PolG Inhibits Glycolysis by Suppressing PKM2 Phosphorylation Resulting Reduced Gastric Cancer Proliferation

Mengzhu Lv

China Medical University First Hospital <https://orcid.org/0000-0002-7804-2636>

Simeng Zhang

China Medical University First Hospital

Yuqing Dong

china medical university first hospital

Liu Cao

China Medical University

Shu Guo (✉ faylv47@126.com)

Department of Plastic Surgery, China Medical University the First Hospital, No.155, Nan Jing Street, HePing District, Shenyang 110001, Liaoning Province, PR China <https://orcid.org/0000-0001-9094-1602>

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Abstract

Background: Gastric cancer (GC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer death. There is a critical need for the development of novel therapies in GC. DNA polymerase gamma (PolG) has been implicated in mitochondrial homeostasis and affects the development of a numerous of cancer types. However, its effects in GC and molecular mechanisms remain to be fully determined.

Methods: GSE62254 dataset was used to predict the impact of PolG on prognostic valule in GC patients. Lentivirus-mediated transduction was used to silence PolG expression. Western blot analysis the knockdown effect. Co-immunoprecipitation analysis was performed to explore the potential molecular mechanism. Analysis of the glycolysis process in GC cells was also performed. Cell proliferation was determined using a CCK-8 (Cell Counting Kit-8) proliferation assay. Cell migration was detected using transwell method. Animal experiment was used to measure the In vivo xenograft tumor growth.

Results: GC patients with low PolG expression have worse OS and pFS . PolG binds to PKM2 and affects the activation of the Tyr105 site phosphorylation, then interfering with the glycolysis of Gastric cancer cells. In vitro tumor formation experiments in mice also confirmed that PolG knockdown GC has stronger proliferation ability. PolG can suppress GC cells growth in both vivo and vitro.

Conclusion: Our study reveals a potential molecular mechanism between PolG and the energy metabolic process of GC tumor cells, suggesting PolG as an independent novel potential therapeutic target for tumor therapy.

Background

One million new cases of gastric cancer (GC) were diagnosed globally in 2018 with an estimated 783,000 deaths (amounting to 1 in every 12 deaths), making it the fifth most frequently diagnosed cancer and the third leading cause of cancer death [1]. Major efforts have been made in GC treatment including targeted therapies such as HER2-targeted trastuzumab, VEGFR2-targeted ramucirumab, and immune checkpoint inhibitors (ICIs) [2]. In addition to these improvements, the complex biology of GC often results in treatment failure and therapeutic resistance [3]. Therefore, it is necessary to further explore novel treatment targets, and adopt comprehensive treatment approaches to deliver better patient outcomes in GC.

Under well-oxygenated conditions, oxidative phosphorylation is the primary way of nutrient catabolism and energy production in most differentiated cells [4]. When the mitochondrial function of tumor cells is impaired, the energy supply capacity of oxidative phosphorylation is reduced which may impact the survival of tumor cells. However, due to changes in metabolic homeostasis, tumor cells have a preference to obtain energy through glycolysis under the same conditions, which is known as the Warburg effect [5, 6].

DNA polymerase gamma (PolG) is the main polymerase of mitochondrial DNA. PolG can affect the stability of mitochondrial DNA and interfere with the expression of proteins synthesized by mitochondrial DNA transcription, affecting mitochondrial homeostasis [7–9]. Studies have reported that knockdown of PolG in bowel cancer can reduce mtDNA content, and increase glucose uptake and lactate secretion, making tumors more resistant to oxidative stress [10]. By systematically analyzing the DDR gene data of hereditary breast cancer patients, missense mutations in PolG were significantly related to the risk of breast cancer [11]. Curcumin can interfere with mitochondrial function by reducing the expression of PolG, thereby inhibiting the development of GC [12, 13]. These findings suggest that PolG may be closely related to the occurrence, development and prognosis of tumors, yet the specific molecular mechanisms of PolG remain to be determined.

