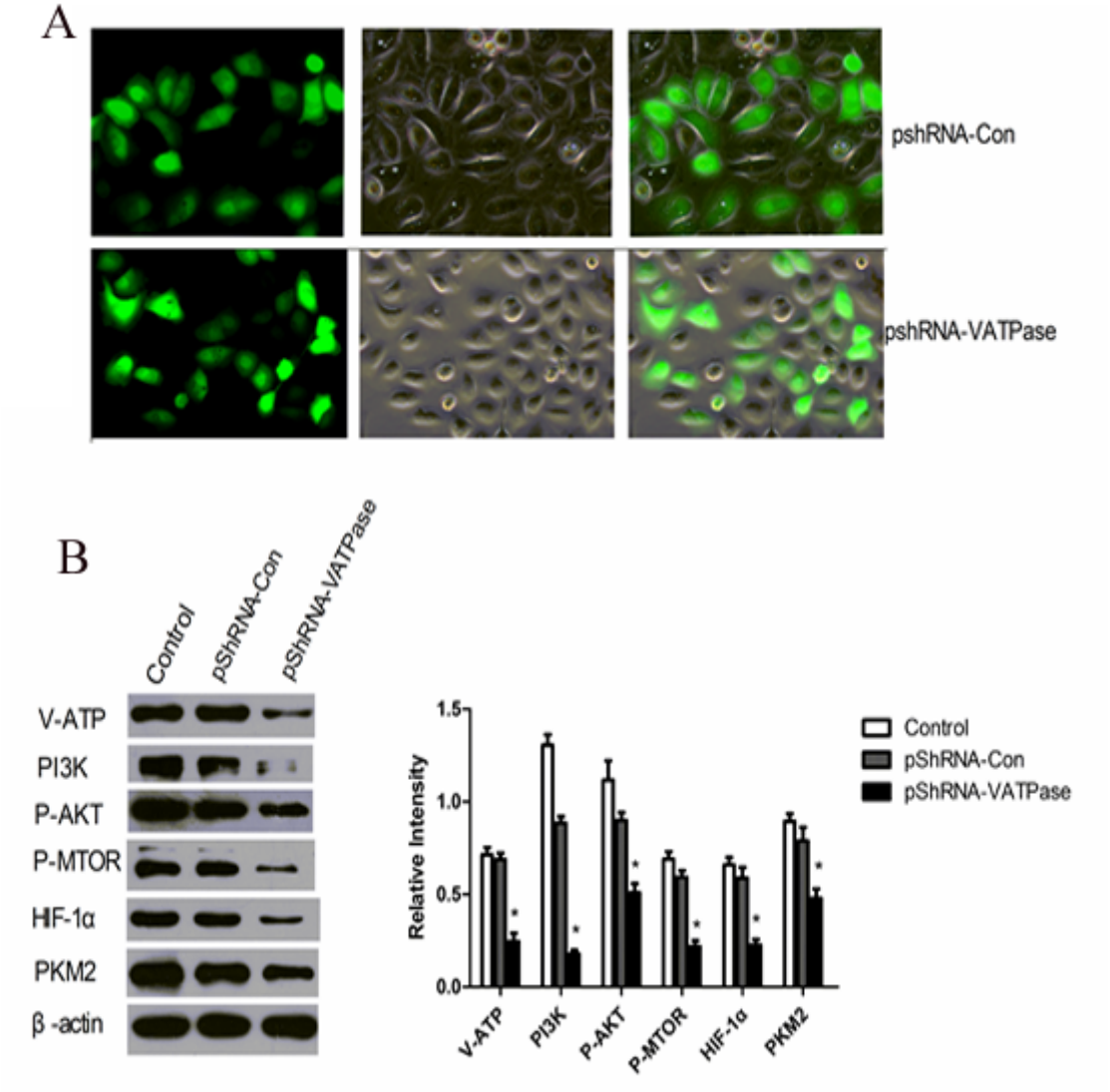


Figure 5

Effects of knockdown of V-ATPases on the SGC7901 cells.

