

Original Article

Metformin inhibits gastric cancer via the inhibition of HIF1 α /PKM2 signaling

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Abstract: Recent evidence suggests that anti-diabetic drug metformin prevents cancer progression, but the mechanism by which metformin inhibits tumor growth remains elusive. In this study, we investigated the anticancer role of metformin in gastric cancer and explored the underlying mechanism. The expression of hypoxia inducible factor 1 α (HIF1 α) and pyruvate kinase M2 (PKM2) in different stages of gastric cancer tissues was detected by immunohistochemistry. Gastric cancer cell viability was evaluated by CCK-8 assay; apoptosis and cell cycle were analyzed by flow cytometry. The expression of PI3K, Akt, HIF1 α , PARP, PKM2 and COX in gastric cancer cells was detected by immunofluorescence and Western blot analysis. We found that HIF1 α and PKM2 protein expression levels were higher in advanced gastric cancer tissues than in gastritis tissues. Metformin reduced gastric cancer cell viability, invasion and migration. Metformin induced apoptosis and cell cycle arrest in part through inhibiting PARP expression. Metformin downregulated PI3K, Akt, HIF1 α , PARP, PKM2 and COX expression. Moreover, overexpression of HIF1 α increased gastric cancer cell viability, invasion and migration. In summary, metformin has profound antitumor effect for gastric cancer by inducing intrinsic apoptosis via the inhibition of HIF1 α /PKM2 signaling pathway.

Keywords: Metformin, gastric cancer, hypoxia inducible factor 1 α , pyruvate kinase M2

Introduction

Gastric cancer (GC) is the second most common cause of cancer-related death worldwide [1, 2]. Tumor metabolism has been shown to play important role in tumorigenesis and tumor development [3]. Effect of tumor microenvironment on tumor metabolism has received more attention recently [4-7]. Hypoxia inducible factor 1 α (HIF1 α) and glucose metabolism cause the change of tumor microenvironment [8, 9]. Targeting HIF-1 and tumor glucose metabolism at several levels reduce the antioxidant capacity of tumors, affect the tumor microenvironment, and sensitize various solid tumors to irradiation [10].

HIF1 α plays a critical role in the regulation of tumor angiogenesis in response to hypoxia [11, 12]. Upon hypoxia, PI3k/Akt/HIF1 α signaling pathway is activated to regulate tumor glucose

metabolism [13]. Metformin, a well-known anti-diabetic drug, has been shown to reduce the incidence of malignancies in patient with diabetes [14]. The use of metformin in patients with type 2 diabetes may reduce the risk of thyroid cancer [15]. A systematic review showed that metformin significantly reduced the occurrence of GC, liver cancer, lung cancer, colon cancer, esophageal cancer and reduced cancer-related mortality [16]. However, the mechanism by which metformin inhibits tumor growth remains elusive.

The aim of this study was to investigate the anti-cancer role of metformin in gastric cancer and explore the underlying mechanism. We detected the expression of HIF1 α and pyruvate kinase M2 (PKM2) in different stages of GC and examined the efficacy and possible mechanism of metformin against human GC cells.

Materials and methods

Reagents

Metformin and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody and Trizol were purchased from Invitrogen (Carlsbad, CA, USA). Annexin V Apoptosis Detection kit FITC was purchased from eBioscience (San Diego, CA, USA). Antibodies against PARP (9532), Akt (9272), and β -actin (4967) were purchased from Cell Signal Technology (Boston, MA, USA). Antibody against PI3K (Y467) and HIF1 α (K377) were purchased from Bioworld Technology (USA). Antibody against HIF1 α (ab113642) and COX (ab33985) were purchased from Abcam (USA). Rabbit secondary antibody was from Cell Signal Technology. PrimeScript™ RT Master Mix and SYBR Premix Ex Taq reagents were purchased from Takara Biotechnology (Dalian, China).

Samples and immunohistochemistry

A total of 20 superficial gastritis, 40 early GC and 40 advanced GC tissues were obtained from patients admitted at The Affiliated Drum Tower Hospital of Nanjing University after the approval of the local ethics committee and informed consent were obtained. Immunohistochemical staining of deparaffinized tumoral and gastritis tissues were performed according to standard protocols using HIF1 α and PKM2 antibody. The staining intensities were graded as 0, 1, 2, and 3 by two pathologists, respectively.

Cell culture

Human GC lines SGC7901 (moderately differentiated) and BGC-823 (poorly differentiated) were purchased from Shanghai Institute of Biochemistry, and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 ng/L penicillin, and 100 ng/L streptomycin at 37°C in 5% CO₂. HIF1 α overexpression plasmid or control plasmid was transfected into BGC823 cells using Lipofectamine 2000 according to the manufacturer's protocol.

Cell viability assay

Cell viability was detected by cell counting kit-8 (CCK-8) assay. Cells were seeded into 96-well

plates at 1×10^4 cells/well and cultured overnight at 37°C. After treatment with metformin at indicated concentrations for 24, 48, 72 h, 10 μ L CCK-8 was added to each well and incubated for 1 h at 37°C. The absorbance was measured at 450 nm. The data were presented as mean \pm SD of triplicate samples from at least three independent experiments. The cell viability was calculated using the following formula: cell viability (%) = $(A_s - A_b) / (A_c - A_b) \times 100\%$, where A_s represents the A value of the experimental well, A_c represents the A value in the control well, and A_b represents the A value of the blank well.

