Original Article Phosphoglycerate kinase 1 gene knockdown suppresses the migration and invasion of glioma cells

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Abstract: Phosphoglycerate kinase 1 (PGK1), a glycolytic enzyme, is overexpressed in several carcinomas and plays an important role in their malignancy. However, expression and function of PGK1 in gliomas are unknown. In the present study, we determined PGK1 protein expression in different World Health Organization (WHO) grade glioma tissues and normal brain tissues by performing immunohistochemical analysis. Small-interfering RNA against PGK1 (si-PGK1) was synthesized and was transiently transfected into 2 human glioblastoma cell lines U-87 MG and U-118 MG. PGK1 mRNA and protein expression after transfection were detected by performing quantitative reverse transcription-PCR (qRT-PCR) and western blotting, respectively. Effect of PGK1 knockdown on the migration and invasion of U-87 MG and U-118 MG cells was determined by performing transwell assay. Further, effect of PGK1 knockdown on the protein expression of beta-catenin and chemokine receptor 4 (CXCR4) was determined by performing western blotting. Immunohistochemical analysis detected high PGK1 protein expression in different WHO grade gliomas. Moreover, upregulated PGK1 protein expression was significantly associated with gliomas having advanced WHO grades. Si-PGK1 transfection successfully silenced PGK1 mRNA and protein expression in U-87 MG and U-118 MG cells, as confirmed by performing qRT-PCR and western blotting, respectively. PGK1 knockdown also suppressed the migration and invasion of U-87 MG and U-118 MG cells and decreased the protein expression of beta-catenin and CXCR4, which promote the invasion and metastasis of many carcinomas. Thus, these results suggested that PGK1 played an oncogenic role in gliomas and could serve as a biomarker for predicting the metastatic progression of gliomas.

Keywords: PGK1, glioma, invasion, migration

Introduction

Gliomas, the most common and aggressive type of primary brain tumours, account for approximately 30% of all brain and central nervous system tumours and 80% of all malignant brain tumours [1]. The most common classification system for gliomas is the World Health Organization (WHO) classification system that classifies gliomas into grades I-IV; this grade is further stratified into well-differentiated lowgrade glioma (WHO grade I-II) and high-grade glioma (WHO grade III-IV) [2]. The overall prognosis of gliomas is closely associated with their WHO grade [3]. High-grade gliomas include anaplastic astrocytoma and glioblastoma multiforme [2] and are associated with poor prognosis even after treatment with combined therapies, including surgery followed by concomitant radiotherapy and temozolomide-based chemotherapy [4]. The median overall survival of patients with anaplastic gliomas (WHO grade III) is approximately 3 years and that of patients with glioblastoma multiforme is poor, i.e., only ~15 months [5]. The major reason underlying the failure of glioma treatment is the invasive nature of glioma cells [4]. Gliomas are complex tumours caused by interactions amongst multiple genes and involve the activation of many oncogenes and inactivation of tumour suppressor genes [6]. Therefore, early diagnosis and novel therapeutic approaches are urgently required for treating gliomas.

Phosphoglycerate kinase 1 (PGK1) is a glycolytic enzyme that catalyzes the conversion of 1, 3-diphosphoglycerate to 3-phosphoglycerate. PGK1 is overexpressed in several carcinomas, including breast, ovarian, pancreatic, colon, and gastric carcinomas, and in neuroblastomas

and plays an important role in their malignancy [7-12]. In vivo and in vitro findings have suggested that PGK1 is crucially involved in the growth and metastasis of malignant gastric tumours and can serve as a prognostic marker and/or be of therapeutic value for preventing the metastasis of these tumours [13]. PGK1 is overexpressed in neuroblastoma cells, and its expression is significantly positively correlated with chemokine receptor 4 (CXCR4) expression, tumour metastasis to the bone marrow, and poor survival [11]. Several proteins associated with PGK1 signalling have been identified, including beta-catenin [14] and CXCR4 [11, 14], which promote the invasion and metastasis of many carcinomas [12, 13]. However, expression and function of PGK1 in gliomas are unknown.

