P4HB and PDIA3 are associated with tumor progression and therapeutic outcome of diffuse gliomas

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Abstract. Diffuse gliomas are the most common type of primary brain and central nervous system (CNS) tumors. Protein disulfide isomerases (PDIs) such as P4HB and PDIA3 act as molecular chaperones for reconstructing misfolded proteins, and are involved in endoplasmic reticulum stress and the unfolded protein response. The present study focused on the role of P4HB and PDIA3 in diffuse gliomas. Analysis of GEO and HPA data revealed that the expression levels of P4HB and PDIA3 were upregulated in glioma datasets. Their increased expression was then validated in 99 glioma specimens compared with 11 non-tumor tissues. High expression of P4HB and PDIA3 was significantly correlated with high Ki-67 and a high frequency of the *TP53* mutation. Kaplan-Meier survival curve and Cox regression analyses showed that glioma patients with high P4HB and PDIA3 expression had a poor survival outcome, P4HB and PDIA3 could be independent prognostic biomarkers for diffuse gliomas. *In vitro*, knockdown of PDIA3 suppressed cell proliferation, induced cell apoptosis, and decreased the migration of glioma cells. Furthermore, downregulation of P4HB and PDIA3 may contribute to improve the survival of patients who receive chemotherapy and radiotherapy. The data suggest that high expression of P4HB and PDIA3 plays an important role in glioma progression, and could predict the survival outcome and therapeutic response of glioma patients. Therefore, protein disulfide isomerases may be explored as prognostic biomarkers and therapeutic targets for diffuse gliomas.

Introduction

Diffuse gliomas (including oligodendrogliomas, astrocytomas, oligoastrocytomas and glioblastomas) are the most common type of primary brain and central nervous system (CNS) tumors. According to cancer statistics, the mortality due to diffuse gliomas is the highest among all brain and CNS tumors (1). The pathogenesis of diffuse gliomas is extremely complicated, and involves the aberrant activation of proto-oncogenes and inactivation of anti-oncogenes (2). The 2016 WHO classification of CNS tumors (2016 CNS WHO) first uses molecular features in addition to histology to define the various tumor entities, and then proposes a concept for how tumor diagnosis should be carried in the molecular era, in regards to *IDH* mutation and *EGFR* amplification (3). Therefore, the discovery of novel molecular biomarkers for diagnosis, prognosis and targeted therapy of diffuse gliomas is urgently needed.

P4HB (prolyl 4-hydroxylase, β polypeptide, also known as PDIA1) and PDIA3 (protein disulfide isomerase family A, member 3, also known as ERp57) are the main members of the protein disulfide isomerase (PDI) gene family, and are identified primarily as enzymatic chaperones for reconstructing misfolded proteins within the endoplasmic reticulum (ER), and are involved in ER stress and the unfolded protein response (UPR) (4). Several studies have linked PDIs to various human cancers, including breast, liver, gallbladder (5), laryngeal (6) and cervical cancer (7). In particular, both P4HB and PDIA3 are highly expressed in hepatocellular carcinoma (8,9) and colon cancer (10), and they are both associated with advanced stage tumors and poor prognosis. Overexpression of P4HB promotes hepatocellular carcinoma progression via downregulation of GRP78 and subsequent upregulation of epithelial-mesenchymal transition (EMT) (11). PDIA3 is overexpressed in invasive ductal breast cancer, and is believed to serve as a marker of aggressiveness (12). Another study reported that PDIA3 was one of the most frequently

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Abbreviations: P4HB, prolyl 4-hydroxylase, β polypeptide; PDIA3, protein disulfide isomerase family A, member 3; qRT-PCR, quantitative real-time polymerase chain reaction; CNS, central nervous system; GBM, glioblastoma; WHO, World Health Organization; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; TCGA, The Cancer Genome Atlas; LGG, lower grade gliomas; GEO, Gene Expression Omnibus; HPA, Human Protein Atlas; OS, overall survival; KPS, Karnofsky Performance Score; HR, hazard ratio; TMZ, temozolomide; ACTB, β-actin; CCK-8, Cell Counting Kit-8

Key words: P4HB, PDIA3, diffuse gliomas, prognosis, therapeutic outcome

upregulated proteins in breast tumor interstitial fluids and bloods, which could serve as a potential serological marker for the early detection of breast cancer (13). However, little is known about whether P4HB and PDIA3 are correlated with glioma progression and treatment outcome.

In the present study, we aimed to investigate the expression and biological significance of P4HB and PDIA3 in human diffuse gliomas, and evaluate the association between gene expression and clinical pathological patient parameters. Additionally, the correlation of P4HB and PDIA3 with the outcome of chemotherapy and radiotherapy was also studied in order to reveal the underlying mechanisms.

Materials and methods

Clinical specimens. Glioma tissue specimens (n=99) were obtained from patients diagnosed with diffuse gliomas undergoing surgical resection at the Department of Neurosurgery of Xiangya Hospital of Central South University from February 2015 to June 2016. After excision, tissue specimens were immediately frozen in liquid nitrogen for subsequent use. All clinicopathological data were assembled according to the classification of 2016 CNS WHO, and the patient information is presented in Table I. Eleven non-tumor brain tissues were obtained from adult patients with craniocerebral injuries, which required partial resections of brain tissue as decompression treatment to reduce intracranial pressure. This study was approved by the Ethics Committees of Central South University and all patients provided written informed consent.