Annexin V-FITC apoptosis assay

Cells were seeded in six-well plates at 4×10^5 cells/well and then treated with different concentrations of metformin for 24 h. Apoptotic cells were detected by flow cytometry using Annexin V-FITC kit according to the instructions.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. After indicated treatments, cells were trypsinized, rinsed with PBS, fixed with 70% ethanol at 4°C overnight, and treated with RNaseA (0.02 mg/ml) in the dark at room temperature for 30 min. Cells were resuspended in 0.05 mg/ml propidium iodide and analyzed with flow cytometry. For each sample, at least 1×10^4 cells were recorded.

Cell invasion assay

Invasion assay was performed using 24-well Transwell units with 8 μ m pore size polycarbonate inserts. The polycarbonate membranes were cultured at 37°C for 1 h. Cells (1×10^4) suspended in 200 μ L of RPMI1640 medium containing 1% fetal bovine serum were seeded in the upper compartment of the Transwell unit. 800 μ L of RPMI1640 medium containing 10% fetal bovine serum was added into the lower compartment as a chemoattractant. After 24 h incubation, cells on the upper side of the membrane were removed, and the cells that migrated through the membrane to the underside were fixed and stained with 0.1% crystal violet. Cell numbers were counted in five separate fields using light microscopy at 400 \times magnification. The data were expressed as the mean

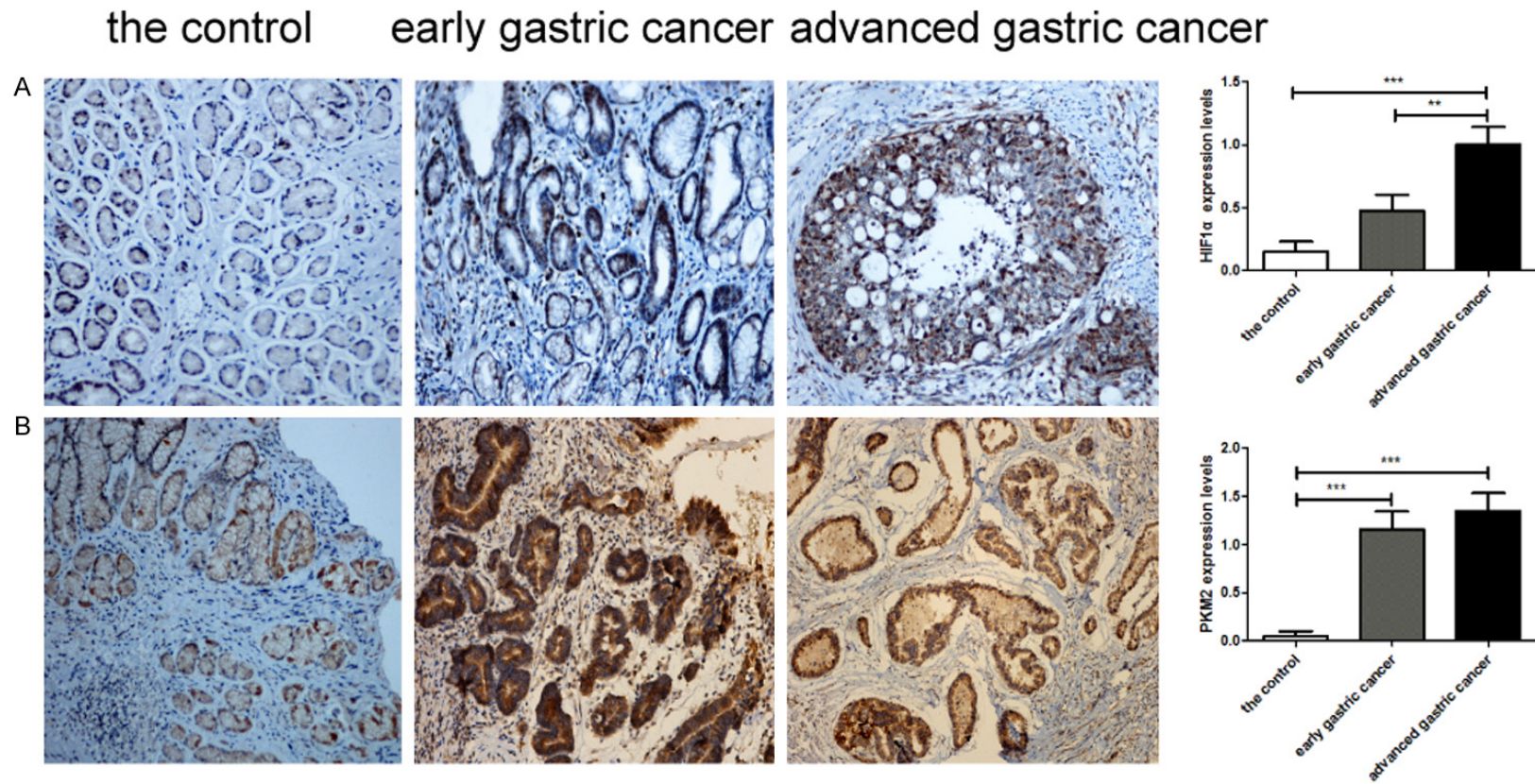


Figure 1. Immunohistochemical analysis of HIF1 α and PKM2 expression in superficial gastritis, early gastric cancer, and advanced gastric cancer tissues. A. Representative images of HIF1 α staining. B. Representative images of PKM2 staining. Original magnification 200 \times . ** P <0.01, *** P <0.001.