Here, we determined PGK1 expression levels in WHO grades I-IV gliomas by performing immunohistochemical analysis. Our results showed that PGK1 expression level increased with an increase in the WHO grade of gliomas and was significantly upregulated in gliomas of advanced WHO grade. *PGK1* knockdown suppressed the migration and invasion of human glioblastoma cell lines U-87 MG and U-118 MG and decreased the protein expression of CXCR4 and beta-catenin. These findings suggested that PGK1 could serve as a biomarker for predicting the metastatic progression of gliomas and/or as a therapeutic target for treating patients with metastatic gliomas.

Materials and methods

Patients and tissue specimens

Paraffin-embedded tissue samples from 36 patients with WHO grades I-IV gliomas were obtained from the archives of the Department of Pathology, First Affiliated Hospital, Jinan University, Guangzhou, China, between 2012 and 2015. Patients receiving adjuvant immunosuppressive treatment, including chemotherapy or radiotherapy before operation, were excluded from the study. Of the 36 tumour samples obtained, 18 samples were categorized as WHO grades I-II gliomas and remaining 18 samples were categorized as WHO grades III-IV gliomas. Surgically resected brain tissues of 6 trauma patients admitted to the hospital during the same period were used as controls (normal brain, NB). Absence of gliosis was confirmed pathologically in all the patients. The final study protocol was approved by the Ethics Committee of our hospital, and informed consent was obtained from each patient included in the study.

Immunohistochemical analysis

Slides were dried overnight at 37°C, dewaxed in xylene, rehydrated using a graded alcohol series, and immersed in 3% hydrogen peroxide methanol solution for 15 minutes to block endogenous peroxidase activity. The slides were then pretreated with an antigen retrieval buffer [citrate buffer (pH 6.0)] at 100°C for 2 minutes and were incubated with 10% normal goat serum at room temperature for 10 minutes to decrease non-specific reactivity. Next, the slides were incubated overnight with anti-PGK1 rabbit polyclonal antibody (ab154613; Cambridge, MA, USA) at 4°C. The slides were then rinsed 3 times with 0.01 M phosphatebuffered saline (PBS, pH 7.4) for 10 minutes, and the primary antibodies were detected using a secondary antibody (Envision; Dako, Glostrup, Denmark) for 30 min at 37°C. Next, the slides were washed with PBS and were stained with 3,3'-diaminobenzidine. Finally, the slides were counterstained using Mayer's hematoxylin, dehydrated, and mounted. Non-neoplastic brain tissues were used as controls. PGK1 expression in different WHO grade tumours was assessed by examining 10 randomised visual fields per section at 400× magnification by using Olympus light microscope (Tokyo, Japan). Five high-power fields were randomly selected per specimen. Results of immunohistochemical analysis were reviewed independently by 2 senior pathologists who were blinded to the outcome of the study. Semiquantitative assessment of target proteins was performed by consensus and involved the determination of the intensity of staining (negative, 0; light yellow, 1; brown, 2; and tan, 3) of each cell and extent of staining (ratio of the number of positive cells to the number of counted cells: ratio of 1, 25%; ratio of 2, 25%-50%; ratio of 3, 51%-75%; and ratio of 4, > 75%) of each random field. Scores for the intensity and extent of staining were multiplied to obtain a weighted score for each patient (maximum possible, 12). For statistical analysis, the weighted scores were grouped into 4 categories, with a score of 0 being considered negative; score of 1-4 (+) being considered weakly positive; and scores of 5-8 (++), 9-12 (+++), and (++)-(+++) being considered highly positive.