TCGA, GEO and HPA data analysis. The Cancer Genome Atlas (TCGA) gene expression data (Illumina HiSeq) plus clinical information for glioma samples were obtained from the TCGA data portal (http://www.cancergenome.nih.gov/). The datasets included 152 GBM and 460 LGG (low-grade glioma) patients, and most of them had received chemotherapy and/or radiation therapy. The gene expression profiles of GSE4290 (14), GSE4271 (15), GSE4412 (16) and GSE43378 (17) were downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database (18). The sample information is presented in Table II. The original CEL files and annotation files of the platform were also downloaded. The gene expression microarrays are based on Affymetrix Human Genome U133 Plus 2.0 Array platform (Affymetrix, Inc., Santa Clara, CA, USA). The Human Protein Atlas (HPA; www.proteinatlas.org) contains information for a vast majority of all human protein-coding genes regarding the expression and localization of corresponding proteins (19). The study focused on 3 cases of normal brain tissues, 12 cases of low- and high-grade glioma tissues from surgical resections of patients. The immunohistochemical (IHC) staining images of P4HB (HPA018884) and PDIA3 (HPA002645) were obtained from the HPA.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. One microgram of total RNA of each sample was reversely transcribed into cDNA under standard conditions by using the PrimeScript[RT] reagent kit with gDNA Eraser (Takara, Shiga,

Table I. Clinical and molecular pathological characteristics of the diffuse glioma patients.

Clinical characteristics	Data
Number of patients, N	99
Oligodendrogliomas, n (%)	14 (14.1)
Oligoastrocytomas, n (%)	10 (10.1)
Astrocytomas, n (%)	49 (49.5)
Glioblastomas with grade IV	26 (26.3)
WHO grade II, n (%)	38 (38.4)
WHO grade III, n (%)	35 (35.3)
Gender, female/male	37/62 (37.4/62.6)
Mean age at diagnosis, years	45.34±1.543
KPS score, >80/≤80 (%)	83/16 (84.7/16.3)
GFAP, low/high (%)	3/80 (3.6/96.4)
Ki-67, low/high (%)	50/33 (60.2/39.8)
MGMT promoter methylation, -/+ (%)	79/2 (97.5/2.5)
IDH mutation, -/+ (%)	49/32 (60.5/39.5)
p53, low/high (%)	37/44 (45.7/54.3)
1p/19q codeleted, -/+ (%)	19/18 (51.4/48.6)

KPS, Karnofsky Performance Score; +, positive; -, negative.

Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the SYBR® Premix DimerEraser™ (Takara) on the LightCycler® 480 system (Roche Diagnostics, Basel, Switzerland). The qRT-PCR reaction included an initial denaturation step at 95°C for 30 sec, followed by 40 cycles of 92°C for 5 sec, 55°C for 30 sec and 72°C for 30 sec. Melting curve analysis was used to determine the specific PCR products. ACTB (β-actin) was used as the internal control for data normalization. Relative quantification of gene expression was calculated by the comparative cycle-threshold (CT) ($2^{-\Delta\Delta CT}$) method. The primer sequences were as follows: P4HB forward primer, 5'-TCGA GTTCACCGAGC AGACAG-3' and reverse primer, 5'-AGCTCTCGGCTGCTG TTTTG-3'; PDIA3 forward primer, 5'-ATGGGCCTGTGA AGGTAGTGG-3' and reverse primer, 5'-TGACCACACCAA GGGGCATA-3'; ACTB forward primer, 5'-CTTCAGGTTCA CCACCCAAGA-3' and reverse primer, 5'-TGAAGGCTCCT CTCTGCTCAT-3'. Primers for P4HB, PDIA3 and ACTB were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Cell culture and transfections. Human glioma cell lines U87 and U251 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified incubator with 5% CO_2. According to the manufacturer's protocol, U87 and U251 cell lines at modest confluence were transfected with 50 nM of either siRNA targeting PDIA3 (si-PDIA3) or the negative control (si-NC) using Lipofectamine RNAiMAX reagent (Invitrogen). The PDIA3 siRNA (5'-GGA CAAGACUGUGGCAUAU-3'), and negative control siRNA

Table II. Clinical information of the glioma samples in the TCGA and GEO datasets.

Clinical data	Datasets				
	GSE4290	GSE4271	GSE4412	GSE43378	TCGA
Sample number, N	176	77	85	50	612
Non-tumor samples, n (%)	23 (13.1)				
Oligodendrogliomas, n (%)	50 (28.4)		11 (12.9)	6 (12)	175 (28.6)
Oligoastrocytomas, n (%)			7 (8.3)		115 (18.8)
Astrocytomas, n (%)	26 (14.8)	21 (27.3)	8 (9.4)	11 (22)	170 (27.8)
Glioblastomas of grade IV, n (%)	77 (43.7)	56 (72.7)	59 (69.4)	32 (64)	152 (24.8)
WHO grade II, n (%)	45 (25.6)			5 (10)	218 (35.6)
WHO grade III, n (%)	31 (17.6)	21 (27.3)	26 (30.6)	13 (26)	242 (39.6)
Gender, F/M		25/52	53/32	16/34	257/355
Mean age (years)		45.48±1.483	44.38±1.678	52.72±2.426	47.29±0.620
KPS score				Yes	Yes
Survival data	No	Yes	Yes	Yes	Yes

F, female; M, male; KPS, Karnofsky Performance Score.

(siN05815122147) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Proliferation assay. Cell proliferation assays were performed with the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. Briefly, U87 and U251 cells were seeded at a density of $1x10^3$ cells per well in 96-well plates, and then transfected with siRNAs and cultured in 100 μl DMEM medium. After a period of time (0, 12, 24, 48 and 72 h), 10 μl of CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The absorbance (A) in each well was measured at 450 nm with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell proliferation was also assessed by colony formation assay. The cells were trypsinized into a single-cell suspension after transfection with siRNAs for 12 h. An equal number of transfected cells was placed respectively in the fresh 6-well plate, and cultured in DMEM containing 10% FBS at 37°C, replacing the medium every 2 days. After ~2 weeks, the cells were fixed using 4% polyoxymethylene and stained with 0.5% crystal violet solution (Beyotime Biotechnology, Shanghai, China). Visible colonies were manually counted and photographed.

