PYGB Promoted Tumor Progression by Regulating Wnt/β-Catenin Pathway in Gastric Cancer

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Abstract
Gastric cancer is one of the most common gastrointestinal malignancy with high mortality in East Asia. Investigation of pathogenic mechanisms of gastric cancer is crucial to develop novel therapeutic strategies and identify new therapeutic candidates. Brain-type glycogen phosphorylase is a glycogen phosphorylase involved in glycogen metabolism, which participates in multiple physiological and pathological processes. Overexpression of brain-type glycogen phosphorylase has been reported in various types of cancer, such as colorectal cancer and non-small cell lung cancer, however, the potential role of brain-type glycogen phosphorylase in gastric cancer remains unclear. Herein, we observed brain-type glycogen phosphorylase expression was significantly elevated in human gastric cancer tissues and positively correlated with the clinical-pathological features including tumor size, lymph node involvement, and tumor, node, metastasis stage of patients with gastric cancer. We further reported brain-type glycogen phosphorylase depletion suppressed the growth of gastric cancer, weakened the epithelial–mesenchymal transformation, and reduced the migration and invasion ability in cell models. We further confirmed brain-type glycogen phosphorylase depletion inhibited tumor growth and lung metastasis in mice. Importantly, we found brain-type glycogen phosphorylase regulated the progression of gastric cancer via Wnt/β-catenin pathway, shedding lights on brain-type glycogen phosphorylase as a promising therapeutic target for drug design and development targeting gastric cancer.

Keywords
gastric cancer (GC), brain-type glycogen phosphorylase (PYGB), epithelial–mesenchymal transformation (EMT), proliferation, migration, invasion

Abbreviations
BSA, bovine serum albumin; CCK-8, Cell Counting Kit-8; EMT, epithelial mesenchymal transformation; EMT, epithelial–mesenchymal transformation; FBS, fetal bovine serum; GC, gastric cancer; HE, hemotoxylin and eosin; IHC, immunohistochemistry; LNI, lymph node involvement; PBST, phosphate buffered saline with Tween-20; PCR, polymerase chain reaction; PFA, paraformaldehyde; PYGB, brain-type glycogen phosphorylase; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; shRNA, short hairpin RNA; TNM, tumor, node, metastasis

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Introduction
Gastric cancer (GC) is considered as one of the major causes of cancer-related deaths worldwide and the most common gastrointestinal malignancy in East Asia.¹² Despite advances in surgical techniques and targeted chemotherapies, the 5-year overall survival rate remains unsatisfactory as most patients with lymphatic metastasis are always diagnosed at advanced stages.³⁻⁵ The molecular biomarkers related to progression and metastasis of GC should be identified and utilized in clinical
**Materials and Methods**

**Human Specimen**

Paired human GC tissues and the corresponding normal tissues were obtained from 57 patients in our hospital by surgical resection. Relevant clinical information was collected and all tissue analysis were consented by all participants. All procedures performed in this study involving human participants were in accordance with the standards upheld by the Ethics Committee of The Second Affiliated Hospital of Chongqing Medical University and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects.

**Cell Culture**

Cell lines used in this study included human GC cell lines (HGC-27, SNU-1, AGS and MKN45) and human gastric epithelial cell line (GES-1). Cell lines were bought from Shanghai Biochemistry and Cell Biology Institute. Cells were maintained in Roswell Park Memorial Institute-1640 culture medium (Life Technologies) with 10% heat-inactivated fetal calf serum (Life Technologies) and 100 μg/mL penicillin/streptomycin at 37 °C in a 5% CO2 humidified incubator (Thermo). Brain-type glycogen phosphorylase short hairpin RNA (shRNA) vectors and a negative control were bought from Gene Pharma. Transfection of PYGB shRNA plasmids was performed using Lipofectamine 3000 according to the manufacturer instruction (Invitrogen). Lentiviruses-mediated PYGB ablation or relative nonscramble control was constructed by GenePharma.

