Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells via the Bcl-2/Bax/caspase-3 signaling pathway

Yunli Zhang, Litao Yang, Guangou Dai, Hu Cao

Department of Abdominal Surgery, Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, China

Received March 26, 2018; Accepted May 15, 2018; Epub December 1, 2018; Published December 15, 2018

Abstract: Gastric cancer (GC) is one of the most common malignancies in the world. Fluorouracil (5-FU) is widely used in the treatment of cancers, but resistance to 5-FU results in the failure of chemotherapy. Phosphoglycerate dehydrogenase (PHGDH) has been reported to play a vital role in the development of 5-FU resistance in cancer cells. However, the exact role of PHGDH and the underlying mechanisms for 5-FU resistance in GC cells remain elusive. In this study, PHGDH expression was much higher in the GC tissues of 5-FU-resistant patients than that in the GC tissues of 5-FU-sensitive patients. Moreover, the expression of PHGDH was obviously increased in BGC823/5-FU cells compared with that in BGC823 cells. 5-FU treatment significantly reduced the viability of BGC823/5-FU cells, in a dose- and time-dependent manner. Furthermore, 5-FU treatment inhibited the proliferation of BGC823/5-FU cells, as evidenced by decreased cell viability and reduced colony-forming ability. The knockdown of PHGDH made possible the inhibitory effect of 5-FU on the proliferation of BGC823/5-FU cells. Furthermore, 5-FU treatment promoted apoptosis of BGC823/5-FU cells, as indicated by increased numbers of TUNEL-positive cells and increased rates of apoptosis. Notably, the promoting effect of 5-FU on the apoptosis of BGC823/5-FU cells was markedly enhanced by PHGDH knockdown. Additionally, 5-FU treatment downregulated Bcl-2 expression and upregulated the expression of Bax and caspase-3, and this effect was remarkably enhanced by PHGDH knockdown. In conclusion, knockdown of PHGDH potentiates 5-FU cytotoxicity in GC cells via the Bcl-2/Bax/caspase-3 signaling pathway.

Keywords: Phosphoglycerate dehydrogenase, fluorouracil, gastric cancer, cytotoxicity

Introduction

Gastric cancer (GC) is a kind of malignant tumor that develops in the lining of the stomach. Globally, GC is the fourth most common type of cancer and the third leading cause of cancer deaths, accounting for approximately 952000 new cases annually [1]. GC is difficult to cure unless it is detected at an early stage. Unfortunately, since GC has no characteristic clinical manifestations in its early stages, most patients with GC are diagnosed at an advanced stage, which is one of the main reasons for its high mortality and poor prognosis [2]. Despite substantial improvements in the diagnosis and treatment of GC in the last decade, the 5-year survival rate of patients with GC remains less than 20% [3]. At present, palliative chemotherapy is widely used for the treatment of GC. Chemotherapy can reduce the tumor size, relieve disease symptoms, and increase survival time [4]. However, most GC cells are resistant to chemotherapy drugs, which leads to the failure of chemotherapy [5]. Thus, developing strategies to improve the chemosensitivity of GC cells has become a hot topic for cancer research.

Phosphoglycerate dehydrogenase (PHGDH) is a key rate-limiting enzyme in the de novo biosynthesis of serine that introduces the glycolytic intermediate 3-phosphoglycerate into serine and glycine [6]. Serine and glycine released in the process provide numerous precursor molecules and other substrates required for the growth and proliferation of cancer cells [7]. Recently, more and more attention has been paid to the role of PHGDH in tumor progression. PHGDH expression has been reported to be upregulated in a number of human cancers, such as epithelioid carcinoma [8], glioma [9], melanoma [10], and breast cancer [11]. Downregulation of PHGDH has been shown to strikingly suppress the proliferation, migration, and...
Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

Invasion abilities of pancreatic cancer cells by inhibiting the expression of cyclin B1, cyclin D1, matrix metalloproteinases-2 and matrix metalloproteinases-9 [12]. Knockdown of PHGDH in breast cancer cells leads to increased oxidant levels, increased apoptosis and loss of breast cancer stem cell enrichment under hypoxic conditions [13]. Notably, PHGDH expression has been negatively correlated with the 5-year survival rate of patients with GC [14]. These observations suggest that PHGDH may be a new target for cancer therapy.