Hoechst staining assay. Apoptotic cells were observed using the Hoechst 33342 staining kit (Beyotime Biotechnology) according to the manufacturer's instructions. Briefly, glioma U87 and U251 cells were seeded in a 12-well plate for 24 h, and then transfected with siRNAs. At 48 h after transfection, the cells were fixed using 4% polyoxymethylene and incubated with 100 μl of 1X Hoechst 33342 solution for 30 min in the dark. After a PBS wash, the cells were visualized and photographed under a fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

Migration assay. Cell migration assay was performed via the scratch method. Glioma U87 and U251 cells were seeded into 6-well plates and allowed to grow to a monolayer. After transfection with siRNAs for 12 h, a single scarification was scratched across the cell layer using a pipette tip. The cells were then maintained in DMEM containing 1% FBS at 37°C. The scratch gap was recorded and photographed using the Leica Microsystems CMS GmbH (D-35578; Germany) at 0, 24 and 48 h after scratching, and the cell migration ability was analyzed.

Statistical analysis. Statistical analyses were performed with SPSS 22.0 software (IBM SPSS, Inc., Chicago, IL, USA). GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for graphing and analysis. Data were exhibited as means ± standard deviation (SD). The Pearson's chi-squared test was used to compare the categorical variables. Regarding the numerical variables, statistical significance of differences between two groups was assessed using two-sided Student's t-test; and comparisons of multiple groups were made by one-way analysis of variance (ANOVA). All experiments were performed in triplicate and P<0.05 was considered to indicate a statistically significant difference. Survival analysis was performed by Kaplan-Meier method with the log-rank (Mantel-Haenszel) test. The risk association of gene expression with several known risk factors was determined using univariate and multivariate Cox regression analyses.

Results

P4HB and PDIA3 are upregulated in glioma datasets. To investigate the implication of protein disulfide isomerases (P4HB and PDIA3) in diffuse gliomas, we first analyzed the gene expression data of gliomas in the GSE4290 dataset, and found that the mRNA expression of P4HB and PDIA3 was significantly increased in the glioma samples compared to that in the non-tumor controls (Fig. 1). Glioblastomas presented with statistically higher P4HB and PDIA3 expression than astrocytomas (both P<0.05) and oligodendrogliomas (both
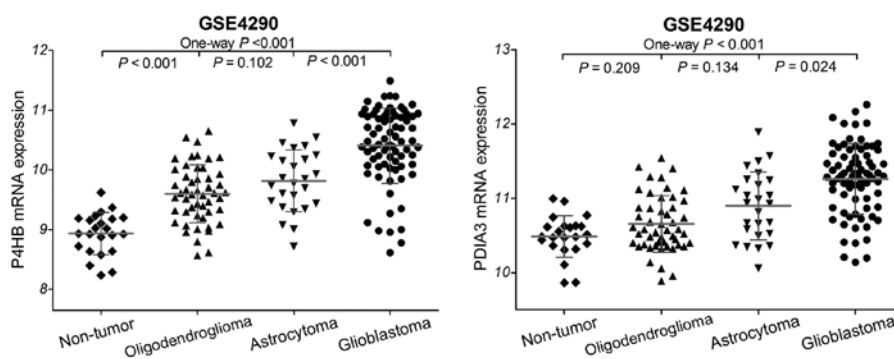
Figure 1. Expression of P4HB and PDIA3 in glioma samples of the GSE4290 dataset.
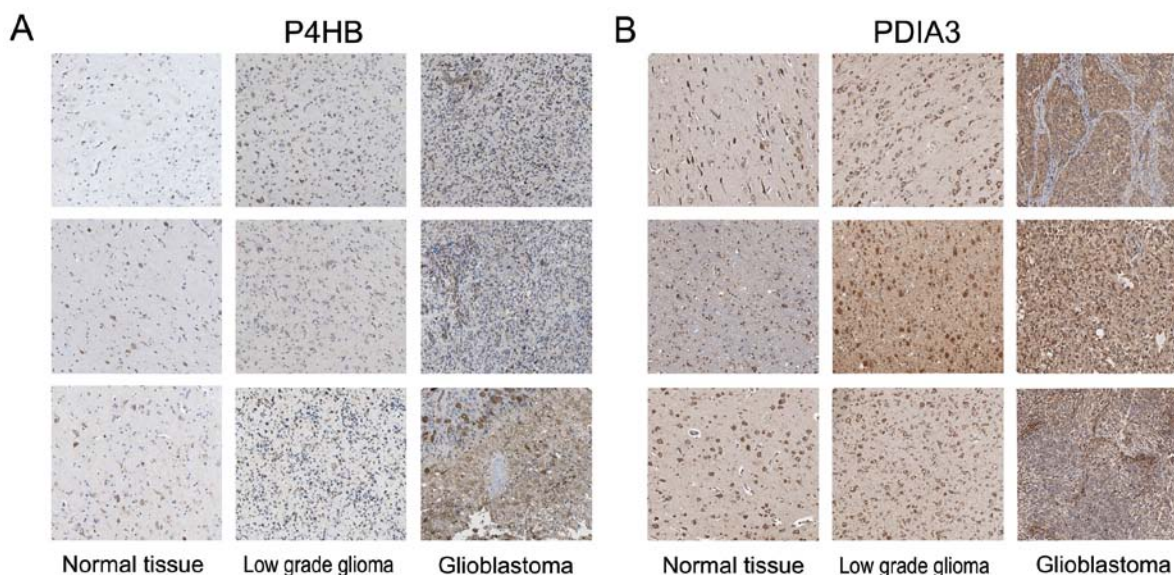

Figure 2. Representative immunohistochemistry images of P4HB (A) and PDIA3 (B) protein in normal brain tissues, low-grade glioma and glioblastoma tissues.