In this study, we explored the potential mechanism of PolG in the growth of GC tumors. Our results suggest that PolG is a tumor suppressor gene that impacts GC cell viability. PolG can also competitively bind to the phosphorylation site of PKM2, to reduce phosphorylation at the PKM2-Tyr105 site, and independently suppress the glycolysis of GC tumors to inhibit tumor growth.

Methods

Data collection and screening

Microarray data of GSE62254 were obtained from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/). GSE62254 data were based on the GPL570 platforms (Affymetrix Human Genome U133 Plus 2.0 Array, 300 GC patients), and 295 samples with both clinical parameters and gene expression data of GC were included in this study. Kaplan Meier-plotter (KMplotter) (<http://www.kmplot.com/>) was used for external validation.

Survival analysis

Survival analysis was performed by Kaplan-Meier (KM) method and log-rank test. The expression levels of hub-genes were separated according to high and low expression based on the median value. The Cox proportional-hazards regression models was applied to perform uni- and multivariate analyses were performed by. A nomogram was set up by the rms package in R, according to the final multivariate COX regression model. The predictive accuracy was assessed by C-Index. The internal validation of nomogram was measured by a calibration curve.

Chemicals and reagents

DMEM medium, FBS, penicillin/streptomycin, and trypsin were purchased from Invitrogen. Sodium butyrate and Puromycin and were purchased from Sigma. Anti-PolG antibodies (EPR7296) were purchased from Abcam. Antibodies specific to β -actin (A1978) were purchased from Sigma. PKM2 (4053s), pPKM2-Tyr105(3827s), Myc(2276s), Flag(2368s) were purchased from Cell Signaling Technology.

Plasmid construction

A lentiviral PolG shRNA was purchased from Genechem (Shanghai, China). The shRNA sequence targeting the human PolG complementary DNA was 5'-TGTCCAGGGAGAGTTTATA-3'. A scrambled shRNA was included as a negative control (NC). The target sequence was inserted into the GV248 lentiviral vector (Genechem). The expression of the Myc-PolG plasmid was constructed by PCR and subcloned into the Myc-pCMV vector(Clontech). The Flag-PKM2 plasmid was purchased from OriGene.

Cell Culture and Transfection

SGC7901, MGC803 and HEK293T cells were cultured in high-glucose DMEM with 10% FBS and 100 units/ml of penicillin/streptomycin, at 37 °C in a 5% CO₂ incubator. Cells were transfection by Lipofectamine 2000 (Thermo Fisher Scientific, 11668019) according to the instructions and harvested 48 h after transfection. 293T cells were used for lentivirus production by transfected with the shRNA-expression vector. The transfected 293T cells supernatant was collected at 24 h, 48 h and 72 h. Following by centrifugation at 1500 g for 30 min, the lentiviral particles were resuspended in PBS and added to the SGC7901 and MGC803 cells for 24 h infection. Stably transfected cell lines were sifted for 5 days in 20 µg/mL puromycin.

Western Blot Analysis

Cells were lysed with an IP lysis buffer supplemented with protease and phosphatase inhibitor cocktails for 30 min on ice. Total protein was harvested by centrifugation at 15,000 rpm for 20 min at 4 °C. Samples were loaded to 10% polyacrylamide gels, separated by SDS-PAGE and transferred to PVDF membranes for 2.5 h at 80-120V. Membranes were incubated through prescribed antibodies at 4 °C overnight after blocked by 5% BSA in TBST for 2 h at room temperature. After 3 times wash with TBST, membranes were incubated with secondary antibody for 2 h at room temperature. At last, bands were analyzed by chemiluminescence detection (Tanon Science & Technology Co., Ltd., Shanghai, China).