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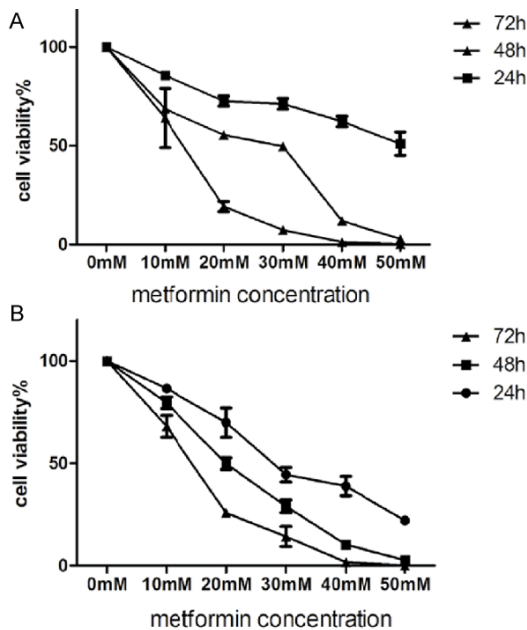


Figure 2. Metformin inhibits the viability of gastric cancer cells. SGC7901 cells (A) and BGC823 cells (B) were treated with metformin (0-50 mM) for 24, 48, 72 h. Cell viability was evaluated by CCK-8 assay.

value of cells in five fields based on three independent experiments.

Cell migration assay

Migration assay was performed using 24-well Transwell units with 8 μ m pore size polycarbonate inserts. The polycarbonate membranes were coated with Matrigel (Becton Dickinson) and cultured at 37°C for 1 h. The next steps were the same as cell invasion assay described above.

Quantitative Real-time PCR

Total RNA was extracted using the Trizol Reagent and subsequently reverse transcribed using the PrimeScript RT Master Mix according to the manufacturer's instructions. Quantitative Real-time PCR was performed with the 7500 Real-time PCR System (Applied Biosystems) using SYBR Premix Ex Taq reagents. PCR cycling conditions were: 40 cycles of 5 s at 95°C, 32-34 s at 60°C. Fold-induction was calculated using the formula $2^{-\Delta\Delta Ct}$. The specific primers were as follows: HIF1 α : sense: 5'-GTAGTGCTG-ACCCTGCACTCAA-3' antisense: 3'-CCATCGGAA-GGACTAGGTGTCT-5'; β -actin: sense: 5'-ACCGA-GCGCGCTACA-3', antisense: 3'-CAGCCGTGG-CCATCTCTT-5'.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl with pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA). The proteins in cell lysates were resolved by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked by 5% non-fat dry milk in Tris buffered saline containing 0.1% Tween-20 for 2 h at room temperature. Then the membranes were incubated with primary antibodies (1:1000 dilutions) overnight, followed by incubation with appropriate HRP-conjugated secondary antibodies (1:5000 dilutions). The blots were detected using Millipore Immobilon Western Chemiluminescent HRP Substrate according to the manufacturer's instructions.

Immunofluorescence

Cells were cultured on 24-well plates, fixed with 4% paraformaldehyde, and blocked for 1 h with 5% normal goat serum, followed by incubation with monoclonal antibodies against HIF1 α (1:200) and COX (1:100) overnight at 4°C. Cells were then rinsed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit or goat anti-mouse secondary antibody. Cells were counter-stained with DAPI (2 μ g/ml) and examined by fluorescence microscopy.