(A) Fluorescence micrographs of pshRNA-V-ATPases group and pshRNA-Con group were taken (×200).

(B)Protein level of indicated factors of PI3K/AKT/mTOR/HIF-1α signaling pathway and PKM2 was analyzed by western blotting.

(*P<0.05)

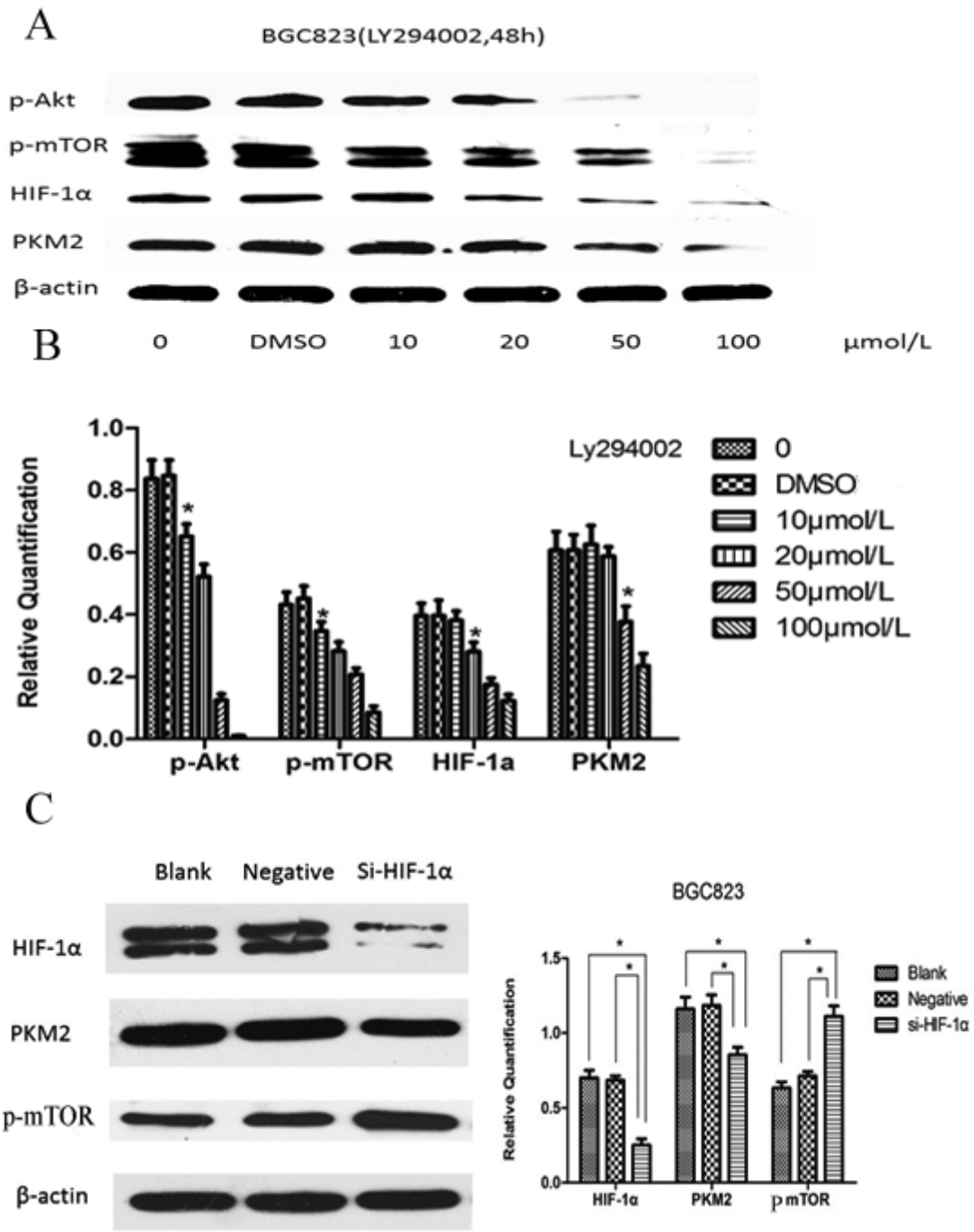


Figure 6

A．Effects of LY294002 on PI3K/AKT/mTOR/HIF-1α signal pathway and downstream PKM2. BGC823 cells were pretreated with LY294002 (0, 10, 20, 50, 100 μmol/L) or with DMSO (0.2%) as a control for 48h and indicated protein levels were assayed by western blot (* P<0.05).

B. Quantitative analysis of protein expressions was performed according to the software of Quantity one.

C：HIF-1α was knocked down in the BGC823 cells. Protein levels of p-mTOR and PKM2 were measured by western blotting analysis (* *P*<0.05).

