

The PDK1/c-Jun pathway activated by TGF- β induces EMT and promotes proliferation and invasion in human glioblastoma

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Abstract. Glioblastoma multiforme (GBM) is the most common primary malignant tumor affecting the human brain. Despite improvements in therapeutic technologies, patients with GBM have a poor clinical result and the molecular mechanisms responsible for the development of GBM have not yet been fully elucidated. 3-phosphoinositide dependent protein kinase 1 (PDK1) is upregulated in various tumors and promotes tumor invasion. In glioma, transforming growth factor- β (TGF- β) promotes cell invasion; however, whether TGF- β directly regulates PDK1 protein and promotes proliferation and invasion is not yet clear. In this study, PDK1 levels were measured in glioma tissues using tissue microarray (TMA) by immunohistochemistry (IHC) and RT-qPCR. Kaplan-Meier analyses were used to calculate the survival rate of patients with glioma. *In vitro*, U251 and U87 glioma cell lines were used for functional analyses. Cell proliferation and invasion were analyzed using siRNA transfection, MTT assay, RT-qPCR, western blot analysis, flow cytometry and invasion assay. *In vivo*, U251 glioma cell xenografts were established. The results revealed that PDK1 protein was significantly

upregulated in glioma tissues compared with non-tumorous tissues. Furthermore, the higher PDK1 levels were associated with a large tumor size (>5.0 cm), a higher WHO grade and a shorter survival of patients with GBM. Univariate and multivariate analyses indicated that PDK1 was an independent prognostic factor. *In vivo*, PDK1 promoted glioma tumor xenograft growth. *In vitro*, functional analyses confirmed that TGF- β upregulated PDK1 protein expression and PDK1 promoted cell migration and invasion, and functioned as an oncogene in GBM, by upregulating c-Jun protein and inducing epithelial-mesenchymal transition (EMT). c-Jun protein were overexpressed in glioma tissues and positively correlated with PDK1 levels. Moreover, our findings were further validated by the online Oncomine database. On the whole, the findings of this study indicate that in GBM, PDK1 functions as an oncogene, promoting proliferation and invasion.

Introduction

The World Health Organization (WHO) has published criteria for the classification and malignancy grade of glioma as being pilocytic (grade I), diffuse (grade II), anaplastic (grade III) and glioblastoma multiforme (GBM; grade IV) (1). GBM is the most malignant tumor affecting the human brain, and the overall prognosis remains very poor. The median survival time is <15 months (2-4). Novel biomarkers and molecular targets have yet to be identified for the improvement of the diagnosis and treatment of human GBM. However, the molecular mechanisms responsible for the development of GBM, which may lead to the identification of novel therapeutic targets, remain unclear.

3-Phosphoinositide dependent protein kinase-1 (PDK1) is a serine-threonine kinase belonging to the AGC kinase family. PDK1 is a transcriptional regulator of the PI3K signaling pathway and activates several downstream proteins (5-7). Furthermore, PDK1 regulates downstream regulators, such as protein kinase B (PKB)/Akt (6,8), rho-associated, coiled-coil-containing protein kinase 1 (ROCK1) (9), β 3 integrin (10), phospholipase C γ 1 (PLC γ 1) (11) and myotonic dystrophy kinase-related CDC42-binding kinase- α (MRCK α) (12). In

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Abbreviations: PDK1, 3-phosphoinositide dependent protein kinase 1; EMT, epithelial-mesenchymal transition; TGF- β , transforming growth factor- β ; TMA, tissue microarray; IHC, immunohistochemistry; SI, staining index; OS, overall survival; MOD, mean optical density; WHO, World Health Organization

Key words: 3-phosphoinositide dependent protein kinase 1, c-Jun, epithelial-mesenchymal transition, invasion, glioblastoma

addition, the most important evidence is derived from clinical data demonstrating that PDK1 is frequently overexpressed in different tumor types, including gallbladder cancer (13), acute myeloid leukemia (14,15), melanoma (16), esophageal squamous cell carcinoma (17) and prostate cancer (18). PDK1 knockdown has been shown to decrease proliferation and induce apoptosis in breast cancer (19) and esophageal cancer (20). PDK1 overexpression increases tumor invasiveness (12,21,22). In xenograft tumor models, PDK1 knockdown has also been shown to affect tumor growth (23) and metastasis (24-26). Notably, PDK1 overexpression is associated with a more aggressive phenotype and a worse prognosis. However, PDK1 expression in human GBM and its biological and clinical significance are not yet fully understood. The transforming growth factor- β (TGF- β) signaling pathway plays an important role in many cancer types, as it promotes the proliferation of malignant tumor cells and promotes migration and invasion by inducing epithelial-mesenchymal transition (EMT) (27). However, the mechanisms through which glioma cells acquire the ability to take advantage of the TGF- β tumor-promoting effects remain elusive. Previous studies have suggested that c-Jun and JunB share extensive homology within the leucine zipper and basic domains, and JunB inhibits cell proliferation and migration by antagonizing c-Jun activity (28,29). Previous findings have also suggested that increasing c-Jun transcriptional activity induces glioma progression (30). Thus, we wished to determine whether PDK1 directly regulates c-Jun to induce EMT in human GBM.

In this study, we found that PDK1 was overexpressed in glioma tissues and was positively associated with glioma grade. The 5-year survival rate of patients with glioma with a high PDK1 expression was significantly lower than that of those with a low PDK1 expression. Cox regression analysis revealed that PDK1 may be used as an independent prognostic factor for patients with glioma. *In vivo*, PDK1 promoted glioma tumor xenograft growth. *In vitro*, further analyses suggested that TGF- β upregulated PDK1 protein expression and PDK1 promoted cell proliferation, migration and invasion, and functioned as an oncogene in malignant glioma, by upregulating c-Jun protein and inducing EMT. Furthermore, our findings were further validated by the online Oncomine database. In Oncomine database, PDK1 and c-Jun proteins were overexpressed in human glioma. Taken together, these results suggest that PDK1 is positively associated with EMT and that the upregulation of c-Jun protein PDK1 accelerates GBM cell invasion. Thus, PDK1 inhibition may prove to be a potential therapeutic strategy for the treatment of GBM.

Materials and methods

Tissue microarrays and cell lines. All tumor tissues were obtained from surgery, and were immediately frozen in liquid nitrogen and stored at -80°C until processed. The construction of tumor tissue microarrays (TMAs) have been described previously (31). In total, 6 non-tumorous and 113 paraffin-embedded tissues from patients with glioma of grade I to IV were used, at the Sun Yat-Sen Memorial Hospital from January, 2005 to January, 2011. Patients with glioma, and with clinicopathological characteristics and follow-up information available, were included. Tissues with lost cores

or insufficient cells were excluded from this study. The TMA consisted of 2007 WHO glioma tissues graded as follows: Grade I (n=10), grade II (n=18), grade III (n=36) and grade IV (n=49) samples. This study was performed in accordance with the policies of the Institutional Research Ethics Committee of Sun Yat-Sen Memorial Hospital. Written informed consent was obtained from the study participants at the Sun Yat-Sen Memorial Hospital of Guangzhou City.

The human U87 (glioblastoma of unknown origin) and U251 glioma cell lines and were purchased from the American Type Culture Collection (ATCC). Furthermore, a previous study suggested that the U87 cell line may be misidentified (32). Of note, however, the U87 cell line was authenticated before use in this study. In this study, PCR amplification of the cell samples was carried out using the STR Multi-amplification kit, (DC2101, Promega, Madison, WI, USA) and the data revealed that the sample cell was the U87 MG cell line based on the ATCC database. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO_2 humidified atmosphere.

Immunohistochemistry (IHC) staining. PDK1, c-Jun, β -catenin and E-cadherin protein expression levels were analyzed by IHC on paraffin-embedded tissue samples as previously described (33,34). Briefly, the specimens were cut into 5- μm -thick sections and baked at 65°C for 30 min. The sections were deparaffinized and antigenic retrieval. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity followed by incubation with 1% bovine serum albumin to block the non-specific binding. PDK1 (ab52893; 1:100 dilution; Abcam, Cambridge, MA, USA), c-Jun (ab32137; 1:200 dilution; Abcam), β -catenin (ab32572; 1:500 dilution; Abcam) and E-cadherin (ab15148; 1:30 dilution; Abcam) antibodies was incubated with the sections overnight at 4°C , respectively. After washing, the tissue sections were treated with biotinylated secondary antibody for 60 min at room temperature. After rinsing with PBS, the slides were immersed for 3-5 min in DAB (3, 3'-diaminobenzidine) (Sigma, St. Louis, MO, USA) solution, then monitored under a microscope. The reaction was terminated with distilled water. The slides were then counterstained with hematoxylin, dehydrated and coverslipped.