Statistical analysis

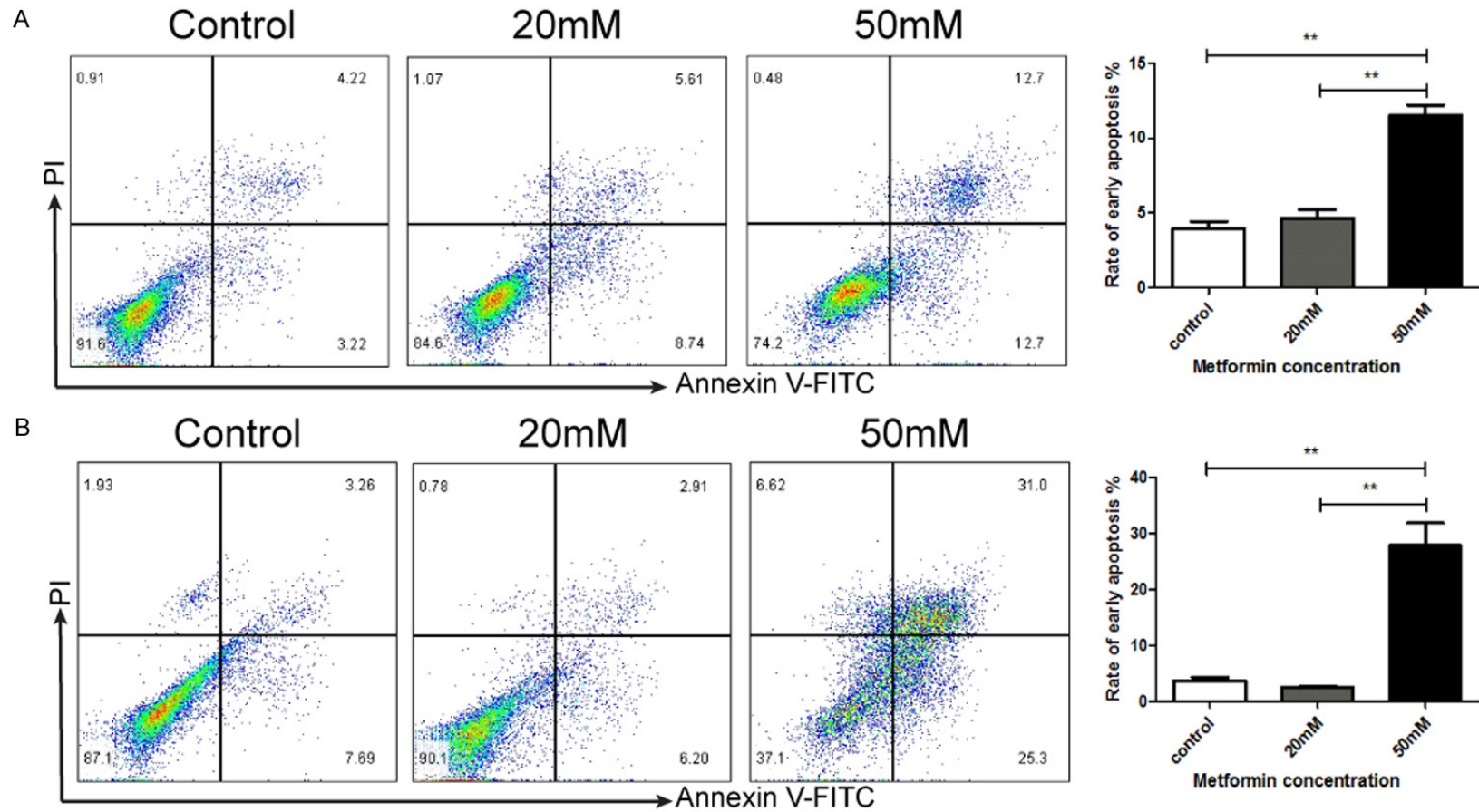
All data were presented as mean \pm SD of three independent experiments at least. Statistical analysis was performed using SPSS22.0 and Prism 5 (GraphPad Software Inc., San Diego, USA). Single factor analysis of variance test was used for comparisons among multiple groups, and *t* test was used for comparisons between two groups. *P* < 0.05 was considered statistically significant.

Results

High expression levels of HIF1 α and PKM2 in GC tissue

We detected the expression of HIF1 α and PKM2 in superficial gastritis, early GC and advanced GC tissues by immunohistochemistry. The expression level of HIF1 α appeared to increase in early GC, but there was no significant difference compared with superficial gastritis. However, the expression level increased significantly in advanced GC compared with

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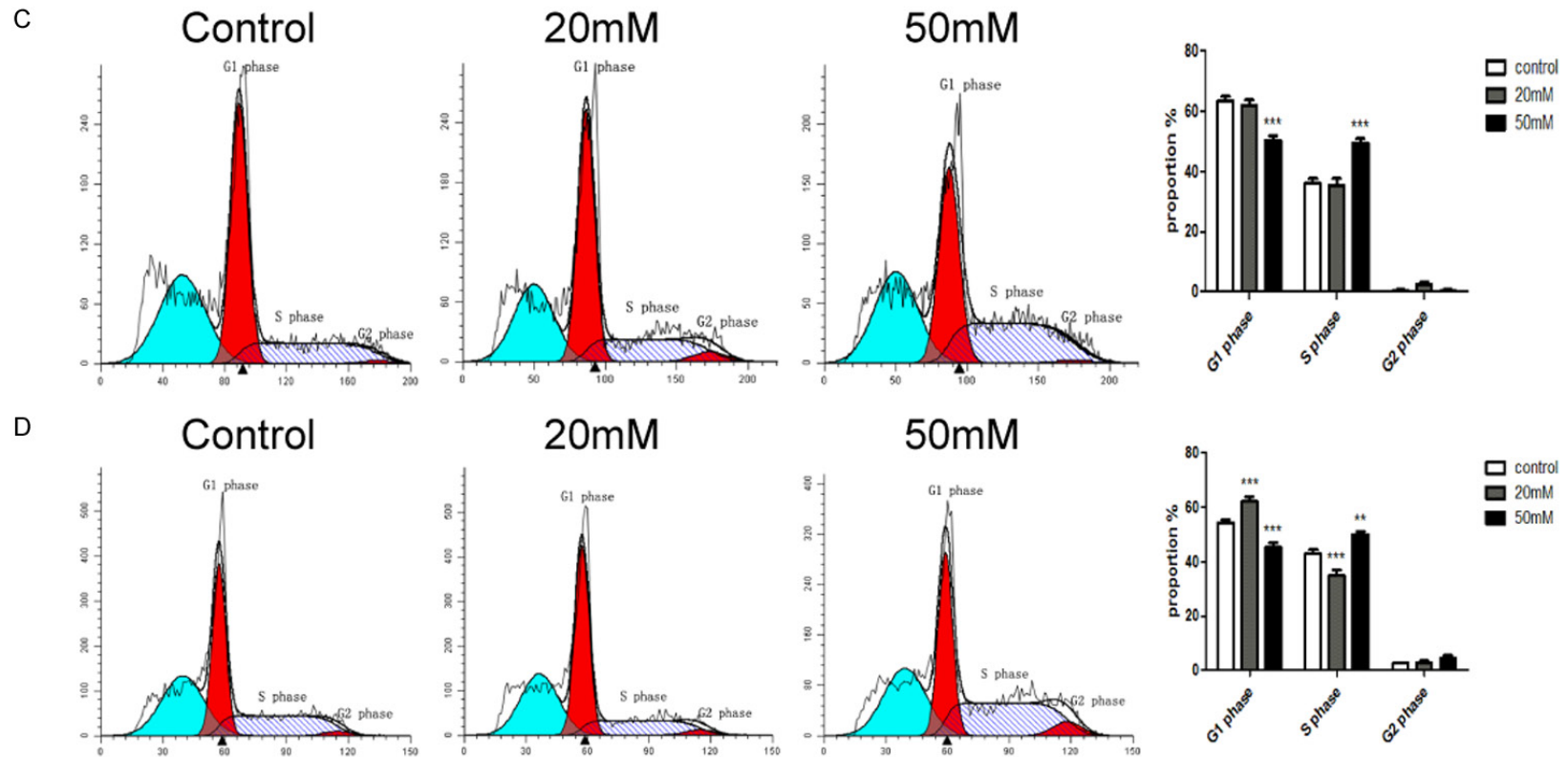