Co-immunoprecipitation analysis

SGC7901 cells were dissolved with IP lysis and incubated with antibody for 2 h. Then protein A/G-Sepharose was added and incubate on a mixer overnight at 4 °C. Then use centrifugation for 5 min at 700 g at 4 °C to connect Beads the next day, and cleaned in IP lysis for 3 times (each time for 10 min). The beads were then resuspended with the loading for WB.

Glucose consumption and lactate production analysis

Cells were seeded in six-well culture plates. The medium was changed into phenol red-free DMEM media after 6 h. Then harvest the media after 48 h. Glucose consumption was measured between the media before and after the 48 h incubation period by an assay kit (Sigma, GAHK20). Extracellular lactate levels were normalized to the protein concentration of the samples by a lactate assay kits (Sigma, MAK065).

Cell proliferation assay

Cell counting Kit-8 (CCK8) (Abbkine, KTA1020) was used to evaluate the cell proliferation ability. Cells were seeded into 96-well plates at a density of 5×10^3 cells/well for 6 h, 24 h, 48 h, 72 h. Then the culture medium was replaced with 90 μ l of basal DMEM and 10 μ l CCK8. After incubation at 37 °C for 3 h, the absorbance was measured using an absorbance reader (TECAN, Switzerland) at 450 nm.

Transwell migration assay

SGC7901 or MGC803 cells (3×10^4) were resuspended with Serum-free DMEM media and seeded into the upper chamber while 10%FBS DMEM with added to the lower chambers (Corning, 3422). After 24 h incubation, invasive cells on the underside were fixed in methanol for 10 min and stained with hematoxylin for 30 min at room temperature. Image was taken by an inverted microscope (Nikon Corp., Tokyo, Japan). Three independent experiments were performed, and five individual fields were counted each for statistical analysis.

In vivo xenograft tumor growth

For the xenograft tumor growth assay, SGC7901 stable cell lines with PolG knockdown were injected subcutaneously into the right flank of 6-week-old male BALB/C nude mice (N = 10). NaB was intraperitoneally injected into mice in the PolG-knockdown group (200 mg/kg) (N = 5). NC group were used for comparative(N = 5). Tumors were cultivated for 14 days. All animal experiments were approved by the Committee of China Medical University.

Statistical analysis

Three independent experiments' values are expressed as the mean $\pm$ standard. Statistical significance was analyzed using a t-test or a one-way analysis of variance. All statistical analysis was performed using SPSS 17.0 and Prism 5.0 software. Values of $P < 0.05$ were defined as statistically significant.

Results

Prognostic value of PolG in GC

GC samples with survival data and gene expression profiles were obtained from the GSE62254 dataset as shown in Table 1. The sample characteristics of GSE62254 were consistent with randomized clinical studies of GC [3, 14, 15]. The prognostic value of PolG was evaluated by Kaplan-Meier analysis which showed that low expression of PolG was associated with worse overall survival (OS, log-rank $P < 0.001$) as well as progression-free survival (PFS, log-rank $P < 0.001$) (Fig. 1A). Furthermore, COX regression analysis indicated that PolG was an independent prognostic factor both in univariate (HR 0.611, 95% CI; 0.432–0.866; $P = 0.006$) and multivariate analysis (HR 0.679, 95% CI; 0.479–0.963; $P = 0.03$) (Table 2). External validation analysis based on KMplotter showed a favorable prognosis of PolG in GC (log-rank $P = 0.004$) (Fig. 1B). To further investigate the predictive value of PolG in a prognostic model, a nomogram of OS which combined the significant prognostic factors identified from multivariate analysis was adopted (Fig. 1C). The centrality-index (C-Index) of OS prediction was 0.75 (95% CI, 0.71 to 0.79). The

calibration curve was applied to reflect the probability of survival at 1 and 3 years, which indicated the consistency between the nomogram prediction and the objective observations (Fig. 1D).

Gene-set enrichment analysis of PolG

The potential molecular mechanisms of high PolG expression in GC subtypes were investigated by gene-set enrichment analysis. The results indicated that DNA replication and cell cycle were the most significantly enriched biological processes associated with PolG expression (Fig. 1E). In contrast, cytochrome P450, chemical carcinogenesis and retinol metabolism were highly enriched in samples with low expression of PolG.