P<0.001). However, no significant difference was observed between astrocytomas and oligodendrogliomas (both P>0.05). These data imply that the mRNA expression of P4HB and PDIA3 is upregulated in diffuse gliomas.

The expression of P4HB and PDIA3 protein in gliomas was analyzed through the on-line Human Protein Atlas (HPA). Immunohistochemical (IHC) staining images of normal brain tissues, low-grade glioma and glioblastoma tissues were obtained from HPA. A photomontage is illustrated in Fig. 2A and B, P4HB and PDIA3 protein exhibited strongly cytoplasmic and membranous staining while assessed by histologic sampling of glioma patients. The expression of P4HB and PDIA3 protein was remarkably higher in the low-grade gliomas and glioblastomas compared to that in the normal tissues. This analysis suggests that there is also strong expression at the protein level of P4HB and PDIA3 within glioma tissues.

High expression of P4HB and PDIA3 is associated with poor survival of glioma patients. Next, we investigated the correlation between gene expression and overall survival (OS) of glioma patients using Kaplan-Meier analysis with a log-rank comparison in the independent glioma gene expression data of the TCGA and GEO datasets. According to the median value of gene expression in tumor samples, glioma patients were divided into two groups: low expression group and high expression group. As shown in Fig. 3A, within TCGA, the overall survival of glioma patients with high P4HB expression (P<0.0001) was significantly worse than that of the low expression patients; survival of glioma patients with high PDIA3 expression (P<0.0001) was markedly worse than patients with low expression. What is more, in several GEO datasets, the overall survival of glioma patients with high P4HB and PDIA3 expression was also significantly worse than that of the low expression patients (P=0.0060 and P=0.0062 in GSE4271, P=0.0223 and P=0.0265 in GSE4412, P<0.0001 and P<0.0001 in GSE43378, respectively; Fig. 3B-D). All together, these results suggest that high expression of P4HB and PDIA3 is correlated with poor survival outcome of diffuse glioma patients.

Furthermore, univariate Cox regression analysis of overall survival of glioma samples within TCGA showed that high expression (P<0.001 for P4HB and P<0.001 for PDIA3), increased age (both P<0.001), high Karnofsky Performance Score (KPS; both P<0.001), high WHO grade (all P<0.001 for II/IV and III/IV), advanced histological type (all P<0.001 for OD/GBM, OA/GBM and A/GBM) were factors associated
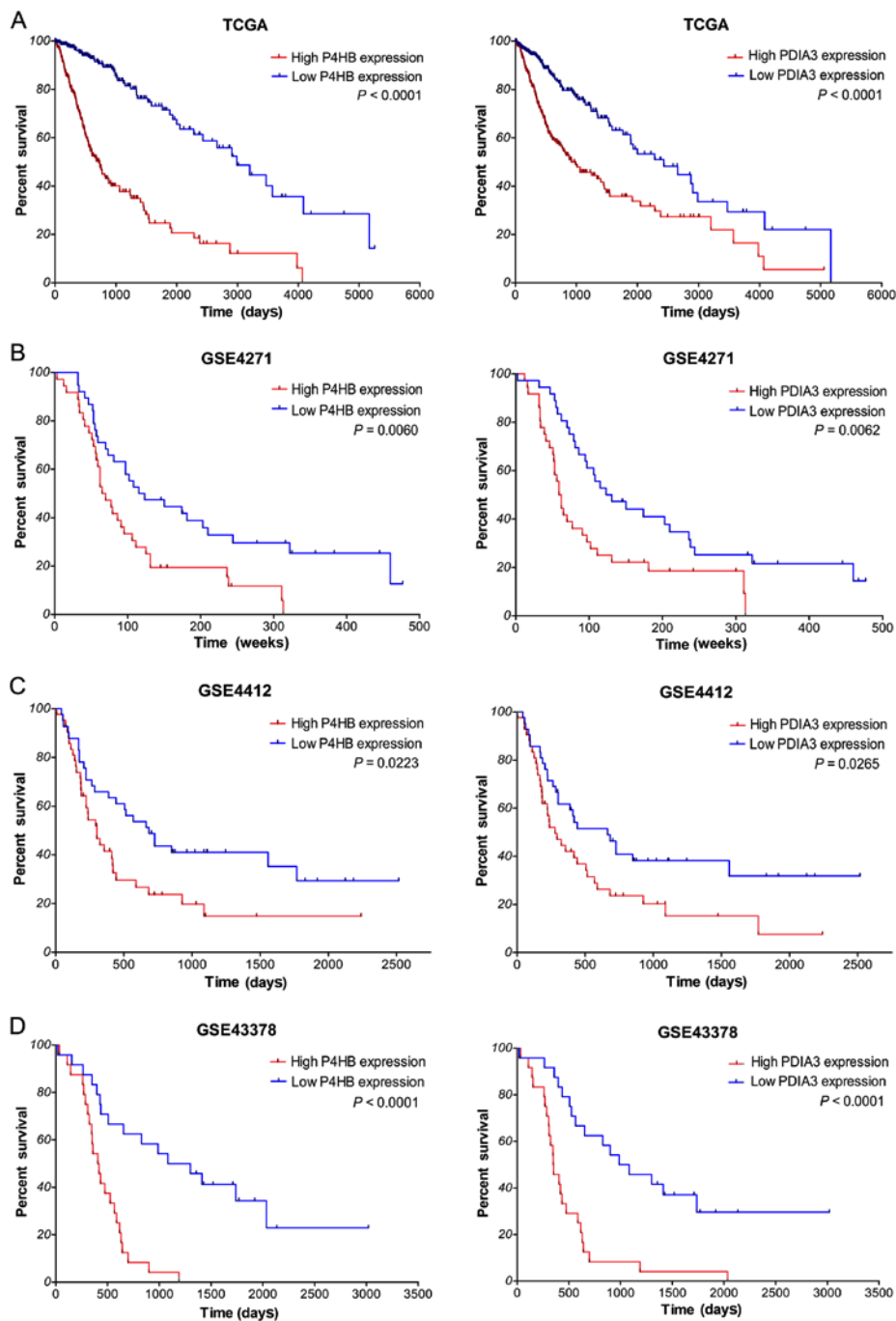
Figure 3. Kaplan-Meier survival curve analysis with a log-rank comparison according to P4HB and PDIA3 expression in glioma samples of TCGA (A) and GEO datasets [GSE4271 (B), GSE4412 (C), and GSE43378 (D)].