Figure 3. Metformin induces apoptosis and cell cycle arrest in gastric cancer cells. SGC7901 cells (A, C) and BGC823 cells (B, D) were treated with metformin (0, 20, 50 mM) for 24 h. The proportions of early apoptosis cells (A, B) and cells in different phases of cell cycle (C, D) were assessed by flow cytometry. ** $P < 0.01$, *** $P < 0.001$.

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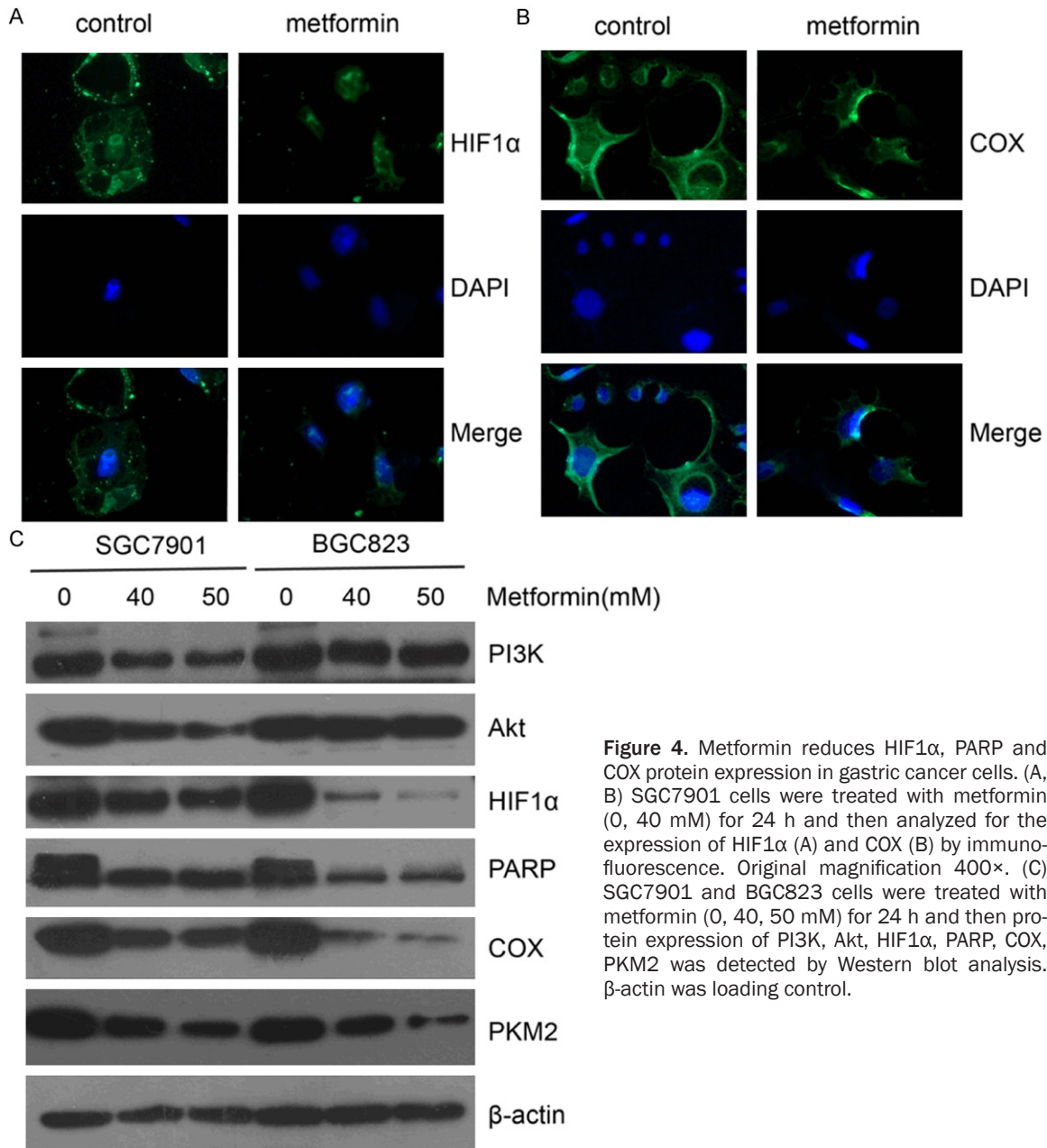