Quantification of staining analysis. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was viewed and scored separately by two experienced pathologists, and the scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining as previously described (33,34).

Isolation of total RNA and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the frozen samples and cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions as previously described (35). RNA was treated with RNase-free DNase I (Roche, Basel, Switzerland). The BcaBest RNA PCR kit (Takara, Dalian, China) was then used to synthesize the cDNA according to the manufacturer's instructions. All primers were synthesized by

Shanghai GenePharma Co., Ltd. (Shanghai, China). Quantitative PCR (qPCR) was carried out using the Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with Real-time PCR Master Mix (SYBR-Green). PCR reactions were performed under the following conditions: Pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min and elongation at 72°C for 10 min. β -actin was used as an internal control. The sequences of the primers used for qPCR are listed as follows: PDK1 forward, 5'-TCAGGACGACGA GAAGCTGTAT-3' and reverse, 5'-AACGTACTGCGCTGTT CC CACG-3'; c-Jun forward, 5'-AGAGCGACGCGAGCC AAT-3' and reverse, 5'-GAGCCCTTATCCAGCCCCGAG-3'. Snail forward, 5'-ATGCCGCGCTCTTCTCGTCAG-3' and reverse, 5'-CCTCGAGGCTCAGCGGGACAT-3'; E-cadherin forward, 5'-GTTACTGATTGGTCTACGAGA-3' and reverse, 5'-ATTGAAATGATCCAGTGCTTG-3'; N-cadherin forward, 5'-TCATGAAATCAACTATGCAAACCC-3' and reverse, 5'-ATTGAAATGATCCAGTGCTTG-3'; ZEB1 forward, 5'-GCACAACCAAGTGCAGAAGA-3' and reverse, 5'-CAT TTGCAGATTGAGGCTGA-3'; TWIST1 forward, 5'-GTCC GGAGTGTTACGAGGAG-3' and reverse, 5'-TGGAGGACCT GGTAGAGGAA-3' and β -actin forward, 5'-CTCCATCCTGG CCTCGCTGT-3' and reverse, 5'-GCTGTCACCTTCAC CGTTCC-3'. The $2^{-\Delta\Delta C_q}$ method was used to quantify the relative mRNA amount (36).

Western blot analysis. For western blot assays, the total cell lysates were prepared in high KCl lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 300 mM KCl, 1 mM EDTA, 0.5% Triton X-100 and 0.5% sodium deoxycholate) with complete protease inhibitor cocktail (Roche Molecular Diagnostics, Branchburg, NJ, USA). The protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Thirty micrograms of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (Roche, Branchburg, NJ, USA). The membranes were treated with 1% blocking solution in TBS for 2 h, the membranes were incubated with primary antibodies anti-PDK1 (ab52893; 1:1,000 dilution; Abcam), c-Jun (ab32137; 1:2,000 dilution; Abcam), Snail (ab82846; 1:300 dilution; Abcam), β -catenin (ab32572; 1:5,000 dilution; Abcam), E-cadherin (ab15148; 1:500 dilution; Abcam) and β -actin (PR0255; 1:2,000 dilution; Zhongshan Jinqiao Company, Beijing, China), at 4°C overnight, followed by anti-rabbit secondary antibody conjugated with HRP (1:5,000; Epitomics, Burlingame, CA, USA) for 2 h. The immunolabeled proteins were detected by BM Chemiluminescence Western Blotting kit (Roche, Branchburg, NJ, USA). The quantification of the western blots was obtained by multiplying the area and intensity of each band using Image J software (NIH, Bethesda, MD, USA).

Lentivirus production and transduction. The PDK1 sequence was amplified from normal human genomic DNA and constructed into the lentivirus expression vector pWPXL (Telebio Biomedical, Shanghai, China) to generate pWPXL-PDK1. pWPXL-PDK1 was transfected into 293T cells (ATCC, Manassas, VA, USA) using Lipofectamine 2000

(Invitrogen). The recombinant lentiviruses were harvested from the supernatant of cell cultures at 48 h post-transfection. The U251 and U87 cells were infected with the recombinant lentivirus-transducing units plus 6 mg/ml Polybrene (Sigma, St. Louis, MO, USA).

RNA interference. The selected siRNA targeting PDK1 was used in this study. The sequences were as follows: siRNA-1 duplex (sense strand, 5'-GGUUGUUGUUGGAGAAGCATT-3' and antisense strand, 5'-UGCUUCUCCAACAACAACCTT-3') and siRNA-2 duplex (sense strand, 5'-GGUCAGUAGUCUUG UAAUATT-3' and antisense strand, 5'-UAUUACAAGACUA CUGACCTT-3'). The siRNAs were synthesized by Shanghai GenePharma Co., Ltd. Approximately 2×10^6 cells per well were seeded in a 6-well plates on the day prior to transfection. Transfection with 50 nmol siRNAs was performed according to the manufacturer's instructions using Lipofectamine 2000 transfection reagent (Invitrogen).

MTT assays. The MTT assays were performed as previously described (14,37). In brief, 1×10^5 cells/well was seeded in 96-well plates with 200 μ l culture medium. Following treatment with TGF- β 1 (10 ng/ml) (Sigma) for 0, 1, 2, 3 and 5 days, the medium was replaced with 200 μ l DMEM/FBS containing 5 mg/ml MTT and incubated at 37°C for 4 h. The supernatant was then discarded, and the cells were lysed in 200 μ l DMSO for 10 min at 37°C. The optical density (OD) values were measured at 490 nm (Epoch Microplate Spectrophotometer; BioTek Instruments, Inc., Winooski, VT, USA).

Invasion and migration assays. The assays were performed as previously described (38,39). The cells (1×10^6) were suspended in 200 μ l serum-free DMEM and seeded in the top chambers of 24-well plates (Corning, New York, NY, USA) coated with 30 μ l Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chambers of the plates were filled with 500 μ l DMEM containing 10% FBS. The cells were allowed to migrate for 48 h at 37°C. Following migration, cells in the top chambers were removed using a cotton swab, and the cells which migrated to the bottom chambers were fixed in 4% paraformaldehyde, and the cells were stained with 0.5% (w/v) crystal violet (Sigma) for 2 h at room temperature. The fixed and stained cells were counted in 5 independent fields under a microscope. At least, 3 chambers were counted for each experiment. For the migration assay, a similar protocol was followed apart from the replacement of the top chamber of the transwell plate with an uncoated chamber. The culture medium in the bottom chamber was replaced with DMEM containing 2.5% FBS, and the cells were allowed to migrate for 12 h.

Wound healing assays. The cells were seeded in 6-well plates and cultured until they reached 80% confluency, and a wound was then created by manually scraping the cell monolayer. The cells were washed twice with PBS to remove the floating cells, and then incubated in DMEM supplemented with 1% FBS. Cell migration was observed at pre-selected time points (0, 6, 24 and 48 h) as previously described (39). Images were acquired with a Nikon DS-5M Camera System (Nikon Instruments Inc., Tokyo, Japan).

Table I. Association between PDK1 levels and various clinicopathological characteristics in 113 glioma specimens.

Variables	No. of cases (n=113)	PDK1 expression		χ^2	P-value
		Low (n=52)	High (n=61)		
Age (years)					
≤50	50	26 (52%)	24 (48%)	1.292	0.2557
>50	63	26 (41.27%)	37 (58.73%)		
Sex					
Female	46	21 (45.65%)	25 (54.35%)	0.0042	0.9485
Male	67	31 (46.27%)	36 (53.73%)		
Tumor size (cm)					
≤5	41	29 (70.73%)	12 (29.27%)	15.82	<0.0001 ^a
>5	72	23 (31.94%)	49 (68.06%)		
WHO grade					
Grade I	10	9 (90.00%)	1 (10.00%)	28.73	<0.0001 ^a
Grade II	18	15 (83.33%)	3 (16.67%)		
Grade III	36	11 (30.56%)	25 (69.44%)		
Grade IV	49	13 (26.53%)	36 (73.47%)		

^aStatistically significant difference (P<0.05) as determined by the Chi-square test.