**Antibodies**

Antibodies used in this study are as follows: PYGB (1:100 for immunohistochemistry [IHC], 1:1000 for immunoblot; ab154969; Abcam), E-cadherin (1:100 for IHC and IF; 14472; CST), Vimentin (1:100 for Immunofluorescence [IF], ab154969; Abcam), E-cadherin (1:100 for IHC, 1:1000 for immunoblot; 51067-2-AP, Proteintech), T cell factor 4 [TCF-4] (1:1000, ab217668, Abcam), c-Myc (1:1000, ab32072, Abcam), Ki67 (1:100, ab15580, Abcam), and GAPDH (1:1,500; 5174, cell signaling technology [CST] Biological Reagents Co, Ltd.).

**Quantitative Real-Time Polymerase Chain Reaction**

RNAs were extracted from tissues and cultured cells using TRIzol (Invitrogen) as illustrated by manufacture protocol. RNAs were subsequently transcribed into complementary DNA through reverse transcription kits (Roche). Quantitative polymerase chain reaction (PCR) was conducted by ABI 7500 real-time PCR system (Applied Biosystems). Primers used are listed in Table 1.

**Immunoblot**

Protein samples were isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10-30 μg/lane), then transferred onto polyvinylidene difluoride membrane (EMD Millipore). The membrane were blocked in 5% bovine serum albumin (BSA) and immersed into indicated primary

### Table 1. Primers.

| Primers     | Sequences                                      |
|-------------|------------------------------------------------|
| PYGB forward| 5'-CCAGACTGCTTCAAGGACATCG-3'                  |
| PYGB reverse| 5'-GTTCCTGATGACCTTCTGTC-3'                   |
| β-Catenin forward| 5'-TGCCAGGATGCCACCCACG-3'            |
| β-Catenin reverse| 5'-GCCATTCATGGGTCTGCT-3'                  |
| TCF-4 forward| 5'-AAGCTTATGCACTACCAACAGGAATG-3'          |
| TCF-4 reverse| 5'-CTCGAGTCATCTCTGCCATGATT-3'             |
| c-Myc forward| 5'-CGTCCTGGGAGGAGGAT-3'                    |
| c-Myc reverse| 5'-CGCTCTATGGCGAAAGT-3'                    |
| GAPDH forward| 5'-CACCCACTCTCCTCCATTTG-3'                 |
| GAPDH reverse| 5'-CCACCACCTGTGTTGCTAG-3'                  |

Abbreviation: PYGB, brain-type glycogen phosphorylase.
Figure 1. Brain-type glycogen phosphorylase was upregulated in human gastric cancer. A, Brain-type glycogen phosphorylase level in GC tissues and in normal tissues were analyzed by qRT-PCR (57 pairs of gastric cancer and nontumor tissues). B, Brain-type glycogen phosphorylase protein levels were determined by immunoblot. C, Immunohistochemistry assay detected upregulated PYGB expression level in GC tissue samples. D, Correlation between overall survival rates and PYGB expression level. E, Relative PYGB expression level in several gastric cancer cell line: HGC-27, SNU-1, AGS, and MKN45, and normal gastric epithelia cell line GES-1 quantified by qRT-PCR (mean ± SD, **P < .01). GC indicates gastric cancer; PYGB, brain-type glycogen phosphorylase; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.
antibodies, followed by 1 hour incubation with horseradish peroxidase-conjugated labelled secondary antibodies (Beyotime Biotechnology Institute). Protein bands detected by ECL kit (Thermo Fisher Scientific, Inc).

Immunofluorescent Staining

To detect EMT markers intensity in human GC cell lines, cells were fixed in 4% paraformaldehyde (PFA), and then blocked in 5% BSA in phosphate buffered saline with Tween-20 (PBST). E-cadherin and Vimentin antibodies were then applied to incubate with cells at 4 °C overnight. After rinsing in PBST for 3 times, secondary antibodies conjugated with Alexa 488 or 594 (Invitrogen) were added. 4',6-Diamidino-2-phenylindole was used to counterstain cell nuclei (Vector Laboratories, Inc). Images were obtained using fluorescent microscope (LSM700, Zeiss model).