Figure 4. Metformin reduces HIF1 α , PARP and COX protein expression in gastric cancer cells. (A, B) SGC7901 cells were treated with metformin (0, 40 mM) for 24 h and then analyzed for the expression of HIF1 α (A) and COX (B) by immunofluorescence. Original magnification 400 \times . (C) SGC7901 and BGC823 cells were treated with metformin (0, 40, 50 mM) for 24 h and then protein expression of PI3K, Akt, HIF1 α , PARP, COX, PKM2 was detected by Western blot analysis. β -actin was loading control.

superficial gastritis and early GC tissues (**Figure 1A**). The expression level of PKM2 increased significantly in early and advanced GC compared with superficial gastritis tissue (**Figure 1B**).

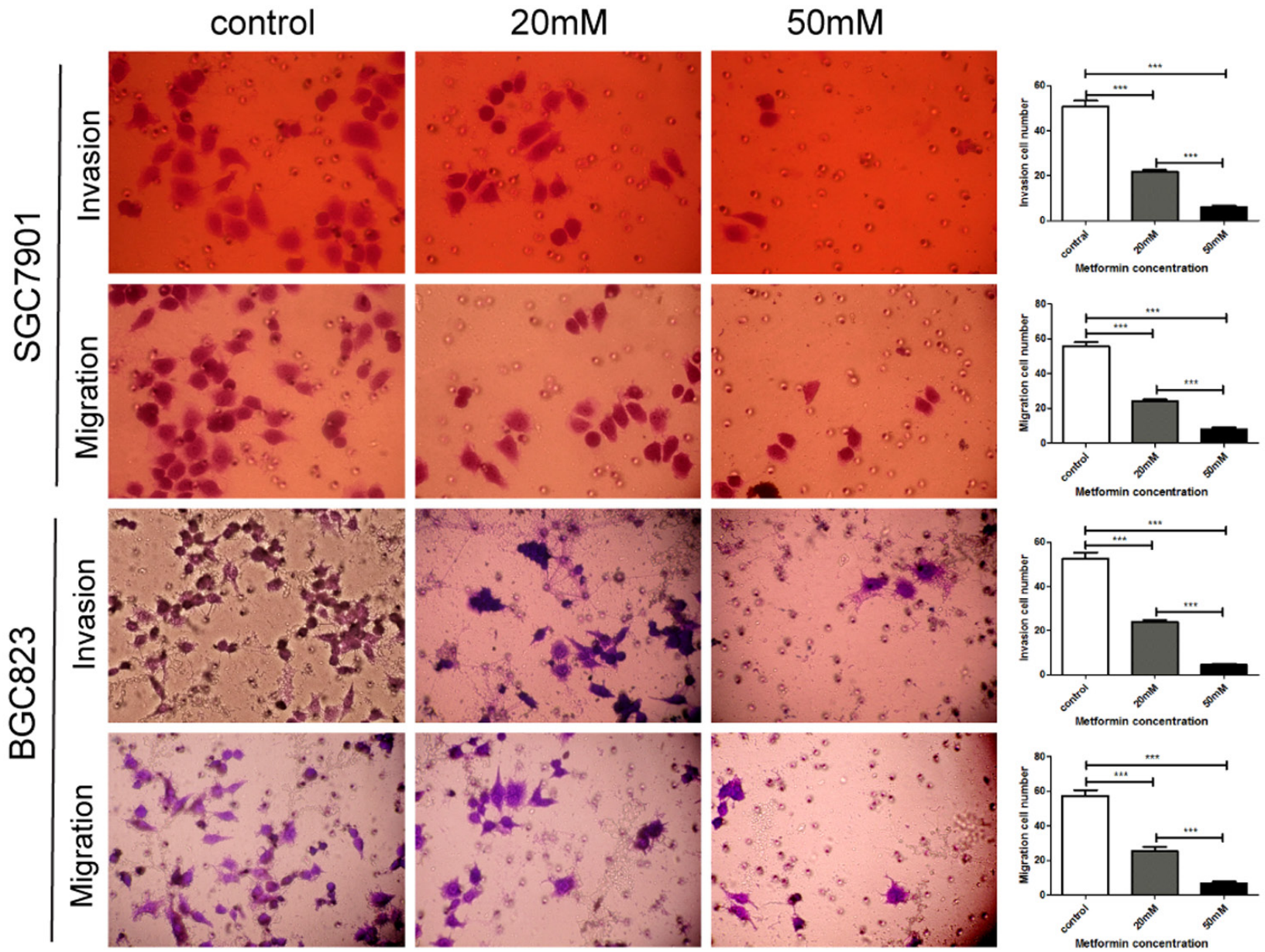
Metformin decreases GC cell viability

We evaluated the effect of metformin on the viability of two GC cell lines: SGC7901 and BGC823. CCK-8 assay showed significant dose- and time-dependent decrease in the viability of SGC7901 and BGC823 cells after metformin treatment (**Figure 2**).