with prognosis (Table III). Subsequent multivariate analysis results revealed that high P4HB and PDIA3 expression (HR, 1.696, P=0.019; HR, 1.395, P=0.043, respectively) are independent prognosis factors for the survival of glioma patients, in addition to increased age, high KPS and grade. Similar results were obtained from the Cox regression analysis within the GSE43378 dataset (data not shown). These data indicate that high expression of P4HB and PDIA3 are independent prognostic biomarkers for diffuse gliomas.

Validation of the P4HB and PDIA3 expression in diffuse glioma tissues. The expression levels of P4HB and PDIA3 were confirmed in our 99 diffuse glioma clinical specimens

and 11 non-tumor brain tissues as detected by qRT-PCR. The primary clinical characteristics of the glioma patients are listed in Table I. Based on the classification of 2016 CNS WHO, our experimental results showed that the relative expression level of P4HB and PDIA3 was significantly upregulated in the diffuse glioma specimens compared with that in the non-tumor tissues (Fig. 4). GBM cases displayed statistically higher expression of P4HB and PDIA3 than the oligodendroglioma (P=0.001 and P<0.001), oligoastrocytoma (P=0.002 and P=0.001), and astrocytoma (P=0.001 and P<0.001). But no significant difference was observed among the oligodendrogliomas, oligoastrocytomas and astrocytomas (all P>0.05). Similar with the above analysis results of the GSE4290 dataset, these results indicate

Table III. Cox regression analyses of *P4HB* and *PDIA3* in glioma samples of TCGA.

	P4HB				PDIA3			
	Univariate model		Multivariate model		Univariate model		Multivariate model	
Variables	HR	P-value	HR	P-value	HR	P-value	HR	P-value
High expression	4.878	<0.001	1.696	0.019	2.53	<0.001	1.395	0.043
Sex, F/M		0.791				0.791		
Increased age	1.077	<0.001	1.035	<0.001	1.077	<0.001	1.031	<0.001
High KPS	0.941	<0.001	0.967	<0.001	0.941	<0.001	0.968	<0.001
Grade II/IV	0.051	<0.001	0.455	0.002	0.051	<0.001	0.126	0.001
Grade III/IV	0.147	<0.001	0.832	0.023	0.147	<0.001	0.335	0.001
Histological types								
OD/GBM	0.073	<0.001	0.285	<0.001	0.073	<0.001		0.120
OA/GBM	0.085	<0.001	0.429	0.005	0.085	<0.001		0.107
A/GBM	0.140	<0.001	0.573	0.020	0.140	<0.001		0.227

HR, hazard ratio; F, female; M, male; KPS, karnofsky performance score; OD, oligodendroglioma; OA, oligoastrocytoma; A, astrocytoma; GBM, glioblastoma.

Table IV. Correlations of gene expression and pathological parameters of diffuse glioma specimens.

	P4HB expression			PDIA3 expression		
Variables	High	Low	P-value	High	Low	P-value
Sex, female/male	22/27	14/35	0.094	18/31	18/31	1
Mean age at diagnosis (years)	45.84±2.197	44.61±2.219	0.695	47.98±2.328	42.94±2.022	0.105
KPS score, >80/≤80	37/12	45/4	0.029	36/13	46/3	0.006
GFAP (low/high)	3/38	0/42	0.074	0/43	3/36	0.064
Ki-67 (low/high)	14/27	36/6	<0.001	17/26	32/7	<0.001
MGMT promoter methylation (-/+)	39/0	40/2	0.168	41/1	37/1	0.943
IDH mutation (-/+)	26/13	23/19	0.273	25/17	23/15	0.927
p53 (low/high)	7/32	30/12	<0.001	9/33	27/11	<0.001
1p/19q codeleted (-/+)	8/7	10/11	0.735	10/7	9/11	0.402

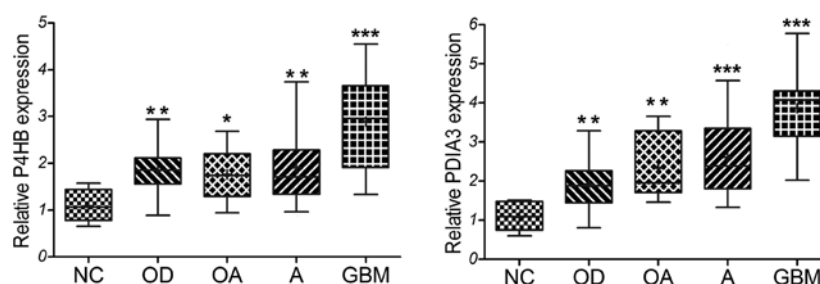KPS, Karnofsky Performance Score. GFAP, glial fibrillary acidic protein.