Immunohistochemistry and Hematoxylin and Eosin Staining

Briefly, specimens were fixed in 4% PFA and cut into 5 μm slices. Tissues were stained with primary antibody targeting PYGB, Ki67, β-catenin, and E-cadherin or hematoxylin and eosin (HE) for histological analysis. And after washing, sections were incubated with secondary antibody for 1 hour and stained with diaminobenzidine.

Sections staining score for PYGB is described as follows: the percentage of positively staining in each section scored as 1 (<25% staining of tumor cells), 2 (25%-50%), 3 (50%-75%), or 4 (>75%). In addition, staining intensity was classified as 0 (negative), 1 (weak), 2 (intermediate), or 3 (strong). Final score was calculated as the intensity score plus the percentage values. Samples scored between 3 and 6 were considered PYGB high expression. Final score <3 was considered PYGB low expression.

**Colony Formation Assay**

Gastric cells were seeded into a 6-well plate ~400 cells per well and cultured for ~2 weeks. Cells were then fixed and stained with crystal violet (Sigma). Cell colony number was then calculated manually.

**Cell Counting Kit-8 Assay**

Control or PYGB knockdown AGS and MKN45 cells were plated into 96-well plates and maintained for 48 hours. Then cells were supplied with Cell Counting Kit-8 (CCK-8) solutions according to manufacturer protocol. The absorbance was measured by a microplate reader at 450 nm wavelength.

**Transwell Assay**

Briefly, ~3 × 10^4 AGS and MKN45 cells were maintained in 20% matrigel in serum-free medium. Cells were seeded into the upper chambers with filters (8.0 μm membrane pores), whereas complete medium with 10% fetal bovine serum (FBS) was added into the lower chamber. Cells tended to migrate into the lower chambers due to FBS; 36 hours postseeding, to quantify the cell number in the lower chambers, cells were fixed and stained with 0.5% crystal violet, and cell numbers were counted manually.

**Wound-Healing Assay**

Cells were seeded in 6-well plates and cultured until 90% confluence. Mechanical lesions were created using a sterile 200 μL tip. Cells were immersed in complete medium to recover the lesion after phosphate buffered saline wash. Images were obtained by an inverted microscope at 0 hour and 24 hours postwound generation. The width of lesion was measured and analyzed.

**Tumor Growth Assays**

All mice experiments received full approval from the Animal Ethics Committee of our hospital. To establish the xenograft model, 5 × 10^6 of AGS cells with or without PYGB stable depletion were subcutaneously implanted into male BALB/c nude mice (5-6 weeks old, Beijing, China). A total of 6 mice were used in each group. Tumor volume was monitored every 3 day; 18 days postinjection, tumors were isolated, weighted, and photographed.

**Table 2. Relationship Between PYGB and Clinicopathological Parameters.**

| Parameters                  | Number of patients | Low (<median) | High (≥median) | P value |
|-----------------------------|--------------------|---------------|----------------|---------|
| Number                      | 57                 | 28            | 29             |         |
| Age (years)                 |                    |               |                |         |
| <60                         | 28                 | 13            | 15             | .689    |
| ≥ 60                        | 29                 | 15            | 14             |         |
| Gender                      |                    |               |                |         |
| Male                        | 31                 | 16            | 15             | .681    |
| Female                      | 26                 | 12            | 14             |         |
| Tumor size                  |                    |               |                |         |
| <3 cm                       | 30                 | 20            | 10             | .005    |
| ≥3 cm                       | 27                 | 8             | 19             |         |
| Lymph node involvement      |                    |               |                |         |
| Absent (pN0)                | 26                 | 17            | 9              | .025    |
| Present (pN+)               | 31                 | 11            | 20             |         |
| TNM stage                   |                    |               |                |         |
| I-II                        | 28                 | 19            | 9              | .005    |
| III-IV                      | 29                 | 9             | 20             |         |
| Lauren histotype            |                    |               |                |         |
| Intestinal                  | 30                 | 14            | 16             | .696    |
| Diffuse                     | 27                 | 14            | 13             |         |
| HP infection                |                    |               |                |         |
| Yes                         | 32                 | 18            | 14             | .223    |
| No                          | 25                 | 10            | 15             |         |

Abbreviations: HP, *Helicobacter pylori*; TNM, tumor, node, metastasis; PYGB, brain-type glycogen phosphorylase.