Cell lines and cell culture

Human glioblastoma cell lines U-87 MG and U-118 MG were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U-87 MG and U-118 MG cells were cultured in Eagle's minimum essential medium or Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μ g/mL). The cells were maintained at 37°C in a humidified 5% CO₂ incubator and were passaged when they reached 90%-95% confluence.

SiRNA synthesis and transfection

SiRNA against *PGK1* (si-*PGK1*) and scrambled siRNA (si-Scr) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For transfection, U-87 MG or U-118 MG cells were plated in a 24- or 6-well plate and were cultured for 24 h until they reached 40%-60% confluence. The cells were then transfected with 50 nM si-*PGK1* or si-Scr by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cells were collected at 48 h after the transfection for performing transwell assay or for extracting RNA or proteins.

RNA extraction and quantitative reverse transcription-PCR

Total RNA was extracted from each group of U-87 MG and U-118 MG cells by using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Next, mRNA was reverse transcribed into cDNA by using PrimeScript RT Reagent kit with cDNA Eraser (Takara, Dalian, China) in a 20-µL reaction, according to the manufacturer's protocol. Equal amounts of cDNA were used as templates for performing quantitative reverse transcription-PCR (gRT-PCR) to detect the expression level of PGK1 relative to that of the 18S rRNA gene (endogenous control). Relative PGK1 expression level was quantified using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) and the following primers: PGK1-F, 5'-CCGAGCCAGCCAAAA-TAGA-3'; PGK1-R, 5'-GCTGGATCTTGTCTGCAACT-TTAG-3': 18S rRNA-F. 5'-CCTGGATACCGCAGCT-AGGA-3'; 18S rRNA-R, 5'-GCGGCGCAATACGA-ATGCCCC-3'. All experiments were performed in duplicate and were repeated 3 times. Fold induction of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

Western blotting

U-87 MG and U-118 MG cells in each group were washed twice with ice-cold PBS and were resuspended in ice-cold RIPA buffer containing 1 mmol/L phenylmethanesulfonyl fluoride and a cocktail of protease inhibitors (dilution, 1:100; Beyotime, Nantong, China). The cell samples were centrifuged at 14,000 rpm and 4°C for 20 min. Supernatants were recovered, and total proteins were quantified using BCA Protein Assay kit (Beyotime). Next, 30 µg protein were loaded and resolved on 10% SDSpolyacrylamide gels and were transferred onto PVDF membranes (Pall, New York, NY, USA), The membranes were blocked with 5% non-fat milk in TBS containing 0.05% Tween-20 (TBST) for 1 h at 37°C and were incubated overnight with anti-PGK1 rabbit polyclonal antibody (1:1000), anti-PGK1 CXCR4 monoclonal antibody (1:100), and anti-beta catenin rabbit monoclonal antibody (1:6000) (all from abcam, Cambridge, MA, USA) at 4°C. The membranes were then washed 3 times with TBST, incubated with secondary antibody for 40 min at 37°C, and visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) after washing 3 times with TBST. GAPDH was used as an internal loading control. The membranes were scanned, and densitometric analysis was performed using Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Specific bands were quantified by drawing same-sized squares around each band to measure its density. Next, value was adjusted using the density of the background around each band. Results of densitometric analysis were expressed as the relative ratio of the target protein to the reference protein.

Transwell migration and invasion assays

Cell migration and invasion were assessed by performing transwell assays. For cell migration assay, U-87 MG and U-118 MG cells were harvested at 24 h after the transfection, and 5 × 10^4 cells in 200 µL 0.1% serum medium were placed in the upper chamber of an insert (pore size, 8 µm; Becton Dickinson Labware). The lower chamber was filled with 10% foetal bovine serum (600 µL). For cell invasion assay, the same density of cells was placed in the upper transwell chambers precoated with Matri-



Figure 1. A. Representative sections showing PGK1 protein expression in NB tissues and glioma tissues belonging to different WHO grades (×400) (a, NB; b, WHO grades I-II gliomas; c, WHO grades III-IV gliomas). B. Immunohistochemical analysis showed that overall PGK1 protein expression was significantly higher in glioma tissues belonging to WHO grades III-IV than in glioma tissues belonging to WHO grades I-II and NB tissues.