Metformin induces apoptosis and cell cycle arrest in GC cells

To elucidate the mechanism by which metformin decreases the viability of GC cells, we wondered whether metformin could induce apoptosis and cell cycle arrest in GC cells. By Annexin V-FITC and PI staining, we observed that metformin increased the proportion of apoptotic cells in SGC7901 and BGC823 cells in a dose dependent manner (**Figure 3A, 3B**). In addition, by flow cytometry analysis, we found that metformin induced cell cycle arrest in SGC7901

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Figure 5. Metformin inhibits the invasion and migration of gastric cancer cells. SGC7901 and BGC823 cells were seeded on transwell for invasion and migration analysis. The numbers of invaded and migrated cells were counted in five separate fields using light microscopy. Original magnification 400×. The data were expressed as the mean value of cells in five fields based on three independent experiments. *** $P < 0.001$.

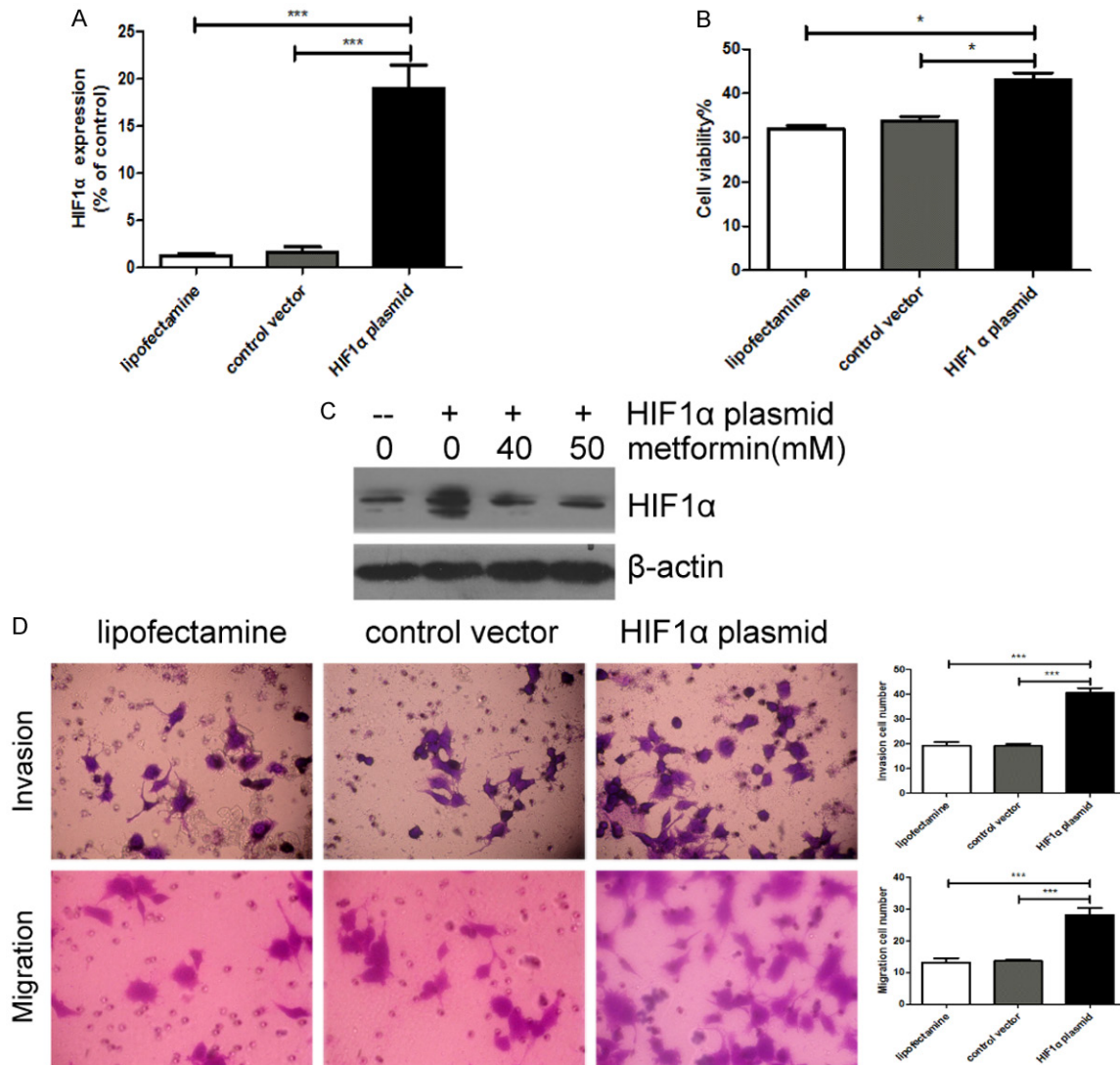


Figure 6. HIF1α over-expression increases the viability, invasion and migration of BGC823 cells. A, C. Lipofectamine, control vector or HIF1α overexpression plasmid were transfected into BGC823 cells, and HIF1α mRNA and protein expression were detected by RT-PCR and Western blot analysis. β-actin was loading control. B. Cell viability was detected by CCK-8 assay. D. The numbers of invaded and migrated cells were counted in five separate fields using light microscopy. Original magnification 400×. The data were expressed as the mean value of cells in five fields based on three independent experiments. * $P < 0.05$, *** $P < 0.001$.

and BGC823 cells in a dose dependent manner (Figure 3C, 3D).