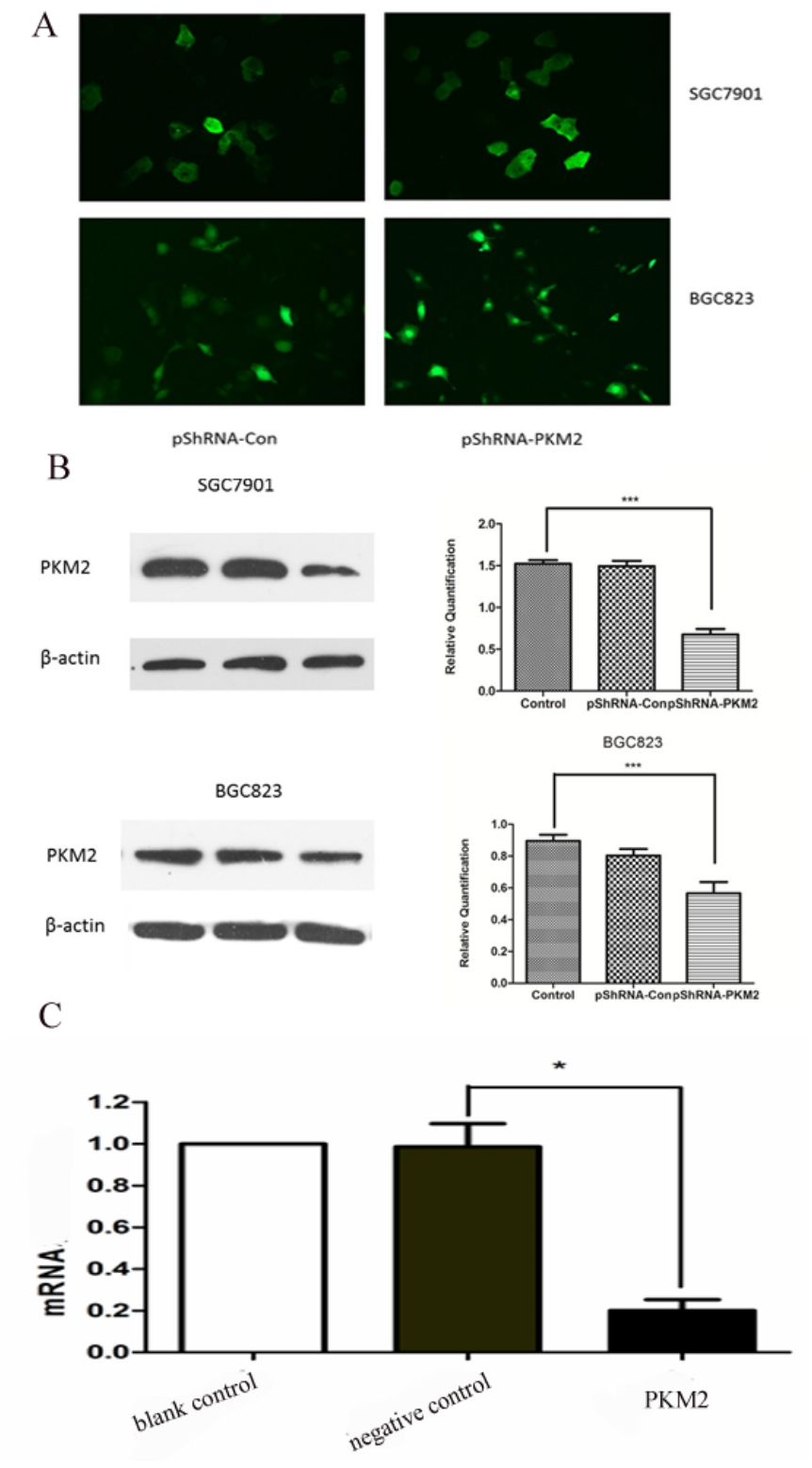


Figure 7

Effects of knocking down PKM2 on the SGC7901 cells and the BGC823 cells.

(A) Fluorescence micrographs of pshRNA-PKM2 group and pshRNA-Con group were taken (×200);

(B) Protein level of PKM2 was analyzed to confirm transfection efficiency (***P<0.01);

(C) Expression of mRNA was evaluated by qRT-PCR. (*P<0.05).

Figure 8

8A-8B. Effects of knockdown of PKM2 on cell viability and migration of the SGC7901 and the BGC823 cells

(A) CCK-8 assay were used to assess effects of knockdown of PKM2 on cell proliferation of the SGC7901 cells and the BGC823 cells.

(B) Transwell assay was used to evaluate the efficiency of knockdown of PKM2 on cell migration of the SGC7901 and the BGC823 cells.

8C-8F. Effects of knockdown PKM2 on cell aerobic glycolysis of gastric cancer cells

(C) Protein levels of GLUT-1 and LDH-A were measured by western blot.

(D) The levels of glucose uptaken rate were detected by spectrophotometry.

(E) The levels of lactate production were detected by the spectrophotometry.

(F) Activity of LDH was analyzed.