Flow cytometry. The cells were cultured in 6-well plates and detected utilizing the Gallios flow cytometer (Beckman Coulter, Miami, FL, USA) for cell cycle assays and a BD flow cytometer (BD Biosciences, San Jose, CA, USA) for apoptosis assays. For cell cycle detection, the propidium iodide (PI) Detection kit (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China) was utilized following the manufacturer's instructions. The cell-cycle raw data was re-analyzed by MutiCycle for windows software (Phoenix Flow Systems, San Diego, CA, USA). Apoptosis was measured using the Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA) (40). An Accuri™ C6 flow cytometer (BD Biosciences) was utilized to quantify the percentage of apoptotic cells.

Tumor implantation. To develop xenograft tumors, approximately 1×10^7 glioma U251 cells were inoculated into the mammary fat pads of six to eight-week-old athymic female nude mice, which were purchased from the Shanghai Experimental Animal Center (Shanghai, China). All animals (18 mice) weighed 23-25 g, and were maintained under SPF conditions at 20-26°C, a relative humidity of 40-70% and a 12-h light-dark cycle. All food was subjected to a high temperature for steam disinfection (60 min, 120°C). All water was acidified by hydrochloric acid and adjusted to a pH between 2.5 and 2.8. All efforts were made to minimize suffering. The mice were examined by palpation for tumor formation for >60 days. After tumors were detected, the tumor size was measured every 7 days using calipers, and tumor volume was calculated as follows: Volume (mm³) = length x width² x 0.5 every 7 days for 8 weeks. The animals were sacrificed when the xenografts reached approximately 1.5 cm in diameter, and tumor engrafts were harvested and weighed. All animal experiments were carried out under

the guide of the Sun Yat-Sen University Committee for Use and Care of Laboratory Animals and approved by the Animal Experimentation Ethics Committee of Sun Yat-Sen University.

Statistical analysis. Statistical analyses were performed using Statistical Package for Social Sciences software for Windows version 13.0 (SPSS, Chicago, IL, USA) or Graphpad Prism software 5.0 (GraphPad Software, San Diego, CA, USA). Associations between the patient clinicopathological characteristics and PDK1 expression were identified using the Chi-square (χ^2) test. Overall survival (OS) was evaluated by Kaplan-Meier analysis and differences between groups was assessed by the log-rank test, while the prognostic significance of the clinicopathological characteristics was determined using Cox regression analyses. OS was calculated as the time from the date of diagnosis to the date of death or the date of the last follow-up (if death did not occur). The correlation between PDK1 and c-Jun protein expression was determined using Pearson's correlation analysis. The comparison of two independent groups was analyzed using Student's t-tests. Multiple group comparisons were analyzed with one-way ANOVA and Tukey's HSD post hoc test. All statistical tests were two-tailed. Errors were the SD of averaged results and P-values <0.05 were considered to indicate statistically significant differences.

Results

Clinicopathological characteristics of the patients with glioma from the TMA and PDK1 expression. To investigate the function of PDK1, we used a TMA containing 113 glioma tissues (including 10 grade I, 18 grade II, 36 grade III and 49 grade IV samples) and 6 non-tumorous tissues and performed IHC to evaluate PDK1 expression and its

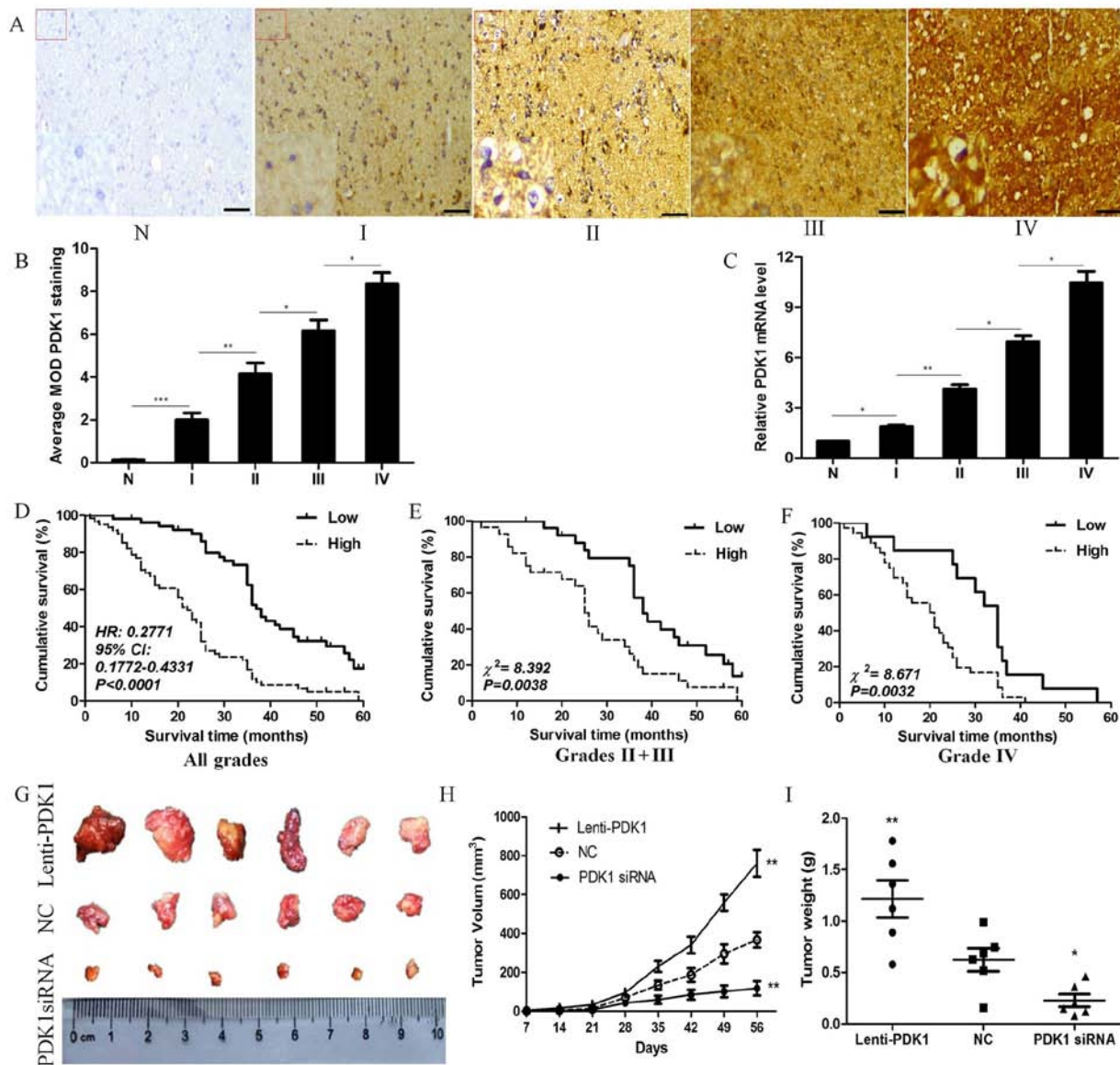


Figure 1. 3-Phosphoinositide dependent protein kinase 1 (PDK1) expression is significantly associated with glioma progression. (A) Paraffin-embedded specimens of 6 non-tumorous brain tissues and 113 glioma specimens (including 10 grade I, 18 grade II, 36 grade III and 49 grade IV tissues) with WHO glioma grade I to IV were stained by immunohistochemistry (IHC). The areas in the boxed regions in the top left corner (magnification, x200) are presented as enlarged images in the lower left corner (magnification, x400) (scale bars, 100 μ m). (B) Comparative quantification of the mean optical density (MOD) of PDK1 staining among nontumorous tissues and glioma specimens of different grades. (C) PDK1 mRNA expression levels were detected by RT-qPCR in a total of 63 tissue samples (10 non-tumorous tissues, and 3 grade I, 9 grade II, 19 grade III, and 22 grade IV glioma tissues). (D) High PDK1 expression was associated with a significantly lower overall survival, compared with a lower PDK1 expression (n=113, $\chi^2=31.71$; P<0.0001). (E and F) A high PDK1 expression was significantly associated with a lower survival compared with a low PDK1 expression in tissues of either the grade II + III subgroup (n=54, $\chi^2=8.392$; P=0.0038) or grade IV subgroup (n=49, $\chi^2=8.671$, P=0.0032). (G) Images of the xenograft tumors retrieved immediately at the end of the experiment at ~8 weeks. (H) Xenograft assay revealed that PDK1 knockdown decreased the volume of the xenograft tumors, while PDK1 overexpression increased significantly tumor volume, compared to the control group. (I) PDK1 knockdown reduced the weight of the xenograft tumors, while PDK1 overexpression significantly increased tumor weight, compared to the control group (*P<0.05, **P<0.01, ***P<0.001, one-way ANOVA).

association with the clinicopathological characteristics. The clinicopathological characteristics of the patients with glioma are shown in Table I. From the 113 patients with glioma, there were 67 males (59.29%) and 46 females (40.71%), with an age ranging from 21 to 79 years (median age, 53.6 years). From the 113 glioma tissues, 41 tumors (36.28%) were ≤ 5.0 cm in size, and the other 72 tumors (63.72%) were >5.0 cm in size. A high PDK1 expression were found to be significantly associated with a large tumor size (>5.0 cm) (P<0.0001) and a higher WHO grade (P<0.0001).