Fluorouracil, also known as 5-FU, is the most widely used cytotoxic drug in the treatment of human cancers. 5-FU can inhibit the synthesis of DNA by interrupting the action of thymidylate synthase. Furthermore, thymidylate synthase methylates deoxyuridine monophosphate to form thymidine monophosphate (dTMP). 5-FU treatment leads to a scarcity of dTMP, ultimately resulting in cell death. Besides, 5-FU induces cell cycle arrest and inhibits RNA synthesis [15]. In the past decades, 5-FU treatment has been shown to markedly reduce GC mortality. However, the efficacy of 5-FU in patients with GC is limited owing to the development of the 5-FU-resistance. Thus, it is important for us to find new strategies for overcoming the 5-FU-resistance in GC.

In this study, we detected the expression of PHGDH in GC tissues and cell lines (BGC823 and BGC823/5-FU cells). Furthermore, we explored the effects of PHGDH knockdown on the proliferation and apoptosis of BGC823/5-FU cells and its underlying molecular mechanisms.

Materials and methods

Patients' characteristics and clinicopathological features

This study received approval from the Ethics Committee of Zhejiang Cancer Hospital. In all, 29 patients with GC who received 5-FU-based chemotherapy signed an informed consent before participating in the project. The treatment was effective in shrinking the primary tumor and reducing malignant pleural effusions. 5-FU-resistant cases were distinguished when the size of the primary tumor increased, pleural effusions increased, or tumor cells metastasized within 12 months, otherwise defined as 5-FU-sensitive (n = 7). The GC tissue samples were obtained from these patients.

Cell culture and transfection

BGC823 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and incubated in an RPMI-1640 medium containing 10% fetal bovine serum (Solarbio, Beijing, China), 100 μg/ml streptomycin (Solarbio) and 100 U/ml penicillin (Solarbio) in a 5% CO2 incubator at 37°C.

A 5-Fu-resistant BGC823 cell line (BGC823/5-Fu) was constructed by screening the parental BGC823 cells in a culture medium containing stepwise increased concentrations of 5-Fu (Solarbio) for 12 months. When they acquired resistance to 1 μM 5-FU, the cells were termed as BGC823/5-Fu cells and used for this study.

To knockdown PHGDH, sh-PHGDH-1 and sh-PHGDH-2 were transfected into BGC823/5-FU cells. Forty-eight hours after transfection, BGC823/5-FU cells were treated with indicated concentrations (0, 1, 2, 4, 8, 16, and 32 μM) of 5-FU for 48 h or exposed to 16 μM 5-FU for different durations (24, 48, and 72 h).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the tissues and cells using TRIzol reagent (Sigma-Aldrich, Shanghai, China) and reverse transcribed to cDNA using cDNA synthesis kit (Roche, Penzberg Germany). PHGDH expression was detected using the ABI Q6 qRT-PCR system (Applied Biosystems Inc, Foster City, CA, USA). The primer sequences used in this study were as follows: PHGDH, forward: 5'-CACATTCTTGGCTTGAAC-3', reverse: 5'-TTATTAGACGGTTATTGTAGTA-3'; β-actin forward: 5'-GTCATTCCAAATATGAGATGCGT-3', reverse: 5'-GCATTACATAATTTCGAGGAAAGCA-3'. The relative expression level of PHGDH was normalized to that of β-actin (housekeeping gene) using the 2^{ΔΔCt} method.

Western blot assay

Proteins were isolated with ice-cold RIPA buffer and the protein concentrations were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Cell lysates containing 30 μg of total protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, and then transferred to polyvinylidene fluoride membranes (Millipore Corporation, Billerica,
Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

MA, USA). Membranes were blocked with 5% skim milk, immunoblotted overnight at 4°C with primary antibodies against PHGDH (Novus Biologicals, Littleton, CO, USA), Bcl-2 (Abcam, Cambridge, MA, USA), Bax (Abcam), caspase-3 (Abcam), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated with horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China) for 2 h at 25°C. Immunoreactivity was detected using ECL (Pierce) and the intensity of the protein bands was quantified with the Image J software.