*p < 0.05.
For migration assessment, mice were killed 5 weeks postinjection and lung tissues were then excised and analyzed.

**Statistics**

GraphPad Prism 6 was utilized for all statistical analyses. Results displayed were generated from 3 independent experiments and shown as the mean ± standard deviation. Student t test was used for significant study. Data are shown as mean ± SD, and P value < .05 was considered statistically significant in this study (*P < .05; **P < .01, and ***P < .001).

**Results**

**PYGB Was Upregulated in Human GC**

To explore PYGB possible involvement in the human GC, we firstly investigated relative PYGB expression levels in 57 paired GC tissues and adjacent normal tissues via quantitative real-time polymerase chain reaction (qRT-PCR), immunoblot, and IHC assays. Interestingly, we observed PYGB was highly expressed in GC tissues compared with that in nontumor tissues (Figure 1A). Immunoblot and IHC assays further confirmed the elevated PYGB level in GC tissues (Figure 1B and C). In addition, IHC assay depicted PYGB was localized in cytoplasm in GC cells.

In consideration of the different PYGB expressions in GC tissues, patients were separated into 2 groups: high PYGB level and low PYGB level (Figure 1C and Table 2). The χ² test revealed that high PYGB expression level in patients with GC was correlated with a lower overall survival rates (*P = .0289; Figure 1D). Furthermore, we analyzed the clinical association between PYGB levels in GC tissues and clinicopathological features. We assessed patient age, gender, tumor size, lymph node involvement (LNI), tumor, node, metastasis (TNM) stage, Lauren histotype, and *Helicobacter pylori* infection, respectively. We found PYGB expression level was significantly associated with tumor size (**P = .005), LNI (*P = .025), and TNM stage (**P = .005) in patients with GC (Table 2).

The PYGB protein was also highly expressed in GC cell lines HGC-27, SNU-1, AGS, and MKN45 compared with that in normal gastric epithelia cell line GES-1 cells (Figure 1E). Consistent with results in patients’ tissues, increased PYGB level was observed in GC cell lines, especially in AGS and MKN45 cells. Together, these results suggested potential oncogenic role of PYGB in GC.

**PYGB Knockdown Suppressed Proliferation of GC Cells**

To further examine the role of PYGB in the GC proliferation, we used PYGB shRNAs to knockdown PYGB level in GC cells, such as AGS and MKN45 cells. In order to ablate PYGB expression, we selected 2 effective PYGB shRNA sequences and transfected AGS and MKN45 cells. We detected reduced PYGB expression using quantitative PCR assays (Figure 2A). We performed colony formation assays and CCK-8 assays to assess the impacts of PYGB knockdown on cell proliferation. Brain-type glycogen phosphorylase shRNAs significantly inhibited AGS and MKN45 cell proliferation as shown in colony formation and the optical density value at 490 nm wavelength through colony formation and CCK-8 assays, respectively (Figure 2B and C).
PYGB Knockdown Inhibited EMT in GC Cells

Epithelial–mesenchymal transformation is a critical process in cancer progression, which results in the acquisition of invasive and metastatic properties. To investigate the effects of PYGB on EMT in GC cells, we examined the expression of EMT-associated markers in both AGS and MKN45 cells using immunofluorescence. Notably, PYGB knockdown led to the upregulation of epithelial cell markers E-cadherin level and downregulation of mesenchymal markers Vimentin in PYGB-depleted cells (Figure 3). Notably, E-catenin was strongly higher in the cell membrane of PYGB-depleted cells than that in control cells (Figure 3). As shown in immunofluorescent staining, loss of PYGB inhibited EMT in GC cells.

PYGB Promoted the Migration and Invasion of GC Cells

Based on our previous results, we further explored PYGB effects on cell migration and invasion of GC cells. Wound healing assays and transwell assays were performed. As indicated in Figure 4A, the wound was wider in PYGB-silencing cells (nonsense control [NC] versus knock down [KD]) both in AGS and MKN45 cells confirming the repression effects of PYGB ablation on cell migration. Moreover, through transwell
assay, we found cell density was reduced upon PYGB ablation, suggesting PYGB depletion significantly blocked cell invasion (Figure 4B). Taken together, our results demonstrated the critical role of PYGB in GC cell migration and invasion.