Figure 4. qRT-PCR analysis of relative P4HB and PDIA3 expression in 99 diffuse glioma specimens and 11 non-tumor brain tissues (NC). [*]P<0.05, [**]P<0.01, [***]P<0.001. P=0.001 for P4HB; P<0.001 for PDIA3 between oligodendroglioma (OD) and GBM; P=0.002 for P4HB; P=0.001 for PDIA3 between oligoastrocytoma (OA) and GBM; P=0.001 for P4HB; P<0.001 for PDIA3 between astrocytoma (A) and GBM.

that P4HB and PDIA3 are highly expressed in diffuse gliomas, especially in GBM.

Correlations of P4HB and PDIA3 expression with pathological characteristics of the glioma patients. To further
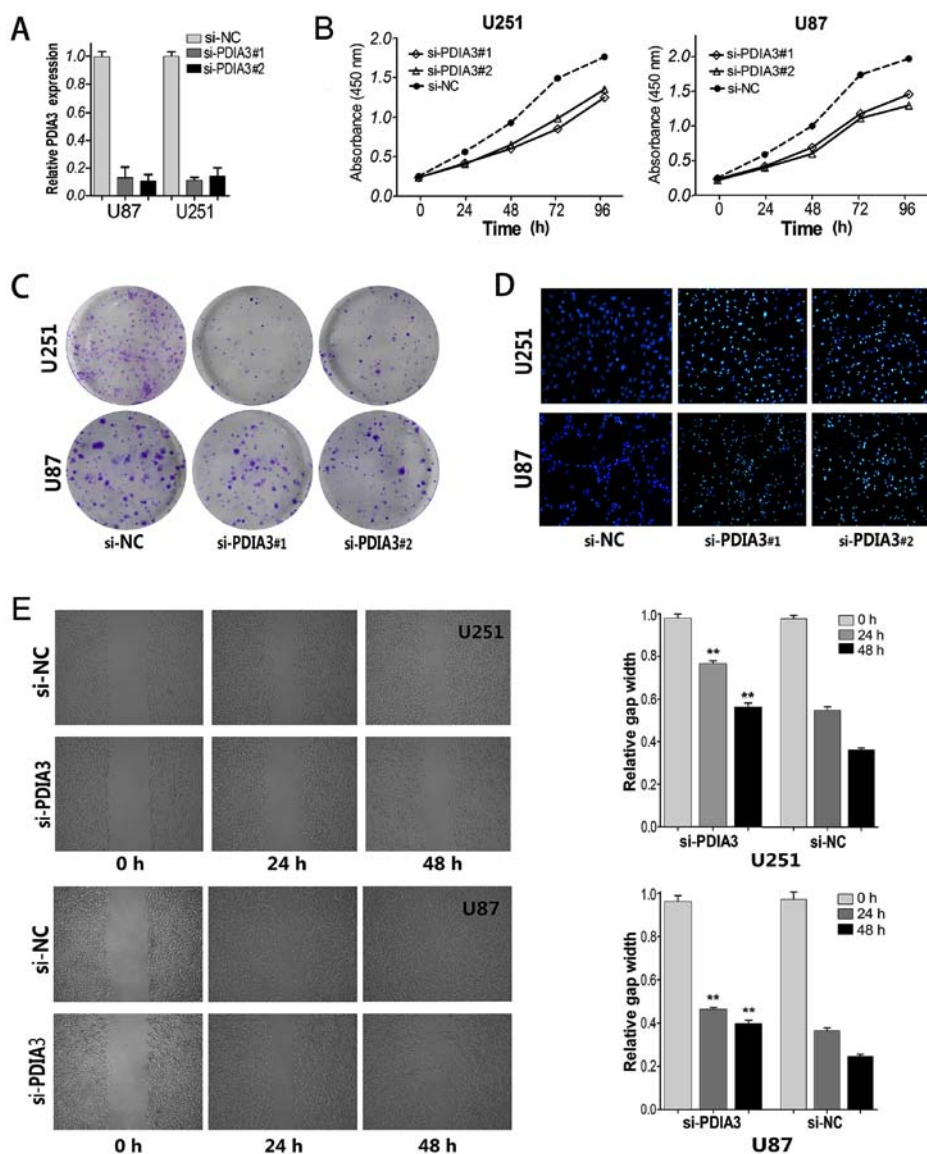
Figure 5. Effects of PDIA3 on glioma cell proliferation, apoptosis and migration *in vitro*. (A) Relative PDIA3 expression in U87 and U251 cells transfected with siRNA against PDIA3 (si-PDIA3) and negative control (si-NC). (B) CCK-8 assay, (C) colony formation assay, (D) Hoechst staining and (E) Scratch assay were performed to test the biological functions of U87 and U251 cells. **P<0.01.

elucidate the correlations between gene expression and the clinical and molecular pathological characteristics of diffuse glioma patients, the median value of relative gene expression was used as a cut-off point. Glioma patients were then divided into low expression group and high expression group. As shown in Table IV, high expression of P4HB and PDIA3 was significantly correlated with high Ki-67 in diffuse glioma specimens (both P<0.001), in despite of a high KPS (P=0.029 and P=0.006). Higher expression of p53 (indicated by *TP53* mutation) was a relative risk factor for glioma patients with high P4HB and PDIA3 expression (both P<0.001). However, no significant relationship was found between expression of the genes and other pathological characteristics including gender, age, GFAP, *MGMT* promoter methylation, *IDH* mutation and 1p/19q codeletion (all P>0.05). As might be expected, Ki-67, a nuclear protein is necessary for cellular proliferation (20). *TP53* gene encodes a tumor-suppressor protein p53, which responds to diverse cellular stresses to regulate the expression of multiple target genes. Mutations in the *TP53* gene are asso-

ciated with a variety of human cancers, including gliomas (21). These findings imply that P4HB and PDIA3 play an important role in tumor progression of diffuse gliomas.