PDK1 expression is significantly associated with glioma progression. To investigate PDK1 expression in glioma and non-tumorous tissues, we performed IHC staining. We found that PDK1 staining was predominant in the cytoplasm. The results revealed a significantly higher level of PDK1 expression in glioma tissues compared with non-tumorous tissues. In the 113 glioma tissues, we found that a high PDK1 expression was significantly increased from glioma grade I to IV (Fig. 1A). Moreover, the results of the comparative quantification of the mean optical density (MOD) of PDK1 staining among the

Table II. Univariate and multivariate analyses of prognostic parameters for survival of patients with glioma.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (years)						
≤50 vs. >50	0.632	0.358-1.369	0.431			
Sex						
Female vs. male	0.751	0.567-3.625	0.512			
Tumor size (cm)						
≤5 vs. >5	0.529	0.332-0.895	<0.001 ^a	0.483	0.298-0.932	<0.001 ^a
PDK1 level						
Low vs. high	1.785	0.685-5.296	<0.0001 ^a	1.367	0.521-5.026	<0.0001 ^a
WHO grade						
II + III vs. IV	0.653	0.298-3.762	<0.0001 ^a	1.173	0.409-2.321	<0.0001 ^a

^aStatistically significant difference (P<0.05) as determined by the Cox regression analyses.

normal tissues and glioma specimens of different grades are summarized in Fig. 1B. The MOD of PDK1 staining increased while glioma progressed from a lower grade to a higher one (P<0.05). To confirm these observations, we examined the PDK1 mRNA level in a total of 63 tissue samples (10 non-tumorous tissues, as well as in 3 grade I, 9 grade II, 19 grade III and 22 grade IV glioma tissues) by RT-qPCR. PDK1 mRNA expression was significantly higher in the glioma than in the normal tissues. Not surprisingly, the PDK1 mRNA level also increased from the lower grade to the higher grade ones (P<0.05; Fig. 1C). These results indicate that PDK1 plays a critical role in glioma initiation and progression.

In order to determine the prognostic value of PDK1, Kaplan-Meier survival analyses were performed for overall survival. It was found that patients with a high PDK1 expression had a significantly lower overall survival, compared with those with low PDK1 expression. In addition, the median survival time of patients whose tumors exhibited high PDK1 expression levels was only 22 months, whereas the median survival time of those with low PDK1 expression levels was 37 months (HR, 0.2771; 95% CI, 0.1772-0.4331, P<0.0001; Fig. 1D) The cumulative 5-year survival rate was 26.23% (16/61) in the low PDK1 expression group, whereas it was only 5.77% (3/52) in the high PDK1 expression group. To further validate these findings, we assessed the prognostic significance in different subgroups of patients with glioma stratified according to the 2007 WHO glioma grade. Of note, a high PDK1 expression was significantly associated with a shorter survival time in the different glioma patient subgroups. The overall survival of patients with a high PDK1 expression was significantly decreased compared with that in those with a low PDK1 expression in either the grade II + III subgroup (n=54, $\chi^2=8.392$, P=0.0038, log-rank; Fig. 1E) or in the grade IV subgroup (n=49, $\chi^2=8.671$, P=0.0032, log-rank; Fig. 1F). Collectively, our data suggest that the PDK1 expression level is significantly associated with the disease outcome.

Univariate Cox regression analyses were used to determine the independence of PDK1 as a prognostic marker of

the survival of patients with glioma. As shown in Table II, overall survival was strongly associated with PDK1 expression (HR, 1.785; 95% CI, 0.685-5.296; P<0.0001), as well as with tumor size (HR, 0.529; 95% CI, 0.332-0.895; P<0.001) and glioma grade (HR, 0.653; 95% CI, 0.298-3.762; P<0.0001). Furthermore, PDK1 expression was also demonstrated to be a useful prognostic biomarker for patients with glioma by multivariate analysis; the survival of the patients was found to be associated with tumor size (HR, 0.483; 95% CI, 0.298-0.932; P<0.001), PDK1 expression (HR, 1.367; 95% CI, 0.521-5.026; P<0.0001) and glioma grade (HR, 1.173; 95% CI, 0.409-2.321; P<0.0001). Taken together, these data suggest that PDK1 may serve as a prognostic predictor for patients with glioma.

To further determine whether PDK1 is associated with tumor growth *in vivo*, we established a tumor implantation model to assess tumor growth using glioma U251 cells. Nude mice were subcutaneously injected with 1×10^7 glioma U251 cells transfected with lenti-PDK1, PDK1 siRNA or negative control. Representative images of the xenograft tumors at 8 weeks are shown in Fig. 1G. The data revealed that PDK1 knockdown decreased the volume of the xenograft tumors, while PDK1 overexpression significantly enhanced tumor volume, compared with control group (P<0.01, Fig. 1H). We also observed similar results regarding tumor weight. PDK1 knockdown reduced the weight of the xenograft tumors, while PDK1 overexpression significantly increased tumor weight, compared with the control group (P<0.05, Fig. 1I). On the whole, these data suggest that an increased PDK1 expression is associated with the progression of GBM.

TGF- β upregulates PDK1, and PDK1 promotes the proliferation and inhibits the apoptosis of glioma cells in vitro. The TGF- β signaling pathway has been shown to play an important role in several cancer types, as it promotes cancer cell proliferation (27). However, the mechanisms through which malignant glioma cells acquire the ability to take advantage of the TGF- β tumor-promoting effects remain elusive. In this study, we found that TGF- β transcriptionally upregulated PDK1 expression