		PGK1 protei		
Characteristic	n	Low expression (-, +)	High expression (++, +++)	P value
Age (years)				0.348
< 55	17	8	9	
≥ 55	19	9	10	
Gender				0.299
Male	16	7	9	
Female	20	11	9	
Tumour diameter (cm)				0.152
< 5	15	7	8	
≥5	21	9	12	
WHO grade				0.010
1-11	18	12	6	
III-IV	18	5	13	

Table 1. Correlation between PGK1 protein expression and
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gel (BD Biosciences). After 24 h of incubation, the cells in the upper chamber of the filter were removed using a cotton swab. Cells on the underside of the filter were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet in 20% ethanol, and counted from 5 randomly selected fields by using a phase-contrast micro-

scope. Migrated cells were photographed at 200× magnification with Olympus light microscope from 5 independent fields in each well. Both the assays were performed in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (IBM, Chicago, IL, USA). Results are presented as mean \pm standard deviation (SD). Independent *t*-test was used to compare means in different groups. *P* values of < 0.05 were considered statistically significant.

Results

PGK1 expression in gliomas of different grades

PGK1 protein expression in different WHO grade glioma tissues and NB tissues was determined by performing immunohistochemical analysis. High PGK1 protein expression was



Figure 2. *PGK1* mRNA and protein levels in U-87 MG and U-118 MG cells transfected with si-*PGK1* and si-Scr. A. *PGK1* mRNA levels in U-87 MG and U-118 MG cells transfected with si-*PGK1* and si-Scr, as detected by performing qRT-PCR. B. Graphs showing PGK1 protein levels in U-87 MG and U-118 MG cells transfected with si-*PGK1* and si-Scr, as detected by performing western blotting. C. Bar graphs showing fold increase in PGK1 protein expression in each group; **P* < 0.05 compared with si-Scr.

observed in advanced WHO grade gliomas (Figure 1A). Weakly positive (+) PGK1 expression was detected in only 14% NB tissues (Figure 1B). Percentages of negative, weakly positive (+), detectably positive (++), and strongly positive (+++) PGK1 protein expression in gliomas of WHO grades I-II were 35%, 30%, 20%, and 15%, respectively (Figure 1B). Percentages of negative, weakly positive (+), detectably positive (++), and strongly positive (+++) PGK1 protein expression in gliomas of WHO grades III-IV were 10%, 5%, 35%, and 50%, respectively (Figure 1B). These results indicated that PGK1 protein expression increased with an increase in the WHO grades of tumours.

Association of PGK1 protein expression with clinicopathological characteristics of patients with gliomas

Table 1 summarizes the association between PGK1 protein expression and clinicopathological characteristics of patients with gliomas. PGK1 protein expression was significantly upregulated in gliomas of advanced WHO grade (*P* < 0.01; **Table 1**). No significant association was observed between PGK1 protein expression and age and gender of patients and tumour diameter (*P* > 0.05 for all; **Table 1**).

Si-PGK1 successfully silenced PGK1 mRNA and protein expression in human glioblastoma cell lines

To attenuate *PGK1* expression, si-*PGK1* was synthesized and transiently transfected into

the 2 human glioblastoma cell lines U-87 MG and U-118 MG and the cells were cultured for 48 h. *PGK1* mRNA expression was successfully downregulated in si-*PGK1*-transfected U-87 MG and U-118 MG cells compared with that si-Scr-transfected U-87 MG and U-118 MG cells (**Figure 2A**). Moreover, PGK1 protein expression was successfully downregulated in si-*PGK1*-transfected U-87 MG and U-118 MG cells compared with that in si-Scr-transfected U-87 MG and U-118 MG cells (**Figure 2B** and **2C**). These results indicated that si-*PGK1* successfully silenced *PGK1* mRNA and protein expression and was suitable for use in subsequent assays.