Effect of PDIA3 on glioma cell proliferation and migration in vitro. To further explore the effects of PDIs on glioma cells, we detected the relative expression level of P4HB and PDIA3 in the cultured glioma cell lines. The U87 and U251 cell lines were selected for further study. Since Sun *et al* (22) has carried out a study of the *P4HB* gene in glioma cells, here, some experiments concerning *PDIA3* gene were performed. As shown in Fig. 5A, knockdown of *PDIA3* significantly reduced the expression levels of *PDIA3* in two cell lines, as compared with negative controls.

The proliferation of glioma cells was determined by CCK-8 and colony formation assays. Results of CCK-8 assay revealed that cell growth was significantly suppressed in the si-PDIA3- transfected cells compared to the negative controls (both P<0.001; Fig. 5B). Consistently, the colony formation
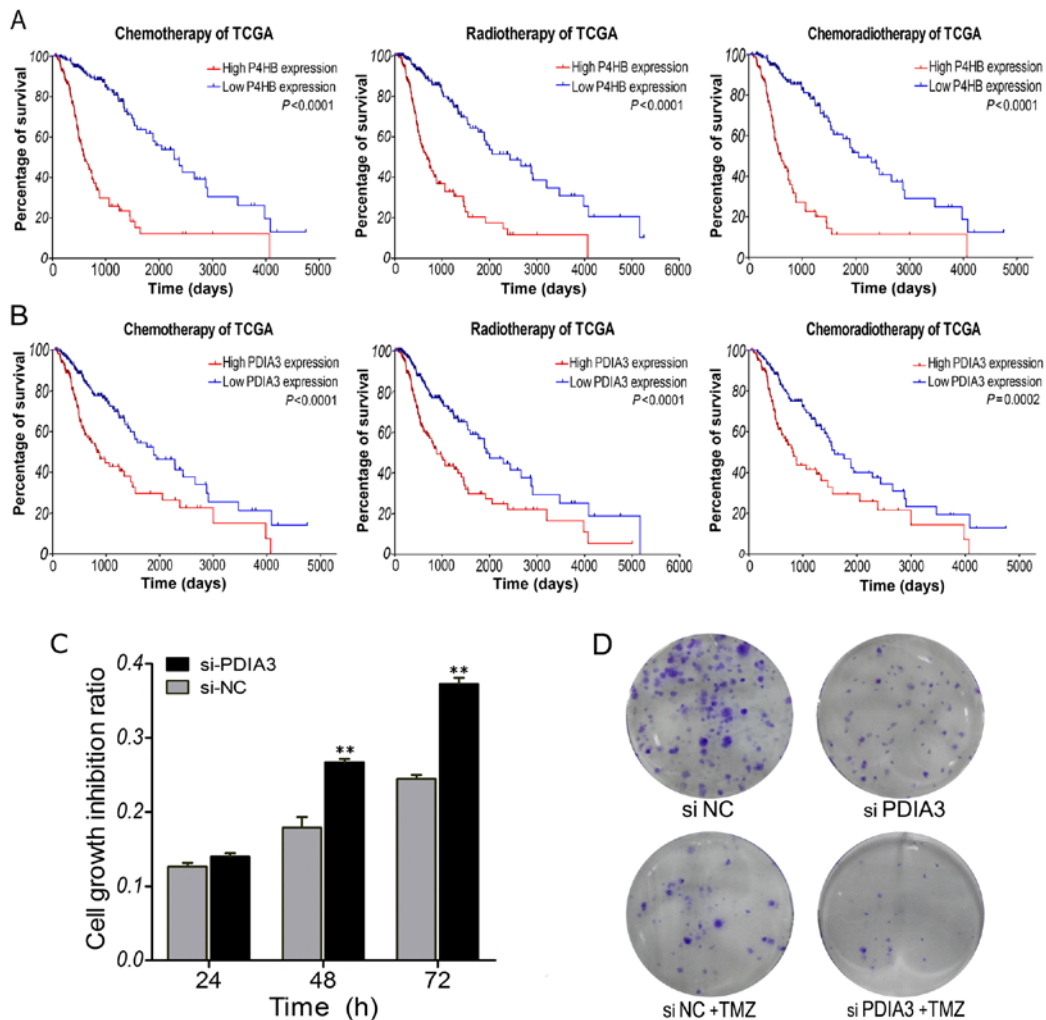
Figure 6. Kaplan-Meier survival curve analysis of glioma patients with chemotherapy (n=330) or radiotherapy (n=388) in TCGA based on the expression of P4HB (A) and PDIA3 (B). Cell growth inhibition test (C) and colony formation assay (D) were performed to determine the biological functions of U251 cells after knockdown of PDIA3 plus temozolomide (TMZ) treatment.

assay showed that the number of colonies was evidently decreased after knockdown of PDIA3 in the U251 and U87 cells (Fig. 5C). After apoptosis detection with Hoechst staining assay, it was shown that the nuclear chromatin condensation of apoptosis in the si-PDIA3-transfected cells was markedly increased compared with the negative controls (Fig. 5D). In addition, a scratch assay was displayed that the migration ability of the two glioma cell lines transfected with si-PDIA3 was significantly reduced in comparison with the negative controls (Fig. 5E). These were consistent with other reports that bacitracin, an inhibitor of PDIs, inhibited glioma cell migration and invasion *in vitro* by decreasing pFAK (phosphorylated focal adhesion kinase) and MMP2 (the secreted matrix metalloproteinase 2) (23). All together, the results indicate that PDIA3 may play a major role in the proliferation, apoptosis and migration of glioma cells.