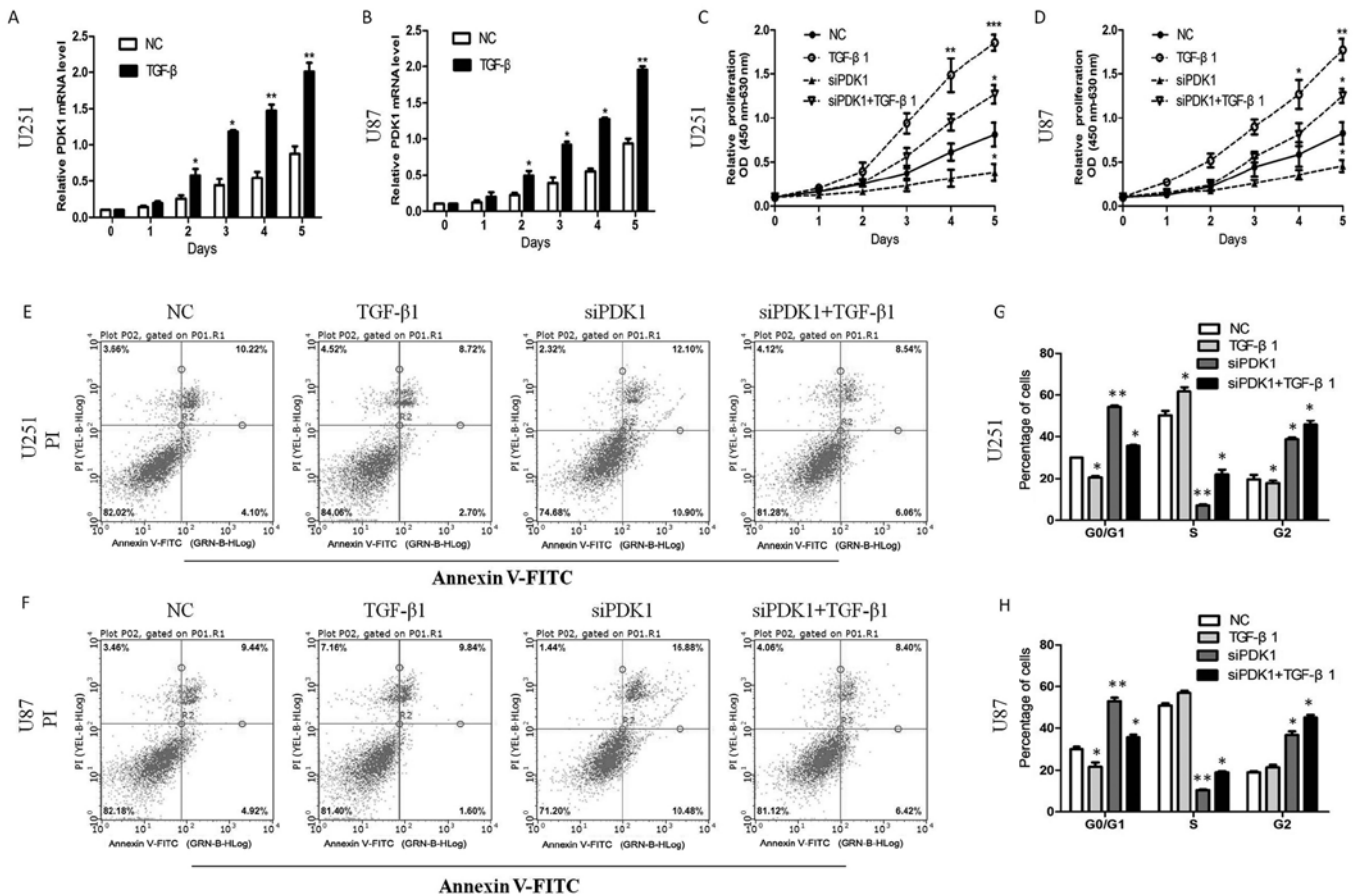


Figure 2. TGF- β upregulates 3-phosphoinositide dependent protein kinase 1 (PDK1) and PDK1 promotes the proliferation and inhibits the apoptosis of glioma cells *in vitro*. (A and B) TGF- β transcriptionally upregulated PDK1 protein in glioma cells. And PDK1 mRNA expression increased after TGF- β treatment (10 ng/ml) at 0, 1, 2, 3, 5 days (* P <0.05, ** P <0.01, Student's t-tests). (C and D) PDK1 knockdown significantly reduced cell proliferation, compared to the control group. However, compared with the PDK1 knockdown group, cell proliferation increased significantly with PDK1 knockdown and TGF- β treatment together (* P <0.05, ** P <0.01, *** P <0.001, one-way ANOVA). (E and F) PDK1 knockdown markedly increased the percentage of apoptotic cells. The apoptosis of the cells decreased markedly by TGF- β treatment. However, the percentage of apoptotic cells decreased markedly following PDK1 knockdown and TGF- β treatment together, compared with PDK1 knockdown alone. (G and H) Glioblastoma cells infected with PDK1 siRNA contained more cells ratio at the G0/G1 phase compared with control cells. In response to TGF- β treatment for 24 h, the cell population in the S phase significantly increased, whereas the accumulation of cells in the G2 phase markedly decreased. However, the cell population in the S phase increased through PDK1 knockdown and TGF- β treatment, compared with PDK1 knockdown alone (* P <0.05, ** P <0.01, one-way ANOVA).

in glioma cells. The PDK1 mRNA level increased following treatment with TGF- β 1 (10 ng/ml) for 0, 1, 2, 3, 5 days (P <0.05; Fig. 2A and B). Furthermore, cell proliferation significantly increased following TGF- β 1 treatment. PDK1 knockdown significantly decreased cell proliferation. However, compared with PDK1 knockdown alone, cell proliferation increased with PDK1 knockdown and TGF- β 1 treatment (P <0.05; Fig. 2C and D). These data suggest that PDK1 promotes glioma cell proliferation, and that PDK1 knockdown significantly inhibits cell proliferation. In addition, TGF- β probably promotes cell viability through the activation of the PDK1 pathway.

To further elucidate the effects of PDK1 on glioma cell viability, we further used the Annexin V/PI double staining method to assess whether interference with PDK1 expression induces cell apoptosis. The results revealed that PDK1 knockdown markedly increased the percentage of apoptotic U251 cells. The apoptotic rates of the U87 cells were similarly increased by PDK1 knockdown. As expected, cells apoptosis markedly reduced by TGF- β treatment. However, the percentage of apoptotic cells decreased markedly following

PDK1 knockdown and TGF- β treatment, compared with PDK1 knockdown alone (Fig. 2E and F). Furthermore, we also used flow cytometry to assess whether PDK1 knockdown inhibits cell cycle progression. As shown in Fig. 2G and H, in the U251 and U87 cells transfected with PDK1 siRNA we observed a greater number of cells in the G0/G1 phase compared with the control cells. In response to TGF- β treatment for 24 h, the cell population in the S phase significantly increased, whereas the accumulation of cells in the G2 phase markedly decreased. However, the cell population in the S phase significantly increased following PDK1 knockdown and TGF- β treatment, compared with PDK1 knockdown alone. These data indicate that PDK1 knockdown induces cell apoptosis by blocking cell cycle progression at the G0/G1 phase, and the cell apoptotic ratio significantly decreases through the activation of the TGF- β /PDK1 pathway.

PDK1 enhances the invasive and migratory potential of glioma cells. To determine whether the effects of PDK1 are associated with migration and invasion, we conducted