PGK1 knockdown suppressed the migration of human glioblastoma cell lines.

The role of PGK1 in regulating the migration of human glioblastoma cell lines U-87 MG and U-118 MG was determined by performing a transwell assay of si-PGK1- or si-Scr-transfected U-87 MG and U-118 MG cells. Significantly less number of si-PGK1-transfected U-87 MG cells passed through the membrane into the lower chamber than si-Scr-transfected U-87 MG cells (Figure 3A). The number of migrating si-Scr- and si-PGK1-transfected U-87 MG cells was 225 \pm 24 and 132 \pm 25, respectively (Figure 3B). The effect of si-PGK1 or si-Scr transfection on the migration of U-118 MG cells was similar to that on the migration of U-87 MG cells (Figure 3C). The number of migrating si-Scr- and si-PGK1-transfected U-118 MG cells was 198 \pm 11 and 105 \pm 30, respectively (Figure 3D).



Figure 3. Effect of *PGK1* knockdown after si-*PGK1* or si-Scr transfection on the migration of U-87 MG and U-118 MG cells. A. Representative images of the migration of U-87 MG cells. B. Average number of migrating U-87 MG cells per field in the indicated experimental groups. C. Representative images of the migration of U-118 MG cells. D. Average number of migrating U-118 MG cells per field in the indicated experimental groups. C. Representative images of the migration of U-118 MG cells. D. Average number of migrating U-118 MG cells per field in the indicated experimental groups. Migration of U-87 MG and U-118 MG cells was measured by performing transwell assay at 48 h after si-*PGK1* or si-Scr transfection. Data are presented as mean \pm SD; **P* < 0.05.

PGK1 knockdown suppressed the invasion of human glioblastoma cell lines

The role of *PGK1* in regulating the invasion in human glioblastoma cell lines U-87 MG and U-118 MG was determined by performing Matrigel-transwell assay after si-PGK1 or si-Scr transfection. Significantly less number of si-PGK1-transfected U-87 MG cells passed through the Matrigel-coated membrane into the lower chamber than si-Scr-transfected U-87 MG cells (Figure 4A). The number of invasive si-Scrand si-PGK1-transfected U-87 MG cells was 118 ± 18 and 62 ± 20 , respectively (Figure 4B). The effect of si-PGK1 or si-Scr transfection on the invasion of U-118 MG cells was similar to that on the invasion of U-87 MG cells (Figure 4C). The number of invasive si-Scr- and si-PGK1-transfected U-118 MG cells was 236 ± 20 and 72 ± 18, respectively (Figure 4D).

PGK1 knockdown decreased the protein expression of beta-catenin and CXCR4

To investigate the mechanisms underlying the regulation of the migration and invasion of U-87

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MG and U-118 MG cells by PGK1, protein expression of beta-catenin and CXCR4, which promote the invasion and metastasis of many carcinomas [11, 14], was determined by performing western blotting. Protein expression levels of beta-catenin and CXCR4 were obviously decreased after si-*PGK1* transfection compared with those after si-Scr transfection in both U-87 MG and U-118 MG cells (**Figure 5**).

Discussion

Gliomas are the most common and aggressive type of brain tumours because of the highly invasive nature of glioma cells. A single glioma cell can invade the brain and often produce secondary lesions at sites distant from the primary tumour [15], thus decreasing the efficacy of surgical resection [16, 17]. Therefore, determining mechanisms underlying the aggressiveness of glioma cells could identify potential targets for effectively treating gliomas. The glycolytic enzyme PGK1 is overexpressed in several malignant tumours, and increased expression of PGK1 is associated with the metastasis of



Figure 4. Effect of *PGK1* knockdown on the invasion of U-87 MG and U-118 MG cells after si-*PGK1* or si-Scr transfection. A. Representative images of the invasion of U-87 MG cells. B. Average number of invasive U-87 MG cells per field in the indicated experimental groups. C. Representative images of the invasion of U-118 MG cells. D. Average number of invasive U-118 MG cells per field in the indicated experimental groups. Invasion of U-87 MG and U-118 MG cells was measured by performing Matrigel-transwell assay at 48 h after si-*PGK1* or si-Scr transfection. Data are presented as mean \pm SD; **P* < 0.05.