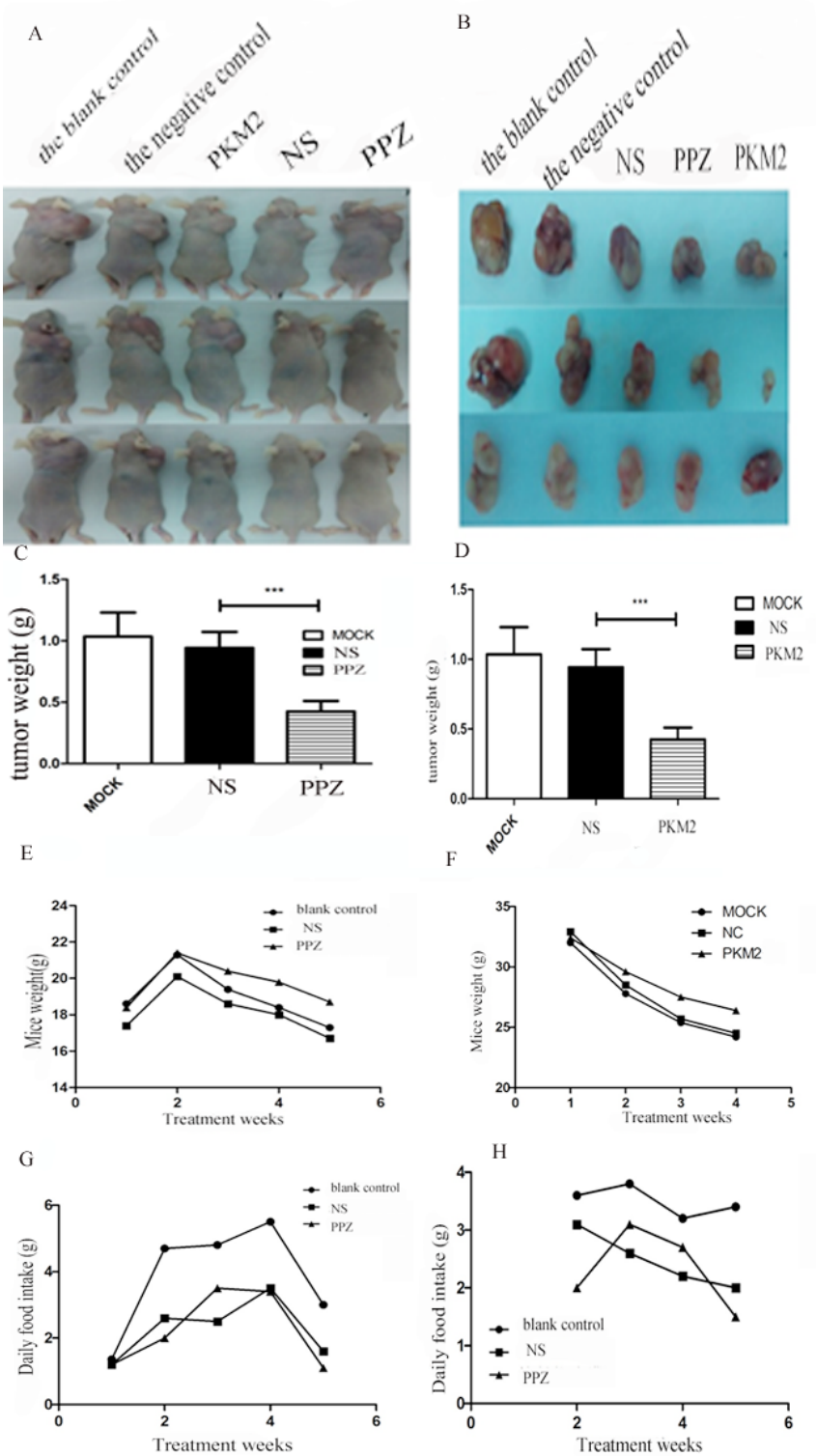


Figure 9

Results of *in vivo* experiments.

(A) Photograph of mice from different groups (P<0.05) .

(B) Photograph of tumor removed from mice sacrificed 5 weeks after treatment (P<0.05).

(C) Weight of tumor of PPI group was significantly lower than control groups. Weight of tumor in MOCK group (g):1.034±0.088. Weight of tumor in NS group (g):0.942±0.059. Weight of tumor in PPI group (g):0.516±0.007. Randomly designed analysis of variance was used for data analyzing (***P<0.01).

(D) Weight of tumor of PKM2 knocked down group was significantly lower than NC group. Weight of tumor in NC group (g):0.982±0.054. Weight of tumor in PKM2 knocked down group (g):0.242±0.046. Randomly designed analysis of variance was used for data analyzing (*P<0.05). (E) Weight loss of mice slowed down in PPI group. Weight of mice was recorded once a week and was compared after five weeks treatment. Weight of mice in MOCK (g):17.3±0.328. Weight of mice in the NS group (g):16.7±0.341. Weight of mice in PPI group (g):18.7±0.228 .Randomly designed analysis of variance was used for data analyzing (*P<0.05).

(F) Weight loss of mice slowed down in PKM2 knocked down group. Weight of mice was recorded once a week and was compared after five weeks treatment. Weight of mice in NC group (g):24.5±0.405. Weight of mice in PKM2 knocked down group (g): 26.8±0.283 .Randomly designed analysis of variance was used for data analyzing (*P<0.05).Randomly designed analysis of variance was used for data analyzing (*P<0.05).