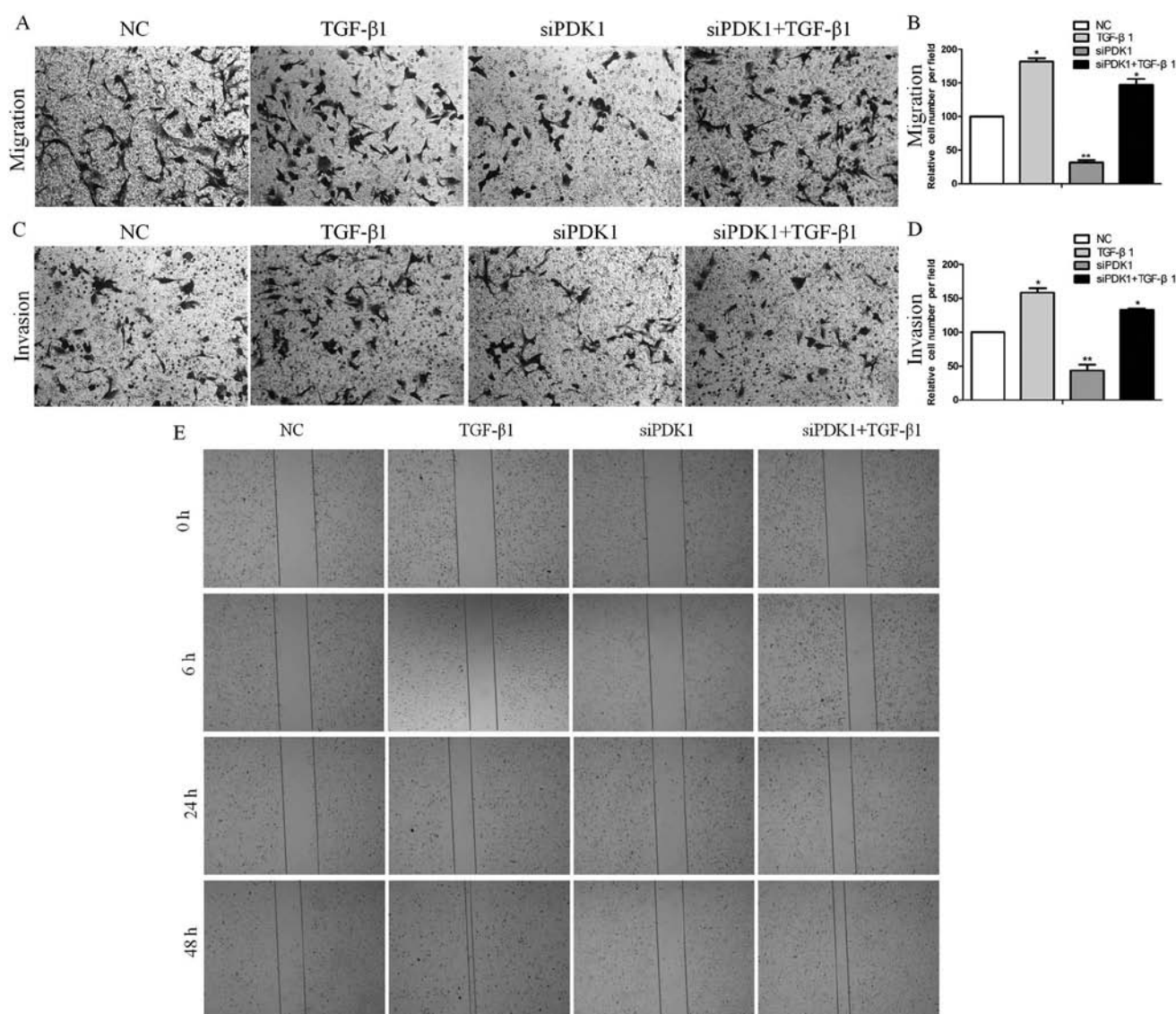


Figure 3. 3-Phosphoinositide dependent protein kinase 1 (PDK1) enhances the invasive and migratory potential of glioma cells. (A and B) Transfection with PDK1 siRNA decreased the number of migrating U251 cells. In response to TGF- β 1 treatment for 24 h, the number of migrating cells increased significantly. However, PDK1 knockdown and TGF- β 1 treatment significantly increased migration ($P<0.05$, $^{**}P<0.01$, one-way ANOVA). (C and D) PDK1 knockdown reduced cell invasion. In response to TGF- β 1 treatment for 24 h, the invasion of the cells increased significantly. However, PDK1 knockdown and TGF- β 1 treatment significantly increased the invasion of cells ($P<0.05$, $^{**}P<0.01$, one-way ANOVA). (E) PDK1 knockdown markedly suppressed migration; however, the migration of cells increased with PDK1 knockdown and TGF- β 1 treatment, as assessed by wound healing assays.

migration and invasion assays using glioma U251 cells transfected with PDK1 siRNA and/or treated with TGF- β . Transfection with PDK1 siRNA decreased the number of migrating cells by approximately 68.5% compared with the control group. In response to TGF- β treatment for 24 h, the migrating cells significantly increased by approximately 81.7% compared with the control group. However, the cells subjected to PDK1 knockdown and TGF- β treatment exhibited a significantly increased migration by only 46.5% compared with the control group (Fig. 3A and B). PDK1 knockdown reduced cell invasion by approximately 42.3% compared with the control group. After TGF- β treatment for 24 h, the invasion of cells increased by approximately 69.8% compared with the control group. However, PDK1 knockdown and TGF- β treatment together significantly increased cell invasion by only 32.6% compared with the control group

(Fig. 3C and D). In agreement with these data, the results of wound healing assays also revealed showed that siRNA knockdown for 48 h markedly decreased cell migration, with less cells migrating into the gap formed in a scratch assay. However, the number of migrating cells increased markedly following TGF- β treatment for 48 h. Both siRNA knockdown and TGF- β treatment had a positive effect on cell migration and probably promoted cell migration through the activation of the TGF- β /PDK1 pathway (Fig. 3E). These data thus suggest that PDK1 is significantly associated with glioma cell migration and invasion.

PDK1/c-Jun pathway is activated by TGF- β and induces EMT and promotes progression in human GBM. To investigate the molecular mechanisms through which PDK1 accelerates GBM cell invasion, the U251 glioma cells were treated with

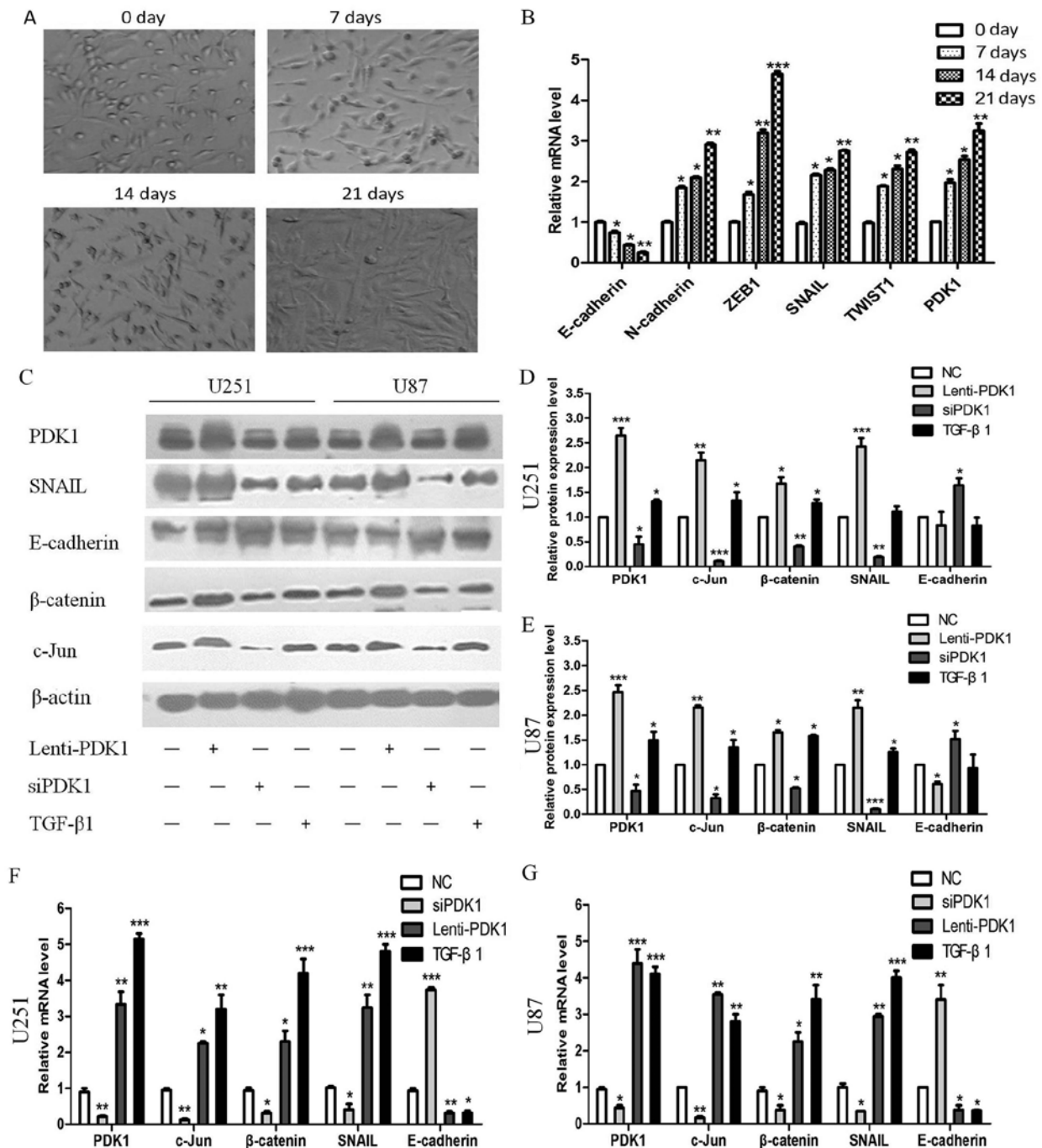


Figure 4. The 3-phosphoinositide dependent protein kinase 1 (PDK1)/c-Jun pathway activated by TGF- β induces EMT and promotes glioblastoma progression. (A) U251 glioma cells gradually became spindle-shaped with TGF- β (10 ng/ml) treatment after 0, 7, 14 and 21 days. (B) The PDK1 expression level gradually increased; the expression of the epithelial cell marker, E-cadherin, gradually decreased, while that of the mesenchymal cell markers, N-cadherin, ZEB1, SNAIL and TWIST1, gradually increased. (C-E) PDK1, c-Jun, SNAIL and β -catenin protein expression increased significantly, but that of the epithelial cell marker, E-cadherin, decreased markedly following treatment with PDK1 overexpression plasmid or TGF- β 1 in U251 and U87 cells. However, the protein levels exhibited opposite effects following transfection with siRNA against PDK1. (F and G) PDK1, c-Jun, SNAIL, β -catenin and E-cadherin mRNA expression exhibited similar effects, as with protein expression (* P <0.05, ** P <0.01, *** P <0.001, one-way ANOVA).