Knocking down of PolG inhibits GC cells proliferation and migration

To elaborate the association of PolG with poor survival in GC patients with low expression of PolG, SGC7901 and MGC803 cells were used to explore the potential roles of PolG in GC cells. A PolG-shRNA lentiviral vector was constructed and used to establish stably transfected PolG-shRNA SGC7901 and MGC803 cell lines. As shown in Fig. 2A, PolG knockdown on SGC7901 and MGC803 cell proliferation was detected through cell counting Kit-8 (CCK-8). The decreased expression of PolG markedly accelerated the growth of SGC7901 and MGC803 cells. Cell proliferation was increased by 55% and 25% in SGC7901 and MGC803 cells of the shPolG group, respectively, compared to the control group ($P < 0.001$). The migration abilities of GC cells were significantly increased in both PolG-knockdown cell lines through transwell analysis ($P < 0.0001$, Fig. 2B);

To further investigate the potential causes of these phenomena, we examined the rates of lactate production and glucose consumption of each cell line. The results showed that the decrease of PolG expression in GC cells increased both lactate production and glucose consumption ($P < 0.001$, Fig. 2C). These data suggest that knockdown of PolG increased the basal glycolytic rate and Warburg effect of tumor cells. Also, KEGG enrichment analysis by GSEA indicated that metabolism-related pathways were highly enriched in the group with low PolG expression (Fig. 2D) which may be due to the impact of PolG on the activity of key kinases involved in tumor metabolism.

PolG inhibits tumor glycolysis by interactions with PKM2

We detected the molecular mechanism of PolG in GC cell glycolysis. Western blot (WB) detection indicated that PKM2-Tyr105 increased significantly after PolG knockdown ($P < 0.01$, Fig. 3A). PKM2 is a main regulator for glycolysis, and PKM2-Tyr105 phosphorylation leads to less kinase activity which can promote the Warburg effect [16]. We then analyzed the potential interactions of PolG with PKM2. Endogenous and exogenous co-immunoprecipitation analysis showed that PolG interacted with PKM2 in SGC7901 cells (Figs. 3B, C). Based on these data, we hypothesized that the observed changes in cellular metabolism caused by PolG knockdown were due to interactions with PKM2, which interfered with the phosphorylation of PKM2-Tyr105.

Replenishing PolG can mimic PKM2 inhibition in reducing the proliferation of GC cells

We confirmed that PolG knockdown promoted GC cells proliferation, and PolG could interact with PKM2. However, PolG knockdown may affect mitochondrial function, leading to a compensatory increase in cellular glycolysis. To further confirm that the changes in GC cell metabolism and cell behavior were due to the interactions between PolG and PKM2, we performed PolG replenishment on knocked-out cell lines. Also, we used NaB to target PKM2 for tumor suppression [17] and to inhibit PKM2 phosphorylation in a controlled experiment.

In the preliminary experiment, we treated the SGC7901 cells with different concentrations of NaB (0–10 mM) for 24 h, and found that after the concentration was increased to 5 mM, the suppression of tumor cells entered the plateau phase, so we finally chose 5 mM as the final stimulation concentration (Fig. 4A). Then we selectively performed PolG supplementation experiments, and added NaB (5 mM) to the medium of the PolG-knockdown cell lines. The Cell proliferation (CCK8, Fig. 4B) and migration (transwell, Fig. 4C) were shown to be significantly reduced. In addition, WB for PKM2-Tyr105 exhibited significantly reduced levels in the PolG supplementation and NaB groups (Fig. 4D).