**PYGB Regulated GC Progression via Wnt/β-Catenin Signaling Pathway**

To further clarify the mechanism underlying PYGB-mediated GC progression, we screened possible signaling pathways related to PYGB regulation in cancer. Interestingly, previous studies reported PYGB-regulated Wnt/β-catenin signaling promoted ovarian cancer progression,25 we hypothesized PYGB might also regulate GC through Wnt/β-catenin signaling. We then validated the association between PYGB and Wnt/β-catenin signaling pathway using AGS and MKN45 cells transiently overexpressed control or PYGB shRNA. As demonstrated in Figure 5A, PYGB ablation remarkably reduced β-catenin, TCF-4, and c-Myc levels assessed by qRT-PCR. Immunoblot assay also depicted decreased β-catenin, TCF-4, and c-Myc protein levels in PYGB knockdown cells (Figure 5B). Taken together, these findings suggested PYGB modulated GC progression by activating Wnt/β-catenin signaling pathway.

**PYGB Facilitated Tumor Growth and Metastasis of GC Cells In Vivo**

Given we observed that PYGB silencing inhibited proliferation, migration, and invasion of GC cells, we further detected...
the potential functions of PYGB in gastric tumor growth in vivo.

AGS and MKN45 cells expressing PYGB shRNA were selected for PYGB stable depletion cell line using puromycin. Then stable cell lines (control and PYGB shRNA) were administered into nude mice, respectively. Tumor growth in these 2 groups were monitored every 3 day: 18 days postinjection, tumors were isolated, weighted, and photographed (Figure 6A). And tumor growth curves were analyzed. We noticed obviously smaller tumor volume in PYGB ablation mice than that in control mice (Figure 6A). And the tumor weight in PYGB-depleted group was lighter. The IHC assay displayed decreased density of PYGB, Ki67, and β-catenin, and elevated E-cadherin level, which were consistent with our former assays (Figure 6B).

Furthermore, we assessed metastasis ability in each group. Lung tissues from 2 groups were isolated, photographed, and stained with HE. We observed decreased lung metastasis in PYGB-depleted group (Figure 6C). In conclusion, data obtained in vivo suggested PYGB facilitated tumor growth and metastasis of GC cells.

PYGB Altered EMT of GC Cells Through WNT Pathway

We further investigated whether PYGB knockdown could induce changes in cell morphology and EMT through an inverted microscope. Interestingly, our data indicated that PYGB knockdown reduced intercellular mass and weakened the spindle morphology, confirming the effects of PYGB on EMT in GC cells (Figure 7A). Additionally, we further detected whether WNT signaling pathway regulated PYGB-dependent EMT. A specific inhibitor targeted WNT pathway, FH535, was applied to GC cells under PYGB depletion, and expression levels of proteins including β-catenin, c-Myc, proliferating cell nuclear antigen, E-cadherin, and matrix metalloproteinase 2 were detected. Consistently with the previous findings, we found FH535 treatment rescued PYGB-mediated changes of EMT biomarkers (Figure 7B), suggesting PYGB regulated EMT of GC cells via WNT pathway.