TGF- β 1 (10 ng/ml). The results revealed that the U251 cells gradually became spindle-shaped at 0, 7, 14 and 21 days (Fig. 4A). Moreover, the PDK1 expression level gradually increased. In addition, the expression levels of the epithelial cell marker, E-cadherin, gradually decreased, while those of the mesenchymal cell markers, N-cadherin, ZEB1, SNAIL and TWIST1 gradually increased (Fig. 4B). These results indicate that TGF- β upregulates PDK1 expression to induce EMT. In

the UCSC database (<http://genome.ucsc.edu>), the transcription factor, JunB, binds to the DNA sequences downstream of the PDK1 gene. Previous studies have also suggested that JunB and c-Jun share extensive homology within the leucine zipper and basic domains, and JunB inhibits cell proliferation and migration by antagonizing c-Jun activity (28,29). It has also been suggested that increasing c-Jun transcriptional activity induces glioma progression (30). Therefore, in this study, we

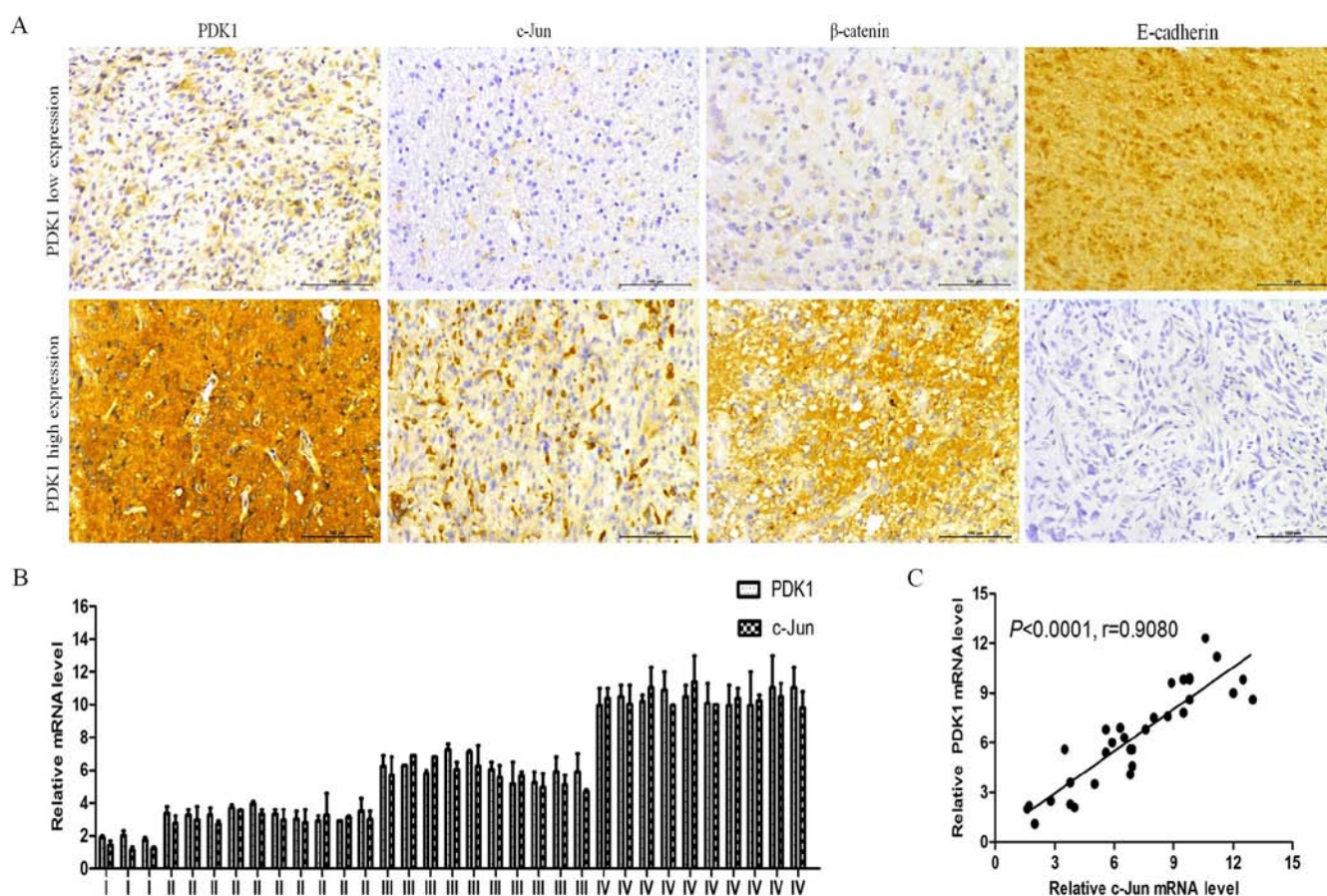


Figure 5. 3-Phosphoinositide dependent protein kinase 1 (PDK1) protein is significantly associated with c-Jun expression in glioma tissues. (A) PDK1, c-Jun, β-catenin and E-cadherin protein expression in human glioma tumor tissues with high or low PDK1 levels. (B) PDK1 and c-Jun mRNA expression level increased from glioma grade I to IV. (C) Levels of PDK1 positively correlated with the c-Jun levels in glioma tumor tissues ($r=0.9080$, $P<0.0001$).

wished to determine whether PDK1 directly regulates c-Jun to induce EMT in human GBM.

Subsequently, following transfection of the U251 and U87 cells with PDK1 overexpression plasmid or treatment with TGF-β1, the protein expression levels of PDK1 and c-Jun increased. In addition, the protein expression levels of the epithelial cell marker E-cadherin decreased markedly in glioma cell lines with PDK1 overexpression or treatment with TGF-β1, while those of the mesenchymal cell markers, Snail and β-catenin, increased. PDK1 and c-Jun protein expression was downregulated significantly when PDK1 expression was inhibited by siRNA, and E-cadherin protein expression increased, while Snail and β-catenin protein expression decreased ($P<0.05$; Fig. 4C-E). Moreover, similar results were obtained for the mRNA expression levels in the U251 and U87 cells ($P<0.05$; Fig. 4F and G).

It should be noted that the PDK1, c-Jun, β-catenin and E-cadherin expression levels in the glioma tissues with high or low PDK1 levels were similar to those found in the cell lines (Fig. 5A). c-Jun and β-catenin protein expression levels were decreased in the tissues with low PDK1 expression, and E-cadherin protein expression was increased. However, c-Jun and β-catenin protein expression increased, and E-cadherin protein expression decreased when PDK1 protein highly expressed. In agreement with these findings, the PDK1 and c-Jun mRNA levels examined increased in the tissues with

glioma grade I to IV, with similar patterns in the glioma tissues (Fig. 5B). PDK1 protein expression also positively correlated with c-Jun expression in the glioma tissues ($r=0.9080$, $P<0.0001$; Fig. 5C). Collectively, these results confirm that PDK1 promotes cell invasion in GBM through the upregulation of c-Jun and the promotion of EMT.

Evidence in the Oncomine database. The Oncomine database (<https://www.oncomine.org/>) provide solutions for individual researchers and multinational companies, with peer-reviewed analytical methods and a powerful set of analysis functions that compute gene expression signatures, clusters, and gene-set modules, automatically extracting biological insights from the data. To further validate the findings of our study, we searched the Oncomine database for PDK1 expression in human glioma (data not shown). A total of 8 datasets demonstrated that PDK1 expression in human glioma was higher compared to that in normal brain tissues. PDK1 overexpression exhibited a significant association with a high WHO glioma grade, recurrence and treatment response. Moreover, PDK1 overexpression was significantly associated with MGMT methylation, EGFR amplification, LDHA mutation and the loss of heterozygosity of chromosome 1p, 10q, 19q. Not surprisingly, 5 datasets revealed that PDK1 overexpression was significantly associated with short survival time during follow-up. Intriguingly, 11 datasets revealed that the protein expression level of c-Jun

was higher in human glioma, compared with normal brain tissues, while 2 datasets demonstrated an association between c-Jun expression and higher-grade human glioma with an approximately 2-fold increase. Furthermore, 6 datasets indicated that the expression of c-Jun was associated with patient survival time during follow-up (data not shown). Collectively, these results obtained from the Oncomine database confirm our findings that PDK1 may upregulate c-Jun protein expression to promote the progression of human glioma.

Discussion

In this study, we examined PDK1 expression in 113 glioma and 6 normal brain tissues using TMA. We found that PDK1 was significantly upregulated in glioma, compared with non-tumorous tissues. Moreover, PDK1 exhibited a significant association with WHO glioma grade, tumor size and survival time. PDK1 protein was an independent prognostic factor. *In vivo*, PDK1 promoted glioma tumor xenograft growth. TGF- β induced tumor cell EMT and increased cell motility, which was associated with migration and invasion. *In vitro*, our data confirmed that TGF- β upregulated PDK1 and PDK1 promoted cell migration and invasion, and functioned as an oncogene in GBM, by upregulating c-Jun and inducing EMT. In glioma tissues, c-Jun protein was increased and positively correlated with the PDK1 levels. In the Oncomine database, PDK1 and c-Jun protein levels were overexpressed in human glioma. Of note, both PDK1 and c-Jun protein expression levels were significantly associated with WHO glioma grade and survival time. Taken together, these results suggest that PDK1 is positively associated with EMT and that the upregulation of c-Jun protein by PDK1 accelerates GBM cell invasion.