Metformin reduces HIF1α, PARP, COX and PKM2 expression in GC cells

Next we evaluated the effects of metformin on PI3k, Akt, HIF1α, PARP, COX, and PKM2 protein

expression in GC cells. Immunofluorescence staining of HIF1α and COX in SGC7901 cells showed significant decrease in HIF1α and COX expression after metformin treatment (Figure 4A, 4B). Western blot analysis showed that metformin inhibited the expression level of PI3K, Akt, HIF1α, PARP, COX and PKM2 in SGC7901 and BGC823 cells (Figure 4C).

Metformin inhibits the invasion and migration of GC cells

To investigate the activity of metformin against tumor metastasis, we examined the effects of metformin on the invasion and migration of GC cells. Transwell assay showed that metformin inhibited the invasion and migration of SGC-7901 and BGC823 cells in a dose dependent manner (**Figure 5**).

HIF1 α over-expression increases the viability, invasion and migration of GC cells

Since metformin inhibited protein expression of HIF1 α in GC cells, we wondered whether HIF1 α might be the key factor to mediate the effects of metformin on GC cells. We transfected HIF-1 α over-expression plasmid into BGC823 cells, and confirmed the expression of HIF1 α by RT-PCR and Western blot analysis (**Figure 6A, 6C**). We found that HIF1 overexpression increased the viability, invasion and migration of BGC823 cells (**Figure 6B, 6D**).

Discussion

In this study, we revealed the high expression of HIF1 α and PKM2 in GC tissues, and found that metformin significantly induced apoptosis, inhibited cell invasion and migration of GC cells. The mechanism by which metformin exhibits anti-tumor activities is through the induction of apoptosis and the inhibition of HIF1 α .

Recent studies showed that overexpression of HIF1 α are implicated in tumorigenesis, tumor chemotherapy resistance, tumor angiogenesis, and tumor glycolysis [17-20]. Increased HIF-1 α level is associated with increased risk of mortality in many human cancers, including gastric cancer [11]. HIF1 α inhibitor inhibited tumor growth and angiogenesis [12]. In this study we found that metformin inhibited the expression of HIF1 α in GC cells, suggesting that metformin may inhibit tumor cell growth and metastasis via HIF1 α inhibition. However, further studies are needed to confirm our conclusion.

Targeting of tumor metabolism is emerging as a novel therapeutic strategy against cancer [21]. According to the "Warburg effect", tumor cells exhibit an increased dependence on glycolytic pathway for ATP generation both in normoxia or hypoxia conditions [22]. PKM2 is an important executor downstream of HIF1 α signaling and acts as the key enzyme of glycolysis [23]. In this

study we found high expression of PKM2 in GC tissues by immunohistochemistry, indicating the important role of glycolysis in the development of GC. Our study showed that metformin inhibited the expression of PKM2 protein, especially in poorly differentiated BGC823 cells. These data suggest that metformin reduces the energy supply of GC by inhibiting HIF1 α /PKM2 pathway.

The most important function of mitochondrial respiratory chain is to generate ATP by oxidation phosphorylation (OXPHOS). After sequential electron transfer, two respiratory chains generate ATP through being catalyzed by the respiratory chain enzyme complexes IV- cytochrome C oxidase (COX). In energy-rich conditions, the mitochondria of tumor cells maintain "well-being" state and effectively shut off apoptotic machinery, resulting in the protection against cell death, even when challenged with toxic drugs. Conversely, when the mitochondria of tumor cells are in the condition of "stress", they induce the apoptosis of tumor cells [24]. One study showed recently that metformin inhibited mitochondrial complex I of cancer cells to reduce tumorigenesis [25]. In this study we found that metformin inhibited the expression level of COX in SGC7901 and BGC823 cells.

Poly(ADP)-ribose polymerase (PARP) plays a crucial role in DNA repair and the maintenance of genome stability. The proteolytic degradation of PARP is caused by a variety of stimuli [26]. In the present study, the expression of PARP was decreased significantly in GC cells treated with metformin. At the same time, cell apoptosis ratio increased remarkably.

In order to confirm that HIF1 α mediates the effects of metformin on GC cell proliferation, apoptosis, invasion, and migration, we transfected HIF1 α overexpression plasmid into BGC823 cells; and found that cell viability, invasion and migration were obviously enhanced in the cells transfected with HIF1 α plasmid. These data indicate that metformin inhibits GC cell proliferation, invasion and metastasis by inhibiting the expression of HIF1 α .

To the best of our knowledge, this is the first report demonstrating HIF1 α /PKM2 signal pathway as a target of metformin in GC cells. Metformin exhibit potent effects to inhibit malignant behaviors of GC cells through decreasing

the expression of HIF1 α and PKM2. However, how metformin inhibits HIF1 α /PKM2 signal pathway is not clear and needs further exploration.

In conclusion, our study provides evidence that metformin inhibits GC growth and metastasis. The main mechanism responsible for the anti-tumor effects of metformin might be inducing intrinsic apoptosis and tumor glucose metabolism via the inhibition of HIF1 α . These findings suggest that metformin is a promising therapeutic agent for GC.

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Disclosure of conflict of interest

None.

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