Survival outcome evaluation of patients with chemotherapy and radiotherapy. To determine the correlations of P4HB and PDIA3 expression signature with the response to chemotherapy and radiotherapy, subset survival analyses were performed with the TCGA dataset, for which therapeutic information was available. It is well known that glioma patients benefit from

temozolomide (TMZ) chemotherapy and radiotherapy. As shown in Fig. 6A and B, the overall survival of the low P4HB expression group was significantly better than that of the high expression group in glioma patients who received chemotherapy (P<0.0001) or radiotherapy (P<0.0001). Moreover, survival of the low-PDIA3 expression patients was noticeably better than that of the high expression patients who received chemotherapy (P<0.0001) or radiotherapy (P<0.0001). This was consistent with a previous report that overexpression of P4HB is related to the development of TMZ resistance, and knockdown of P4HB or inhibition of PDI activity using bacitracin sensitizes glioma cells to TMZ *in vitro* and *in vivo* (22). Furthermore, the TMZ sensitivity of glioma U251 cells was determined by CCK-8 and colony formation assays. After TMZ treatment, cell growth inhibition ratio was significantly increased in the si-PDIA3-transfected cells compared to the negative controls (Fig. 6C). The number of colonies was remarkably reduced after knockdown of PDIA3 plus TMZ treatment in the glioma cells (Fig. 6D). These results suggest that glioma patients with low P4HB and PDIA3 expression could benefit more from chemotherapy and radiotherapy. High expression of P4HB and PDIA3 may be indicators of poor response to adjuvant chemoradiotherapy.

Discussion

In the present study, we first demonstrated that the expression of P4HB and PDIA3 was frequently upregulated at the mRNA and protein levels in diffuse gliomas. High P4HB and PDIA3 expression was significantly correlated with high Ki-67 and more *TP53* mutations. These findings imply that high expression of P4HB and PDIA3 plays an important role in diffuse glioma progression. Furthermore, survival curve and Cox regression analysis showed that glioma patients with high P4HB and PDIA3 expression had a poor survival outcome, P4HB and PDIA3 could be independent prognostic biomarkers for diffuse glioma patients.

Many cancer-related pathologies are associated with the deregulation of endoplasmic reticulum (ER) homeostasis or the induction of ER stress and unfolded protein response (24). Protein disulfide isomerases (PDIs) act as molecular chaperones involved in ER stress and unfolded protein response, and have been extensively studied during the past decade. Dysregulation of PDI expression, post-translational modification or enzymatic activity could cause many human diseases, such as neurodegenerative disorders, diabetes and cardiovascular disease (4,25). Recently, emerging evidence indicates that PDIs are associated with tumor progression and could be potential molecular targets for cancer therapy (26). For instance, positive expression of PDIA3 was associated with tumor progression and poor postoperative survival of gallbladder cancer patients (5). PDIA3 expression was upregulated in cervical cancer, and could serve as a prognostic marker (7). Knockdown of PDIA3 suppressed cell invasion in HeLa cells and inhibited lung metastasis in a xenograft mouse model (7). PDIA3 modulated STAT3 activity in radioresistant laryngeal tumor cells, and an increase in the PDIA3-STAT3 complex was associated with poor prognosis in laryngeal cancer (6).

The biological significance of PDIA3 was explored with glioma cells *in vitro*. Through knockdown of PDIA3 in U87 and U251 cells, experimental results showed that the cell growth and colony formation ability were significantly suppressed, apoptosis was induced and migration ability was markedly decreased. Previous studies have reported that PDIA3 may be a substrate for caspase-3 and -7 during etoposide-induced apoptosis in acute myelocytic leukemia cells (27). Both P4HB and PDIA3 possess Bak-dependent pro-apoptotic function via inducing mitochondrial outer membrane permeabilization (28). Knockdown of PDIA3 inhibited cell proliferation by inducing G1/S arrest in breast cancer cells (29). Moreover, cell surface PDIs are associated with cancer invasion and metastasis. The expression of P4HB and PDI3 protein was found to be significantly higher in axillary lymph node metastatic breast tumor compared with primary breast tumor (30). PDI-mediated disulfide bond formation regulated the enzyme activity and secretion of MMP9 (matrix metallopeptidase 9), a main proteinase degrading extracellular matrix and facilitating metastasis and tumor angiogenesis (31).

Finally, we found that glioma patients with low P4HB and PDIA3 expression benefit more from chemotherapy and radiotherapy. Knockdown of PDIA3 enhanced TMZ sensitivity of glioma cells. This was consistent with the report that P4HB is involved in the development of TMZ resistance (22). These findings imply that high expression of P4HB and PDIA3 indicates a poor response to adjuvant chemoradiotherapy. Similarly, recent studies have reported that knockdown of P4HB or inhibition of PDI activity using bacitracin enhanced cisplatin cytotoxicity in ovarian cancer resistant A2780 cells (32). PACMA 31, one of the propynoic acid carbamoyl methyl amide (PACMA) active analogs, acts as a novel irreversible inhibitor of PDIs, which exhibits tumor targeting ability and significant anticancer activity in ovarian cancer models, without causing toxicity to normal cells (33). Other studies have shown that the presence of autoantibodies to PDIA3 favors the development of an efficient and specific T-cell response against PDIA3 in colon cancer patients (34). It may be relevant for the design of novel therapeutic strategies.

Taken together, the present study demonstrated that both P4HB and PDIA3 were upregulated in diffuse gliomas. High expression levels of P4HB and PDIA3 was found to be associated with tumor progression, and could be independent prognostic biomarkers for diffuse glioma. *In vitro*, knockdown of PDIA3 suppressed cell proliferation, induced apoptosis and decreased migration of glioma cells. Furthermore, downregulation of P4HB and PDIA3 may contribute to improve the survival of patients who receive chemotherapy and radiotherapy. These findings imply that protein disulfide isomerases could be explored as therapeutic targets for diffuse gliomas.

Acknowledgements

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