Figure 5. Protein expression levels of beta-catenin and CXCR4 in both U-87 MG and U-118 MG cells after *si-PGK1* and si-Scr transfection. A. Graphs showing beta-catenin and CXCR4 protein levels after *si-PGK1* and si-Scr transfection, as determined by performing western blotting. B. Graphs showing a fold increase in the protein expression levels of beta-catenin in each group. C. Graphs showing a fold increase in the protein expression levels of CXCR4 in each group. *P < 0.05 compared with si-Scr.

colon and gastric cancers [7-12, 14]. However, the role of PGK1 in gliomas is unclear. Therefore, the present study investigated *PGK1* protein expression and impact of *PGK1* knockdown on the invasion and metastasis of glioma cells in vitro.

We found that PGK1 was overexpressed in gliomas of different grades and that PGK1 protein expression was significantly upregulated in gliomas of advanced WHO grade, which was consistent with the findings of previous studies. Increased PGK1 expression has also been detected in breast, ovarian, pancreatic, colon, and gastric carcinomas and in neuroblastomas [7-12]. We also investigated the role of PGK1 in the invasion and metastasis in 2 human glioblastoma cell lines U-87 MG and U-118 MG. We

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observed that PGK1 knockdown suppressed the migration and invasion of both U-87 MG and U-118 MG cells. The function of PGK1 were also been investigated in other malignancies. PGK1 overexpression dramatically increased the invasiveness of gastric cancer cells [14]. Moreover, PGK1 overexpression promoted the progression and metastasis of gastric cancer in a mouse model [13]. In addition, PGK1 expression promoted the radioresistance of a human glioma cell line U251 [18]. In Lewis lung carcinoma cells, PGK1 overexpression decreased COX-2 expression, thus affecting PGE2 expression, cell invasion, angiogenesis, and immune functions and inhibiting tumour progression [19]. Our results indicated that PGK1 played an oncogenic role in gliomas.

Further, we found that PGK1 knockdown decreased the protein expression of beta-catenin and CXCR4. Beta-catenin is a key downstream component of canonical Wnt signalling pathway and plays an important role in regulating tumour cell invasion and metastasis [20]. The relationship between PGK1 and beta-catenin has also been investigated in gastric cancer. A study showed that PGK1 overexpression increased beta-catenin levels in gastric cancer cell line MKN45 [14]. CXCR4 is a 7transmembrane G protein-coupled chemokine receptor, and CX-CR4 overexpression in cancer cells contributes to their invasion and metastasis [21]. The relationship between PGK1 and CXCR4 has also been investigated in gastric and cervical cancers. Some studies have shown that PGK1 overexpression increases CXCR4 levels [14, 22]. Therefore, our result that PGK1 regulates the protein expression of beta-catenin and CXCR4 is in accordance the results of previous studies. However, specific mechanisms underlying the regulation of beta-catenin and CXCR4 by PGK1 should be investigated in further studies.

In conclusion, PGK1 protein expression was significantly upregulated in gliomas of advanced WHO grade. Moreover, *PGK1* knockdown suppressed the migration and invasion of human glioblastoma cell lines and decreased the protein expression of beta-catenin and CXCR4, which promote the invasion and metastasis of many carcinomas [11, 14]. Thus, these results indicated that PGK1 played an oncogenic role in gliomas.

Disclosure of conflict of interest

None.

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