(G)Food intake was recorded everyday and the daily intake was analyzed once a week. Daily food intake of mice in MOCK group (g):3.87±0.751. Daily food intake of mice in NS group (g): 2.28±0.404. Daily food intake of mice in PPI group (g): 2.24±0.518 (P 0.05).

(H) Daily food intake of mice in MOCK group (g): 3.1±0.412. Daily food intake of mice in NS group (g): 2.28±0.271. Daily food intake of mice in PPI group (g): 2.16±0.322 (P 0.05).

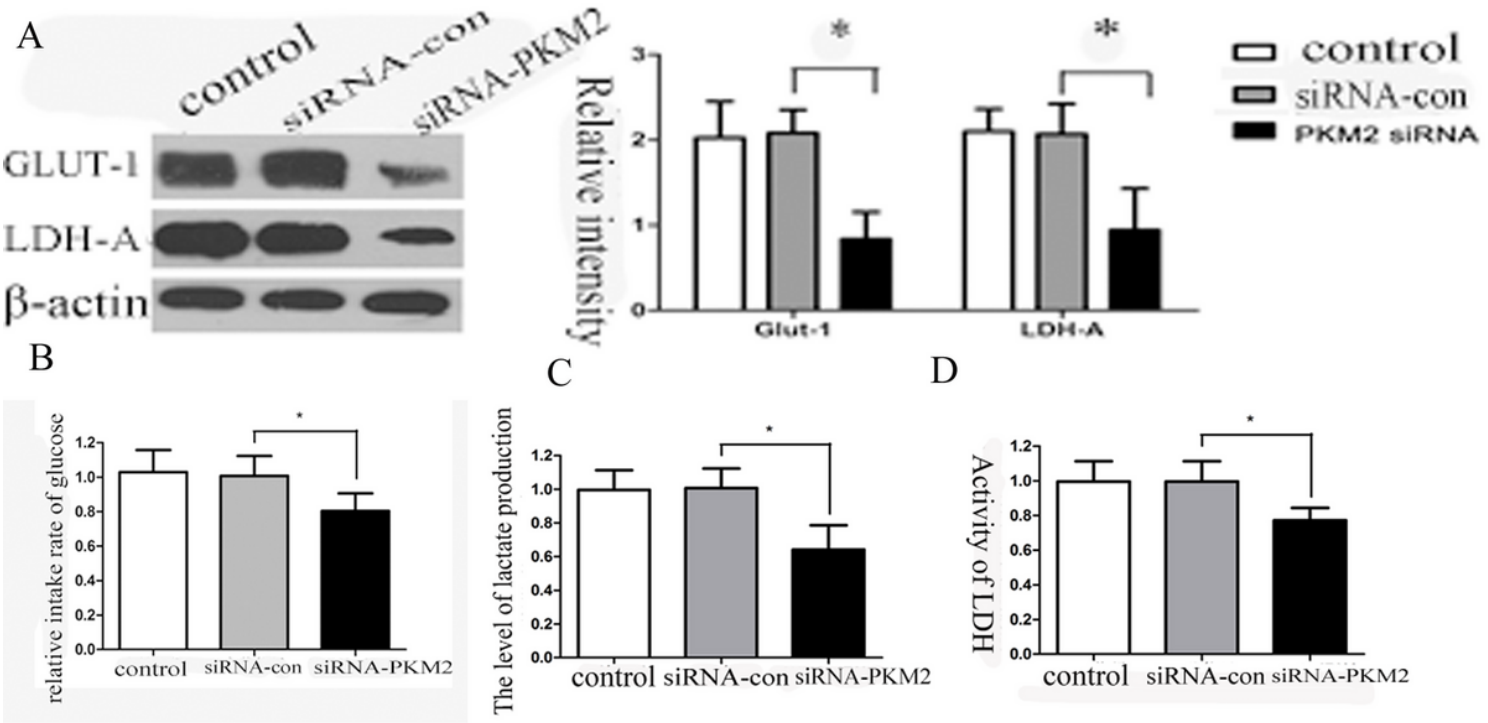
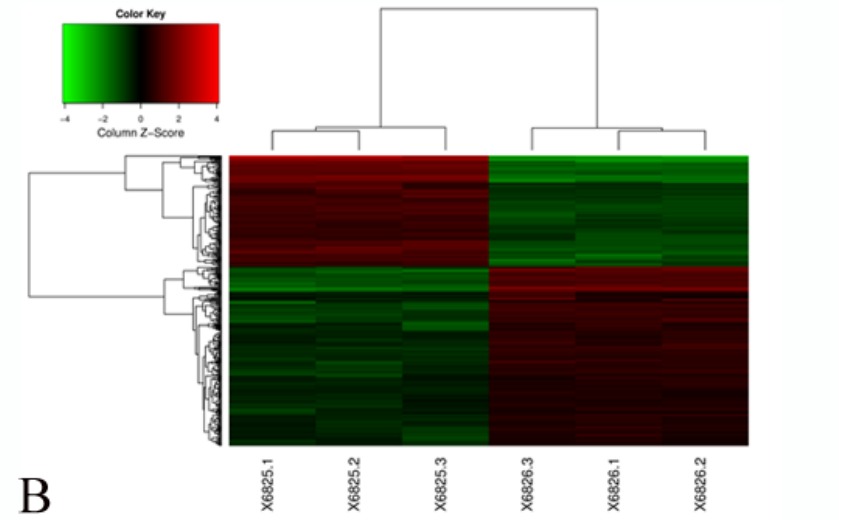