PolG knockdown promotes GC cell growth in vivo

To explore the impact of PolG knockdown in promoting cells growth in vivo, we injected the stably PolG knockdown SGC7901 cell lines and NC into nude mice. NaB was intraperitoneally injected into mice in the PolG-knockdown group (200 mg/kg) for comparative observation [18]. PolG knockdown in SGC7901 cells was shown to be larger than the NC group, and an intraperitoneal injection of NaB was found to suppress tumor growth (Fig. 5A). Furthermore, compared with the NC and NaB groups, the PolG-knockdown group had larger tumor weights ($P < 0.001$, Figs. 5B and C). These data indicated that PolG knockdown promoted GC cells growth in vivo.

Discussion

Through online GC data analysis, we found that GC patients with low PolG expression have worse OS and pFS, but the molecular mechanism remains unclear. PolG knockdown in GC cells in vitro, showed increased proliferation and migration capabilities compared to control cells. KEGG enrichment analysis of GSEA suggested that these phenomena are closely related to the effect of PolG on the reprogramming of tumor cell metabolism.

In previous studies, it was shown that PolG is a mitochondrial DNA polymerase, which plays an important role in maintaining the stability of mitochondrial DNA. The abnormality of PolG could directly affect the function of the respiratory chain which is composed of proteins synthesized by transcription of mitochondrial DNA and downstream signal modulation of cellular metabolism [19–21]. It has also been reported that PolG promotes metabolic reprogramming in various types of tumor cells by affecting

mitochondrial function in tumor cells [11–13]. Although more than 90% of the cellular ATP is produced by mitochondria in normal differentiated cells, tumor cells undergo the Warburg effect by relying on aerobic glycolysis as their primary energy source. Metabolic reprogramming is an important feature during tumorigenesis and development. Glycolysis is an insufficient way of ATP production that allows tumor cells to uptake more nutrients, and synthesizes organic molecules to support their proliferation and invasion [22, 23].

In this study, for the first time we confirmed the molecular interaction between PolG and the glycolysis of GC cell. This mechanism is closely related to the inhibition of phosphorylation at PKM2-Tyr105. Recently, researches shown that PKM2 is upregulated in tumor cells, and that PKM2-Tyr105 site phosphorylation can prevent its tetramer formation which directly impacts the kinase activity of PKM2 and enhances the Warburg effect [16, 24–26]. In the current study, both glucose consumption and lactate production were increased in PolG-knockdown GC cells. We investigated the molecular mechanism through which PolG can interact with PKM2 and affect the phosphorylation of PKM2-Tyr105. Moreover, we performed PolG replenishment for PolG-knockdown GC cells and experiments using PKM2 inhibitors (NaB). These experiments showed reduced proliferation and migration abilities. These data confirmed that PolG can suppress the energy metabolism of tumor cells by inhibiting PKM2 phosphorylation, further validating PolG as a potential therapeutic target.

However, our study did not include a comprehensive investigation on the specific mechanism through which PolG affects phosphorylation of Tyr105 after binding to PKM2. We also did not determine the balance between the effect of PolG on PKM2 and the specific effects on mitochondrial function. In future studies, we will continue to elucidate the potential role of PolG in GC.

Conclusions

Our present study revealed that PolG is an independent factor in the treatment of GC. PolG can interact with PKM2 and affects the activation of the Tyr105 site phosphorylation, then suppress the energy metabolism of GC cells, at last interfering the GC cells growth in both vivo and vitro. PolG may be a potential therapeutic target for GC treatment.

Abbreviations

GC	Gastric cancer
PolG	DNA polymerase gamma
PKM2	pyruvate kinase M2
VEGF	vascular endothelial growth factor
ICIs	immune checkpoint inhibitors
KM	Kaplan-Meier method
OS	overall survival
PFS	progression-free survival
CCK-8	cell counting Kit-8

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Committee of China Medical University.

Consent for publication

Not applicable

Availability of data and materials

All data analyzed and displayed in the present manuscript are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Mengzhu Lv carried out all the experiments, collection and analysis of data. Shu Guo and Liu Cao performed experimental guidance and data analysis. Simeng Zhang and Yuqing Dong contributed to animal experiment. All authors read and approved the final manuscript.