Glioma, comprising approximately 80% of all primary malignant brain tumors, is the most prevalent type and results in poor clinical outcomes (41-43). According to the WHO classification, gliomas are classified into four histopathological grades, ranging from grade I to IV. Glioblastoma (grade IV) is one of the most malignant forms of human brain tumor (1). In treatment, the approaches mainly include surgical resection, radiotherapy, chemotherapy, targeted therapy and multiple therapies in combination for the glioma (44). Despite marked progression in therapies, glioma prognosis remains unsatisfactory, and patients who suffer from GBM, the most aggressive type of glioma, have a poor prognosis. Therefore, identifying novel biomarkers may aid in the better understanding of the mechanisms of carcinogenesis and in the development of targeted treatments, which may thus improve the prognosis of patients with glioma. However, the current methods used to characterize glioma are inadequate. The establishment of molecular biomarkers may identify variables required to improve tumor characterization, and may aid in the identification of specific targets for improved treatment with essential prognostic value for glioma patient survival.

PDK1 is a serine-threonine kinase belonging to the AGC kinase family. An increasing amount of data suggest that PDK1 plays a pivotal role in the regulation of cell migration and several signaling pathways activated by growth factors and hormones and activates members of the AGC family of protein kinases (5,6,16,17). PDK1 has been demonstrated to

be crucial for the regulation of each step of cell migration, by activating several proteins, such as PKB/Akt (6,8), ROCK1 (9), β 3 integrin (10), PLC γ 1 (11) and MRCK α (12). Moreover, PDK1 regulates cancer cell invasion as well, thus representing a possible target with which to prevent cancer metastasis. Furthermore, PDK1 protein has been recognized as a key regulator in breast cancer (45), melanoma (16), gallbladder cancer (13), ovarian cancer (46,47), colorectal cancer (48), gastric cancer (49), pancreatic cancer (50,51), prostate carcinoma (52) and acute myeloid leukemia (15). In breast cancer, the activation of SGK1 protein by PDK1 contributes to the maintenance of residual mTORC1 activity through direct the phosphorylation and inhibition of TSC2 (53). Moreover, PDK1 phosphorylation is frequently upregulated in breast cancer with the concomitantly increased phosphorylation of downstream kinases, including Akt, mTOR, p70S6K, S6 and Stat3 (54). PDK1 directly induces PLK1 phosphorylation, which in turn induces MYC phosphorylation and protein upregulation. PDK1/PLK1/MYC signaling is critical for cancer cell growth and survival, and PDK1/PLK1 knockdown suggests an effective therapeutic approach (55). In human esophageal squamous cell carcinoma (ESCC), PDK1 protein is expressed in the cytoplasm, but is not expressed in adjacent non-cancerous tissues. In addition, a high PDK1 expression was found to be closely associated with an advanced tumor stage, positive lymph node metastasis and high histological grade (17). In summary, targeting PDK1 may prove to be an effective approach with which to inhibit cancer progression towards a more invasive and metastatic phenotype.

However, to the best of our knowledge, few studies to date have investigated the expression and significance of PDK1 in glioma tissues and molecular function, particularly as regards the PDK1 pathway regulating GBM invasion and patient prognosis. The results of this study indicated that PDK1 was upregulated in GBM. The PDK1 protein and mRNA levels were significantly upregulated in glioma, compared with non-tumorous tissues. Furthermore, PDK1 overexpression was significantly associated with a large tumor size (>5.0 cm) and higher grade, and a shorter survival time. The overall survival of patients with a high PDK1 expression was significantly decreased compared with that of those with a low PDK1 expression in either the grade II + III subgroup or grade IV subgroup. Our data also indicated that PDK1 protein was an independent prognostic factor. Xenograft assay revealed that PDK1 knockdown decreased the volume of xenograft tumors, while PDK1 overexpression significantly increased tumor volume. These results are in agreement with those of a previous study (14), which demonstrated that PDK1 overexpression was found in >40% of patients with acute myeloid leukemia. PDK1 overexpression occurred uniformly throughout the leukemic population, including putative leukemia-initiating cells. Moreover, PDK1 overexpression was closely associated with the increased phosphorylation of PKC isoenzymes and the inhibition of PKC strongly inhibited the survival advantage of PDK1-overexpressing cells (14).

PDK1 has multiple complex roles in tumor biology. PDK1 has been reported to be involved in the inhibition of apoptosis and the promotion of growth in lung cancer (56). PDK1 protein has also been shown to be closely associated with the proliferation, apoptosis and invasion of esophageal cancer

cells (20). Although PDK1 has been shown to be present in multiple tumors, its functional impact on tumor progression remains largely unknown. The findings of this study suggested that PDK1 expression increased following TGF- β 1 treatment. Furthermore, cell proliferation significantly increased through the upregulation of PDK1 by TGF- β 1. However, PDK1 knockdown significantly reduced cell proliferation. In addition, PDK1 knockdown induced apoptosis by blocking cell cycle progression at the G0/G1 phase.

To determine whether PDK1 is involved in migration and invasion, we conducted migration and invasion assays using cells transfected with PDK1 siRNA. PDK1 knockdown significantly decreased cell migration and invasion, compared with the control group. However, both siRNA knockdown and TGF- β treatment had a significant positive effect on cell migration and promoted cell migration through the activation of the TGF- β /PDK1 pathway. In agreement with these findings, the results of wound healing assay also demonstrated similar effects of PDK1 on cell invasion.

To investigate the molecular mechanisms through which PDK1 accelerates cell invasion, we treated U251 cells with TGF- β 1. The results revealed that the U251 cells gradually became spindle-shaped during EMT, while PDK1 expression increased gradually. These results suggested that TGF- β may upregulate PDK1 to induce EMT. In the UCSC database (<http://genome.ucsc.edu>), the transcription factor JunB binds to the DNA sequences downstream of the PDK1 gene. JunB and c-Jun share extensive homology within the leucine zipper and basic domains, and JunB inhibited cell proliferation and migration by antagonizing c-Jun activity (28,29). Therefore, we wished to determine whether PDK1 directly regulates c-Jun to induce EMT in human GBM. In breast cancer cells, although ATF-3 interacts with c-Jun and JunB proteins, and regulates their expression, a heterodimer complex of only ATF-3/c-Jun forms at the AP-1 site of the MMP-13 promoter and activates its gene expression upon TGF- β treatment (29). Extracellular signals can induce the post-translational modifications of c-Jun, resulting in altered transcriptional activity and target gene expression. This activates a number of cellular processes, such as proliferation, apoptosis, survival, tumorigenesis and tissue morphogenesis (57,58). In the present study, following treatment with PDK1 overexpression plasmid or TGF- β , PDK1 and c-Jun protein expression increased. In addition, the protein expression of the epithelial cell marker, E-cadherin, decreased, while that of the mesenchymal cell markers, Snail and β -catenin, increased. PDK1 and c-Jun protein were down-regulated significantly when PDK1 expression was inhibited by siRNA, and E-cadherin protein increased, but Snail and β -catenin protein decreased. IHC staining in tumor tissues with high or low PDK1 levels revealed similar expression patterns found in the cell lines. In agreement with these findings, the PDK1 and c-Jun mRNA expression levels studied by RT-qPCR increased from glioma grade I to IV with similar patterns in glioma tissues. Moreover, c-Jun expression positively correlated with the PDK1 levels in tumor tissues. In the Oncomine database, the data confirmed that PDK1 and c-Jun protein promote human glioma progression.

In conclusion, the findings of this study confirmed the prognostic potential of PDK1 expression in human GBM and revealed the pro-migratory and pro-invasive functions of this

protein in GBM cells through the PDK1/c-Jun pathway activated by TGF- β .

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL and XX conceived and designed the experiments; DL, XX, JL and CC performed the experiments; DL and WC analyzed the data; DL and XX wrote the manuscript; FW and YX were involved in data collection. FL conceived, designed and supervised the study, and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed in accordance with the policies of the Institutional Research Ethics Committee of Sun Yat-Sen Memorial Hospital. Written informed consent was obtained from the study participants at the Sun Yat-Sen Memorial Hospital of Guangzhou City. All animal experiments were carried out under the guide of the Sun Yat-Sen University Committee for Use and Care of Laboratory Animals and approved by the Animal Experimentation Ethics Committee of Sun Yat-Sen University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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