**Cell counting kit-8 (CCK-8) assay**

The viability of BGC823/5-FU cells was determined using the CCK-8 assay (Solarbio) in accordance with the manufacturer’s instructions. At 48 h after transfection, BGC823/5-FU cells were seeded in a 6-well plate and incubated with 2 μM 5-FU. After 2 weeks of incubation, the clones were fixed in methanol for 20 min, followed by staining with 0.1% crystal violet for 30 min at room temperature. The number of cell colonies with a diameter larger than 0.1 mm was counted under an optical microscope (Leica Microsystems, Wetzlar, Germany).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

A TUNEL assay was carried out to assess cell apoptosis in BGC823/5-FU cells using an In Situ Cell Death Detection Kit (Roche). At 48 h after transfection, BGC823/5-FU cells were incubated with 16 μM 5-FU for 48 h. BGC823/5-FU cells were fixed for 1 h in 4% paraformaldehyde, followed by permeation with 0.1% Triton X-100 for 6 min. Then, BGC823/5-FU cells were stained with the TUNEL reaction mixture for 1 h at 37°C. After washing with phosphate buffered saline (PBS), the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Solarbio) for 5 min at room temperature in the dark. The percentage of TUNEL positive cells was quantified using a confocal microscope (Leika, Germany).

**Flow cytometry**

Apoptosis of BGC823/5-FU cells was evaluated by flow cytometry using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Solarbio). In brief, BGC823/5-FU cells transfected with sh-PHGDH were incubated with 16 μM 5-FU for 48 h. BGC823/5-FU cells were rinsed with PBS and resuspended in a binding buffer (1×). Subsequently, BGC823/5-FU cells were stained for 10 min with Annexin V-FITC and incubated for 5 min with PI in the dark at room temperature. After resuspending in PBS, cells were analyzed by flow cytometry.

**Statistical analysis**

All data were presented as the mean ± standard deviation (SD). The Student’s t-test or one-
Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

way analysis of variance was utilized for statistical analyses of the data. All statistical analyses were performed using SPSS statistical software version 20.0 (SPSS Inc, Chicago, IL, USA). A P-value of 0.05 or less was considered statistically significant.

Results

PHGDH is significantly upregulated in 5-FU-resistant GC tissues and cells

To investigate the role of PHGDH in the development of 5-FU resistance in GC cells, the mRNA and protein expression levels of PHGDH in the GC tissues of 5-FU-sensitive (n = 22) and 5-FU-resistant patients (n = 7) were determined by RT-qPCR and Western blot assays. Results showed that the mRNA and protein expression levels of PHGDH were much higher in the GC tissues of 5-FU-resistant patients than that in the GC tissues of 5-FU-sensitive patients (Figure 1A and 1C). To confirm the differential expression of PHGDH, we determined the mRNA and protein levels of PHGDH in BGC823 and BGC823/5-FU cells. Compared with the BGC823 cells, the mRNA and protein levels were markedly increased in the BGC823/5-FU cells (Figure 1B and 1D). Therefore, these findings suggest that PHGDH expression is markedly upregulated in 5-FU-resistant GC tissues and cells.

Knockdown of PHGDH inhibits the proliferation of BGC823/5-FU cells

To investigate the effect of PHGDH on the proliferation of BGC823/5-FU cells, we performed loss-of-function experiments by transfecting BGC823/5-FU cells with sh-PHGDH-1, sh-PHGDH-2, or sh-NC. As illustrated in Figure 1A and 1B, the inhibitory effects of sh-PHGDH-1 and sh-PHGDH-2 on PHGDH mRNA and protein levels were validated by RT-qPCR and western blot analysis, respectively. The mRNA and protein levels of PHGDH were much higher in the sh-NC group than those in the sh-PHGDH group, especially in the sh-PHGDH-1 group (Figure 2A and 2B). Therefore, the sh-PHGDH-1 plasmid was selected for the following experiments. At 48 h after transfection, BGC823/5-FU cells were incubated with the indicated concentrations (0, 1, 2, 4, 8, 16, and 32 μM) of 5-FU for 48 h or treated with 16 μM 5-FU for different duration (24, 48, and 72 h). As detected by the CCK-8 assay, the 5-FU treat-
Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