Figure 10

Indicated protein levels of tumor tissues in different group (MOCK, NS and PPIs) were measured by western blotting indicating that PPIs might inhibit the expressions of PI3K, p-AKT, p-mTOR, HIF-1α and PKM2 to a certain extent.

A



B

pathway 分析结果			
Gene Set Name	基因数	显著性概率	基因名
KEGG_MAPK_SIGNALING_PATHWAY	13	9.90E-06	TGFBR2,RAC1,MAPK9,FGF2,IL1A,RAP1A,PAK2,ATF4,MAP3K2,DDIT3,DUSP5,STMN1,PPM1A
KEGG_GLYCINE_SERINE_AND_THREONINE_METAETABOLISM	6	9.90E-06	SHMT1,SHMT2,CBS,PHGDH,PSAT1,PSPH
KEGG_PATHWAYS_IN_CANCER	14	9.90E-06	TGFBR2,RAC1,MAPK9,FGF2,VEGFA,IL6,KITLG,IL8,BIRC5,APPL1,LAMC1,CDK2,RALB,FZD10
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	6	3.53E-05	MARS,WARS,AARS,GARS,CARS,YARS
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERAERACTION	10	1.38E-03	TGFBR2,IL1A,VEGFA,IL6,KITLG,IL8,CCL5,LIFR,IL20RB,TNFRSF21
KEGG_ALANINE_ASPARTATE_AND_GLUTAMATE_ME_METABOLISM	4	3.85E-03	GLS,ASNS,ALDH5A1,GPT2
KEGG_COLORECTAL_CANCER	5	3.85E-03	TGFBR2,RAC1,MAPK9,BIRC5,APPL1
KEGG_FOCAL_ADHESION	8	3.93E-03	RAC1,MAPK9,RAP1A,PAK2,VEGFA,LAMC1,VAV3,CAV1
KEGG_PRION_DISEASES	4	4.19E-03	IL1A,IL6,LAMC1,CCL5
KEGG_P53_SIGNALING_PATHWAY	5	4.38E-03	CDK2,RCHY1,MDM4,PMAIP1,SESN2

Figure 11

Gene chip experiment was carried and found that MAPK pathway is the downstream of PKM2. (A) Gene chip cluster diagram. (B) Gene set enrichment analysis was conducted.