Discussion

Gastric cancer exhibits a high incidence and poor prognosis in Eastern Asian countries, particularly in China. The high distal
metastasis and chemotherapy resistance result in high mortality of patients with GC.3,27 It is therefore urgently to develop novel therapeutic strategies and identify new pharmaceutic targets. In decades, a series of breakthroughs have been achieved in the field of nanomaterial development for GC.28,29 In addition, considerable efforts have been devoted to targeted therapy with the aim to improve the prognosis. Importantly, in this study, we observed significantly elevated expression of a glycogen phosphorylase named PYGB in human GC tissues at messenger RNA and protein levels. According to clinical-pathological analysis of patients with GC, we further found the correlation between tumor size, LNI, TNM stage, and PYGB expression levels, indicating PYGB might be considered as a novel therapeutic target for the treatment of GC. Through a series of in vitro assays, we discovered PYGB affected the proliferation, migration, invasion, and EMT of GCs, suggesting the influence on GC progression. Similar to our findings, multiple studies have demonstrated the involvement of PYGB in cancer progression and metastasis.14,23-24 Breast cancers utilize hypoxic glycogen stores via PYGB to stimulate metastatic phenotypes.14 In prostate cancer, PYGB depletion inhibited tumor growth and stimulated the apoptosis of cancer cells.9 Brain-type glycogen phosphorylase small interfering RNA suppressed the cell proliferation in osteosarcoma.12 These studies, together with our findings, confirmed the universal role of PYGB in progression of various types of cancers. However, the precise mechanisms remained unclear.

Previous studies reported PYGB knockdown contributed to the apoptosis of prostate cancer cells by mediating cleaved-poly (ADP-ribose) polymerase, cleaved-caspase-3, Bax, and Bcl-2 expression levels.9 Another study also reported PYGB could suppress ROS production and further inhibiting the apoptosis and necrosis in E coli cells.12 In this study, we investigated a series of subcellular events caused by PYGB depletion. In the future study, we should further clarify whether PYGB affected the apoptosis of prostate cancer cells as well as investigate the influence of PYGB on ROS production in GC cells. Here, we observed PYGB regulated progression of GC via Wnt/β-catenin pathway. Whereas in prostate cancer, PYGB was reported to regulate multiple cellular processes via NF-kB/Nrf2 pathway.13 How a glycogen phosphorylase, PYGB, mediates cancer progression and metastasis via different signaling pathways also requires further study. Given the important role of PYGB in metabolism,8,16,30 we speculated PYGB downregulation led to abnormal metabolism of tumor cells and thus inhibiting the progression of GC. Our hypothesis should be further explored in the future.

Figure 6. Brain-type glycogen phosphorylase facilitates tumor growth and metastasis of gastric cancer cells in vivo. A, Representative image of tumor and tumor growth curve in control or PYGB depleted AGS cells group were shown. Tumor volume and weight displayed relatively smaller tumor in PYGB depleted group. B, PYGB, Ki67, E-cadherin, and β-catenin expression levels in excised xenograft tumor tissues in each group were analyzed by IHC analysis. C, Lung tissues in AGS cells infected with control or PYGB reduction were isolated, photographed, and calculated. And HE staining was conducted in each group (mean ± SD, **P < .01). HE indicates hemotoxylin and eosin; IHC, immunohistochemistry; PYGB, brain-type glycogen phosphorylase; SD, standard deviation.
Figure 7. Brain-type glycogen phosphorylase regulated EMT of gastric cancer cells through WNT pathway. A, The morphological differences of gastric cancer cells between the control group and the PYGB knockdown group were obtained by inverted microscope. Scale bar indicates 100 μm. B, The expression of the indicated proteins upon the treatment of control shRNA, PYGB shRNA, and the combination of PYGB shRNA and FH535 of AGS and MKN45 cells was detected through immunoblot assays. Relative expression was quantified (mean ± SD, ****P < .001). EMT indicates epithelial–mesenchymal transformation; PYGB, brain-type glycogen phosphorylase; SD, standard deviation; shRNA, short hairpin RNA.
In summary, we found significant elevated PYGB levels in human GC tissues. We discovered PYGB expression level was positively correlated with clinical characteristics including tumor size, LNI, and TNM stage. Our findings further indicated PYGB modulated proliferation, migration, invasion, and EMT of GC cells via Wnt/β-catenin signaling pathway in vitro and promoted tumor growth of GC cells in mice. These findings shed light on PYGB as a novel promising therapeutic target for deciphering pathogenesis of GC, providing new insights into development of new strategies in diagnosis and treatment from bench to clinic.

Authors’ Note
CL conceived and designed the experiments. BNX analyzed and interpreted the results of the experiments. KZ performed the experiments. All data generated or analyzed during this study are included in this published article. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (approval no. 20170618). All patients provided written informed consent prior to enrollment in the study.

Declaration of Conflicting Interests
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