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Not applicable

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

(A) Overall Survival (OS) and Progression-Free Survival (PFS) of PolG in the GSE62254 cohort by Kaplan-Meier (KM) analysis. (B) External validation of OS of KMplotter cohort by KM analysis (log-rank $P = 0.004$). (C) The parameters of individual patients correspond to the axis of each variable to obtain the points displayed above the model. The sum of the point of a single variable is reflected in the total points on the axis. The probability of survival at 1 and 3 years was determined by correspondence between the

total points and survival axis. (D) Probability of survival at 1 and 3 years in the GSE62254 GC cohort. (E) Gene set enrichment analysis of the biological processes associated with PolG expression.

Figure 2

PolG knockdown inhibits proliferation and migration of GC cells. The effect of PolG knockdown by shRNA or negative control (NC) in SGC7901 and MGC803 cells. (A) Proliferation was performed by cell counting Kit-8, $^{**}P < 0.01$. (B) Migration abilities were determined by the transwell assay, $^{****}P < 0.0001$.

(C) Lactate production ($***P < 0.001$) and glucose consumption ($**P < 0.01$) were elevated in POLG-knockdown group. (D) KEGG enrichment analysis by GSEA in the low PolG expression group.

Figure 3

PolG inhibits PKM2 phosphorylation by interactions with PKM2. (A) Western blot (WB) analysis of the total and Tyr105 phosphorylation of PKM2 in stable PolG-knockdown SGC7901 cells. Quantification of protein expression (Tyr105 phosphorylation of PKM2) (A) Data are shown as the mean $\pm$ S.E.M. of $n \geq 3$

technical replicates, and are representative of three independent experiments, $**P < 0.01$. (B, C) Co-immunoprecipitation (IP) of endogenous PolG with PKM2 in SGC7901 cells. Cell lysates were subjected to IP using anti-PKM2(rabbit) or anti-PolG(mouse) and unrelated rabbit (or mouse) IgG as a control. Precipitates were subjected to WB analysis with anti-PolG or anti-PKM2. A portion of the whole-cell lysates (WCLs) or the input for IP were subjected to IB analysis. (D, E) SGC7901 cells were co-transfected with expression plasmids encoding Myc-tag or Myc-tagged PolG, and Flag-tagged PKM2 as indicated. Cells were lysed and subjected to IP with an anti-Myc (or anti-Flag) antibody. The resulting precipitates were subjected to WB analysis with anti-Flag (or anti-Myc) antibody. A portion of the WCLs of the input for IP were also subjected to IB analysis.

Figure 4

Replenishment of PolG and PKM2 inhibitors can suppress tumor proliferation in PolG-knockdown cells. (A) SGC7901 cells were treated with different concentration of butyrate(0-10mM) for 24 h and the number of viable cells in response to the treatment was determined by CCK-8 cell viability assay (n = 5). Data are presented as the mean $\pm$ S.E.M. *p < 0.05, **p < 0.01. (B) Proliferation was determined using a CCK-8, Myc-PolG and NaB were respectively compared with the knockdown group, *p < 0.05, **p < 0.01. (C) Transwell assays and quantification of migration ability in SGC7901 and MGC803 cells were undertaken, ****P < 0.0001. (D) WB analysis of total and Tyr105 phosphorylation of PKM2 expressing in SGC7901 cells transfected with shRNA and treated with myc-PolG or NaB.

Figure 5

SGC7901 xenografts treated with stable knockdown of PolG or with simultaneous intraperitoneal injection of NaB. (A) Mice were sacrificed and photographed at day 14. (B) PolG knockdown promoted xenograft tumor growth. At 14 days after injection, tumors were removed and photographed. (C) The

tumor weight was calculated on day 14. Data are presented as the mean $\pm$ SD (n = 5), ***P < 0.001 versus control.

Supplementary Files

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