5-FU treatment significantly reduced the viability of BGC-823/5-FU cells in a concentration-dependent manner (Figure 2C). Thus, a dose of 16 μM of 5-FU was chosen for the following experiments. Furthermore, the viability of BGC823/5-FU cells was markedly reduced at 48 and 72 h post 5-FU treatment (Figure 2D). Simultaneously, a colony formation assay was performed on BGC823/5-FU cells transfected with sh-PHGDH-1, following exposure to 2 μM 5-FU for 2 weeks. The 5-FU treatment obviously decreased the colony-forming ability of the BGC-823/5-FU cells, which was significantly enhanced by the knockdown of PHGDH (Figure 2E). Collectively, these results indicated that the knockdown of PHGDH inhibited the proliferation of the BGC823/5-FU cells.

Knockdown of PHGDH promotes apoptosis of BGC823/5-FU cells

To explore the effect of PHGDH knockdown on the apoptosis of BGC823/5-FU cells, we measured the number of apoptotic BGC823/5-FU cells using the TUNEL assay and flow cytometry. At 48 h post transfection, BGC823/5-FU cells were treated with 16 μM 5-FU for 48 h. The results demonstrated that 5-FU treatment promoted apoptosis in BGC823/5-FU cells as evidenced by the increased numbers of TUNEL-positive cells and increased rates of apoptosis. Notably, the promoting effect of 5-FU on the apoptosis of BGC-823/5-FU cells was prominently enhanced by the knockdown of PHGDH (Figure 3A and 3B). Taken together, these results suggest that the knockdown of PHGDH promotes apoptosis in BGC823/5-FU cells.

Knockdown of PHGDH downregulates Bcl-2 expression and upregulates the expression of Bax and caspase-3

To further confirm whether PHGDH participates in 5-FU-mediated apoptosis by modulating the Bcl-2/Bax/caspase-3 signaling pathway, we performed RT-qPCR and Western blot assays to analyze the expression of Bcl-2, Bax, and caspase-3 in BGC823/5-FU cells transfected with sh-PHGDH-1, following exposure to 16 μM 5-FU for 48 h. 5-FU treatment significantly downregulated the mRNA and protein levels of Bcl-2 and upregulated those of Bax and caspase-3, and this effect was markedly potentiated by the knockdown of PHGDH (Figure 4A-D). Taken together, our findings revealed that the knockdown of PHGDH downregulated Bcl-2 expression and upregulated the expression of Bax and caspase-3.

Discussion

Important findings in the last decade have revealed that metabolic reprogramming may play an essential role in the occurrence and development of human cancers [16]. Tumor growth is maintained via aerobic glycolysis, also called the Warburg effect [17]. Moreover, metabolic reprogramming promotes the accumulation of biomass and the growth of cancer cells through modifications in the genomes of metabolic enzymes [18]. Conversely, the inhibition of metabolic enzymes can repress the growth of...
Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

5-FU treatment markedly increased BGC823/5-FU cell apoptosis, which was prominently enhanced by the knockdown of PHGDH. Additionally, 5-FU treatment strikingly down-

Figure 4. Knockdown of PHGDH downregulates Bcl-2 expression and upregulates the expression of Bax and caspase-3. At 48 h after transfection with sh-PHGDH-1, BGC823/5-FU cells were exposed to 16 μM 5-FU for 48 h. The mRNA expression levels of Bcl-2 (A), Bax (B), and caspase-3 (C) were examined by RT-qPCR. (D) The protein levels of Bcl-2, Bax, and caspase-3 were detected by western blot analysis. *P<0.05, **P<0.01.

cancer cells. Therefore, research on metabolic enzymes holds great promise.

Resistance to chemotherapy drugs often occurs during the treatment of cancers and can be attributed to most cancer-related deaths. Growing evidence suggests that PHGDH plays a crucial role in the development of drug resistance. For instance, Zaal and colleagues demonstrated that PHGDH upregulated inbortezomib (BTZ)-resistant cell lines (BTZ/7 and BTZ/100 cells). Moreover, BTZ resistance is closely correlated with the overexpression of PHGDH [19]. Zhang and colleagues suggested that the inhibition of PHGDH caused doxorubicin-induced oxidative stress and increased the sensitivity of triple-negative breast cancer cells to doxorubicin [11]. Jing and colleagues found that PHGDH was highly expressed in cervical adenocarcinoma, which was associated with tumor size and prognosis. Knockdown of PHGDH in HeLa cells obviously suppressed cell proliferation and enhanced their sensitivity to cisplatin through the downregulation of Bcl-2 and the upregulation of cleaved caspase-3 [20]. However, the role of PHGDH in the development of 5-FU-resistance in GC cells remains unclear. In our study, the expression of PHGDH was much higher in the GC tissues of 5-FU-resistant patients than in the GC tissues of 5-FU-sensitive patients. Furthermore, the expression of PHGDH was strikingly increased in BGC823/5-FU cells compared with that in the BGC823 cells. In addition, 5-FU treatment inhibited the proliferation of BGC823/5-FU cells, as evidenced by decreased cell viability and reduced colony-forming ability. Notably, the knockdown of PHGDH made possible the inhibitory effect of 5-FU on the proliferation of BGC823/5-FU cells.

Apoptosis is a genetically coded program of cell death that maintains homeostasis, which is strictly regulated by polygenes [21]. Chemotherapeutic agents exert their curative effect by inducing apoptosis in cancer cells. Thus, the induction of apoptosis in cancer cells has become a hotspot for the treatment of cancer [22]. Under normal conditions, the pro- and anti-apoptotic proteins are metastable in organisms [23]. The Bcl-2 protein family is a core member of the apoptosis gene family, which is regarded as the primary modulator of cell apoptosis [24]. Bax, a pro-apoptotic protein, functions as an apoptotic activator and induces the opening of the mitochondrial voltage-dependent anion channel, which results in the release of cytochrome c and activation of caspase [25]. Bcl-2, an anti-apoptotic protein, can restrain cell apoptosis by binding pro-apoptotic proteins, such as Bax [26]. Caspase-3, the main executioner caspase in apoptosis, is activated by proteolytic cleavage, which in turn induces apoptosis [27]. In this study, the results of a TUNEL assay and flow cytometry revealed that 5-FU treatment markedly increased BGC823/5-FU cell apoptosis, which was prominently enhanced by the knockdown of PHGDH. Additionally, 5-FU treatment strikingly down-
Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

regulated the mRNA and protein levels of Bcl-2 and upregulated those of Bax and caspase-3, and this effect was markedly potentiated by knockdown of PHGDH.

Taken together, our results suggest that PHGDH expression is upregulated in 5-FU-resistant tissues and cells. The knockdown of PHGDH represses cell proliferation, promotes cell apoptosis, and restores 5-FU sensitivity in BG-C823/5-FU cells. Moreover, the knockdown of PHGDH downregulates Bcl-2 expression and upregulates the expression of Bax and caspase-3. Therefore, these findings suggest that 5-FU resistance in GC cells might be combated by targeting PHGDH, which may serve as a novel therapeutic target for GC.

Acknowledgements

This work was supported by the Medical Science and Technology Project of Zhejiang Province (grant no. 2017196977).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yunli Zhang, Department of Abdominal Surgery, Zhejiang Cancer Hospital, 1 Banshan East Road, Hangzhou 310022, Zhejiang Province, China. Tel: 86-571-88128031; E-mail: zhang_yunli@sohu.com

References

Knockdown of PHGDH potentiates 5-FU cytotoxicity in gastric cancer cells

title myeloma is associated with increased serine synthesis. Cancer Metab 2017; 5: 7.